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A CLEAN UP STEP OF FAT CONTENT PREVIOUS TO TRACE METAL CHARACTERIZATION IN MUSSEL TISSUES BY INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY.

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Abstract

Inductively coupled plasma mass spectrometry analysis of trace and major elements of mussel tissues can be quickly and accurately analyzed after cleaning up the interfering fat content before the sample is digested in a microwave oven. Making use of experimental designs, the clean up procedure was achieved by the extraction of 1 g of freeze-dried tissue sample stirred with 5 ml of dichloromethane during five minutes. The microwave assisted digestion of the fat free samples was carried out with 0.2 g of tissue sample, 15 ml of 7.0% nitric acid with a power of 980 W during 18 min. The analytical method efficiency (accuracy and precision) was evaluated with a CRM: (NIST 2977, mussel tissue) and real mussel samples analyzed previously. The results confirmed the accuracy of the analysis by agreement with the previous results but the precision was significantly improved. The developed method allows operating routinely permitting to large numbers of samples to be quickly screened for trace metals.

Keywords: Trace metals, mussel tissue, clean up, microwave-assisted digestion, inductively coupled plasma mass spectrometry.

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1. Introduction

Bivalve molluscs, mainly mussels (*Mytilus galloprovincialis*), result very suitable for biomonitoring the aquatic environment due to their outstanding characteristics as their abundance, their suitable size, sedentary state, ease of collection, transplantation, maintenance in the laboratory, their wide distribution and their filter feeding activity that favours the bioaccumulation of contaminants [1]. For that reason, they have been widely used as sentinel organisms for the evaluation of coastal contamination [2 - 4].

As a consequence of the increasing environmental and toxicological requirements, several trace metal profiling studies in mussel tissues have been exhaustively performed [5 -7] by using inductively coupled plasma mass spectrometry (ICP-MS) as sample analysis method. This instrumental technique has several advantages, such as the ability to determine several trace metal ions simultaneously, very low detection limits and high repeatability [8]. However, ICP-MS technique is liable to show spectral interferences, e.g. isobaric, doubly charged, and polyatomic interferences in addition to chemical interferences from the matrix [9 – 11]. For that reason, the presence of interfering compounds as fat content and its organical residues should be minimized.

The two key steps on this ICP-MS analysis approach are the clean up and the digestion. The requirements of a suitable clean up method are the quantitative elimination of the interfering fats without significant losses in the content of the analytes. In the case of the digestion, the requirements usually involve total recoverable processes. A third way could be the elimination of the interferents during the digestion process owing to a hard oxidative process. In this sense, microwave-assisted extractions (MAE) systems are widely used as digestion systems for several environmental matrixes including mussel tissues [12 - 14]. However, before the routine use of this acid digestion procedure, the optimisation of the chemical and instrumental variables must be undertaken preferably by

experimental design [15] in order to assure the fitness of the digestion procedure. When ICP-MS is used, we would rather avoid hydrochloric acid due to the interference of the classic argide $^{40}Ar^{35}Cl$ with the monoisotopic ^{75}As [16]. In this sense, chemical (HNO_3 concentration) and instrumental variables (radio frequency power and digestion time) were finally optimised and the goodness of the analytical method was checked using different certified reference materials However, at high nitric acid concentration, high pressure and high temperature (such as microwave systems), it was observed an increase in the oxidant power of the reaction mixtures, which oxidizes a greater amount of compounds, generating a wider variety of organical residues [17]. Different literature works have reported the reaction products of protein and fat contents after microwave acid digestion like benzoic, oxalic, picric, terephthalic, and m- and p- nitrobenzoic acids [17, 18]. These products are sources of carbon which may give space charge effects or salt build up on the orifice of the interface sampler cone. Another isobaric interference comes from the formation of $^{40}Ar^{12}C$ which makes the analysis of ^{52}Cr difficult [16]. Finally, since the trace metal standards are usually made in 1.0% HNO_3, either the use of a higher concentration of HNO_3 or the presence of a random amount of organic residuals can introduce a bias in the metal analysis. In fact, unexpected higher relative standard deviations were pointed out critically in a previous work [19], and consequently we consider that a previous clean up step should be designed and implemented to get rid of the fats and to improve the quality of the analytical results.

The optimisation of the solvent extraction of the mussel tissues in a rotary shaker was the chosen procedure to eliminate the fats. In this case, we initially studied the suitability of different solvents to extract the fats making use of Fourier Transform infrared spectroscopy (FT-IR) and Energy Disperse X-ray spectroscopy (ED-XRF) to characterise the extracted fraction. Later on, based on the best solvents we studied the extraction of the

fat content in the rotary shaker, and the recovery of trace metals in the MAE by means of full factorial and central composite designs [20].

Finally, the effectiveness of the analytical method was proven in order to test the accuracy and precision by re-analysing exactly the same real individuals collected at 10 locations of Bay of Biscay, northern Spain [19]. The mean concentration and standard deviation were compared for both without and with clean up step developed in here.

2. Materials and methods

Real samples pre-treated (freeze-dried mussel tissues) and collected by Bartolome in 2009 [19] were used in the analytical method development and optimisation defined in here. The stabilisation of these real samples is an important step to preserve material characteristics, thus avoiding any physico-chemical degradation. Proper storage conditions are essential to guarantee that the material remains unaltered for a long time. As soon as the previous monitoring study was finished [19], the samples were stored in containers at -42ºC [21].

2.1. Reagents

All reagents were of analytical-reagent grade and ultrapure water (18.2 MΩ cm^{-1} at 25 ºC) obtained from a Milli-Q® Element A10 system (Millipore™, Bedford, USA) was employed. The volumetric glassware was grade A and was calibrated at laboratory temperature.

1000 mg L^{-1} Alfa Aesar (Karlsruhe, Germany) Specpure Plasma Standard stock solutions of As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Sb, Se, Sn, V and Zn were used. Internal standard solutions (Be, Sc, In and Bi) were purchased from Alfa Aesar (Specpure, Karlsruhe, German). Nitric acid (69%, Tracepur) was provided by Merck (Darmstadt, German). Dichloromethane was used as extracting agent and provided by Labscan (HPLC grade, Barcelona, Spain).

For the evaluation of the efficiency of the analytical method, the SRMs 2977 (mussel tissue) were purchased from NIST (Gaithersburg, USA).

2.2. Instrumentation

A Planetary Mono Mill Pulverisette 6 (Fritsch GmbH, Oberstein, Germany) agate ball mill and a Laborette 27 rotary cone sample divider (Fritsch GmbH, Oberstein, Germany) were used for the preparation of the mussel samples after stabilizations at room temperature.

The qualitative analyses were performed using spectroscopic techniques. According to infrared analysis, all FTIR spectra were collected in the middle infrared region (400 - 4000 cm^{-1}) recording 40 scans per spectrum at a spectral resolution of 4 cm^{-1}. A Jasco 6300 FTIR spectrometer was used for this purpose.

The elemental measurements were carried out using a portable Röntec (at the moment, Bruker AG) ArTAX μ-EDXRF with an X-ray tube with molybdenum anode working at a maximum voltage of 50 KV and a maximum current of 0.6 mA. The X-ray fluorescence is detected by means of a thermoelectrically cooled Si-drift (XFlash) detector which has an active area of 5 mm^2 and 8 μm beryllium window. The operating conditions during the μ-EDXRF measurements were fixed at 1800 s, at a voltage of 50 KV and a current of 0.6 mA.

In order to perform the quantitative analysis, microwave assisted digestion was carried out in a close microwave device Multiwave 3000 (Anton Paar, Graz, Austria) equipped with 8 Teflon vessels and temperature controllers.

Inductively coupled plasma with mass detector (7700x, Agilent Technologies, Palo Alto, USA) was used for trace metal determinations using a MicroMist micro-uptake glass concentric nebulizer (Glass Expansion, West Melbourne, Victoria, Australia) in a class 100 clean room. In order to reduce MO^+ formation in the plasma, the spray chamber was Peltier cooled at 2ºC. Finally, standard nickel cones (sample and skimmer) were generally

used. Operating conditions are shown in Table 1. The acquisition masses and integration times (Table 1) provided more than sufficient sensitivity to meet all certified values. The optimization of the ICP-MS conditions was achieved by adjusting the torch position and tuning for reduced oxide and doubly charged ion formation with a standard tuning solution containing 1.0 $\mu g l^{-1}$ of ^{7}Li, ^{24}Mg, ^{59}Co, ^{89}Y, ^{140}Ce and ^{205}Tl in 1.0% HNO_3. This equipment includes a collision cell (He gas, ORS^3 system, Agilent Technologies ©) for discriminate spectral interferences with high performance for all the trace metals considered in here. In addition, EPA 6020 [22] recommendations were followed for interference overcoming such as correction equations for arsenic, lead or cadmium.

2.3. General analytical procedures

Rigorous cleaning procedures of all the laboratory ware and other equipment that comes into contact with samples must be employed in order to avoid contamination of samples. All glassware and plastic ware were washed with a common detergent and thoroughly rinsed with Elix quality water (Millipore, Bedford, MA, USA). After that, all the laboratory ware was soaked into a clean dilute HNO_3 (10%) bath for 24 h. Afterwards, the material was rinsed with Milli-Q quality water (Milli-Q Element A10, Millipore, Bedford, MA, USA).

For the clean up, 1.0 g of each sample was weighed (after determination of percent moisture) in the extraction tube and 5 ml of dichloromethane (100%) was added. After that, the extraction was carried out at 1.0 rpm for 5.0 min in a rotary shaker. This methodology is operationally based on the procedure defined by the NOAA [23]. Once the agitation period was over, the mixture was centrifuged and the supernatant was weighed after evaporation at 65ºC (% fat content).

On the other hand, the solid residue (mussel tissue) was dried at 65ºC until constant weight and stored at room temperature in a desiccator until acid treatment. Then, 0.2 g of this solid residue was exactly weighed and digested using 15 ml of HNO_3 (7.0%) +

microwave assisted digestion. The extraction vessel was placed in the microwave oven and the digestion program was run according to the experimental designs. All samples were brought to final volume of 100 ml using Milli-Q and analyzed by ICP-MS with a final HNO_3 percentage of 1.0%.

3. Results and Discussion

3.1. Qualitative spectroscopic analysis

FT-IR or μ-EDXRF methods are well suited for elemental and molecular analysis studies on biological systems. The possible aims of analysis include structural proteins, lipids, enzymes, domains thereof or nucleic acid derivatives [24]. For the analyst who has a laboratory-scale or even a portable equipment or possibility to use synchrotron facilities, the key task is the formation of thin layers from the liquid or solid biological samples.

Different extracting agents were tested in this work such as dichloromethane, hexane and different alcohols. A standard extraction procedure defined by NOAA [23] was followed. ≈1.0 g of mussel tissue was extracted using 15 ml of each extracting agent for 1 hour to guarantee complete extraction. Then, the solution is poured drop by drop into a pressed KBr powder pellet (a total pressure of 10 tons was applied and the resulting pellets had a diameter of 10 mm and a width of 1 mm) and stand for the evaporation of solvent in a flux cabin. After that, XRF was performed to qualitatively elucidate the presence of trace metal in the final extracts. In addition, FT-IR was used as qualitative analysis for final extracts characterization.

Figure 1 shows examples of the FT-IR spectra obtained for the final extracts: (a) in dichloromethane and (b) in methanol. Figure 1a shows the comparison between the sample (A) and a standard of triglyceride (B) extracted in dichloromethane. The spectrum of triglyceride is characterized by a C=O band in 1740 – 1750 cm^{-1}, the stretching of the methylene, occurred at 2850 – 2960 cm^{-1} with a C=C-H band at 3020 cm^{-1}, and other bands such as C-H at 1464, 1379 and 725 cm^{-1} and C-O at 1240, 1165 and 1103 cm^{-1} also

appeared; this fact produced a specific profile being the intermediate C=O band the most intense one. On the other hand, proteins (Figure 1b) were characterized by the amide I and II bands at 1650 and 1550 cm^{-1}, sometimes, the amide III band at 1450 cm^{-1}, and finally, the stretching N-H band at 3350 cm^{-1}.

Finally, the XRF spectra obtained in each case are shown in Figure 2 (a) and (b) considering the raw signal. With the purpose of excluding the noise, the final spectral range (830-1024 channels, 40.28 – 50 KeV) was not included. As can be seen, the final alcoholic extracts showed qualitatively the presence of trace metals like As, Fe or Zn. This fact did not happen in the final extracts using dichloromethane or hexane. For that reason, alcohols tested were not considered as extracting agents in the optimisation procedures.

3.2. Optimisation of the mussel tissue extraction and microwave-assisted digestion

According to the conclusion obtained from XRF and IR analysis, it can be assumed that dichloromethane or hexane could be promising alternatives as the extractant agents as they could allow the quantitative extraction of lipid compounds without extracting trace metals. In order to optimize the extraction procedure, several variables were established such as: % hexane : dichloromethane (100:0 – 0:100%), B: volume (5 – 20 mL), C: agitation (1 – 9 rpm) and D: time (5 – 120 min) were studied in the extraction experiments. The efficiency of the extraction step was defined as the maximum recovery of the lipid compounds in the extraction procedure, i.e., the maximum residue weight obtained from the evaporated extract at 65ºC. This procedure was based on those proposed by NOAA [23] to treat approximately 1.0 g of mussel tissue sample. After that clean up, the dried solid residue obtained (0.2 g) was acid digested by a microwave assisted system. Nitric acid concentration (1 – 10% v/v), microwave power (280 – 1120 w) and digestion time (5 – 20 min) were the variables and the range values considered in the optimization of the microwave-assisted digestion. In this case, the analytical response to take into account was the maximum concentration of each trace metal.

First and to find the main factors affecting the extraction step and the microwave-assisted digestion, a two-level full factorial designs (2^n + 3 replicates of central point) were carried out for the extraction and digestion, respectively. The analysis of the responses was carried out with The Unscrambler® program and it concluded that the variable time (min) was not significant (p level > 0.05) and the minimum value was selected (t = 5 min). On the other hand, all the digestion variables considered (Nitric concentration, power supply and digestion time) showed a significant effect on the maximum response (p level < 0.05) [25].

The previous designs were extended to a central composite design in order to build up the response surfaces and to obtain the instrumental conditions that define the maximum response for both extraction and digestion procedures. Table 2 shows the extension to central composite design defined by The Unscrambler® program. The response surfaces were also calculated with The Unscrambler® for all the responses and the significant effects of variables were also reassured ($p<0.05$).

From the results obtained, it was possible to plot the response surfaces as a function of the most significant variables for maximum solid extract and each trace metal considered, respectively (Figures 3 and 4). From those plots, we could simultaneously deduce the optimum conditions within the factor space, i.e. without any extrapolation. In this sense, a % hexane : dichloromethane (0% : 100%), t (5.0 min), agitation (1.0 rpm) and volume (5.0 ml) were defined from the surface responses obtained in Figure 3. On the other hand, a microwave power of (P) 980 w, digestion time of (t) 18 min and a nitric acid concentration of (C) 7.0% v/v were obtained for the microwave assisted digestion. As an example, Figure 4 shows the response surfaces for As, Cr and Zn when the P variable is fixed at the optimum level of the CCD (980 w). Similar strategy and plots were obtained for the rest of the trace metals.

3.3. Analytical performance

Using the optimized conditions, analytical figures of merit including detection limit and precision of replicate measurements, were determined. The limit of detection (LOD) was calculated following the IUPAC rules, defined as blank signal + 3 S.D., where S.D. is the standard deviation of 5 measurements of a blank. The LODs estimated for the mussel samples were Cd, Co, Cr, Cu, Ni and V (<10 ng g^{-1}), As, Sb, Se and Sn (<20 ng g^{-1}) Hg (<25 ng g^{-1}), Mn (<0.5 μg g^{-1}), Pb (<0.1 μg g^{-1}) and Zn (<2.0 μg g^{-1}).

Precision of the method (%RSD) within-a-day (n=6) and among days (n=6) was lower than 5.0% for all trace metals and concentrations tried.

The accuracy of the analytical procedure was verified analysing the certified material NIST SRM 2977 (mussel tissue). These experimental data were compared to other data collected from those measured by Bartolome with the same SRM 2977 [26]. This analytical method defined by Bartolome included microwave acid digestion but not any clean up step. The results obtained are shown in Table 3 for each trace metal and a comparison with the corresponding certified values was performed by using the Z function defined as:

$$Z = \frac{(\text{Certified value} - \text{Experimental value})}{\sigma} \quad (1)$$

where σ was defined as: $\sigma = \sqrt{\frac{S_{\exp}^2}{n} + S_{cert}^2 + S_{longterm}^2}$ [27], being S_{exp} the standard deviation of the analysis and S_{cert} the standard deviation of the certified value. $S_{longterm}$ is the standard deviation obtained in different days, and since that value is not available $2S_{exp}$ was used [27]. As it is known, the lack of trueness is considered if the Z values are out of $-2 < Z < 2$.

Experimental data are in good agreement with the certified values as it can be concluded from the Z value obtained because they match the trueness criteria. However, it could be critically concluded that the concentrations obtained with a clean up step (2977B) showed a higher trueness degree than the previous experiments defined by Bartolome for

all the trace metals (2977A). This fact was concluded since the Z values were closer to 0 values (Table 3). Moreover, statistical tests were performed to investigate whether the differences obtained between the experimental and the certified values were significant from the raw experimental results (X_{mean}, S_{exp} and μ_{CRM}, σ_{CRM}). The t-test indicated that both results were similar ($p< 0.05$) and, therefore, the results of trace metals could be assumed to be accurate. This fact assessed a recovery closer to 100% for all the trace metals considered and confirmed the initial qualitative spectroscopic analysis where no trace metal losses were detected in the clean up step. Finally, one additional improvement was obtained in the uncertainty term in the instrumental analysis by using a clean up step because a better standard deviation of instrumental replicates was obtained in all cases (Table 3).

3.5. Application to real samples

Samples collected in the sampling sites defined in a previous study [19] were re-analyzed again with this new analytical method. The same pre-treatment strategy was followed that in that study. Metal body concentrations were calculated and expressed on a dry tissue weight basis (i.e. mg Kg^{-1} dry weight) with correction of the extract content determined in each sample for adequate comparison with other experimental results.

In this work, similar tendencies were obtained in the monitoring profiles that those obtained previously. Therefore, the maximum values in each sampling point were enhanced during spring summer levels (April – July) for all trace metals except for As and Hg (February 2004). Additionally, the mean concentrations obtained for the trace metals were similar in both studied (Figure 5). Again, an important improvement was obtained in the uncertainty of the instrumental measurements because the standard deviations of the replicates for each sample were around 10% lower than those obtained by using the analytical method without clean up [19]. This fact could be explained by reducing the

inaccuracies provided from the fat compounds because the extractable fat content determined was ranged from 10 to 25% in the samples producing an important background effect for ICP-MS determinations.

4. Conclusions

A new friendly and low time consuming methodology for trace metal determination in mussel tissues was established in this work by submitting the freeze-dried, grounded and homogenised samples to an extraction step (clean up) previous to microwave assisted digestion of the solid residue obtained and ICP-MS quantification. The use of extraction step was proven to be a feasible and recommendable alternative, reducing the volume of the reagents and the amount and variety of digestion residues as inaccuracy sources in the ICP-MS analysis. In addition, it was provided a simple method for the simultaneous preparation of numerous samples.

Comparing with the procedure reported previously [19] the present analytical procedure lead to reduce the complexity of the extracting solution, avoid the use of concentrated reagents, reduce the possibility of sample contamination, and reduce the errors associated to the dilution steps. Another advantage of the proposed procedure is not to involve the use of high acid nitric contents. The use of diluted acids was proven to be a feasible and recommendable alternative, reducing the volume of the reagents and the amount and variety of digestion residues. The method had been validated by determining the trace metals in CRM 2977. The proposed procedure can be applicable for the determination of trace metal ions mussel samples with similar detection limits and accuracy but higher precision.

Last but not least, the clean up optimized allows reducing costs and time consuming in maintenance operations (cleaning of cones, lenses, etc) by significantly increasing the matrix tolerance for the direct analysis of the acid extracts. The combination of a friendly clean up step and acid digestion optimized conditions simplifies matrix effect

on the ICP-MS analysis while delivering consistently accurate results and superior performance in routine applications such as mussel samples monitoring for trace metals.

5. Acknowledgements

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Table 1. ICP-MS operating and acquisition parameters.

RF power (W)	1550
Plasma gas flow (l min^{-1})	15
Carrier gas flow (l min^{-1})	0.85-0.90
Sample flow rate (ml min^{-1})	0.1
He flow rate (ml min^{-1})	4.3
Extraction lens 1 (V)	2
Extraction lens 2 (V)	-140
Omega bias (V)	-30
Omega lens (V)	1
Cell input (V)	-34
QP focus	2
Cell output (V)	-30
Octopole RF (V)	150
Octopole bias	-6
QP bias	-3
Data acquisition	(Dwell time, 300 ms)
Sweeps per replicate	8
Replicates	3
Detection mode	Peak hopping
Isotopes	^{75}As, ^{114}Cd, ^{59}Co, ^{52}Cr, ^{63}Cu, ^{202}Hg, ^{55}Mn, ^{56}Ni $^{208, 207, 206}$Pb, $^{78, 82}$Se, ^{118}Sn, ^{123}Sb, ^{51}V and ^{66}Zn

Table 2. Experimentation proposed by central composite design.

#	Levels				
	-α	-1	0	1	+α
	Microwave assisted digestion				
P (w)	280	448	700	952	1120
t (min)	5	8	12.5	17	20
C (% v/v)	1.0	2.8	5.5	8.2	10
	Extraction step				
% Hexane	0	25	50	75	100
rpm	1	3	5	7	9
t (min)	5	33.7	62.5	91.2	120
V (ml)	5	8.7	12.5	16.2	20

Table 3. Results obtained (mg Kg^{-1}) in the trace metal analysis of NIST 2977 A: collected in [26], B: in here.

	Cert.	2977 A		2977 B	
		Exp.	Z	Exp.	Z
As	8.8 ± 0.9	14 ± 2	-1.2	9.0 ± 0.1	-0.2
Cd	0.18 ± 0.01	0.32 ± 0.01	-6.1	0.17 ± 0.01	0.4
Co	0.5 ± 0.1	0.44 ± 0.01	0.6	0.49 ± 0.01	0.1
Cr	3.9 ± 0.5	4.6 ± 0.5	-0.6	4.00 ± 0.02	-0.2
Cu	9.4 ± 0.5	13 ± 3	-0.6	9.5 ± 0.1	-0.2
Hg	0.10 ± 0.01	0.12 ± 0.09	-0.1	0.11 ± 0.01	-0.4
Mn	23.9 ± 0.3	23 ± 2	0.2	23.86 ± 0.02	0.1
Ni	6.1 ± 0.2	6.2 ± 0.3	-0.2	6.07 ± 0.02	0.1
Pb	2.3 ± 0.1	2.0 ± 0.3	0.5	2.22 ± 0.04	0.6
Sb	0.048			0.07 ± 0.02	-0.5
Se	1.8± 0.2			1.6 ± 0.2	0.4
Sn	1.5 ± 0.3			1.2 ± 0.3	0.4
V	1.1	1.1 ± 0.1		1.09 ± 0.03	0.2
Zn	135 ± 5	163 ± 21	-0.6	132 ± 1	0.5

Figure Captions

Figure 1. FTIR spectra obtained for in dichloromethane (a): A - sample and B - standard oil (triglyceride) and in methanol (b): A – sample and B, C - two kinds of fish glue (collagen).

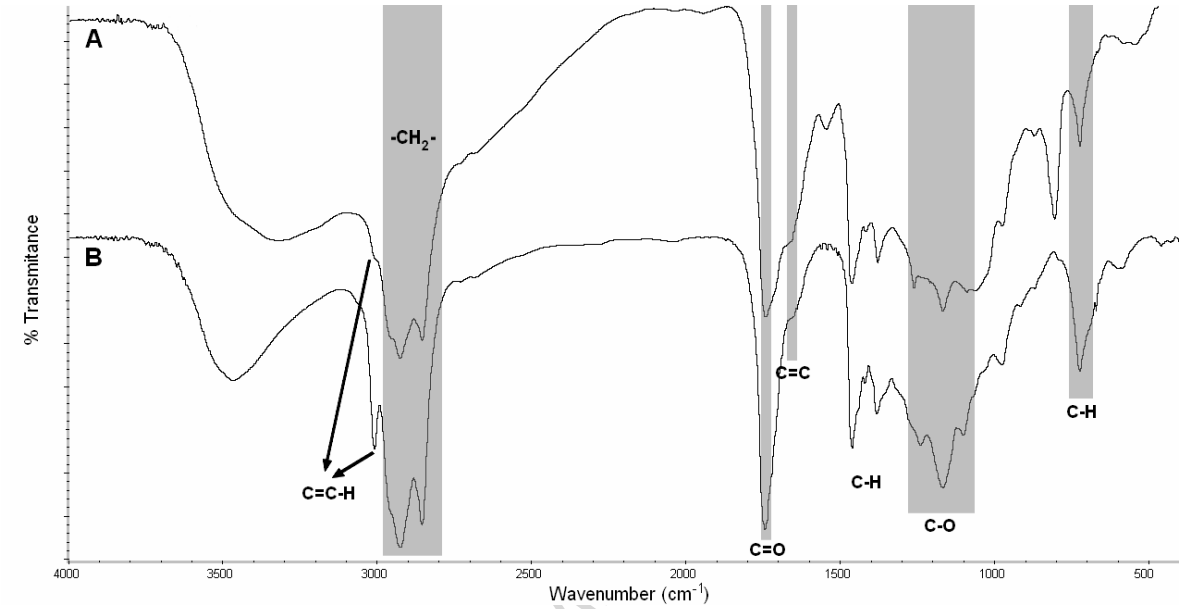
Figure 2. XRF spectra obtained for different extracting agents.

Figure 3. Response surfaces defined by %hexane vs. rpm (a) and volume vs. time (b) for solid residue obtained in the extraction step.

Figure 4. Response surfaces defined by nitric acid concentration and digestion time for the As (a), Cr (b) and Zn (c) responses followed.

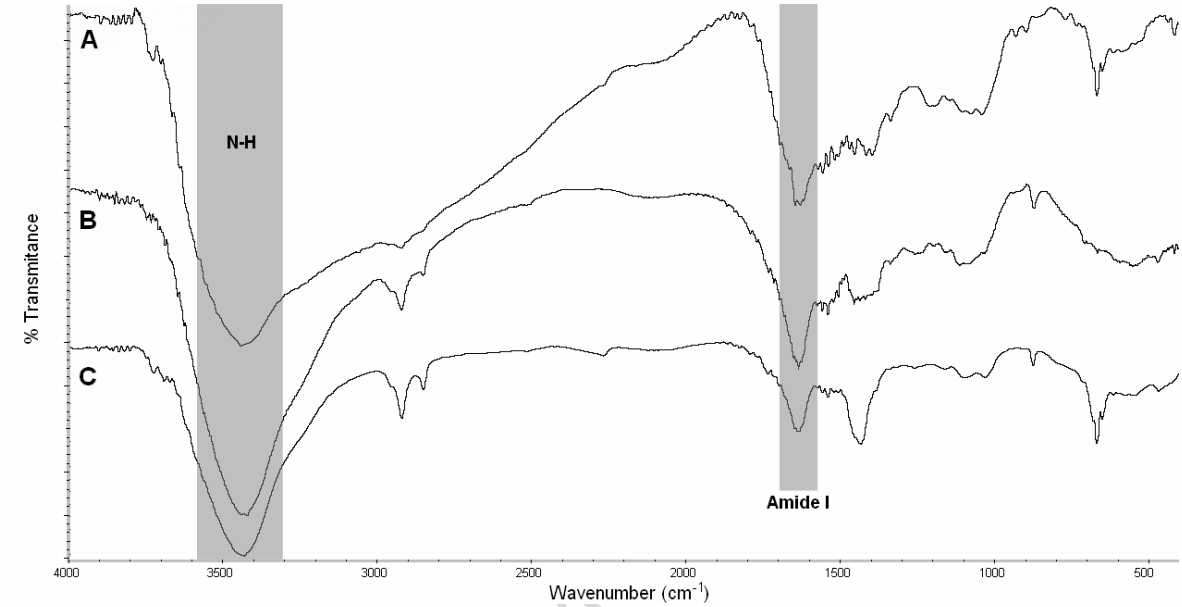
Figure 5. Mean trace metal concentration (log C) obtained with clean up and no clean up analytical methods (N = 10 from the same stations defined by Bartolome [19]).

Figure 1



(a)

Figure 1



(b)

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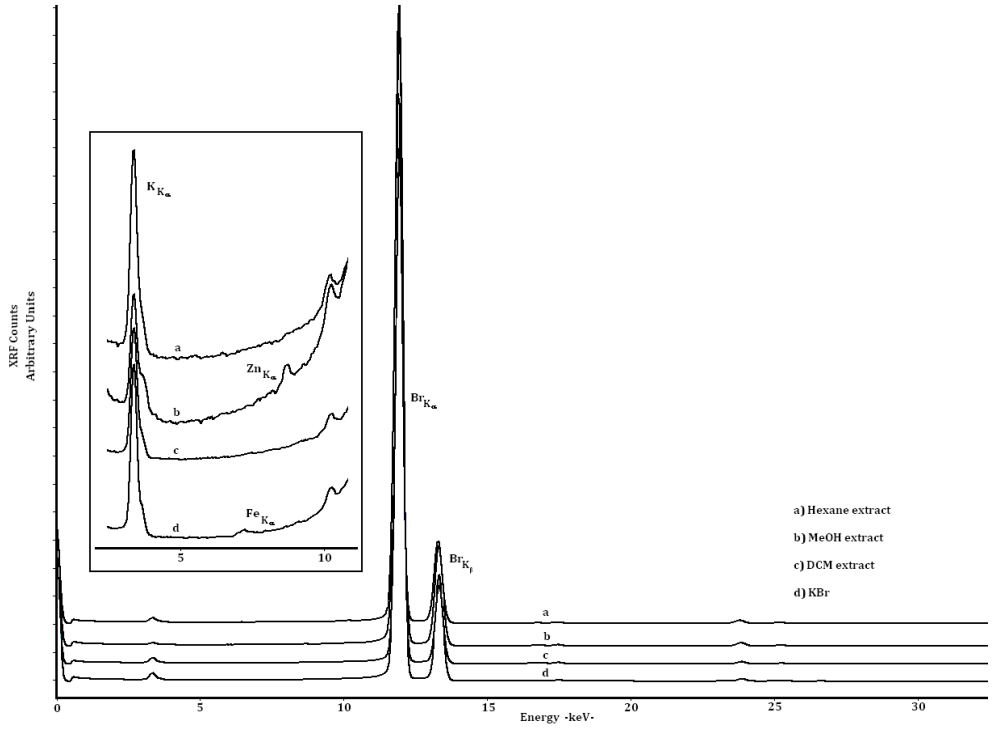
a.

Figure 2



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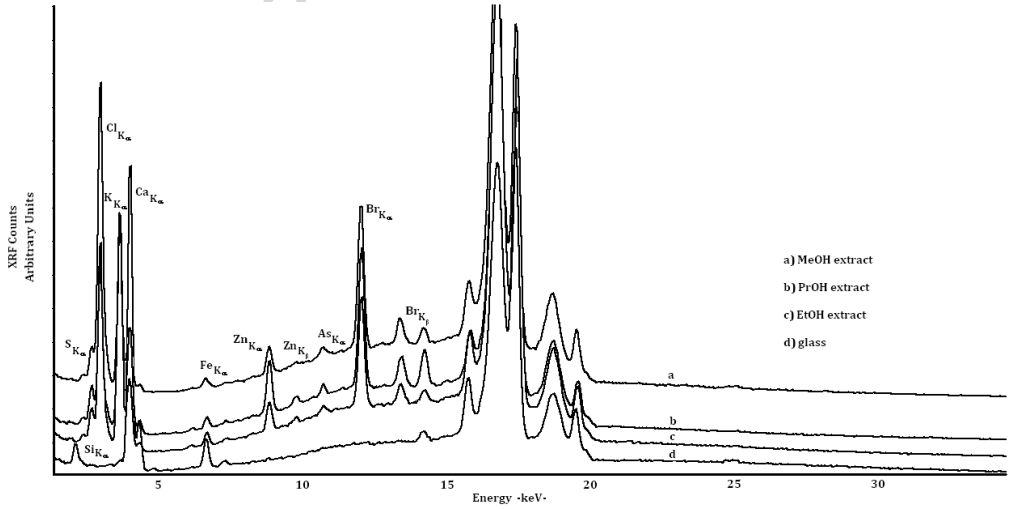

(b)

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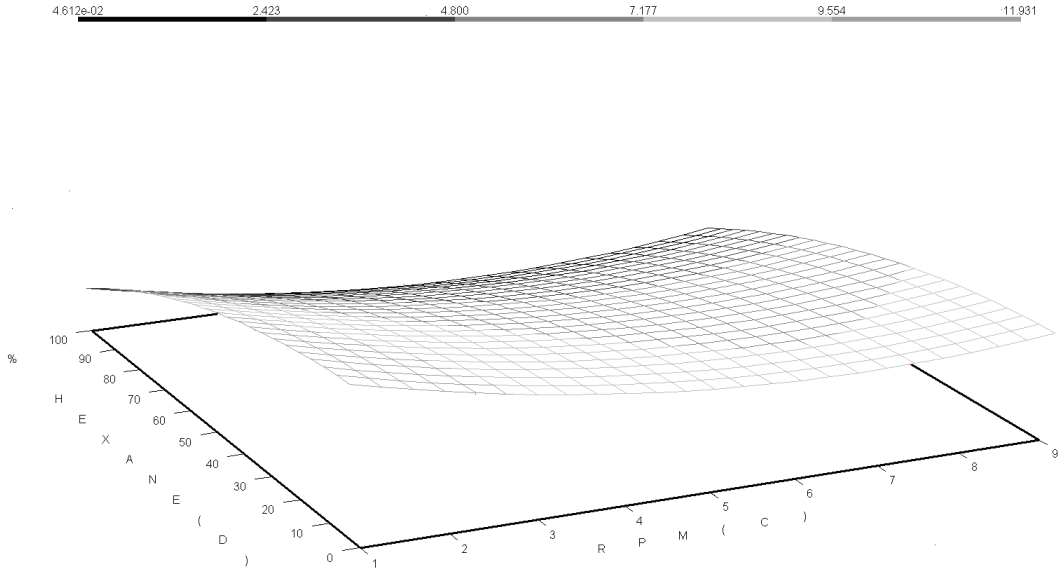
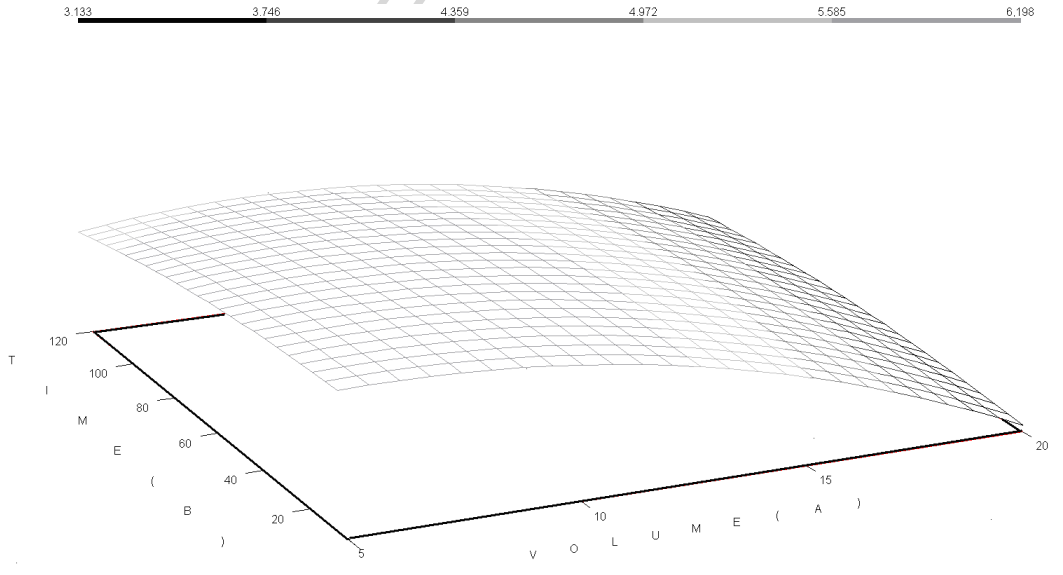
Figure 3



(a)



(b)

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Figure 4

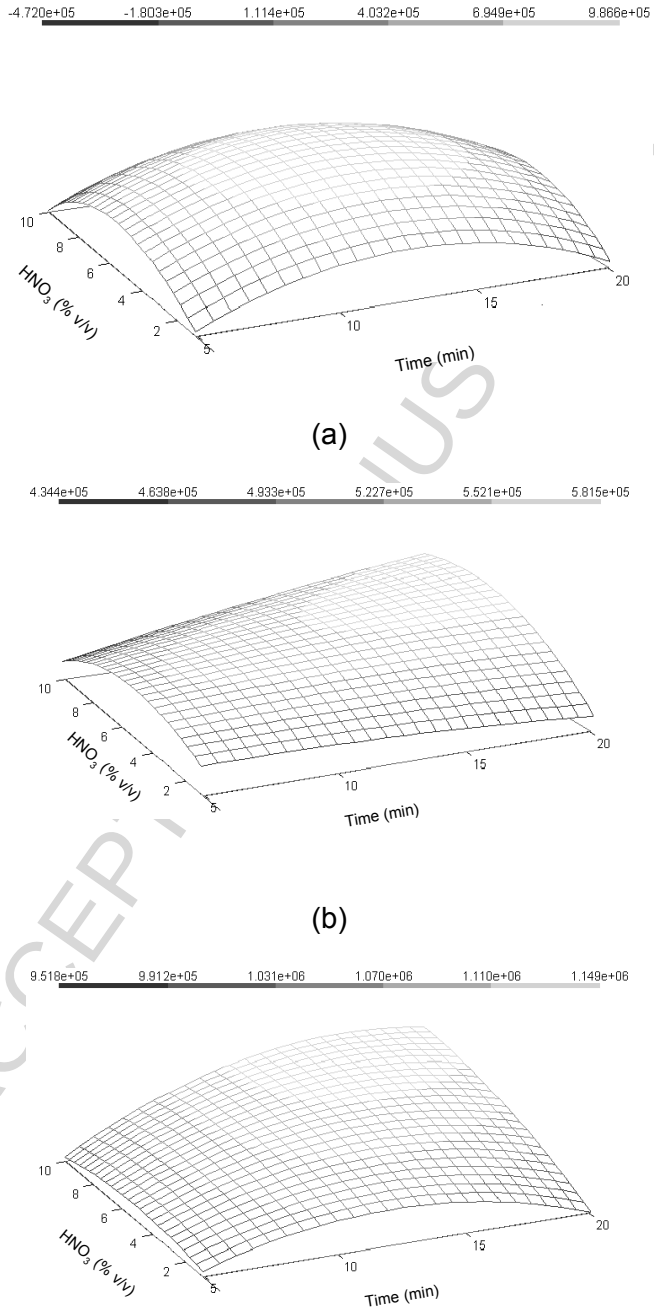

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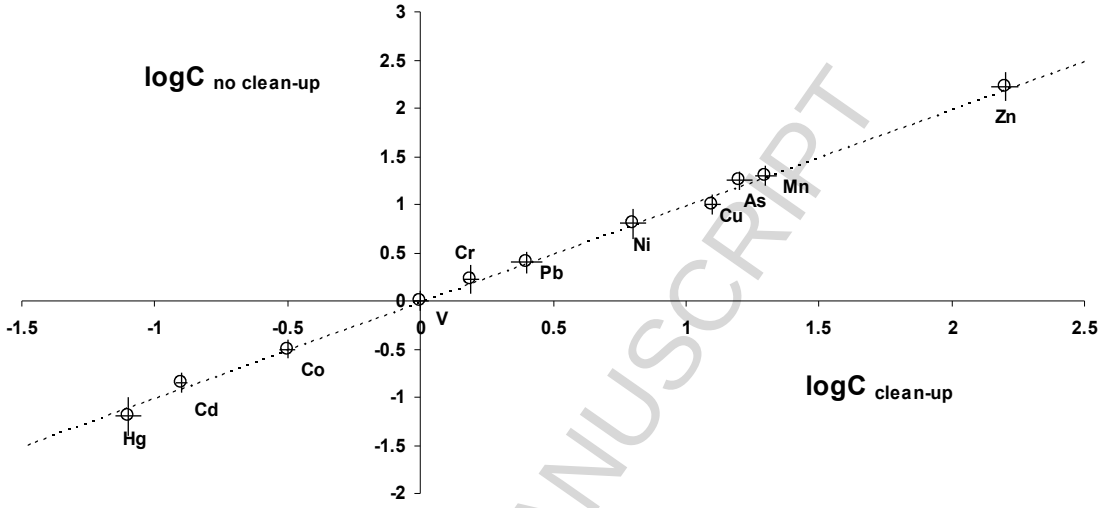
Figure 5



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