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Use of enzymatic hydrolysis for the multi-element determination in mussel soft tissue by inductively coupled plasma-atomic emission spectrometry

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Abstract

A systematic evaluation of different variables affecting the enzymatic hydrolysis of mussel soft tissue by five enzymes, three proteases (pepsin, pancreatin and trypsin), lipase and amylase, has been carried out for the determination of trace elements (As, Al, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn) by inductively coupled plasma-atomic emission spectrometry (ICP-AES). Enzymatic hydrolysis methods offers advantages such as a less species alteration, safer laboratory conditions and a less contaminant wastes. The enzymatic hydrolysis was performed in an incubation camera Boxcult with orbital and horizontal shaker. Variables affecting the enzymatic hydrolysis process were simultaneously studied by applying a Plackett–Burman design (PBD). For a confidence interval of 95%, the significant factors for all enzymes and for most of the elements were the pH, the incubation temperature and the ionic strength. These significant factors were optimized later by using a central composite design (CCD), which gave optimum conditions at pH of 1, incubation temperature of 37 °C and ionic strength fixed by sodium chloride at 0.2 M when using pepsin. For pancreatin, trypsin, lipase and amylase there were found two different optimum condition sets. The first one involves the use of a 0.5 M phosphate buffer (ionic strength), at a pH of 6 and at an incubation temperature of 37 °C, which allows the quantitative extraction of Al, Cr, Mn, Pb and Zn. The second conditions set employees a 0.1 M phosphate buffer (ionic strength), a pH of 9 and an incubation temperature at 37 °C, and it results adequate to extract As, Cd, Cu, Fe and Ni. Analytical performances, repeatability of the over-all procedure and accuracy, by analyzing DORM-1, DORM-2 and TORT-1 certified reference materials, were finally assessed for each enzyme. Good agreement with certified values has been assessed for most of the elements (As, Cd, Cr, Cu, Mn, Ni, Pb and Zn) when using trypsin, pepsin and/or pancreatin, except for Cd and Pb in DORM-1 and DORM-2 because of the certified contents in such certified reference materials are lower than the limit of detection (0.10 and 0.16 $\mu g\, g^{-1}$ for Cd and Pb, respectively, for the use of trypsin).

Keywords: Enzymatic hydrolysis; Multi-element determination; Mussel soft tissue; Inductively coupled plasma-atomic emission spectrometry

1. Introduction

Conventional sample pre-treatments of solid biological materials prior metals determination by atomic spectrometric techniques involve a total decomposition of the biological sample matrix. These procedures are oxidative methods, which use concentrated mineral acids and moderate/high temperature and pressure conditions. Some disadvantages of these methods such as analyte losses by volatilization or metal contamination under these extreme pH and temperature conditions are commonly reported, mainly when using heating in open vessels [1]. In addition, it must be noticed the corrosive environment associated with the use of acid digestion procedures as well as the large volume of acid wastes. Therefore, sample pre-treatment methods such as the slurry sampling technique, the ultrasound/microwave assisted acid leaching, or the enzymatic hydrolysis, can be useful

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alternative sample pre-treatments to conventional acid mineralization/digestion processes [2]. Additionally, the acid leaching treatments and, specially, the enzymatic hydrolysis procedures are less likely to lead species alteration [3]. However, the possibility of species conversion can occur, as it has been reported by Pardo-Martínez et al. [4] for arsenic speciation. Since this fact, enzymatic hydrolysis procedures or enzymatic digestions have been commonly used for metal speciation studies in solid biological and environmental materials [3]. The enzyme used will depend on the sample type, and for proteinaceous materials, a protease or trypsin are adequate, while for carbohydrate-based material, cellulase and amylase is preferred [3]. Therefore, since the first use of Lipase Type III and Protease Type XIV by Forsyth and Marshall for alkyllead speciation [5], different enzyme type have been used and several organometallic compounds have been successfully isolated, mainly organomercury compounds [6] and arsenic [4,7] and selenium species [6,8]. However, the application of enzymatic digestion as a previous stage for multi-element determination is scarce, and few works dealing with total elements contents in enzymatic digests can be found in literature. Pronase E, a non-specific protease enzyme isolated from *Streptomyces Griseus* and containing at least ten proteolytic components [9], has been used to assess total Se in blood serum [10] and As, Cd, Cu, Fe, Mg, Pb and Zn in mussels [11]. In addition, lipase, cellulase and Protease Type XIV have been investigated in a partial enzymatic digestion procedure combined with a high pressure homogenization for releasing selenium from biological materials prior a slurry sampling—electrothermal atomic absorption spectrometric method [12,13]. According with Abou-Shakra et al. [10], the enzymatic digestion leads to an important reduction in the level of the protein matrix, and these procedures can be useful even for biological liquid samples. Additionally, the small polypeptides obtained after breaking down proteins do not affect the instrument stability, and a high degree of long-term stability can be achieved when using inductively coupled plasma techniques.

The aim of the current work has been the investigation of the effects of different enzymes (pepsin, pancreatin, trypsin, lipase and α-amylase) on the releasing of trace elements from mussel soft tissue. A systematic study of the variables affecting the enzymatic hydrolysis of mussel soft tissue was done by using an experimental design approach. In addition, the proposal of optimal enzymes mixtures to release most of the elements from mussel soft tissue was discussed too.

2. Experimental

2.1. Apparatus

Optima 3300 DV inductively coupled plasma-atomic emission spectrometer (Perkin-Elmer, Norwalk, USA) equipped with an autosampler AS 91 (Perkin-Elmer). Incubation camera model Boxcult from Selecta (Barcelona, Spain) with controlled temperature range between 5 and 57 °C (temperature homogenization of ±2.5% and temperature stability of ±0.5%), coupled with an agitator Rotabit with orbital and horizontal shaker and speed control in the 20–230 rpm range (Selecta). Centrifuges Centromix and Centronic (Selecta). ORION 720A plus pH-meter with a glass-calomel electrode (ORION, Cambridge, UK). Samsung domestic microwave oven (Seoul, Korea), programmable for time and microwave power. 25 ml laboratory-made PTFE closed decomposition vessels, suitable for work at low pressures. Statgraphics Plus V 5.0 for Windows, 1994-1999 (Manugistics Inc., Rockville M.D) chemometrics package.

2.2. Reagents

All chemicals used were of ultrapure grade and laboratory water was of resistance 18 MΩ cm^{-1} (Millipore Co., Bedford, MA, USA). Multi-element standard solutions, 1.000, or 10.000 g l^{-1} (Merck, Poole, Dorset, UK). Trypsin, isolated from porcine pancreas, and α-amylase, isolated from *Bacillus subtilis*, (Merck). Pepsin, isolated from porcine stomach mucosa, and pancreatin, from porcine pancreas, (Sigma, St. Lois MO, USA). Lipase Type VII, isolated from *Candida rugosa*, (Sigma). AnalaR nitric acid 70.0% and hydrochloric acid, 37% (J.T. Baker B.V., Deventer, Holland). Sodium hydroxide, potassium dihydrogen phosphate, potassium hydrogen phosphate and sodium chloride (Merck). DORM-1 (dog-fish muscle), DORM-2 (dog-fish muscle) and TORT-1 (lobster hepatopancreas) certified reference materials (National Research Council of Canada).

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

2.3. Mussel samples

Fresh mussel samples (*Mytilus galloprovincialis*) were collected from mussel farms at Ría de Arousa estuary. All studies were performed using all mussel soft tissue (muscle and gill) by preparing a pool with all the mussel soft tissues from each mussel farm. Mussel soft tissue from mussels collected at each location were mechanically blended, homogenized, and lyophilized. Dry mussel soft tissue was subjected to a pulverization process using a vibrating ball mill (mean particle size around 30 μm after measuring by laser diffraction spectrometry) and they were kept into polyethylene bottles with hermetic seals inside a dessicator and at room temperature.

2.4. Microwave acid digestion

Dry mussel soft tissue were subjected to an optimized microwave acid digestion procedure (details given in reference [11]). Additionally, different analytical blanks were prepared in order to known possible contamination of samples

and to determine detection and quantification limits after microwave-assisted acid digestion.

2.5. Enzymatic hydrolysis procedure

Around 0.2 g of mussel soft tissue was weighted into the centrifuge tubes and 20 mg of enzyme (lipase, trypsin and pancreatin) dissolved in 7 ml of the potassium dihydrogen phosphate/potassium hydrogen phosphate (PDHP/PHP) buffer solution (0.1 M at pH 9 for As, Cd, Cu, Fe and Ni or 0.5 M at pH 6 for Al, Cr, Mn, Pb and Zn) was added. The mixtures were incubated at 37 °C with an orbital–horizontal shaking at 200 or 50 rpm (trypsin or pancreatin, respectively) for 24 h. After centrifugation at 3000 rpm for 15 min, the supernatant was made up to 10 ml.

The experimental procedure was similar when using α-amylase: around 0.2 g of mussel soft tissue, 20 mg of enzyme dissolved in 7 ml of the potassium dihydrogen phosphate/potassium hydrogen phosphate (PDHP/PHP) buffer solution (0.1 M at pH 9 for As, Cd, Cu, Fe and Ni or 0.5 M at pH 6 for Al, Cr, Mn, Pb and Zn), but the incubation (37 °C and 50 rpm for the orbital–horizontal shaker) was carried out for 6 h.

Finally, the procedure for pepsin uses a hydrochloric acid/sodium chloride solution at pH 1 and sodium chloride concentration at 0.2 M. The enzymatic hydrolysis was carried out at 37 °C with an orbital–horizontal shaking at 50 rpm for 6 h. The extracts were made up to 10 ml after a centrifugation stage.

Analytical blanks (buffer solution and enzyme) were obtained to know the possible metal contamination and to determine the detection and quantification limits. In addition, blanks (buffer solution and sample) were performed to know the amount of metal extracted by the action of the buffer media.

2.6. ICP-AES measurements

The elements were determined in the acid and enzymatic digests by ICP-AES (axial configuration) without dilution, using the operating conditions and emission wavelength lines given in Table 1. Preliminary studies on the use of scandium as internal standard revealed lack of accuracy for some elements. In addition, the use of aqueous calibration led to non accurate results. Therefore, five points—standard addition, covering analyte concentration ranges between 0 and 10 mg l^{-1} for Al, Cu, Fe, Mn and Zn (0, 2.5, 5.0, 7.5 and 10.0 mg l^{-1}) and between 0 and 2 mg l^{-1} for As, Cd, Cr, Ni and Pb (0, 0.25, 0.50, 1.00 and 2.00 mg l^{-1}), was used to carry out the measurements. For all measurements, r^2 values higher than 0.995 were obtained.

2.7. Variables affecting the enzymatic hydrolysis of mussel soft tissue

It is well-known that enzymes show optimum operating temperature and pH conditions, implying an increase of the rate of enzyme denaturizing at high temperatures and a change in the ionic state of the amino acids residues of the enzyme in function of the pH [14]. These two variables, temperature and pH, were investigated together with the ionic strength, the enzyme mass, the reaction volume, the incubation (enzymatic hydrolysis) time and the speed of the orbital–horizontal shaker. The variable pH was fixed by using a PDHP/PHP buffer solution for all enzymes, except when using pepsin, for which hydrochloric acid was used. The variable *ionic strength* is related to the concentration of the buffer system used to fix the pH. Since PDHP/PHP was used as buffer solution for all enzymes, except for pepsin, the ionic strength was related to the concentration of PDHP/PHP. However, as the extreme acidic pH needed when using pepsin, the ionic strength was studied by using different sodium chloride concentrations. An eighth variable, called *dummy*, was also considered in the study. Dummy factors are imaginary variables for which the change from one level to another is not supposed to cause any physical change [15].

Table 1
Operating conditions for ICP-AES

Radiofrequency power (W)	1300
Gas flows (l min^{-1})	
Plasma	15.0
Auxiliary	0.5
Nebulizer	0.8
Nebulizer type	Cross flow
Peristaltic pump speed (ml min^{-1})	1.5
Stabilisation time (s)	45
Number of replicates	4
Detection wavelengths (nm)	
Al	396.153
As	197.197
Cd	228.802
Cu	327.393
Cr	267.716
Fe	238.204
Mn	257.610
Ni	231.604
Pb	224.688
Zn	206.200

The response variables were the percentage of metal recovery, according to the following equation:

$$\%\text{Recovery} = \frac{C_{\text{enzymatic hydrolysis}}}{C_{\text{acid digestion}}} \times 100 \quad (1)$$

where $C_{\text{enzymatic hydrolysis}}$ is the metal concentration obtained after the enzymatic hydrolysis procedure and $C_{\text{acid digestion}}$ is the metal concentration found after an acid digestion assisted by microwave energy. As ten metals are studied, ten response variables will be obtained.

Table 2
Experimental field definition for the Plackett–Burman design[a]

	Variables	Low level (−)	High level (+)
Enzyme mass (mg)	*W*	5	20
Reaction volume (ml)	*V*	3	7
Hydrolysis temperature (°C)	*T*	25	50
Hydrolysis time (h)	*t*	6	24
Orbital–horizontal shaker speed (rpm)	*S*	50	200
pH[b]		6	9
Ionic strength (sodium dihydrogenphospahte concentration) (M)[c]	IS	0.05	0.20
Dummy factor	*D*	−1	+1

[a] The mussel soft tissue mass was 0.20 g for all experiments.
[b] The low and high levels were 1.0 and 4.0 when using pepsin.
[c] The ionic strength were related to sodium chloride at 0.34 and 0.0 M for the high and low levels, respectively, when using pepsin.

2.8. Buffer blanks preparation

In order to determine the amount of metal released by leaching effect of buffer system solutions, the following blanks were prepared:

(1) a solution of 0.5 M PDHP/PHP at pH 9 (As, Cd, Cu, Fe, Ni and Zn) or 0.1 M PDHP/PHP at pH 6 (Al, Cr. Mn and Pb), subjected both to an incubation at 37 °C with an orbital–horizontal shaking speed of 50 rpm for 6 h (hydrolysis by α-amylase).
(2) a solution of 0.1 M PDHP/PHP at pH 9 (As, Cd, Cu, Fe, Ni and Zn) or 0.5 M PDHP/PHP at pH 6 (Al, Cr. Mn and Pb), subjected both to an incubation at 37 °C with an orbital–horizontal shaking speed of 200 rpm for 24 h (hydrolysis by lipase, trypsin and pancreatin)
(3) a solution of hydrochloric acid/0.2 M sodium chloride solution (pH 1) subjected to an incubation at 37 °C with an orbital–horizontal shaking speed of 50 rpm for 6 h (hydrolysis by pepsin for all elements).

3. Results and discussion

3.1. Plackett–Burman design to study significant variables affecting enzymatic hydrolysis procedures

The effects of changing these variables from a low value to a high value were simultaneously studied by applying a Plackett–Burman design (PBD) as a screening method [16]. Therefore, five PBDs ($2^8 \times 3/64$, type III resolution design, for 8 factors, 4 d.f., 12 runs and 2 replicates) were constructed, one PBD for each enzyme type investigated (pepsin, trypsin, pancreatin, lipase and amylase). All experiments were carried out by duplicate with 0.25 g of a lyophilized mussel soft tissue. Table 2 lists the low (−) and high (+) levels of each variable for the PBDs. The minimum and maximum values for some variables (pH, temperature and ionic strength) were chosen taking into account the possibility of enzyme inactivation or denaturation at some conditions. The experimental conditions offered by the PBD when using trypsin are shown in Table 3 (similar matrices, not given, were obtained when using the other enzymes).

Table 3
Plackett–Burman design for the significant variables determination when using trypsin

	Variables								Metal recovery (%)									
	pH	IS	*V*	*W*	*T*	*S*	*t*	*D*	As	Al	Cd	Cu	Cr	Fe	Mn	Ni	Pb	Zn
1	+	−	+	−	−	−	+	+	60	1	46	32	23	5	15	36	36	3
2	+	+	−	+	−	−	−	+	62	1	37	33	6	4	17	56	57	6
3	−	+	+	−	+	−	−	−	34	1	52	40	28	3	16	29	66	12
4	+	−	+	+	−	+	−	−	63	1	64	40	7	5	15	61	22	6
5	+	+	−	+	+	−	+	−	62	1	37	23	59	4	19	58	60	9
6	+	+	+	−	+	+	−	+	56	1	68	33	74	3	12	56	36	1
7	−	+	+	+	−	+	+	−	40	5	54	29	67	2	18	38	61	35
8	−	−	+	+	+	−	+	+	50	3	52	38	27	6	25	54	66	24
9	−	−	−	+	+	+	−	+	50	3	43	20	17	4	8	62	69	22
10	+	−	−	−	+	+	+	−	63	1	45	16	7	7	21	101	63	9
11	−	+	−	−	−	+	+	+	46	3	49	37	16	3	38	59	65	29
12	−	−	−	−	−	−	−	−	54	4	40	33	18	5	26	60	60	32

The metal recoveries given are the mean of two independent experiments.

The values for the response variables (metal percentage) are also listed in Table 3.

Table 4 shows the statistically significant effect (95% confidence interval) of each variable on the releasing of trace metals from mussel tissue when using each enzyme type. The negative sign means that the change of the variable from the low level to the high level produces a decrease on the efficiency of the metal releasing, while a positive sign means that this change increases such efficiency. From Table 4, it can be observed that there are a few variables affecting the enzymatic hydrolysis by pancreatin, while the number of significant factors increases when using trypsin, pepsin, lipase and α-amylase. This fact can be explained taking into account that pancreatin is an enzyme extracted from porcine pancreas and it is available without purification. Therefore and according to the United States Pharmacopeias (USP), pancreatin isolated from porcine pancreas is a mixture of different enzymes, mainly trypsin, lipase and α-amylase [17]. Since these three main enzymes catalyze very different bounds, it appears that the combined effect by them leads to high metal extraction yields when using very different enzymatic conditions.

The variables *enzyme mass* (*W*) and *reaction volume* (*V*) are non significant factors for most of the elements and enzymes, except for trypsin. Anyway, if these two factors are significant, their effects on the metal releasing is always positive, meaning that metal extraction is higher when increasing both.

The variable *hydrolysis time* (*t*) was found as non significant for most of the elements and enzymes, specially when using pancreatin, pepsin and α-amylase. This fact could be attributed to inactivation of the enzymes or lack of stability after a certain time. Additionally, chemical equilibriums between unhydrolyzed proteins and hydrolyzed derivates from proteins are established after a certain time, and a decrease on enzymatic hydrolysis can be expected. This means that although longer hydrolysis times are reported in literature, an efficient metal releasing can be achieved with short times.

The variable *orbital–horizontal shaking speed* (*S*) has resulted non significant for most of the cases, and it offered a negative effect when it is significant.

Although the variable *incubation temperature* (*T*) could be though as a very important variable, its statistical significance was little, and it can be considered as non significant when hydrolyzing mussel soft tissue with trypsin and pancreatin. The statistical significance of this variable increases when using pepsin (positive effect for As and Cu, and negative effect for Cr), lipase (negative effect for releasing As, Fe and Ni) and α-amylase (negative effect for Al, Cu, Fe, Ni, Pb and Zn).

The pH and the *ionic strength* (IS) were the most statistically significant variables, and their effect could be positive or negative in function of the enzyme type, and mainly, in function of the released element. The significance of the ionic strength is attributed to the fact that enzymatic catalysis depends on the movement of charged molecules relative

Table 4
Effects of changing values from low to high levels

	W	*V*	*T*	*t*	*S*	pH	IS	*D*
Trypsin								
Al	+	+	NS	NS	NS	NS	NS	NS
As	NS	+	NS	+	NS	NS	+	NS
Cd	+	+	NS	NS	NS	+	+	NS
Cu	NS	+	NS	NS	NS	+	+	NS
Cr	+	+	NS	NS	NS	+	+	NS
Fe	NS	NS	NS	+	NS	+	NS	NS
Mn	NS	+	NS	NS	NS	−	−	NS
Ni	NS	+	NS	+	NS	−	+	NS
Pb	NS	+	NS	+	NS	NS	−	NS
Zn	NS	+	NS	NS	NS	NS	NS	NS
Pancreatin								
Al	NS	NS	NS	NS	NS	NS	NS	NS
As	+	NS	NS	NS	−	+	NS	NS
Cd	NS	+	NS	NS	−	NS	NS	NS
Cu	NS	NS	NS	+	−	+	NS	NS
Cr	NS	+	NS	NS	NS	NS	+	NS
Fe	NS	NS	NS	NS	NS	+	NS	NS
Mn	NS	+	NS	NS	NS	−	NS	NS
Ni	NS	+	+	NS	NS	NS	NS	NS
Pb	NS	NS	NS	NS	−	NS	−	NS
Zn	NS	+	NS	NS	NS	NS	−	NS
Pepsin								
Al	NS	NS	NS	NS	NS	−	+	NS
As	+	NS	+	−	−	−	−	NS
Cd	NS	NS	NS	NS	NS	NS	NS	NS
Cu	NS	+	+	NS	NS	−	NS	NS
Cr	NS	NS	−	NS	NS	−	NS	NS
Fe	NS	NS	NS	NS	NS	−	+	NS
Mn	NS	NS	NS	NS	−	NS	NS	NS
Ni	NS	NS	NS	NS	−	NS	+	NS
Pb	NS	+	NS	+	−	−	NS	NS
Zn	NS	NS	NS	NS	−	NS	NS	NS
Lipase								
Al	−	+	NS	NS	NS	−	NS	NS
As	NS	NS	−	NS	NS	+	−	NS
Cd	NS	+	NS	NS	NS	NS	+	NS
Cu	NS	NS	NS	NS	NS	NS	+	NS
Cr	NS	NS	+	NS	NS	NS	+	NS
Fe	NS	NS	−	NS	NS	+	NS	NS
Mn	NS	NS	NS	+	NS	−	NS	NS
Ni	+	+	−	NS	NS	+	−	NS
Pb	+	NS	NS	+	NS	−	−	NS
Zn	−	NS	NS	+S	NS	−	−	NS
α-Amylase								
Al	NS	NS	−	NS	NS	−	NS	NS
As	+	NS	NS	NS	NS	+	NS	NS
Cd	NS	NS	NS	−	NS	+	+	NS
Cu	NS	NS	−	+	NS	+	−	NS
Cr	NS	+	+	−	−	NS	+	NS
Fe	NS	+	−	NS	NS	+	NS	NS
Mn	NS	−	NS	NS	NS	−	NS	NS
Ni	+	+	−	NS	−	NS	+	NS
Pb	NS	NS	−	NS	−	+	NS	NS
Zn	+	NS	−	NS	NS	−	NS	NS

Positive sign (+) means that the effect of the variable on the response is positive. Negative sign (−) means that the effect of the variable on the response is negative. NS means non-significant.

Table 5
Experimental field definition for the central composite design[a]

Variable	High level (+)	Low level (−)	Central level	High star level (+)	Low star level (−)
Pepsin					
Hydrolysis temperature (°C)	50	30	40	56.8	23.2
pH[b]	4.0	1.5	2.75	4.85	0.65
Ionic strength (sodium chloride concentration) (M)	0.3	0.1	0.2	0.4	0.02
Trypsin, pancreatin, α-amylase and lipase					
Hydrolysis temperature (°C)	50	30	40	56.8	23.2
pH[b]	9	6	7.5	10	5
Ionic strength (PDHP/PHP concentration) (M)	0.5	0.2	0.35	0.6	0.1

[a] The mussel soft tissue sample mass was 0.2 g for all experiments.
[b] The low and high levels were 1.0 and 4.0 when using pepsin.

to each other. Thus, both the binding of charged substrates to enzyme and the movement of charged groups within the catalytic active site is influenced by the ionic composition of the medium. This influence appears to be more important than the temperature.

Finally, the *dummy factor* (*D*) is not significant for any case and this means that there are not any systematic error neither unknown variables affecting the system under study.

Therefore, variables such as pH, ionic strength and hydrolysis temperature were considered for a further optimization process, while the variables less significant (enzyme mass, reaction volume, hydrolysis time and orbital–horizontal shaking speed) were fixed at convenient values in accordance with the negative or positive effect for each enzyme. These values were 7 ml for the reaction volume; 20 mg for enzyme mass, except when using lipase (5 mg); 24 h for the enzymatic time, except for pepsin and α-amylase (6 h) and 50 rpm for the orbital–horizontal shaking, except for trypsin and lipase (200 rpm).

3.2. Optimization of significant variables by central composite designs

The significant variables were simultaneously optimized by applying a central composite design [18] (CCD) 2^3 + star with 5 error d.f., 1 center, 2 replicates and 32 runs. Since pepsin offers the best catalytic action at low pH, which implies the use of sodium chloride to fix the ionic strength of the medium, a CCD was constructed for optimize the mussel soft tissue hydrolysis by pepsin. The rest of enzymes were optimized by using the same CCD, which upper and lower values (cube points), and star values (star points) and central values (central points) listed in Table 5. A CCD matrix for the use of pepsin is shown in Table 6. The response variables (percentages of released metals) are also listed in the table. After performing the CCD experiments, the statistical evaluation of quadratic terms have resulted significant for all cases, which implies responses surfaces with curvature, some of them shown in Fig. 1.

Table 6
Central 2^3 + star orthogonal composite design ($n = 32$) for pepsin

Run	Variables			Metal recovery (%)									
	pH	IS (M)[a]	*T* (°C)	Al	As	Cd	Cu	Cr	Fe	Mn	Ni	Pb	Zn
1	2.75	0.22	40.00	8	61	51	41	21	9	82	16	50	69
2	1.50	0.10	30.00	16	66	52	76	17	16	87	23	87	97
3	4.00	0.10	30.00	6	57	44	36	17	8	73	11	45	60
4	1.50	0.34	30.00	36	65	49	44	20	16	87	21	51	91
5	4.00	0.34	30.00	7	56	43	38	17	8	73	15	45	57
6	1.50	0.10	50.00	19	67	50	44	19	17	79	19	55	84
7	4.00	0.10	50.00	6	50	43	36	17	7	72	13	43	58
8	1.50	0.34	50.00	18	63	52	42	19	21	78	18	50	87
9	4.00	0.34	50.00	7	60	43	35	17	7	74	13	45	57
10	0.65	0.22	40.00	9	62	47	41	71	11	75	13	49	72
11	4.85	0.22	40.00	5	50	42	34	16	8	68	8	40	54
12	2.75	0.02	40.00	16	59	48	39	18	11	87	16	48	64
13	2.75	0.42	40.00	12	62	48	40	18	9	77	45	46	64
14	2.75	0.22	23.18	8	58	47	41	18	54	76	14	46	98
15	2.75	0.22	56.82	8	63	45	38	18	9	75	15	47	61
16	2.75	0.22	40.00	8	60	54	42	21	10	81	15	50	68

Metal recoveries given are the mean of 2 replicates.
[a] Sodium chloride concentration.

3.2.1. Enzymatic hydrolysis of mussel soft tissue by pepsin

The careful study of the results from the CCD revels that all elements are released from mussel soft tissue under the same pH, temperature and ionic strength conditions. These conditions are a pH of 1, an incubation temperature of 37 °C and sodium chloride at 0.2 M).

3.2.2. Enzymatic hydrolysis of mussel soft tissue by trypsin, pancreatin, lipase and α-amylase

From Fig. 1 it can be seen that metals are extracted from mussel soft tissue under two different operating conditions. The first conditions set is the enzymatic hydrolysis at pH 6, fixed by using PDHP/PHP at 0.5 M, and at 37 °C as incubation temperature. Under these conditions, metals such as Al, Cr, Mn, Pb and Zn are released from mussel soft tissue, some of then at a 100% yield. The second set of conditions involves the use of a higher pH and a lower concentration for the buffer system: a pH at 9, fixed with 0.1 M PDHP/PHP, and an incubation temperature of 37 °C. The metal released under these conditions are As, Cd, Cu, Fe and Ni. The different behavior of the released elements at different pH and ionic strengths could be related to other chemical equilibriums occurring at the same time that the enzymatic hydrolysis. Such equilibriums could be the formation of hydroxide complexes which lead to an increase on the metal releasing at certain conditions when working at low pH (pH 6 instead than pH 9).

The optimized hydrolysis conditions for each enzyme were applied to a mussel soft tissue three times and the metal percentage extracted by using each enzyme is shown in Table 7. It can be seen that an enzymatic hydrolysis with trypsin leads to quantitative yields for As, Cd and Cr. Pepsin and pancreatin, other two proteolytic enzymes, can be successfully used to extract Zn, and Cr and Ni, respectively. Finally, the enzymatic digestion with α-amylase and lipase offer quantitative results for Cr. The relative easiness to extract Cr using all enzymes, even lipase and α-amylase, leads to think that Cr is not bound to any particular cytosolic structure.

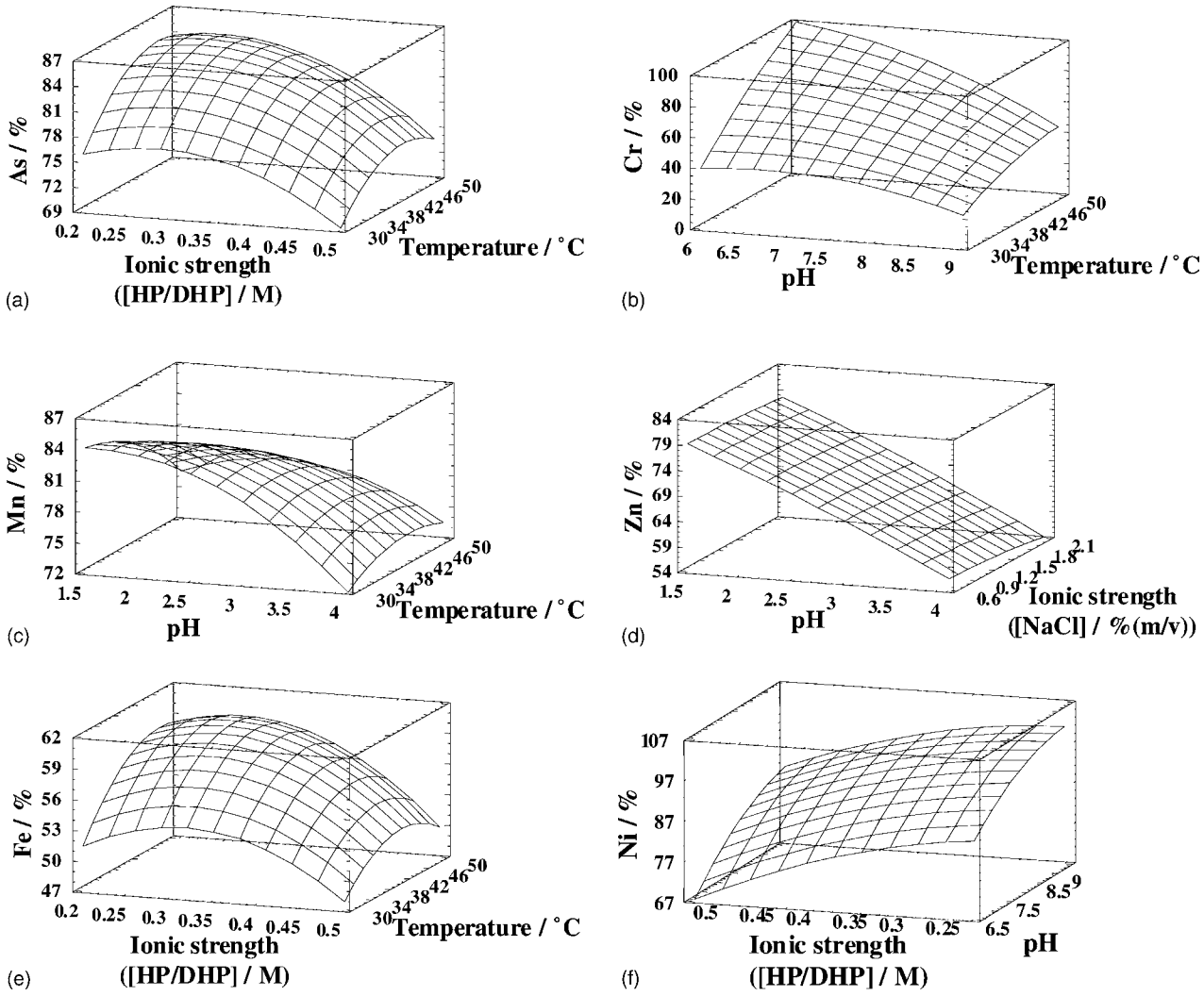

Fig. 1. Some estimated response surfaces from the central composite design: trypsin (a, b), pepsin (c, d) and pancreatin (e, f).

Table 7
Mean metal percentages by enzymatic hydrolysis and by buffer solutions (PDHP/PHP, pH 6, PDHP/PHP, pH 9 and hydrochloric acid/sodium chloride, pH 1)

	Metal percentage (%) ($n = 3$)									
	Pepsin		Trypsin		Pancreatin		Lipase		α-amylase	
	E[a]	B[b]	E[a]	B[c]	E[a]	B[c]	E[a]	B[c]	E[a]	B[d]
Al	36	14	23	3	26	3	15	3	13	3
As	69	36	92	27	79	27	74	27	68	40
Cd	54	14	96	26	80	26	62	26	70	35
Cu	77	20	72	18	50	18	29	18	50	13
Cr	73	21	108	9	104	9	104	9	105	5
Fe	55	20	34	8	64	8	62	8	48	10
Mn	88	43	81	24	49	24	62	24	38	28
Ni	46	21	38	8	105	8	66	8	84	49
Pb	89	34	81	14	84	14	30	14	65	46
Zn	98	43	75	10	23	10	14	10	17	10

[a] With enzyme under optimum conditions.
[b] Without enzyme (hydrochloric acid/0,2 M sodium chloride, 37 °C, 50 rpm for 6 h).
[c] Without enzyme (0.5 M PDHP/PHP at pH 6, 37 °C, 200 rpm for 24 h, for Al, Ba, Cr, Mn, Pb and Zn; and 0.1 M PDHP/PHP at pH 9, 37 °C, 200 rpm for 24 h, for As, Cd, Cu, Fe and Ni).
[d] Without enzyme (0.5 M PDHP/PHP at pH 6, 37 °C, 50 rpm for 6 h for Al, Ba, Cr, Mn, Pb and Zn; and 0.1 M PDHP/PHP at pH 9, 37 °C, 50 rpm for 6 h, for As, Cd, Cu, Fe and Ni).

3.3. Effect of the buffer solution

A study on the effect of the buffer solution on the extraction of metals from the samples was also considered. Since three different buffer systems were used and optimum orbital–horizontal shaking speed and hydrolysis time were different for some enzymes and metals, five different blanks (given in Section 2.8.) were prepared and analyzed. Table 7 shows the result obtained, expressed as metal percentages, where it can be seen the metal leached by the buffer solution can reach a 40% (As, buffer system conditions for α-amylase), while the metal extraction by the buffer solution is unimportant in some cases, as Cr using buffer solution and conditions for α-amylase, lipase, trypsin and pacreatin, and Ni using buffer solution and conditions for pacreatin (metal extraction lower than 5%). The highest metal extraction yields are those related with a buffer solution for pepsin (hydrochloric acid and sodium chloride). This fact is easily expected because of hydrochloric acid has been reported as an excellent agent to extract metals from biological samples [11].

3.4. Comparison to manufacture standard conditions

A comparison between the released elements concentrations by using the optimized conditions for each enzyme and the standard conditions recommended by the manufacture for each enzyme was established. These standard conditions were a pH of 2 and a temperature of 37 °C for pepsin; pH and temperature of 8 and 25 °C, respectively, for trypsin; pH and temperature of 6 and 25 °C, respectively, for α-amylase; a pH of 7.2 and a temperature of 37 °C for lipase; and finally, pH and temperature at 7.5 and 40 °C, respectively, for pancreatin. Other variables affecting the process were fixed at convenient values. Results, plotted in Fig. 2, show that only Cr is quantitatively extracted (94.0%) when using α-amylase, otherwise, the metal extraction yields were considerably lower than those obtained when using optimized conditions. It has been proved that the optimization of enzymatic hydrolysis conditions is needed to guarantee quantitative metals extractions.

3.5. Mixture of enzymes

Since similar incubation temperature, pH and ionic strength conditions have been found for some enzymes (trypsin, pancreatin, α-amylase and lipase) for releasing some elements, the possibility of using a mixture of enzymes was considered. Several combinations between a protease enzyme (trypsin or pancreatin) and α-amylase and lipase were added to samples, whose were incubated at optimum enzymatic hydrolysis conditions. These mixtures were chosen taking in mind that the enzymes did not offer antagonist effects. Results are listed in Table 8, where it can be seen that elements such as As, Cd and Cr, which were quantitatively extracted by trypsin, are now extracted with trypsin/α-amylase and trypsin/lipase combination, and with pancreatin/α-amylase too. Ni, which was efficiently released from mussel soft tissue by pancreatin, is quantitatively extracted by pancreatin/α-amylase and pancreatin/lipase. Elements such as Mn and Pb, whose were not quantitatively extracted by pancreatin or trypsin, are now extracted by the mixture trypsin/lipase, trypsin/α-amylase or pancreatin/α-amylase. This fact can be explained taking in mind that each enzyme type catalyzes a certain chemical bond in certain biomolecules, and metals could be bound to a huge biomolecules types.

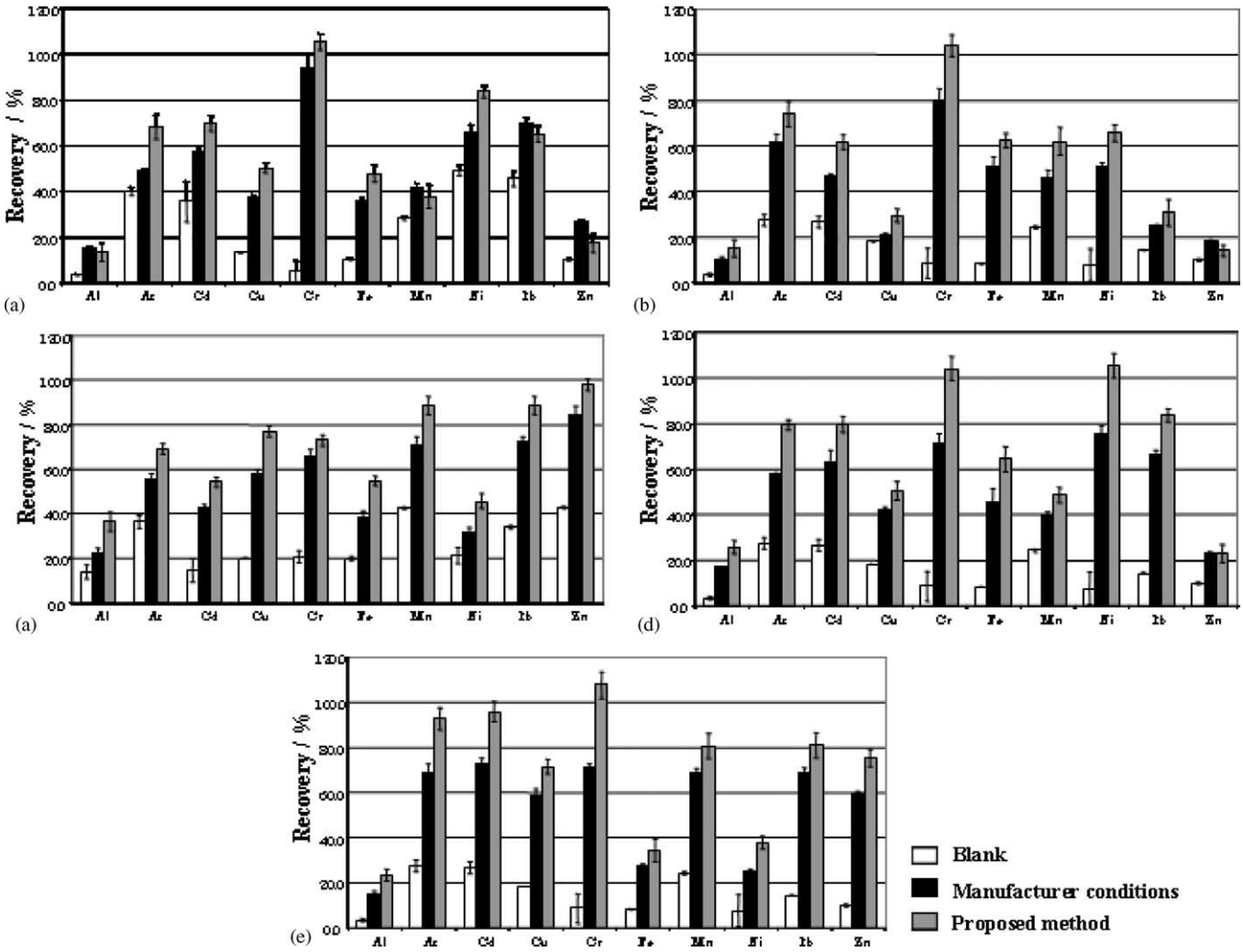

Fig. 2. Recovery of metals obtained by using the optimized enzymatic procedure (■) and the operating conditions recommended by the manufactures (■) for α-amylase (a); lipase (b); pepsin (c); pancreatin (d) and trypsin (e).

However, there was not found any increase on the Al, Cu, Fe and Zn releasing for the use of any enzyme mixture. These four elements were not quantitatively extracted by action of any enzyme alone, except Zn when using pepsin. That could be because other chemical equilibriums, presumably formation of hydroxide complexes, could occur when these metals are being released from the different cytosolic structures.

Table 8
Metal percentages by mixture of enzymes

	Metal percentage (%) (n = 3)			
	Trypsin + α-amylase	Trypsin + lipase	Pancreatin + α-amylase	Pancreatin + lipase
Al	30 ± 2	28 ± 4	27 ± 2	25 ± 2
As	94 ± 5	96 ± 7	89 ± 4	83 ± 4
Cd	88 ± 6	92 ± 5	91 ± 6	86 ± 4
Cu	70 ± 4	65 ± 3	58 ± 2	61 ± 4
Cr	98 ± 5	91 ± 2	104 ± 11	101 ± 4
Fe	41 ± 2	36 ± 1	71 ± 3	65 ± 5
Mn	87 ± 2	93 ± 5	57 ± 3	52 ± 3
Ni	44 ± 3	41 ± 2	93 ± 6	88 ± 9
Pb	90 ± 5	87 ± 4	91 ± 9	85 ± 4
Zn	84 ± 4	79 ± 4	39 ± 2	28 ± 2

The ratio of enzymes was 1:1 (20/20 mg each one).

Table 9
Certified and found concentration values of elements determined in three certified reference materials

	DORM-1		DORM-2		TORT-1	
	Certified value ($\mu g\,g^{-1}$)	Found value ($\mu g\,g^{-1}$)	Certified value ($\mu g\,g^{-1}$)	Found value ($\mu g\,g^{-1}$)	Certified value ($\mu g\,g^{-1}$)	Found value ($\mu g\,g^{-1}$)
Enzymatic hydrolysis by trypsin						
As	17.7 ± 2.1	16.0 ± 0.4	18.0 ± 1.1	17.1 ± 0.7	24.6 ± 2.2	21.4 ± 0.7
Cd	0.086 ± 0.012	–[b]	0.043 ± 0.008	–[b]	26.3 ± 2.1	25.0 ± 1.1
Cu	5.22 ± 0.33	3.70 ± 0.20	2.34 ± 0.16	2.17 ± 0.07	439 ± 22	121 ± 12
Cr	3.60 ± 0.40	2.65 ± 0.25	–[a]	4.93 ± 0.20	2.4 ± 0.6	2.8 ± 0.3
Mn	1.32 ± 0.26	1.44 ± 0.06	3.66 ± 0.34	2.66 ± 0.26	23.4 ± 1.0	19.9 ± 0.4
Enzymatic hydrolysis by pancreatin						
Cr	3.60 ± 0.40	3.15 ± 0.18	–[a]	4.32 ± 0.48	2.4 ± 0.6	2.9 ± 0.2
Ni	1.20 ± 0.30	1.40 ± 0.12	19.4 ± 3.1	16.3 ± 0.6	2.3 ± 0.3	2.6 ± 0.1
Pb	0.40 ± 0.12	–[b]	0.065 ± 0.007	–[b]	10.4 ± 2.0	10.4 ± 0.4
Enzymatic hydrolysis by pepsin						
Cu	5.22 ± 0.33	4.40 ± 0.12	2.34 ± 0.16	2.26 ± 0.06	439 ± 22	120 ± 14
Cr	3.60 ± 0.40	3.10 ± 0.13	–[a]	5.22 ± 0.31	2.4 ± 0.6	2.9 ± 0.1
Mn	1.32 ± 0.26	1.56 ± 0.10	3.66 ± 0.34	3.17 ± 0.08	23.4 ± 1.0	20.4 ± 0.7
Pb	0.40 ± 0.12	–[b]	0.065 ± 0.007	–[b]	10.4 ± 2.0	9.0 ± 0.3
Zn	21.3 ± 1.0	21.4 ± 0.2	25.6 ± 2.3	22.3 ± 0.8	177 ± 10	167 ± 7

The enzymatic hydrolysis was carried out at optimized parameters. The statistical data are based on 4 measurements of 5 replicates.
[a] Not certified.
[b] <LOD.

3.6. Repeatability and accuracy

The repeatability of the over-all procedure has been assessed by preparing eleven times an enzymatic digest from a same mussel soft tissue sample using single enzymes. Each enzymatic digests was analysed once and the RSD values were lower than 10% for all cases (figures not given).

Accuracy of the proposed methods was assessed by analyzing different certified reference materials (CRM), DORM-1, DORM-2 and TORT-1. These CRMs were subjected to the optimized procedures five times, and the different metal concentrations were determined four times by ICP-AES. Results in Table 9 show that As, Cd and Cr concentrations in all CRMs studied are within the certified concentration ranges when using trypsin, except for Cd in DORM-1 and DORM-2, for which Cd concentration is lower than the LODs of the methods, and for Cr in DORM-1. Additionally, the Cu concentration in DORM-2 and Mn in DORM-1 agrees with certified contents when using trypsin, while accurate results have not been obtained when analyzing TORT-1 after enzymatic hydrolysis with trypsin or pepsin. Enzymatic hydrolysis by pancreatin leads to accurate results for Cr, Ni and Pb (Pb concentrations in DORM-1 and DORM-2 are lower than the LOQs of the methods). Enzymatic hydrolysis by pepsin leads to accurate results for Cr, Mn, Pb and Zn.

4. Conclusions

Enzymatic hydrolysis procedures lead to quantitative recoveries for many elements from mussel soft tissue, avoiding the use of corrosive chemicals and obtaining a non oxidizing solution in which the metals and organometallic species could not altered. The enzymatic digests can be easily analyzed by different analytical atomic techniques such as ICP-AES, inductively coupled plasma-mass spectrometry (ICP-MS), electrothermal atomic absorption spectrometry (ETAAS) or flame atomic absorption spectrometry (FAAS).

As mussel soft tissue is a proteinaceous material, the use of proteases (trypsin, pepsin and pancreatin) offers the highest percentages of released metals. However, there are some elements, such as Al, Cu, Fe and Zn, which do not offer quantitative recoveries by using some protease. This could be attributed to chemical reaction with hydroxyl radicals–ions (alkaline medium when using trypsin or pancreatin) and the main protein hydrolysis equilibrium could be affected. This assumption agrees with the fact that these metals are better released, even the extraction is quantitative for Zn, when using pepsin (this enzyme acts at an acid pH, around 1).

There are two different operating conditions when using trypsin and pancreatin: the first needs a low pH, around 6, and a high ionic strength and it results advantageous for releasing Cr, Mn, Pb and Zn. The second operation conditions uses a high pH (9) and low ionic strength and it is adequate to quantitatively extract As, Cd and Ni. Further studies would be desirable to elucidate the different metal releasing yields when using an enzyme at different pHs.

The percentage of released Cr is quantitative (recovery defined by Eq. (1) close to 100%) for the use of all enzymes except pepsin. The efficient Cr extraction when using α-amylase or lipase must be explained under the assumption

that most of the Cr is not bound to any cytosolic structure or biomolecule in cells.

The possibility of using different enzymes to hydrolyze certain biomolecules and to release the trace elements associated with is limited because the catalytic action of the different enzymes is overlay. This could be attributed to the fact that the products of the hydrolysis due to a certain enzyme can show enzymatic activity on other biomolecules.

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