

Analytical, Nutritional and Clinical Methods

# Determination of Cd, Cu, and Zn in fish and mussel by AAS after ultrasound-assisted acid leaching extraction

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## Abstract

An ultrasound-assisted solid–liquid extraction procedure by using diluted mixed acid solution was developed for determination of cadmium, copper and zinc in fish and mussel samples. The effects of several parameters such as nitric acid concentration, hydrochloric acid concentration, hydrogen peroxide concentration, leaching solution volume, and sonication time have been investigated. A 30-min sonication, 56 °C operating temperature and 6 mL of 1:1:1 HNO<sub>3</sub>(4 M):HCl(4 M):H<sub>2</sub>O<sub>2</sub>(0.5 M) were used for 0.5 g of dried sample. Cadmium and copper were determined by graphite furnace atomic absorption spectrometry, and zinc was determined by flame atomic absorption spectrometry. The results obtained from the proposed procedure were evaluated by comparison with the results obtained by microwave-assisted digestion. Ratio (%) of metal amount obtained from leaching technique to amount obtained from digestion technique for cadmium, copper and zinc ranged from 92% to 114% for fish and from 88% to 103% for mussel samples. The MDL were 0.02, 0.13 and 0.63 mg kg<sup>-1</sup> for cadmium, copper and zinc, respectively. The accuracy of the developed method was investigated by analyzing a dogfish muscle certified reference material (DORM-2). Recoveries were obtained in the order of 80.9 ± 0.3 and 87.2 ± 0.6%.

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## 1. Introduction

At present, many countries enforce regulations on contamination level of toxic metals in food and food products, especially seafood. These regulations require precise and accurate analytical method for the determination of metals (Codex Alimentarius, 2005; US FDA, 1993). Conventional wet acid digestion and microwave acid digestion methods are widely used for the mineralisation of biological samples prior to the determination by atomic absorption spectrometry, for example AOAC standard methods (AOAC International, 2002). The severe disadvantage of these methods is that concentrated acids or oxidizing reagents cause the matrix interference in the determination step. In addition, the procedure is tedious, large time and high energy consuming, and contamination can be occurred (Buldini,

Ricci, & Sharma, 2002; Sun, Waters, & Mawhinney, 1997). Other drawback is nitrous vapors formation after organic matrix destruction stage is high carcinogenic.

A sample preparation method, called “acid leaching extraction” has been employed to avoid the previous inconveniences (Azouzi, Cervera, & Guardia, 1998; Bermejo-Barrera, Moreda-Pineiro, & Bermejo-Barrera, 2001; Bermejo-Barrera, Moreda-Pineiro, Muniz-Naveiro, Gomez-Fernandez, & Bermejo-Barrera, 2000; Bermejo-Barrera, Muñoz-Naveiro, Moreda-Piñeiro, & Bermejo-Barrera, 2000; Bermejo-Barrera, Muniz-Naveiro, Moreda-Pineiro, & Bermejo-Barrera, 2001; Watson, 1984; Yebra-Biurrun & Garcia-Garrido, 2001). The procedure involves the partial dissolution of metals in the diluted acid solution without sample destruction compared to the digestion techniques. The amount of acid used can be reduced, the generated nitrous vapors can probably be excluded, and the cooling stage is not required, so that the operation time is diminished (Bendicho & Lavilla, 2003). The procedure

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has been employed for the determination of metals in various types of samples, for examples, seafood (Azouzi et al., 1998; Bermejo-Barrera et al., 2000; Bermejo-Barrera, Muñiz-Naveiro et al., 2000; Bermejo-Barrera et al., 2001; Bermejo-Barrera, Muniz-Naveiro et al., 2001; Domínguez-González, Moreda-Piñeiro, Bermejo-Barrera, & Bermejo-Barrera, 2005; Mendez, Alava, Lavilla, & Bendicho, 2002), food containers, plants (Ruiz-Jimenez, Luque-Garcia, & Luque de Castro, 2003), environmental samples (Al-Merey, Al-Masri, & Bozou, 2002), and biological samples (Capelo, Maduro, & Vilhena, 2003; Seferinoglu et al., 2003).

The acid leaching extraction method consists of two principal techniques, e.g., microwave-assisted acid leaching and ultrasound-assisted acid leaching. The method development for both techniques has been reported continuously. The most significant variables affecting the accuracy and precision of the measurement are sample mass, nitric acid concentration, hydrochloric acid concentration or hydrogen peroxide, and exposure time to microwave energy or sonication time.

The development of the microwave-assisted acid leaching method for extraction of trace elements from seafood products was reported (Bermejo-Barrera et al., 2000; Link, Walter, & Kingston, 1998). A mixture of HCl, HNO<sub>3</sub>, and H<sub>2</sub>O<sub>2</sub> was used for quantitative extraction of Ca, Cr, Cu, Fe, Mg, Mn, Pb, and Zn.

The development of ultrasound-assisted acid leaching procedure is nowadays of interest (Priego-Capote & Luque de Castro, 2004). The comparison of the ultrasound-assisted extraction and microwave-assisted digestion has also been published recently, for examples, the determination of metals in plant samples (Filgueiras, Capelo, Lavilla, & Bendicho, 2000), in vegetables (Nascentes, Korn, & Arruda, 2001), in environmental samples (Sastre, Sahuquillo, Vidal, & Rauret, 2002), in soil samples (Al-Merey et al., 2002). The method developments were proposed, e.g., the use of a mixture of HNO<sub>3</sub>, HCl, and H<sub>2</sub>O<sub>2</sub> solution to extract multi-elements from mussel samples (Azouzi et al., 1998; Balarama Krishna & Arunachalam, 2004; Bermejo-Barrera, Muñiz-Naveiro et al., 2000; Bermejo-Barrera et al., 2001; Bermejo-Barrera, Muniz-Naveiro et al., 2001; Yebra-Biurrun, Cancela-Pérez, & Moreno-Cid-Barinaga, 2005), the ultrasound-assisted acid leaching for the Cd and Pb determination in foods (Aleixo et al., 2004). Moreover, the application of ultrasound-assisted acid leaching procedure with a flow injection technique has been also reported for the determination of Cu in mussel samples (Moreno & Yebra, 2002), Cd and Pb in plants (Ruiz-Jimenez et al., 2003). In addition, ultrasound extraction shows advantageous features for element speciation. Both organic and aqueous extraction media have been used for separation of organometallic and inorganic species from solid matrix (Ashley, 1998; Bendicho & Lavilla, 2003; Marin, Lopez-Gonzalez, & Barbas, 2001; Rio-Segade & Bendicho, 1999). Most of the authors mentioned that a formula of acid leaching solution was not able to

extract many elements simultaneously. But, the analytical results for the metal determination by the acid leaching and digestion methods showed a good agreement. In addition, the results obtained from acid leaching techniques were accurate and sufficiently precise.

Acid leaching extraction method usually involves the use of diluted acid to extract elements from solid samples, providing a significant speeding up. So far, analytical results are very promising, and new developments are expected on the topics addressed in the present work. Various ultrasound-assisted extraction procedures have been reported for mussel tissue samples, but the procedure for fish tissue samples although not yet sufficiently exploited. The main objective of this research is to develop the acid leaching extraction technique to investigate the simultaneous quantitative extraction of Cu, Cd, and Zn in fish and mussel tissues. The optimization of the extraction conditions and the method validation were performed.

## 2. Experimental

### 2.1. Apparatus

A microwave digestion unit and accessories (Multiwave, Paar Physica), a centrifuge (Centaur 2, Sanyo), and an ultrasonic bath (28H Ney Ultrasonik) were used for the sample pre-treatment. A flame atomic absorption spectrometer (AAAnalyst 100, Perkin–Elmer Instrument) and a graphite furnace atomic absorption spectrometer (4110 ZL, Perkin–Elmer Instrument) were used to determine metal concentrations. The analytical conditions are summarized in Table 1.

### 2.2. Materials and reagents

De-ionized water was obtained from the purification via cation and anion exchange resin columns, the conductivity of the de-ionized water was less than 4  $\mu\text{S cm}^{-1}$ . All glassware (borosilicate) and plasticware (low density polyethylene, LDPE) were soaked in 2% (v/v) HCl for 2 h, rinsed with de-ionized water, soaked in 2% (v/v) HNO<sub>3</sub> for 2 h, and then rinsed with de-ionized water several times.

All reagents were standard analytical grade and used without further purification. De-ionized water was used for all preparation of solutions. Acid solutions (Lab Scan) were prepared using volumetric glassware. Working standard metal solutions were prepared from stock solutions of 1000 mg L<sup>-1</sup> (BDH Laboratory Supplies) by diluting to the required concentration by 1% (v/v) HNO<sub>3</sub>.

### 2.3. Sample fortification

Due to a very low contamination level of cadmium and copper, the fortified samples were prepared by the following procedure in order to facilitate the experimental operation. Fresh Siamese Tigerfish and mussel tissues were carved to small pieces and soaked into a solution contain-

Table 1

GFAAS conditions for the determination of Cd and Cu and FAAS conditions for the determination of Zn in acid digests and acid leachates from fish and mussel samples

Element	Operating conditions	Graphite furnace temperature program				
		Step	Temperature (°C)	Ramp (s)	Hold (s)	Ar flow rate (mL/min)
Cd	Wavelength 228.8 nm	Drying 1	110	1	30	250
	Slit width 0.7 nm	Drying 2	130	15	30	250
	Injection volume 20 $\mu$ L	Pyrolysis	500	10	20	250
		Atomization	1500	0	5	0
		Cleaning	2450	1	3	250
Cu	Wavelength 324.8 nm	Drying 1	110	1	30	250
	Slit width 0.7 nm	Drying 2	130	15	30	250
	Injection volume 20 $\mu$ L	Pyrolysis	1200	10	20	250
		Atomization	2000	0	5	0
		Cleaning	2450	1	3	250
Zn	Wavelength 213.9 nm Slit width 0.7 nm Lamp current 15 mA Air flow rate 10 L/min C <sub>2</sub> H <sub>2</sub> flow rate 3.0 L/min	–	–	–	–	–

ing 100 Cu(II) and 100 mg L<sup>-1</sup> Cd(II) for 2 days at 4 °C. All of soaked samples were washed with de-ionized water for 5–6 times, and homogenized. Samples were dried at 100 °C for 20 h, ground to small particle size ( $\leq 300 \mu\text{m}$ ), and put into a LDPE bottle, and kept in a desiccator.

#### 2.4. Microwave digestion

A 0.2 g of dried samples was weighted into quartz vessels. Concentrated HNO<sub>3</sub> (5.0 mL) and concentrated HCl (0.50 mL) were added into each vessel. Then, the vessels were capped with Teflon seal and energy was applied following the power program in Table 2. After the digestion was completed, the vessels were cooled until the temperature of each vessel lower than 50 °C. The solution was transferred into a volumetric flask and made up to 10 mL with de-ionized water. This solution was kept in a LDPE bottle at 4 °C before measurement. The reagent blank was prepared in the same manner. The metals in the final solution were determined by FAAS and GFAAS. The analytical results were used to compare with the results from acid leaching techniques.

#### 2.5. Acid leaching extraction

A 0.2 g of dried samples was weighted into a plastic tube and a mixture of HNO<sub>3</sub>, HCl, and H<sub>2</sub>O<sub>2</sub> was added. The blank leaching was performed by using de-ionized water as leaching solution. The appropriate formula of leaching

solution, its volume, sonication times and temperatures were investigated. The mixture was sonicated in an ultrasonic bath for 15–60 min at ambient temperature. The mixture was centrifuged at 3000 rpm for 10 min. The liquid phase was transferred to a 10 mL volumetric flask. The solid residue was washed with 2 mL of de-ionized water, then centrifuged at 3000 rpm for 15 min, transferred to the same volumetric flask and the volume was adjusted to 10 mL with de-ionized water. This solution was kept in a LDPE bottle at 4 °C before measurement. The metals in the leachates were determined by FAAS and GFAAS. The results were compared with the digestion ones.

##### 2.5.1. Effect of nitric acid concentration and hydrochloric acid concentration

The fortified samples were used to investigate the effect of nitric acid concentration. Leaching solutions were prepared by fixing concentration of HCl and H<sub>2</sub>O<sub>2</sub> (4 and 0.5 M, respectively) and varying concentration of HNO<sub>3</sub> (0, 0.5, 1, 2, 3, and 4 M). While the effect of hydrochloric acid concentration was studied by fixing concentration of HNO<sub>3</sub> and H<sub>2</sub>O<sub>2</sub> (4 and 0.5 M, respectively) and varying concentration of HCl (0, 0.5, 1, 2, 3, and 4 M). The ratio of the mixture of HNO<sub>3</sub>, HCl, and H<sub>2</sub>O<sub>2</sub> was fixed at 1:1:1. Samples were sonicated at ambient temperature for 15 min.

##### 2.5.2. Effect of hydrogen peroxide

Leaching solution without H<sub>2</sub>O<sub>2</sub> (1:1:1 HNO<sub>3</sub>(4 M):HCl(4 M):H<sub>2</sub>O) was used to investigate the effect of hydrogen peroxide by comparing with the previous results obtained from the leaching solution with H<sub>2</sub>O<sub>2</sub>.

##### 2.5.3. Effect of leaching solution volume

The volume of the leaching solution was varied with 2, 4, and 6 mL of 1:1:1 HNO<sub>3</sub>(4 M):HCl(4 M):H<sub>2</sub>O<sub>2</sub>(0.5 M). The results could be attributed to optimize the solvent volume used to leach the elements from fish and mussel samples.

Table 2

Power program of microwave digestion

Step	Power (W)	Time (min)
1	100	5
2	600	5
3	1000	10
4	0	15

#### 2.5.4. Effect of temperature and sonication time

The ultrasound exposure time was varied from 15 to 30 and 60 min at different temperatures.

#### 2.6. Determination of metals in fish and mussel samples

The optimum conditions were applied with real samples, fish and mussel. The fresh samples were homogenized and dried as described previously without fortification. The leaching procedure was the same as mentioned above. Cu and Cd were determined by GFAAS and Zn was determined by FAAS. The precision was studied by measurement 10 replicates of leachates. The precision is expressed in term of relative standard deviation (RSD).

### 3. Results and discussion

Acquisitive results from acid leaching extraction technique were compared with results of microwave digestion. Subsequently a term of “ratio (%) of metal amount obtained from leaching technique to amount determined by digestion technique” was employed. The ratio (%) was defined by Eq. (1)

$$\text{Ratio}(\%) = \frac{M_{\text{leach}}}{M_{\text{digest}}} \times 100 \quad (1)$$

where  $M_{\text{leach}}$  is the amount of analyte from acid leaching extraction technique ( $\text{mg kg}^{-1}$  of sample) and  $M_{\text{digest}}$  is the amount of analyte from microwave digestion technique ( $\text{mg kg}^{-1}$  of sample).

In order to confirm the retention of metals in the fortified samples and the strength of leaching solution, the leaching by using de-ionized water was considered. In this study samples were treated with 6 mL of de-ionized water and the results were compared with the ones from leaching by 6 mL of 1:1:1  $\text{HNO}_3$ (4 M): $\text{HCl}$ (4 M): $\text{H}_2\text{O}_2$ (0.5 M) solution. The overall found ratios were less than 15%.

#### 3.1. Effect of nitric acid concentration

To investigate the effect of nitric acid concentrations, the concentrations of hydrochloric acid and hydrogen peroxide were constant, 4 M for HCl and 0.5 M for  $\text{H}_2\text{O}_2$  and the range of 0–4 M nitric acid was used for fortified samples.

The ratio (%) in function of nitric acid concentration was shown in Fig. 1.

Fig. 1(a) showed the constant ratio (%) of Cu and Cd that is close to 100% when 0–4 M nitric acid was used. But in case of Zn, nitric acid concentration showed a small influence on the leaching. The ratio (%) was inferior of 90 when leaching solution consisted of 2 M nitric acid. The results obtained from mussel samples in Fig. 1(b) showed the similarity as fish samples. The ratio (%) was approached to 100% when 0–4 M nitric acid was used. These results indicated that the concentration of nitric acid was not a significant variable.

#### 3.2. Effect of hydrochloric acid concentration

Similar to the study of nitric acid concentration, the effect of hydrochloric acid concentrations was considered. In this case, concentrations of nitric acid and hydrogen peroxide were fixed at 4 and 0.5 M, respectively, and the range for 0–4 M hydrochloric acid was used. The ratio (%) in function of hydrochloric acid concentrations for fish and mussel samples are shown in Fig. 2.

Fig. 2(a) showed no significant effect of hydrochloric acid concentration for Cu and Cd leaching in fish samples. The obtained ratio (%) of Zn were similar to the results of Zn leaching in Fig. 1. When hydrochloric acid concentrations were 4 M, the ratio closed to 100% was obtained. For mussel samples in Fig. 2(b), the results showed that more than 90% relations were obtained when leaching solutions consisted of 0–4 M hydrochloric acid.

#### 3.3. Effect of hydrogen peroxide

To test the effect of hydrogen peroxide on acid leaching efficiency, the leaching solution without hydrogen peroxide (6 mL of 1:1:1  $\text{HNO}_3$ (4 M): $\text{HCl}$ (4 M): $\text{H}_2\text{O}$ ) was used. The results for fish and mussel samples were summarized in Table 3. It was found that hydrogen peroxide was a significant variable only effecting Cu leaching for both samples.

When applying the two means *t*-test (Miller & Miller, 1993) and comparing the *t* statistics with the critical value for 95% confidence and 6° of freedom that is 2.447, it was found that the mean results for Cu leaching from the two methods were significantly different. This indicated that

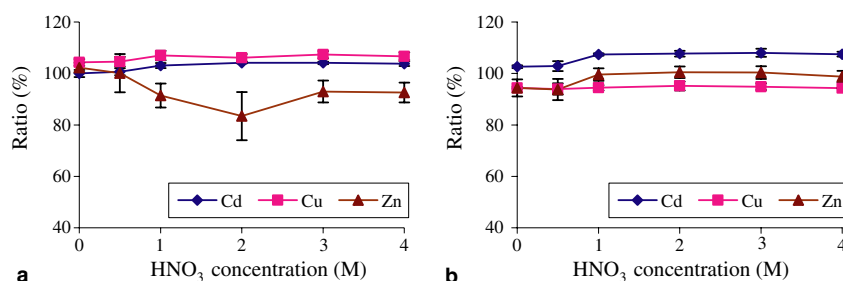


Fig. 1. Effect of nitric acid concentration on the determination of Cd, Cu, and Zn in: (a) fish samples and (b) mussel samples (0.20 g of samples, 6 mL of leaching solution,  $n = 4$ ).

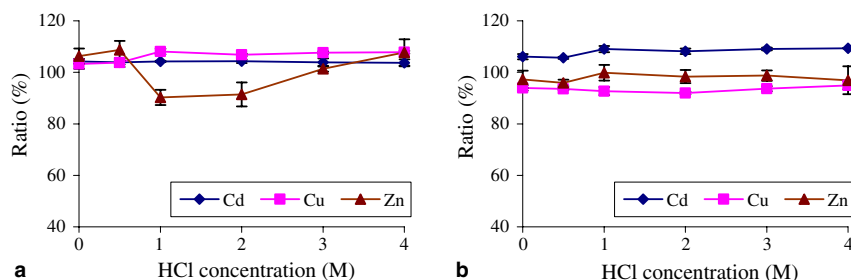


Fig. 2. Effect of hydrochloric acid concentration on the determination of Cd, Cu, and Zn in: (a) fish samples and (b) mussel samples (0.20 g of samples, 6 mL of leaching solution,  $n = 4$ ).

Table 3  
Effect of hydrogen peroxide on acid leaching efficiency

Elements	Fish			Mussel		
	Ratio (%) <sup>a</sup>		$t^b$	Ratio (%) <sup>a</sup>		$t^b$
	With 0.5 M H <sub>2</sub> O <sub>2</sub>	Without H <sub>2</sub> O <sub>2</sub>		With 0.5 M H <sub>2</sub> O <sub>2</sub>	Without H <sub>2</sub> O <sub>2</sub>	
Cd	104.22 ± 0.32	103.98 ± 0.59	0.72	109.40 ± 0.46	109.30 ± 0.46	0.31
Cu	107.32 ± 1.44	94.65 ± 1.58	11.85	95.16 ± 2.34	86.19 ± 0.72	7.33
Zn	98.24 ± 0.52	97.03 ± 2.47	0.96	103.17 ± 1.82	103.70 ± 1.76	0.42

<sup>a</sup> Mean ratio (%) ± confidence interval at 95%,  $n = 4$ .

<sup>b</sup> Two means,  $t$ -test value.

organic matters (protein amino acids) had important role in controlling the release of Cu. H<sub>2</sub>O<sub>2</sub> could dissociate to hydroxyl radical (OH<sup>•</sup>) that could attack proteins, carbohydrates and polyunsaturated fatty acids present in the samples. Hence, it improved the efficiency of the Cu extraction (Azouzi et al., 1998; Goodman, Green, & McPhail, 1984). On the other hand, H<sub>2</sub>O<sub>2</sub> was an insignificant variable for Cd and Zn leaching from both fish and mussel samples.

Regarding to above discussion, the concentrations of the used acids did not affect the leaching efficiency. A mild and appropriate condition of leaching solution should be 1:1:1 HNO<sub>3</sub>(0.5 M):HCl(0.5 M):H<sub>2</sub>O<sub>2</sub>(0.5 M). This acid mixture was used for the extraction of the metals from real sample. The results showed that the ratio (%) was not satisfied. From literature review, many authors strongly recommended the moderate concentration of both acids whose the values were ranging from 1 to 3 M in final solution (Azouzi et al., 1998; Bermejo-Barrera, Muñiz-Naveiro et al., 2000; Bermejo-Barrera et al., 2001; Bermejo-Barrera, Muniz-Naveiro et al., 2001). Successful leaching of Cu and Zn was achieved, but the Cd was partially leached. The

higher concentration was used, the better efficiency was found (Bermejo-Barrera, Muñiz-Naveiro et al., 2000). Thus, a formula of leaching solution as 1:1:1 HNO<sub>3</sub>(4 M):HCl(4 M):H<sub>2</sub>O<sub>2</sub>(0.5 M) was chosen for further experiments.

### 3.4. Effect of acid solvent volume

Two, four and six milliliters of 1:1:1 HNO<sub>3</sub>(4 M):HCl(4 M):H<sub>2</sub>O<sub>2</sub>(0.5 M) solution were applied for fish and mussel samples. Analytical results were shown in Fig. 3. For both samples, acid leaching efficiency for Cd, Cu, and Zn were strongly affected by leaching solution volume, especially in case of Cu and Zn. Nearly 100% relation was found when 6 mL of acid solvent was used.

### 3.5. Effect of temperature

It was recommended that the temperature during sonication played an important role on increasing acid leaching efficiency due to the dissolution of metal in leaching solution

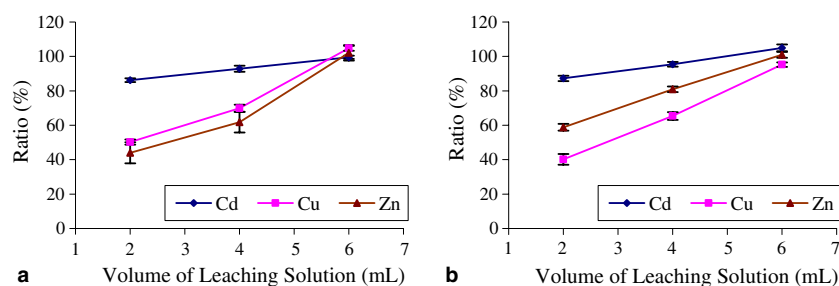


Fig. 3. Effect of acid solvent volume to leach elements in: (a) fish samples and (b) mussel samples (0.20 g of samples were treated with 1:1:1 HNO<sub>3</sub>(4 M):HCl(4 M):H<sub>2</sub>O<sub>2</sub>(0.5 M)).

Table 4  
Effects of temperature and sonication time (0.20 g of samples, 6 mL of 1:1:1 HNO<sub>3</sub>(4 M):HCl(4 M):H<sub>2</sub>O<sub>2</sub>(0.5 M))

Samples	Element	Ratio (%) <sup>a</sup>			
		27 °C		56 °C	
		15 min	15 min	30 min	60 min
Fish	Cd	ND	ND	ND	ND
	Cu	ND	29.69 ± 2.48	90.63 ± 3.73	62.50 ± 2.48
	Zn	100.67 ± 3.91	–	–	–
Mussel	Cd	16.13 ± 12.82	70.97 ± 2.56	88.71 ± 2.56	90.32 ± 5.13
	Cu	32.94 ± 15.60	79.91 ± 2.60	99.30 ± 4.83	92.06 ± 1.11
	Zn	101.01 ± 1.34	–	–	–

ND = not detectable.

<sup>a</sup> Mean ratio (%) ± confidence interval at 95%, *n* = 4.

by increasing temperature (Lorentzen & Kingston, 1996). The result obtained from the leaching by 1:1:1 HNO<sub>3</sub>(4 M):HCl(4 M):H<sub>2</sub>O<sub>2</sub>(0.5 M) were shown in Table 4. The concentration of Cd and Cu in leachates increased when high operating temperature was applied but not sufficient for quantitative extraction.

For mussel samples, the amounts of Cu and Cd in leachates at high temperature were higher than ambient temperature. The analytical performances implied that operating temperature was an important parameter for Cu and Cd leaching. In case of Zn in fish and mussel samples, the leaching efficiency was high. Therefore, the leaching at high temperature was not necessary.

### 3.6. Effect of sonication time

The optimized conditions were applied to real samples without fortification. The obtained ratios (%) for Cd and Cu were slightly lower than 80%. To increase leaching efficiency for Cd from fish and Cu from mussel, the effect of sonication time was evaluated. The exposure time was increased from 15 to 30, and 60 min. In Table 5, the ratio (%) slightly increased for all cases. Cu was the only element that decreased its extraction efficiency when the sonication time was increased from 30 to 60 min. This effect was not found in the case of Cd. However, concentrations of Cd and Cu in samples were failed down slightly when sonication time was increased from 30 to 60 min. This phenomenon might be occurred by re-adsorption (Moreno & Yebra, 2002). Cd and Cu in samples might be adsorbed on sample surface. Thus 30 min was chosen as operating time for Cu and Cd acid leaching.

Table 5  
RSD (%) of Cd, Cu and Zn, (0.20 g of samples, 6 mL leaching solution)

Elements	Fish		Mussel	
	Concentration (mg kg <sup>-1</sup> )	RSD (%)	Concentration (mg kg <sup>-1</sup> )	RSD (%)
Cd <sup>a</sup>	0.06	12.73	0.55	1.10
Cu <sup>a</sup>	1.16	2.85	4.24	3.04
Zn <sup>b</sup>	15.45	6.63	52.30	2.77

<sup>a</sup> Measured by GFAAS, *n* = 6.

<sup>b</sup> Measured by FAAS, *n* = 10.

### 3.7. Precision

The RSD (%) was used to define the precision. Under the optimum parameters, Cd and Cu were leached with six independent batches and Zn was leached with 10 independent batches. The precision of each metal was shown in Table 5.

For environmental and food samples the precision is dependent on the samples matrix, the concentration of analytes, and on the analysis technique. It can vary between 2% and more than 20%. The observed RSD (%) for both samples were less than 15% and ranged in the acceptable precision value according to the AOAC standard (AOAC International, 2002).

### 3.8. Linearity

All working standard solutions were prepared from stock solutions 1000 mg L<sup>-1</sup>. The calibration curves were run (*n* = 5) under the used conditions of the instruments. The linearity of an analytical method is its ability to elicit test results that are directly, or by means of well-defined mathematical transformations, proportional to the concentration of analytes in samples within a given range. The concentration range (*R*<sup>2</sup>) were 2–10 (0.999), 10–50 (0.999), and 0.5–2.5 mg L<sup>-1</sup> (0.998) for Cd, Cu, and Zn, respectively.

### 3.9. Limit of detection (LOD) and method detection limit (MDL)

The LOD is defined as three times of the standard deviation of 10 measurements of a reagent blank. In this study, the reagent blank was prepared by dilution 6.00 mL of leaching solution (1:1:1 HNO<sub>3</sub>(4 M):HCl(4 M):H<sub>2</sub>O<sub>2</sub>(0.5 M)) to 10 mL with de-ionized water. The method detection limit is defined by Eq. (2)

$$MDL_{(mg/kg)} = \frac{LOD_{(mg/L)} \times V_{(L)}}{m_{(kg)}} \quad (2)$$

where *V* = volume of leachates (L), *m* = mass of samples (kg).

The limit of detection of Cd, Cu and Zn were 0.41, 2.56, and 0.01 μg L<sup>-1</sup>, and the method detection limits were 0.02,

Table 6

Analytical results obtained for the determination of Cd, Cu and Zn in a certified sample of dogfish muscle tissue, DORM-2 by AAS using acid leaching extraction method

Element	Certified values (mg kg <sup>-1</sup> )	Acid leaching (n = 12)			Microwave digestion (n = 8)	
		Found values (mg kg <sup>-1</sup> ) <sup>a</sup>	Recovery (%)	Relative error (%)	Found values (mg kg <sup>-1</sup> ) <sup>a</sup>	Relative error (%)
Cd <sup>b</sup>	0.043 ± 0.008	0.066 ± 0.010	153.5 ± 0.8	53	0.068 ± 0.020	58
Cu <sup>b</sup>	2.34 ± 0.16	2.04 ± 0.10	87.2 ± 0.6	13	2.06 ± 0.08	12
Zn <sup>c</sup>	25.6 ± 2.3	20.7 ± 0.6	80.9 ± 0.3	19	21.9 ± 2.2	14

<sup>a</sup> Mean ± confidence interval at 95%.

<sup>b</sup> Measured by GFAAS.

<sup>c</sup> Measured by FAAS.

0.13, and 0.63 mg kg<sup>-1</sup>, respectively. The calculated LOD and MDL of Cd, Cu and Zn were sufficiently low to allow the determination of these elements in real samples.

### 3.10. Accuracy

The accuracy was studied by using DORM-2 (dogfish muscle tissue, *Squalus acanthias*) certified reference material from National Council of Canada. DORM-2 was treated with the optimum conditions, which were 6 mL of 1:1:1 HNO<sub>3</sub>(4 M):HCl(4 M):H<sub>2</sub>O<sub>2</sub>(0.5 M), 30 min sonication time, 56 °C. The results were shown in Table 6.

Table 6 showed that, the recoveries (%) by leaching technique of DORM-2 for Cu and Zn ranging from 80 to 87%. The proposed procedure exhibited satisfactory results for analysis of DORM-2 for Cu and Zn with relative error less than 20%, whilst the result for Cd was found positive bias, which probably contaminated from the reagents and equipment used in the process. The blank analysis gave high background signal for Cd. In addition, the certified value of Cd was closed to the method detection limit value of the method that might cause quite an error. However, the recovery from acid leaching technique and the one from microwave digestion technique were not significantly different; the percent difference was only 3%.

## 4. Conclusion

An acid leaching procedure for the extraction of cadmium, copper and zinc provided a simple method for the simultaneous preparation of numerous samples. The elemental analysis in fish and mussel samples was carried out using flame atomic absorption spectrophotometer and graphite furnace atomic absorption spectrophotometer. Six milliliters of 1:1:1 HNO<sub>3</sub>(4 M):HCl(4 M):H<sub>2</sub>O<sub>2</sub>(0.5 M) was a suitable leaching solution. The metals could be quantitatively recovered from 0.20 g of samples. A 30-min sonication time could be used due to a maximum ratio (%) of metal amount obtained from leaching technique to amount determined by digestion technique.

The acid leaching extraction method had been demonstrated to be a rapid sample pre-treatment procedure. It could be carried out in one run in not over 50, 30 min for the ultrasonic process and 20 min for the centrifugation stage as compared with more than 1 h for the microwave

digestion method. Comparing with the procedure reported previously (Azouzi et al., 1998; Balarama Krishna & Arunachalam, 2004; Bermejo-Barrera, Muñiz-Naveiro et al., 2000; Bermejo-Barrera et al., 2001; Bermejo-Barrera, Muniz-Naveiro et al., 2001; Yebra-Biurrun et al., 2005), the present procedure lead to reduce the complexity of the leaching solution, avoid the use of concentrated reagents, reduce the possibility of sample contamination, and reduce the sonication time from 120 to 30 min. Another advantage of the proposed procedure is not to involve the use of high ultrasonic energy. The method had been validated by determining the metals in DORM-2. The recoveries (%) of copper and zinc were 80.9 ± 0.3% and 87.2 ± 0.6%. The proposed procedure can be applicable for the determination of trace metal ions in fish and mussel samples with low detection limit, high accuracy and high precision. However, the found value from leaching technique of cadmium were higher than the certified value but very near to the found value from microwave digestion technique. The proposed method should be re-validated for cadmium determination.

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## References

- Aleixo, P. C., Junior, D. S., Tomazelli, A. C., Rufini, I. A., Berndt, H., & Krung, F. J. (2004). Cadmium and lead determination in foods by beam injection flame furnace atomic absorption spectrometry after ultrasound-assisted sample preparation. *Analytica Chimica Acta*, 512, 329–337.
- Al-Merey, R., Al-Masri, M. S., & Bozou, R. (2002). Cold ultrasonic acid extraction of copper, lead and zinc from soil samples. *Analytica Chimica Acta*, 452, 143–148.
- AOAC International, (2002). *AOAC official method of analysis*. Arlington: AOAC International.
- Ashley, K. (1998). Ultrasonic extraction of heavy metals from environmental and industrial hygiene samples for their subsequent determination. *Trends in Analytical Chemistry*, 17, 366–372.
- Azouzi, H. E., Cervera, M. L., & Guardia, M. D. L. (1998). Multi-elemental analysis of mussel samples by atomic absorption spectrometry after room temperature sonication. *Journal of Analytical Atomic Spectrometry*, 13, 533–538.

- Balarama Krishna, M. V., & Arunachalam, J. (2004). Ultrasound-assisted extraction procedure for the fast estimation of major, minor and trace elements in lichen and mussel samples by ICP-MS and ICP-AES. *Analytica Chimica Acta*, 522(2), 179–187.
- Bendicho, C., & Lavilla, I. (2003). Ultrasound-assisted metal extractions. *Encyclopedia of Separation Science*, 3, 4421–4426.
- Bermejo-Barrera, P., Moreda-Pineriro, A., & Bermejo-Barrera, A. (2001). Sample pre-treatment methods for the trace elements determination in seafood products by atomic absorption spectrometry. *Talanta*, 57, 969–984.
- Bermejo-Barrera, P., Moreda-Pineiro, A., Muniz-Naveiro, O., Gomez-Fernandez, A. M. J., & Bermejo-Barrera, A. (2000). Optimization of a microwave-pseudo-digestion procedure by experimental designs for the determination of trace elements in seafood products by atomic absorption spectrometry. *Spectrochimica Acta Part B-Atomic Spectroscopy*, 55, 1351–1371.
- Bermejo-Barrera, P., Muñoz-Naveiro, O., Moreda-Piñeiro, A., & Bermejo-Barrera, A. (2000). Experimental designs in the optimisation of ultrasonic bath – acid-leaching procedures for the determination of trace elements in human hair samples by atomic absorption spectrometry. *Forensic Science International*, 107(1–3), 105–120.
- Bermejo-Barrera, P., Muniz-Naveiro, O., Moreda-Pineriro, A., & Bermejo-Barrera, A. (2001). The multivariate optimisation of ultrasonic bath-induced acid leaching for the determination of trace elements in seafood products by atomic absorption spectrometry. *Analytica Chimica Acta*, 439, 211–227.
- Buldini, P. L., Ricci, L., & Sharma, J. L. (2002). Review, recent applications of sample preparation techniques in food analysis. *Journal of Chromatography A*, 975, 47–70.
- Capelo, J. L., Maduro, C., & Vilhena, C. (2003). Discussion of parameters associated with the ultrasonic solid–liquid extraction for elemental analysis (total content) by electrothermal atomic absorption spectrometry. An overview. *Ultrasonics Sonochemistry*, 10, 3–8.
- Codex Alimentarius, (2005). Available from <http://www.codexalimentarius.net>.
- Domínguez-González, R., Moreda-Piñeiro, A., Bermejo-Barrera, A., & Bermejo-Barrera, P. (2005). Application of ultrasound-assisted acid leaching procedures for major and trace elements determination in edible seaweed by inductively coupled plasma-optical emission spectrometry. *Talanta*, Available online 22 January 2005, in press.
- Filgueiras, A. V., Capelo, J. L., Lavilla, I., & Bendicho, C. (2000). Comparison of ultrasound-assisted extraction and microwave-assisted digestion for determination of magnesium, manganese and zinc in plant samples by flame atomic absorption spectrometry. *Talanta*, 53, 433–441.
- Goodman, B. A., Green, H. L., & McPhail, D. B. (1984). An electron paramagnetic (EPR) study of the adsorption of copper complexes on montmorillonite and imogolite. *Geochimica et Cosmochimica Acta*, 48, 2143–2150.
- Link, D. D., Walter, P. J., & Kingston, H. M. (1998). Development and validation of the new EPA microwave-assisted leach method 3051A. *Environmental Science and Technology*, 32, 3628–3632.
- Lorentzen, E. M. L., & Kingston, H. M. (1996). Comparison of microwave-assisted and conventional leaching using EPA method 3050B. *Analytical Chemistry*, 68, 4316–4320.
- Marin, A., Lopez-Gonzalez, A., & Barbas, C. (2001). Development and validation of extraction methods for determination of zinc and arsenic speciation in soils using focused ultrasound. Application to heavy metal study in mud and soils. *Analytica Chimica Acta*, 442, 305–318.
- Mendez, H., Alava, F., Lavilla, I., & Bendicho, C. (2002). Ultrasonic extraction combined with fast furnace analysis as an improved methodology for total selenium determination in seafood by electrothermal-atomic absorption spectrometry. *Analytica Chimica Acta*, 452, 217–222.
- Miller, J. C., & Miller, J. N. (1993). *Statistics for analytical chemistry* (third ed.). West Sussex: Ellis Horwood Limited.
- Moreno, A., & Yebra, M. C. (2002). Flow injection determination of copper in mussels by flame atomic absorption spectrometry after on-line continuous ultrasound-assisted extraction. *Spectrochimica Acta Part B-Atomic Spectroscopy*, 57, 967–974.
- Nascentes, C. C., Korn, M., & Arruda, M. A. C. (2001). A fast ultrasound-assisted extraction of Ca, Mg, Mn and Zn from vegetables. *Microchemical Journal*, 69, 37–43.
- Priego-Capote, F., & Luque de Castro, M. D. (2004). Analytical uses of ultrasound I. Sample preparation. *Trends in Analytical Chemistry*, 23(9), 644–653.
- Rio-Segade, S., & Bendicho, C. (1999). Ultrasound-assisted extraction for mercury speciation by the flow-injection-cold vapor technique. *Journal of Analytical Atomic Spectrometry*, 14, 263–268.
- Ruiz-Jimenez, J., Luque-Garcia, J. L., & Luque de Castro, M. D. (2003). Dynamic ultrasound-assisted extraction of cadmium and lead from plants prior to electrothermal atomic absorption spectrometry. *Analytica Chimica Acta*, 480, 231–237.
- Sastre, J., Sahuquillo, A., Vidal, M., & Rauret, G. (2002). Determination of Cd, Cu, Pb and Zn in environmental samples: microwave-assisted total digestion versus aqua regia and nitric acid extraction. *Analytica Chimica Acta*, 462, 59–72.
- Seferinoglu, M., Paul, M., Sandström, A., Köker, A., Toprak, S., & Paul, J. (2003). Acid leaching of coal and coal-ashes. *Fuel*, 82(14), 1721–1734.
- Sun, D., Waters, J. K., & Mawhinney, T. P. (1997). Microwave digestion with HNO<sub>3</sub>–H<sub>2</sub>O<sub>2</sub>–HF for the determination of total aluminum in seafood and meat by inductively coupled plasma atomic emission spectrometry. *Journal of Agricultural and Food Chemistry*, 45, 2115–2119.
- US FDA (1993). *Guidance documents for trace elements in seafood*. Washington DC: US Food and Drug Administration.
- Watson, C. (1984). Sample preparation for the analysis of heavy metals in foods. *Trends in Analytical Chemistry*, 3, 25–28.
- Yebra-Biurrun, M.C., Cancela-Pérez, S., Moreno-Cid-Barinaga, A. (2005). Coupling continuous ultrasound-assisted extraction, preconcentration and flame atomic absorption spectrometric detection for the determination of cadmium and lead in mussel samples. *Analytica Chimica Acta*, Available online 29 December 2004, in press.
- Yebra-Biurrun, M. C., & Garcia-Garrido, A. (2001). Continuous flow systems for the determination of trace elements and metals in seafood. *Food Chemistry*, 72, 279–287.