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ARTICLE *in* ANALYTICAL CHEMISTRY · APRIL 2007

Impact Factor: 5.64 · DOI: 10.1021/ac061379w · Source: PubMed

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Feasibility of Pressurization To Speed Up Enzymatic Hydrolysis of Biological Materials for Multielement Determinations

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The feasibility of pressurized solvents (liquids at a high pressure and/or high temperature without the subcritical point being reached) has been newly investigated to accelerate enzymatic hydrolysis processes of mussel tissue for multielement determinations. The target elements (Al, As, Cd, Co, Cu, Fe, Hg, Li, Mn, Pb, Se, Sr, V, and Zn) were released from dried mussel tissue by action of two proteases (pepsin and pancreatin), and they have been evaluated by inductively coupled plasma optical emission spectrometry (ICP-OES). Variables inherent to the enzymatic activity (pH, ionic strength, temperature, and enzyme mass) and factors affecting pressurization (static time, pressure, and number of cycles) were simultaneously studied by applying a Plackett−Burman design (PBD) as the screening method. Results showed that pH, ionic strength, and temperature were the most statistically significant factors (confidence interval of 95%) under pressurized conditions for pepsin, while pH and ionic strength affected pancreatin activity. This means that metal extraction is mostly attributed to enzymatic activity. The static time (enzymatic hydrolysis time) was found statistically nonsignificant for most of the elements, meaning that the hydrolysis procedure can be finished within a 2−15 min range. For pepsin, optimized conditions (pH 1.0, temperature 40 °C, pressure 1500 psi, static time 2 min, and number of cycles 3) gave quantitative extractions for As, Cd, Co, Cu, Hg, Li, Mn, Pb, Se, Sr, V, and Zn. The pepsin mass was 0.05 g, and the solution was Milli-Q water at pH 1.0 (adjusted with hydrochloric acid). For pancreatin, quantitative recoveries were only reached for As, Cd, Cu, Li, Pb, and Sr at room temperature, at a pressure of 1500 psi, for a static time of 2 min and a number of cycles of 3. The extraction solution was a 0.3 M potassium dihydrogen phosphate/potassium hydrogen phosphate buffer at a pH of 7.5 working at room temperature. Around 0.5 g of diatomaceous earth was used as dispersing agent for hydrolyses with either enzyme. Analytical performances, such as limits of detection and quantification and repeatability of the overall procedure, have been established. Finally, accuracy of the methods was assessed by analyzing seafood certified reference materials (GBW-08571, DORM-2, DOLT-3, TORT-2), fatty tissues certified reference materials (BCR 185, NIST 1577b), and fibrous certified reference materials (BCR 62, GBW-08501).

Since the first use of enzymatic hydrolysis by Carpenter[1] to extract metals from human liver and kidney tissues, different enzymes, mainly proteolytic enzymes, have been investigated and applied to metal and organometal extraction from biological matrixes.[2] Most of the enzymes are hydrolytic type, and the catalytic reaction consists in introducing water at specific bounds of the substrate and breaking down such bounds under controlled pH, temperature, and ionic strength conditions.[3] The moderate acidity and temperature make enzyme-based sample pretreatments useful for metal speciation in biological and environmental materials.[2,4] This is mainly because organometallic species alterations are less expected under such soft operating conditions. Moreover, the absence of oxidizing reagents or concentrated mineral acids commonly needed in wet acid digestion procedures and a noncorrosive environment and noncorrosive wastes make methods based on enzymes also attractive for total metal determination. A complete review on different enzymes (lipases, amylases, and proteases) and substrates for both total metal determination and metal speciation can be found in ref 2.

Thermostatic incubation has been by far the most common way to carry out the enzymatic hydrolysis process. For such conventional procedures and when total metal contents are being determined, the literature shows hydrolysis times within 1−24 h

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10.1021/ac061379w CCC: $37.00
Published on Web 02/02/2007

mainly for hydrolyzing selenium-enriched yeast,[5] blood,[6] or plant and animal tissues[7,8] for selenium determinations, or hair, mussel soft tissue, and seaweed for multielement determinations.[9−11] The large pretreatment times for conventional enzymatic hydrolysis are attributed to the fact that the enzyme must disrupt cell membranes before chemically attacking the cytosolic content. Cell membranes are mainly constituted by proteins and polar lipids in which phospholipids and sterols form a lipid bilayer and the proteins are embedded at irregular intervals. This structure, called the *fluid mosaic model*, leads to a very stable and strong array. If cell membranes are disrupted just before or at the same time as enzymes chemically attack the cell cytosol, an important reduction in the hydrolysis time can be expected. Wolf et al.[12] first reported that sonication allows rapid cell membrane disruptions. These authors used ultrasound energy for a rapid isolation of metallothioneins from biological materials. Under this assumption, ultrasound energy can be used to disrupt cell membranes before enzymatic hydrolysis. Therefore, some accelerated ultrasound−enzymatic hydrolysis procedures have been recently reported[2] by using ultrasound baths (UBs)[11,13] for total metal extraction and ultrasound probes (UPs)[14,15] for selenium and arsenic speciation. Enzymatic hydrolysis times have been reduced from several hours to 30 min when using UB devices and even to 1−3 min for the use of UPs. These results allow the expectation of a promising future for the combination of ultrasound irradiation and enzymes for biological sample pretreatments.[2]

Since successful results have been obtained for the use of ultrasound, speeding up enzymatic hydrolysis can be carried out by applying enough energy to disrupt cell membranes before enzymatic attack of the cytosolic content. Pressurized liquid extraction (PLE) is a well-established methodology for the extraction of organic compounds[16,17] and organometallic species[18] from solid biological and environmental samples. This technique is based on the use of solvents, commonly deionized water, organic solvents, and diluted organic acids such as acetic acid, at high pressure and/or temperature without the critical point being reached. The system consists of a stainless steel extraction cell in which the sample is placed and where the programmed parameters (temperature, pressure, static time, and extraction steps) are kept at their specified values by electronically controlled heaters and pumps.[17] By pressurizing the sample cell (from 4 to 20 MPa), it is possible to keep the solvent in a liquid phase even at relatively high extraction temperatures (the critical point for water has been taken to be 374 °C and 221 psi). This leads to a very efficient contact between the solvent molecules and the solid particles, cell membrane disruptions occurring by the action of solvent in very short times, commonly within 5−10 min.

Moreover, cell membrane disruption by pressurization and the use of PLE for assisting enzymatic hydrolysis can offer other advantages over the use of ultrasound: (1) a centrifugation or filtration stage to isolate the enzymatic digest from the pellet is avoided because the solid sample (pellet) remains inside the extracting cell while the enzymatic digest is collected in external vials, and (2) PLE devices are fully automatized pieces of equipment and allow processing of several samples (up to 24).

In this work, the feasibility of pressurization to speed up enzymatic hydrolysis procedures was newly studied. Mussell soft tissue was used as substrate to develop the methods. However, the feasibility of the optimized procedures was proved by analyzing certified reference materials from marine origin (GBW-08571, mussel tissue; DORM-2, dog-fish muscle; DOLT-3, dog-fish liver; and TORT-2, lobster hepatopancreas), certified reference materials with high fat contant (BCR 185, bovine liver; NIST 1577b, bovine liver), and fibrous certified reference materials from botanical origin (BCR 62, olive leaves; GBW-08501, peach leaves). Hydrolytic proteases (pepsin and pancreatin) were investigated under pressurized conditions to quantitatively release target trace elements (Al, As, Cd, Co, Cu, Fe, Hg, Li, Mn, Pb, Se, Sr, V, and Zn).

EXPERIMENTAL SECTION

Apparatus. An Optima 3300 DV inductively coupled plasma atomic emission spectrometer (Perkin-Elmer, Norwalk, CT) equipped with an autosampler, AS 91 (Perkin-Elmer), was employed. A Dionex ASE-200 system (Sunnyvale, CA) equipped with stainless steel extraction cells of 11 mL (Sunnyvale) and cellulose filters (D28, 1.983 cm diameter, Dionex) was used. An Ethos Plus microwave labstation (Milestone, Sorisole, Italy), delivering a maximum power and temperature of 1000 W and 300 °C, respectively, and internal temperature control, was used to assist the acid digestion processes. This piece of equipment uses 100 mL closed Teflon vessels with Teflon covers and an HTC adapter plate and HTC safety springs (Milestone). An ORION 720A plus pH meter with a glass−calomel electrode (ORION, Cambridge, U.K.) was used for pH measurements. A laboratory blender, Stomacher 400 (Seward Medical Ltd., London, U.K.), was used to blend and homogenize mussel samples. During the blending process, samples were contained in Stomacher closure bags 6041/CLR (Seward). A LYPH-LOCK 6 L freeze-dry system, model 77530, from Labconco Corp. (Kansas City, MO) was used to freeze-dry mussels. A vibrating ball mill, Retsch (Haan, Germany), equipped with zircon cups (15 mL in size) and zircon balls (7 mm diameter), was used to pulverize dried mussels. The chemometrics package was Statgraphics Plus V 5.0 for Windows, 1994−1999 (Manugistics Inc., Rockville, MD).

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Table 1. Microwave-Assisted Acid Digestion Program[a]

step	temp/°C	time/min	power/W
1	90	2.5	1000
2	140	6.0	1000
3	200	5.0	1000
4	200	10.0	1000

[a] Venting time of 50 min.

Reagents. All chemicals used were of ultrapure grade, and were diluted using ultrapure water of resistance 18 MΩ cm^{-1} obtained from a Milli-Q purification device (Millipore Co., Bedford, MA). Multielement standard solutions were prepared by combining stock standard solutions (1.000 or 10.000 g L^{-1}) supplied by Merck (Poole, Dorset, U.K.). Pepsin from porcine stomach and pancreatin from porcine pancreas were from Sigma (St. Louis, MO). Diatomaceous earth, 95% SiO_2, used as the dispersing agent, was from Aldrich (Milwaukee, WI). AnalaR nitric acid (69.0%) and hydrochloric acid (37%) were from Panreac (Barcelona, Spain). Sodium dihydrogen phosphate, disodium hydrogen phosphate, and sodium chloride were from Merck. GBW-08571 (mussel tissue) and GBW-08501 (peach leaves) CRMs were supplied from Standard Materials of Soils Components (Harbin, China). DORM-2 (dog-fish muscle), DOLT-3 (dog-fish liver), and TORT-2 (lobster hepatopancreas) CRMs were from National Research Council of Canada (Ottawa, Canada). BCR 185 (bovine liver) and BCR 62 (olive leaves) were from Community Bureau of Reference-Commission of European Communities (Brussels, Belgium). NIST 1577b (bovine liver) was from National Institute of Standards and Technology (Gaithersburg, MD).

To avoid metal contamination, all glassware and plastic ware were washed, kept for 48 h in 10% (v/v) nitric acid, and then rinsed several times with ultrapure water before use.

Mussel Samples. Mussel samples (*Mytilus galloprovincialis*) were collected from mussel rafts located at Ría de Arousa estuary (Galicia, northwest Spain). Studies were performed using all soft mussel tissue (muscle and gill) by preparing a mussel pool with all the mussels from each mussel raft. After mechanical blending, homogenization, and the freeze-dry process, the dry mussel samples were pulverized by using a vibrating ball mill and were kept in polyethylene bottles with hermetic seals.

Microwave-Assisted Acid Digestion. Microwave-assisted acid digestion was carried out by adding 2 mL of nitric acid (69%, m/m), 1 mL of hydrogen peroxide (33%, m/v), and 6 mL of ultrapure water to 0.5 g of pulverized mussel sample. The reactors were then subjected to the microwave program listed in Table 1. After acid digestion was completed, the acid digests were made up to 20 mL with ultrapure water. At least two different blanks were performed for each set of microwave-assisted acid digestions.

Pressurized Enzymatic Hydrolysis. Around 0.25 g of sample and 0.05 g of enzyme (pepsin or pancreatin) were weighed and then mixed thoroughly with 0.50 g of diatomaceous earth (DE) as the dispersing agent. The extraction cells were filled with this mixture and fitted with a cellulose filter. The extracting solutions were Milli-Q water at pH 1.0 (adjusted with diluted hydrochloric acid) when using pepsin or a sodium dihydrogen phosphate/disodium hydrogen phosphate (NaDHP/DNaHP) buffer solution (0.30 M/0.30 M) at pH 7.5 for the use of pancreatin. A 60% PLE flush volume was used throughout. The cells were purged for 60 s with N_2 after the pressurization, and the extract (enzymatic digest) was collected in precleaned glass vials. Finally, the enzymatic digests were diluted to exactly 20 mL, placed into precleaned plastic bottles and analyzed within 1−6 h after preparation. Because enzymatic digests were not acidified, enzymatic digests were stored at −20 °C if they were not analyzed just after preparation. In these cases, segmentation of the samples after enzymatic digest melting was not observed. The pressurized conditions (PLE extraction conditions) are summarized in Table 2. At least two different blanks were performed for each set of PLE conditions.

Table 2. Pressurized Enzymatic Hydrolysis Conditions

flush volume solvent/%	60
N_2 purge time/s	60
cell size/mL	11
diatomaceous earth mass/g	0.5
extraction temperature/°C	50
pressure/psi	1500
static time/min	2
number of cycles	3
solvent composition[a]	
pH[b]	1.0
ionic strength[c]	

[a] Ultrapure water for pepsin and sodium dihydrogen phosphate/disodium hydrogen phosphate buffer for pancreatin. [b] pH of 7.5 for pancreatin. [c] 0.3 M for pancreatin.

Table 3. Operating ICP-OES Conditions

radiofrequency power/W		1300
gas flow rate/L min^{-1}	plasma	15.0
	auxiliary	0.5
	nebulizer	0.8
nebulizer type		cross-flow
peristaltic pump speed/mL min^{-1}		1.5
stabilization time/s		45
number of replicates		5
detection wavelength/nm	As	197.197
	Cd	228.802
	Co	228.616
	Cu	327.393
	Li	670.784
	Hg	194.168
	Mn	257.610
	Pb	224.688
	Se	196.026
	Sr	407.771
	V	292.402
	Zn	206.200

Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) Measurements. Elements were measured by ICP-OES (axial configuration) without dilution, using the plasma operating conditions and emission wavelength lines listed in Table 3. Since ICP-OES response changes when aspirating the NaDHP/DNaHP (pancreatin hydrolysis) and HCl (pepsin hydrolysis) extracting solutions, the standard addition method was used as calibration technique. Standard addition graphs covered analyte concentration ranges from 0 to 10 mg L^{-1} for Al, Cu, Fe, Mn, and Zn, between 0 and 2 mg L^{-1} for As, Cd, Co, Hg, Li, Pb, Se, and V, and between 0 and 0.2 mg L^{-1} for Sr.

RESULTS AND DISCUSSION

Description of Variables Affecting Pressurized Enzymatic Hydrolysis. From literature concerning enzyme-based sample pretreatments, temperature, pH, and ionic strength are the most reported main variables affecting enzymatic hydrolysis.[2,3] However, it must be noted that a couple of these parameters could not be important when the procedure is assisted with ultrasound. For instance, none of them appears to be important when using ultrasonic probes, and quantitative results for selenium species can be obtained in nonbuffered media and at room temperature.[14] Moreover, these factors inherent to enzymatic activity and the enzyme mass as well as variables affecting pressurization, such as pressure, static time, and number of cycles, were investigated. A last variable, called the *dummy factor*, was also considered. Dummy factors are imaginary variables for which the change from one level to another is not supposed to cause any physical change,[19] and they are commonly used to evaluate the possible systematic error and/or the existence of an important variable that was not taken into account. For all experiments, sample masses of 0.25 g thoroughly dispersed with 0.5 g of diatomaceous earth were used. In addition, a blank was prepared for each set of operating conditions. Finally, for all experiments, the PLE flush volume was fixed at 60%. The flush volume is the volume of fresh solvent used to clean the extraction cell after static extraction. Flush volume should be large enough to remove the extracted analytes and to clean the system.

The *pH* was adjusted by using a sodium dihydrogen phosphate/disodium hydrogen phosphate buffer solution at different concentrations for pancreatin. Meanwhile, the pH was fixed by adding hydrochloric acid when using pepsin. Similarly, the variable *ionic strength* is related to the concentration of the buffer system used to fix the pH. Since sodium dihydrogen phosphate/disodium hydrogen phosphate was used as the buffer solution for pancreatin, the ionic strength was related to the concentration of sodium dihydrogen phosphate/disodium hydrogen phosphate. However, as an extremely acidic pH was needed for pepsin, the ionic strength was related to different sodium chloride concentrations.

To know whether quantitative metal extraction was achieved, the samples were previously subjected to a conventional microwave-assisted acid digestion process (Table 1), and metal concentrations in both acid digests and enzymatic digests were expressed as the percentage of released element according to the equation

$$\text{recovery} = ([\text{M}]_{\text{enzymatic hydrolysis}}/[\text{M}]_{\text{acid digestion}}) \times 100$$

where $[\text{M}]_{\text{enzymatic hydrolysis}}$ is the metal concentration obtained after the pressurized enzymatic hydrolysis procedure (1−24 in Table S1, S2, or S3, Supporting Information) and $[\text{M}]_{\text{acid digestion}}$ is the metal concentration found after microwave-assisted acid digestion. The percentages of metal released were also the response variables when an experimental design approach for optimization was applied.

Plackett−Burman Screening Design for Statistically Significant Variable Study. The statistical significance of the variables commented on above was simultaneously evaluated by applying a $(2^8 \times 3)/32$ type III resolution design, a Plackett−Burman design (PBD), for eight factors, four degrees of freedom, twelve runs, and two replicates (one PBD for each enzyme investigated). Table 4 shows the field definition of the variables, i.e., the low (−) and high (+) levels assigned to each variable. The values were chosen according to instrumental (Dionex 200) limitations and enzyme properties; e.g., pepsin works at low pH values and pancreatin at neutral to alkaline pH values, and the temperature must not be high (lower than 50 °C). The PBD experimental conditions and the values for the response variables when using each enzyme are listed in Tables S1 and S2 for pepsin and pancreatin, respectively.

Table 4. Experimental Field Definition for the PBD[a]

variable	symbol	low level (−)	high level (+)
enzyme mass/mg	*W*	5.0	20.0
pressure/psi	*P*	500	1500
temperature/°C	*T*	18	50
static time/min	*t*	2.0	5.0
number of cycles	*C*	1	5
pH[b]	pH	6.0	9.0
ionic strength (sodium dihydrogen phosphate disodium hydrogen phosphate concentration)/ M[c]	IS	0.05	0.20
dummy factor	*D*	−1	+1

[a] The mussel mass was 0.25 g for all experiments. [b] The low and high levels were 1.0 and 3.0 when using pepsin. [c] The ionic strength were related to sodium chloride at 2.0% and 0.0% (m/v) for the high and low levels, respectively, when using pepsin.

At a 95.0% confidence interval a minimum *t* value of 2.46 was obtained after an iterative process by the Statgraphics routine.[20] It is stated that variables with *t* values higher than ±2.46 must be considered as statistically significant factors. Moreover, each metal percentage (11 variable responses related to 11 target elements as shown in Tables S1 and S2), an additional response variable, obtained by calculating the mean percentage of the 11 metal percentages for each run or experiment, was also taken into account. This last response variable, named the mean percentage in Tables S1 and S2, was first considered for statistical evaluation. Figure 1a shows the main effect Pareto chart for pepsin when using the mean percentage as the response variable. It can be seen that the pH, ionic strength, and temperature are statistically significant factors at a confidence level of 95%. These three variables are directly related to enzymatic activity, which means that although the hydrolysis process has been assisted by pressurization, the nature of the metal release is attributed to enzymatic activity. The effect of pH is negative, meaning that pepsin hydrolysis is enhanced at a low pH (1.0). Similarly, ionic strength offers higher metal percentages when using ultrapure water (sodium chloride concentration of 0%, m/v). The effect of the variable temperature is positive, meaning a better pepsin activity at high temperature (50 °C). Results when using each metal percentage as the response variable are similar to those obtained for the mean percentage (Figure 1a). This fact can be visualized in Table 5, where it can be seen that the variables *pH*

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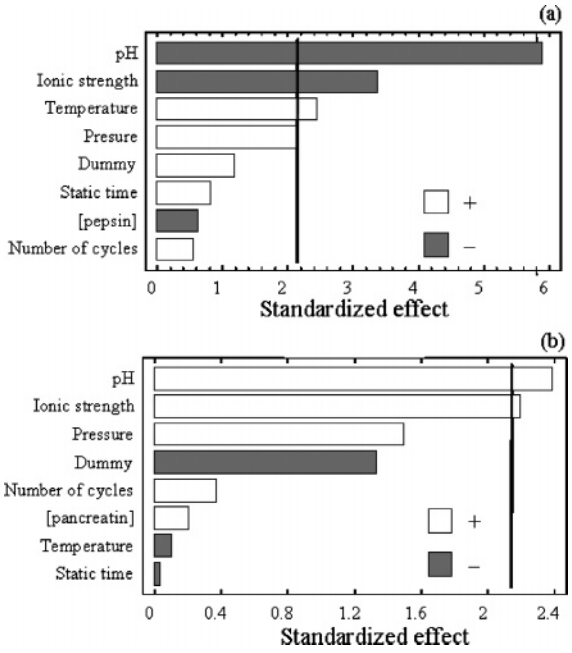

Figure 1. Main effect Pareto charts for pressurization−enzymatic hydrolysis using mean metal percentages for pepsin (a) and pancreatin (b).

and *ionic strength* are significant (negative effect) for most of the elements, while *temperature* is significant and positive for most of them. After removal of those variables less statistically significant (enzyme mass, number of cycles, static time, and dummy) the two-order interaction Pareto chart (not given) shows statistical significance for the two-order interactions pH/ionic strength and pH/pressure, keeping the main effect of pH, ionic strength, and temperature statistically significant. The statistical significance of these couples of variables means that there is a dependent relationship between them and they must be simultaneously evaluated. However, since pH and ionic strength offer a negative effect, meaning that pH must be fixed at the lowest value (1.0) and the solution must be ultrapure water (sodium chloride concentration of 0%, m/v), these two variables were fixed at the low value from Table 4. Finally, as temperature and pressure were not dependent variables (two-order interactions for these variables were not statistically significant), both were further optimized by a univariate approach.

Concerning the use of pancreatin, Figure 1b shows the main effect Pareto chart for the mean percentage as the response variable. It can be seen that pH and ionic strength are statistically significant variables at a confidence level of 95%. Similarly, from Table 5 it can be concluded that these two variables are also statistically significant for several elements. It must be noted that temperature is not statistically significant for any element (Table 5). This finding agrees with those previously reported for ultrasound-assisted pancreatin hydrolysis, for which temperature was an unimportant parameter.[10] From the two-order interaction Pareto chart, after removal of statistically nonsignificant variables (not given), a significant interaction between the variables pH and ionic strength can be seen. Therefore, these two variables will be further optimized by a multivariate approach.

Finally, the following can be considered for the nonstatistically significant variables: (1) The variable *static time* was found as nonsignificant for most of the elements and enzymes. Since this variable has been studied within the 2−7 min range, a static time (hydrolysis time) of 2 min can be fixed for both pepsin and pancreatin. This time is enough to guarantee an efficient chemical attack by enzymes after a fast cell membrane disruption under pressurized conditions. (2) The variable *enzyme mass* was statistically significant for some cases with a negative effect, i.e., pepsin for Al, As, and Li and pancreatin for Li (Table 5). Therefore, the low pepsin or pancreatin masses (5 mg) were chosen as optimum. (3) Similarly, the number of cycles was not statistically significant for most of the cases, although the effect of this variable is positive when significant (Table 5). Therefore, the number of cycles was fixed as 3 for the use of both enzymes. (4) Since temperature and pressure were not statistically significant when using pan-

Table 5. Effects of Changing Values from Low to High Levels[a]

	W	P	T	t	C	pH	IS	D
				Pepsin				
As	−	+	NS	NS	NS	−	−	NS
Cd	NS	NS	+	NS	NS	−	−	NS
Cu	NS	+	+	NS	NS	NS	−	NS
Li	−	NS	NS	+	NS	NS	NS	NS
Mn	NS	NS	NS	NS	NS	−	NS	NS
Pb	NS	+	+	NS	NS	−	−	NS
V	NS	+	+	+	+	−	−	NS
Zn	NS	NS	NS	NS	+	−	−	NS
mean	NS	NS	+	NS	NS	−	−	NS
				Pancreatin				
As	NS	NS	NS	−	NS	+	+	NS
Cd	NS	NS	NS	NS	NS	NS	−	NS
Cu	NS	+	NS	NS	NS	NS	+	NS
Li	−	NS	NS	NS	+	+	NS	NS
Mn	NS	NS	NS	NS	+	−	NS	NS
Pb	NS	+	NS	NS	NS	NS	+	NS
V	NS	NS	NS	NS	NS	+	−	NS
Zn	NS	NS	NS	NS	+	NS	NS	NS
mean	NS	NS	NS	NS	NS	+	+	NS

[a] A positive sign (+) means that the effect of the variable on the response is positive. A negative sign (−) means that the effect of the variable on the response is negative. NS means nonsignificant.

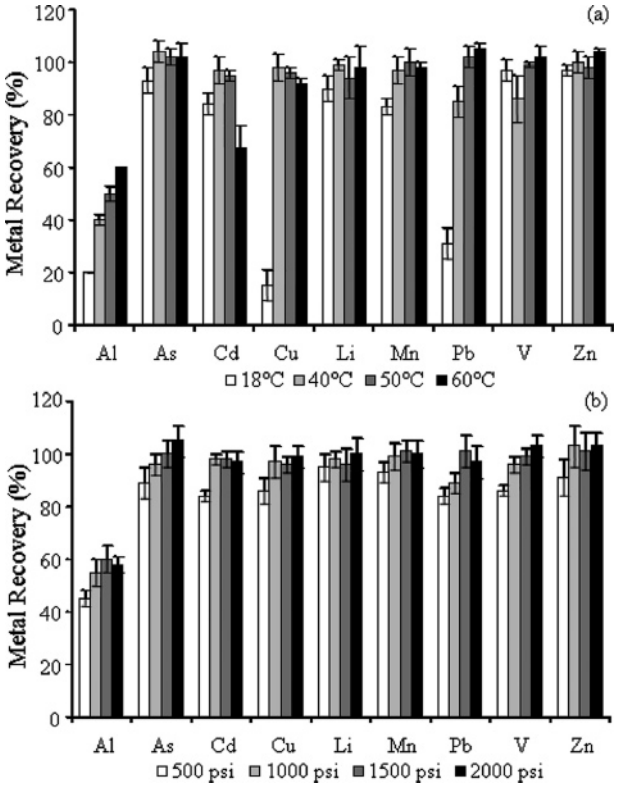

Figure 2. Effect of temperature (a) and pressure (b) on metal percentages when using pepsin in pressurized enzymatic hydrolysis.

creatin, values of 18 °C (room temperature) and 1500 psi were selected for temperature and pressure, respectively. (5) Finally, it must be noted that the *dummy factor* was not significant for any case, meaning that there are no systematic errors nor unknown variables affecting the system under study.

Optimization of Significant Variables. Since there was no interaction observed between temperature and pressure for the use of pepsin, these two variables were optimized following a univariate approach. Temperatures of 40, 50, and 60 °C (30 °C is not allowed by PLE software) were tested. Similarly, experiments at room temperature (around 18 °C) were also considered. After the remaining variables were fixed as above, three replicate trials were performed at each temperature. Results for some elements are shown in Figure 2a. It can be seen that temperature increases the metal percentage for all elements, obtaining quantitative results for all elements when the temperature is 40 and 50 °C. For some elements such as Cd, there is a decrease in the metal percentage for a temperature of 60 °C. Therefore, a temperature of 40 °C was chosen as optimum. This temperature is close to the reported temperature for pepsin when using conventional thermostatic baths and even ultrasound assistance, around 37 °C.[10,13]

Pressurized enzymatic hydrolysis with pepsin was carried out at different pressures (500, 100, 1500, and 2000 psi). Results in Figure 2b show quantitative metal percentages for most of the elements pressurizing was done at 1000 psi or higher. Therefore, a pressure of 1500 psi was finally selected.

For the use of pancreatin, the variables pH and ionic strength were simultaneously optimized by applying an orthogonal 2^2 + star central composite design (CCD) with five error degrees of

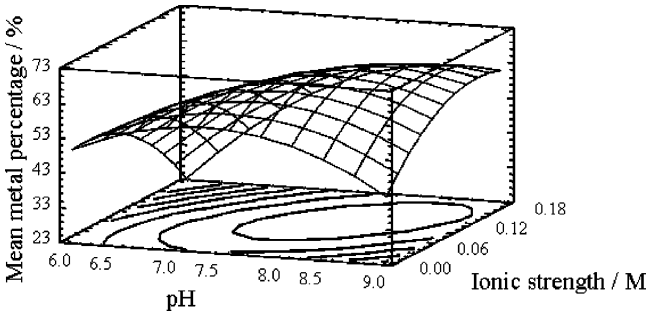

Figure 3. Estimated response surface for pressurized enzymatic hydrolysis with pancreatin using mean metal percentages.

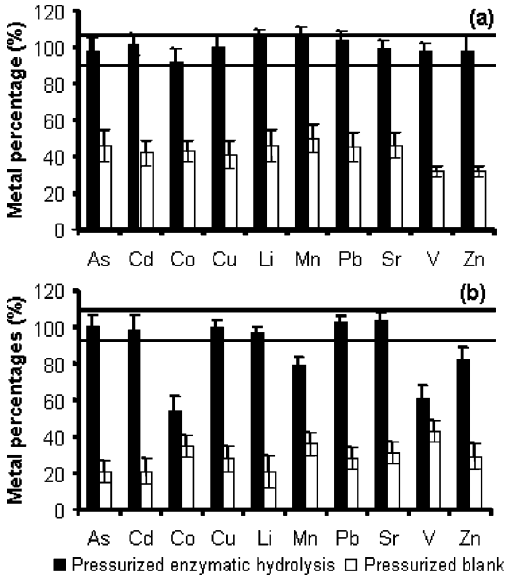

Figure 4. Metal recovery percentages from mussel samples by using the pressurized enzymatic procedure and the leaching effect of buffer solutions (blanks) for pepsin (a) and pancreatin (b). Horizontal lines at 90 and 110% indicate the metal percentage ranges within quantitative recoveries are considered.

freedom, two centers, four replicates, and twenty-four runs. A CCD is based on full, or fractional, two-level factorial designs by centre point replication and inclusion of an axial portion[19] and they are commonly used to find the optimum values of the different variables under study. Since higher metal percentages were obtained at higher values of the ionic strength (sodium dihydrogen phosphate/disodium hydrogen phosphate concentration at 0.2%, m/v), the field definition for this variable was changed from 0.05−0.20% (m/v) to 0.025−0.30% (m/v). The low and high values for pH were the same as those listed in Table 4. Table S3 (Supporting Information) gives the CCD matrix and the response variables (percentages of released metals) for each experiment. The statistical evaluation of quadratic terms was significant for many cases, especially when the response variable was the mean of metal percentages (Figure 3). This implies responses surfaces with curvature, as Figure 3 and a careful study of the results lead to compromise values of 7.5% and 0.3% (m/v) for pH and ionic strength, respectively.

Effect of the Buffer Solution. To confirm that metal extraction from mussel tissue is mainly attributed to pepsin or pancreatin activity, the optimized pressurized enzymatic hydrolysis conditions

Table 6. Analysis of Certified Reference Materials after Pressurized Enzymatic Hydrolysis

Marine Origin

	GBW−08571			DORM-2		
	found value/μg g^{-1}			found value/μg g^{-1}		
	certified value/μg g^{-1}	pepsin	pancreatin	certified value/μg g^{-1}	pepsin	pancreatin
As	6.1 ± 1.1	6.5 ± 0.2	6.2 ± 0.2	18.0 ± 1.1	18.9 ± 0.6	18.0 ± 0.3
Cd	4.5 ± 0.5	4.2 ± 0.1	3.8 ± 0.2	0.043 ± 0.008	<LOD	<LOD
Co	0.94 ± 0.06	0.96 ± 0.10	0.51 ± 0.07	0.182 ± 0.031	0.242 ± 0.006	0.075 ± 0.009
Cu	7.7 ± 0.9	7.5 ± 0.5	7.7 ± 0.4	2.34 ± 0.16	2.49 ± 0.20	2.32 ± 0.08
Hg	0.067 ± 0.008	<LOD	<LOD	4.64 ± 0.26	4.51 ± 0.05	3.60 ± 0.12
Li	0.8[a]	0.88 ± 0.04	0.84 ± 0.03	[b]		
Mn	10.2 ± 1.8	9.0 ± 1.0	8.64 ± 0.86	3.66 ± 0.34	3.22 ± 0.19	2.77 ± 0.11
Pb	1.96 ± 0.09	2.01 ± 0.06	2.00 ± 0.05	0.065 ± 0.007	< LOD	< LOD
Se	3.65 ± 0.17	3.79 ± 0.08	1.47 ± 0.10	1.4 ± 0.09	1.1 ± 0.09	0.53 ± 0.09
Sr	12.8 ± 1.1	12.7 ± 0.1	12.5 ± 0.1	[b]		
Zn	138 ± 9	138 ± 3	121 ± 4	25.6 ± 2.3	24.4 ± 0.8	20.9 ± 0.9

	DOLT−3			TORT−2		
	found value/μg g^{-1}			found value/μg g^{-1}		
	certified value/μg g^{-1}	pepsin	pancreatin	certified value/μg g^{-1}	pepsin	pancreatin
As	10.2 ± 0.5	9.8 ± 0.3	10.1 ± 0.2	21.6 ± 1.8	20.5 ± 0.9	20.9 ± 0.8
Cd	19.4 ± 0.6	19.7 ± 0.3	18.6 ± 0.4	26.7 ± 0.6	26.5 ± 0.3	26.3 ± 0.4
Co	[b]			0.51 ± 0.09	0.59 ± 0.11	0.38 ± 0.08
Cu	31.2 ± 1.0	31.5 ± 0.6	32.0 ± 0.5	106 ± 10	107.4 ± 2.8	99.0 ± 3.1
Hg	3.37 ± 0.14	3.42 ± 0.13	2.06 ± 0.08	0.27 ± 0.06	< LOD	< LOD
Mn	[b]			13.6 ± 1.2	13.3 ± 1.0	11.4 ± 1.0
Pb	0.32 ± 0.05	0.38 ± 0.02	0.35 ± 0.01	0.35 ± 0.13	0.44 ± 0.06	0.28 ± 0.07
Se	7.06 ± 0.48	7.07 ± 0.33	6.33 ± 0.25	5.63 ± 0.67	5.34 ± 0.60	2.81 ± 0.56
Sr	[b]			45.2 ± 1.9	44.6 ± 1.1	44.7 ± 109
V	[b]			1.64 ± 0.19	1.48 ± 0.02	1.02 ± 0.07
Zn	88.6 ± 2.4	85.7 ± 1.6	82.2 ± 1.0	180 ± 6	177 ± 3	163 ± 4

High Fat Content and Botanical Origin

	BCR 185 (bovine liver)			NIST 1577b (bovine liver)		
	found value/μg g^{-1}			found value/μg g^{-1}		
	certified value/μg g^{-1}	pepsin	pancreatin	certified value/μg g^{-1}	pepsin	pancreatin
As	0.024 ± 0.003	<LOD	<LOD	0.05[a]	<LOD	<LOD
Cd	0.298 ± 0.025	0.276 ± 0.015	0.280 ± 0.013	0.50 ± 0.03	0.51 ± 0.01	0.48 ± 0.01
Co	[b]			0.25[a]	0.21 ± 0.01	0.13 ± 0.01
Cu	189 ± 4	192 ± 2	129 ± 1	160 ± 8	162 ± 2	85 ± 4
Hg	0.044 ± 0.003	<LOD	<LOD	[b]		
Li	[b]			[b]		
Mn	9.3 ± 0.3	9.3 ± 0.1	9.5 ± 0.1	10.5 ± 1.7	9.0 ± 0.3	10.7 ± 0.1
Pb	0.501 ± 0.027	0.490 ± 0.009	0.402 ± 0.05	0.129 ± 0.004	<LOD	<LOD
Se	0.446 ± 0.013	0.428 ± 0.002	<LOD	0.73 ± 0.06	0.68 ± 0.03	0.42 ± 0.03
Sr	[b]			[b]		
Zn	142 ± 3	143 ± 2	141 ± 3	127 ± 16	116 ± 1	118 ± 3

	GBW-08501 (peach leaves)			BCR 62 (olive leaves)		
	found value/μg g^{-1}			found value/μg g^{-1}		
	certified value/μg g^{-1}	pepsin	pancreatin	certified value/μg g^{-1}	pepsin	pancreatin
As	0.34 ± 0.06	<LOD	<LOD	[b]		
Cd	0.018 ± 0.008	<LOD	<LOD	0.10 ± 0.02	<LOD	<LOD
Co	0.25[a]	0.23 ± 0.04	0.14 ± 0.02	[b]		
Cu	10.4 ± 1.6	8.8 ± 0.4	4.6 ± 0.3	46.6 ± 1.8	44.3 ± 0.5	5.8 ± 0.1
Hg	0.046 ± 0.012	<LOD	<LOD	0.28 ± 0.02	<LOD	<LOD
Li	[b]			[b]		
Mn	75.4 ± 5.4	77.9 ± 2.4	75.1 ± 1.3	57.0 ± 2.4	58.0 ± 0.6	56.3 ± 1.0
Pb	0.99 ± 0.08	1.03 ± 0.01	0.95 ± 0.01	25.0 ± 1.5	25.4 ± 1.0	23.8 ± 0.8
Se	0.04[a]	<LOD	<LOD	[b]		
Sr	61.6 ± 7.8	31.8 ± 1.0	5.8 ± 0.5	[b]		
Zn	22.8 ± 2.5	22.0 ± 1.2	22.2 ± 1.1	16.0 ± 0.7	15.5 ± 0.2	15.3 ± 0.6

[a] Informative value. [b] Not certified.

for both enzymes were applied to a mussel tissue four times with and without enzyme (enzymatic digest and enzymatic blank, respectively). The metal amount extracted without enzyme is attributed to the leaching action of the buffer solution (ultrapure water at pH 1.0 for pepsin or 0.3% (m/v) sodium dihydrogen phosphate/disodium hydrogen phosphate buffer at pH 7.5). Results are plotted in Figure 4, where it can be seen that most of the metals, except V and Zn, can be leached from the sample until 40−50% when using pepsin and at percentages lower than 43% (V) for the use of pancreatin. These blank results are similar to those obtained after ultrasound assistance, lower than 60% for pepsin and lower than 50% for pancreatin.[13] From Figure 4 it can be concluded that, for the use of pepsin, quantitative recoveries are reached for all metals only if the enzyme (pepsin) is present. Similarly, quantitative recoveries for As, Cd, Cu, Li, Pb, and Sr were obtained when using pancreatin. These results confirm that metal release from mussel is mainly attributed to enzyme (pepsin/pancreatin) activity. The low number of elements released by pancreatin can be attributed to chemical reactions with hydroxyl anions (alkaline medium needed for pancreatin) that can affect the main protein hydrolysis equilibrium. Similar results have been obtained when using conventional enzymatic hydrolysis and ultrasound−enzymatic hydrolysis methods.[10,13]

Analytical Performance. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated, and LOD and LOQ values were low enough to determine As, Cd, Co, Cu, Li, Mn, Pb, Sr, V, and Zn in mussel raft samples. LODs and LOQs for other elements such as Hg and Se were evaluated. The values were not low enough for the determination of these elements in the mussel samples used, although since the Hg and Se contents in some certified reference materials were high, the procedure offered good LOQs for the determination of both metals in such materials.

The repeatability of the overall procedure has been obtained by preparing 11 times a pressurized enzymatic digest from DOLT-3 certified reference material for each enzyme type. Each enzymatic digest was analyzed once, and the RSD values were 3%, 2%, 10%, 2%, 5%, 4%, 8%, 5%, 5%, 2%, 6%, and 2% for As, Cd, Co, Cu, Li, Hg, Mn, Pb, Se, Sr, V, and Zn, respectively, when using pepsin and 2%, 2%, 2%, 4%, 3%, and 3% for As, Cd, Cu, Li, Pb, and Sr, respectively, when using pancreatin.

The accuracy of the proposed methods was assessed by analyzing different CRMs from marine origin (GBW-08571, DORM-2, DOLT-3, TORT-2), CRMs with high fatty content (BCR 185, NIST 1577b), and fibrous CRMs from botanical origin (BCR 62, GBW-08501). These CRMs were subjected to the optimized procedures five times, and the different metal concentrations were determined four times by ICP-OES. The results in Table 6 show that As, Cd, Co, Cu, Li, Hg, Mn, Pb, Se, Sr, and Zn concentrations in CRMs from marine origin are within the certified concentration ranges when using pepsin, except for Cd and Pb in DORM-2, for which Cd and Pb concentrations are lower than the LOQs of the method. It can be seen that Hg and Se, elements which were not studied during the optimization process because of their low concentration in the mussel samples used, are quantitatively extracted when using pepsin. Accurate results have been also found when analyzing bovine liver and botanical CRMs, except for Sr in peach leaves CRM, after pressurized enzymatic hydrolysis with pepsin. It must be noticed that accuracy could not be tested for As and Hg because of the low concentration of these elements in BCR 185, NIST 1577b, BCR 62, and GBW-08501 CRMs. Similarly, accuracy for Li and Sr in some CRMs could not be assessed because the CRMs do not give certified/indicative values for these elements. Pressurized enzymatic hydrolysis of CRMs from marine origin with pancreatin has led to accurate results only for As, Cd, Cu, Li, Pb, and Sr (again, Cd and Pb concentrations in DORM-2 were lower than the LOQs of the method). For Hg and Se, extractions were not quantitative in the CRMs, showing recoveries within 40−90% for Se and lower than 70% for Hg. However, poor accuracy has been found when analyzing fatty and botanical CRMs by pressurized enzymatic hydrolysis with pancreatin, and accurate concentrations were only found for Cd, Mn, and Zn in all bovine liver and botanical CRMS tested, and for Pb in NIST 1577b, BCR 62, and GBW-08501 CRMs.

CONCLUSIONS

The application of pressurized conditions to assist enzymatic hydrolysis procedures has been found feasible to shorten the long hydrolysis times involved with conventional thermostatic devices. Since metal recoveries achieved after pressurization with enzymes are higher than those obtained after pressurization without enzymes (blanks), it can be concluded that metal extraction can be attributed to both pressurization and enzymatic activity. The number of elements quantitatively released from mussel soft tissue is high when using pepsin. This fact has been proved after analyzing different CRMs from marine origin. In addition, pressurization of enzymatic hydrolysis by pepsin has been found successful to extract trace elements from fatty and fibrous materials. This conclusion can be reached since accurate results were obtained after analyzing CRMs with high fat content (bovine liver) and fibrous CRMs (peach and olive leaves). However quantitative recoveries for the use of pancreatin were only obtained for As, Cd, Cu, Li, Pb, and Sr when CRMs from marine origin were analyzed. After analysis of fatty and botanical CRMs, good accuracy was only assessed for Cd, Mn, and Zn. Therefore, it can be concluded that the use of pepsin leads to better results in order to extract several trace elements from biological materials (seafood, fatty and fibrous samples). The number of elements quantitatively released from mussel soft tissue is high when using pepsin. However, quantitative recoveries for the use of pancreatin were only obtained for As, Cd, Cu, Li, Pb, and Sr. Enzymatic hydrolysis with pepsin can be complete after three cycles of 2.0 min each at 40 °C and 1500 psi. Since hydrolysis is carried out at high temperature (40 °C), PLE device software requires 5.0 min to reach the programmed temperature. Therefore, the total pressurization−enzymatic hydrolysis time for pepsin is around 12 min. By using pancreatin, it is possible to finish the enzymatic hydrolysis process in 6 min (three cycles of 2.0 min each) at room temperature and 1500 psi. Because centrifugation or filtration steps are omitted when using the PLE piece of equipment, the total times commented on above are the operational times needed to complete the enzymatic hydrolysis procedures. These times are shorter than those reported for conventional enzymatic hydrolysis procedures (from 1 to 24 h) and also lower than those reported when the procedure is assisted with an ultrasound bath (around 30 min, without considering centrifugation). Moreover, with the reduction of treatment time, the number of released elements is

higher than the number of elements extracted under conventional enzymatic hydrolysis with the same enzymes (pepsin and pancreatin) and the same matrix (mussel soft tissue).[10] Therefore, the use of pressurization conditions to assist enzymatic hydrolysis is a promising methodology that must be fully exploited.

ACKNOWLEDGMENT

We thank Consellería de Educación y Ordenación Universitaria, Xunta de Galicia, for the financial support offered (Grant PGIDT01PXI10301IF) for the Dionex ASE-200 system purchase.

SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review July 27, 2006. Accepted November 9, 2006.

AC061379W