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EVALUATION OF GEL ELECTROPHORESIS TECHNIQUES AND LASER ABLATION-INDUCTIVELY COUPLED PLASMA-MASS SPECTROMETRY FOR SCREENING ANALYSIS OF Zn- AND Cu-BINDING PROTEINS IN PLANKTON

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Abstract

The determination of metal-binding proteins in plankton is of relevant importance because of its role in the photosynthesis, fundamental in the biogeochemical cycle of the oceans and different ecosystems. We have elaborated a new strategy for the screening of Cu- and Zn containing proteins in plankton based on the separation of proteins by use of Blue-Native PAGE (BN-PAGE) consisting of a non-denaturing Tris-Tricine system and detection of metals in the proteins by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). For comparison, denaturing PAGE based on Tris-Glycine and Tris-Tricine systems and Anodic Native PAGE have been investigated too. A high number of proteins bands with MW between 20 and 75 kDa were obtained by using Tris-Glycine PAGE but the detection of metals by LA-ICP-MS was unsuccessful because of the lost of metals from proteins during separation process. Different protein extraction, purification and preconcentration methods were evaluated focused on both issues: to get the best extraction and characterization of proteins and to maintain the integrity of metal-protein bindings in the plankton sample. The use of 25 mM Tris/HCl and a protease inhibitor as extraction buffer with subsequent ultrafiltration and acetone precipitation was the most efficient means of sample preparation. Two Cu- and Zn- proteins were detected, a protein band corresponding to the MW of 60 kDa and another no well resolved band with MW between 15 and 35 kDa.

Keywords Gel electrophoresis, laser ablation, ICP-MS, metal-binding proteins, plankton

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Introduction

Plankton constitutes one of the major reservoirs of the atmospheric CO_2. Its CO_2 uptake capacity depends on the photosynthesis regulated by enzymatic reactions. It is estimated that about 30%-50% of the CO_2 that is currently emitted is taken up by the ocean. The amount of CO_2 that stays in the ocean for longer time scales is important, because this effect mitigates the rising CO_2 concentration in the atmosphere, influencing the climate change [1, 2].

Plankton is exposed to many metals present in their environment. Trace metals are included in plankton biochemical cycles and may have significant effects on the various trophic levels in aquatic food webs. Some metals (Cu, Zn, Fe), are essential micronutrient for phytoplankton communities and are involved in enzymatic reactions that affect photosynthesis. Others (Pb, Hg, Cd) may displace essential trace metals and interfere in proper functioning of enzymes. The role of these metals is fundamental in the biogeochemical cycle of the oceans and the ecological success of different ecosystems [3]. For example, carbonic anhidrase plays an important role in the inorganic CO_2 uptake for the photosynthesis in phytoplankton. Carbonic anhydrase is a Zn enzyme but in the oceans, where Zn is nearly depleted, marine diatoms use Cd or Co as a catalytic metal atom [4]. Other Zn metalloenzymes such as alkaline phosphatase, which allows phytoplankton to acquire P from organic compounds, also suffer this replacement of one essential element by another [5].

In accordance with the above mentioned the determination of metal-proteins in plankton is of relevant importance and still an analytical challenge. The species are often unstable and concentrations are so low, even in the sub-ng g^{-1} level in some cases. For this reason, sensitive and selective analytical techniques should be used. One- or two-dimensional polyacrylamide gel electrophoresis (1D- or 2D-PAGE) with subsequent laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) has been used increasingly for metalloproteins analysis as it has been reviewed [6-11].

In metalloproteins analysis is very important to maintain the integrity of metal-protein binding during all the process. When the elements are part of the proteins and are strongly bound to them, the binding is not broken during electrophoresis process. However, metal-binding proteins in which metal-protein interactions are of lower affinity can lose the metal during electrophoresis process including sample preparation and post-separation procedures [9]. Many authors recommend the use of Native-PAGE instead of denaturing PAGE, to avoid metal-protein binding losses during electrophoresis separation [12-15]. Raab et al. [16] have evaluated the gel electrophoresis conditions for the separation of metal-tagged proteins by GE-LA-ICP-MS, focusing on the stability of metal-protein binding during GE and post-separation methods. Regarding to post-separation process, they found that staining of the gel prior to LA-ICP-MS is not recommended, since most protein-bound metal is lost during the staining procedure (except with covalently bound), and should be avoided prior to ablation.

In previous investigations [17-18], we have demonstrated that the detection of metals bound to the proteins depends, not only on the nature of the electrophoretic process (naturing or non-denaturing) and post-separation gel treatment, but also on the trailing ion chosen and current applied in the electrophoretic method used. We studied some aspects of the separation process in an attempt to find the suitable

electrophoretic procedure for two proteins with different metal-protein affinity (superoxide dismutase containing Cu and Zn, and alcohol dehydrogenase containing Zn) to maintain the integrity of the metal-protein complex and detect successfully the metal bound to each protein. Different electrophoretic techniques both denaturing and non-denaturing were applied. In the denaturing ones, tricine was the best option as trailing ion due to the fact that it seems to have less affinity for Cu and Zn than glycine and preserves better the metal-protein binding due to a slower movement of protein. With respect to current used, it was observed that as higher current is applied the possibility of metal-protein binding losses is higher. Non-denaturing conditions were demonstrated to be more suitable for the later determination of Zn-containing proteins by LA-ICP-MS, being BN-PAGE (based in a Tris/Tricine system) the best electrophoretic procedure for metals such as Zn non-covalently complexed by proteins.

One of the steps of vital importance to obtain reproducible and well resolved results in electrophoresis process is the sample preparation. Recently Schmidt et al. have studied the influence of protein sample preparation and electrophoresis running conditions on metal-protein bindings by electrospray ionization mass spectrometry (ESI-MS), ICP-MS and ultrafiltration [19]. They studied the effect of protein preparation procedure (based on extraction, precipitation, washing and desalting) and 1D- and 2D-PAGE buffer systems on metal-protein-bindings for different pure proteins to which heavy metals such as Cu(II) and Zn(II) were previously added. They found that nearly all buffer systems used in PAGE process changed the metal binding state of the proteins. Especially an SDS containing protein extraction buffer, the 1D-PAGE running buffer, and gel-staining solutions suppressed the metal binding capacities of egg lysocyme protein (Lys). Acetone can be recommended as precipitating agent whereas trichloroacetic acid (TCA) and ammonium sulphate strongly diminish the metal binding capacity of produced protein fractions. In the ESI-MS measurements, the acetate salts of the metals led to the highest binding stoichiometries of metal/proteins complexes compared to other salts.

The objective of this work is to develop an analytical methodology for successful screening of metal-containing proteins in plankton. To this aim, the influence of the different steps in the electrophoresis process (sample preparation, separation, post-separation treatments) with subsequent LA-ICP-MS metals detection was evaluated. The key steps are focused on both issues: to get the best extraction and characterization of proteins and to maintain the integrity of metal-protein bindings in a certified plankton sample used as model system.

Experimental

Instrumentation

Gel electrophoresis was carried out with a MiniProtean® 3 electrophoresis cell connected to a PowerPac™ basic power supply. The gels were vacuum dried prior to LA-ICP-MS measurements with a model 583 gel dryer. All the electrophoretic instrumentation was from Bio-Rad Laboratories (Hercules, USA).

A Nd-YAG LA system operating at 213 nm (UP-213, New Wave Research, Huntington, UK) was coupled to an ICP ion source mass spectrometer (Perkin Elmer Elan DRC-e, Toronto, Canada). Nickel cones were used. Prior to all experiments, the ICP-MS instrument was optimized for routine multi-

elemental analysis following the manufacturer's instructions: the nebulizer gas flow rate, oxides, lens voltage and daily performance of the instrument were optimized aspirating a solution containing Rh, Mg, Pb, Ba and Ce (10 μg L^{-1} of each) and autolens calibration was optimized aspirating a solution of Be, Co, In and U (10 μg L^{-1} of each). The LA set-up was optimized as described elsewhere [20]. Typical operating parameters for solution measurements by ICP-MS and LA-ICP-MS measurements are summarized in Table 1. For LA-ICP-MS measurements, each electrophoretic unstained gel lane was completely and continuously scanned by the laser beam (at a scan translation velocity of 60 μm s^{-1}) in order to study possible metal losses in different parts of the gel. The dried gels were cut in two pieces of a size to fit in the ablation chamber. The gel pieces were glued to the support using double-sides adhesive tape. This operation was required to prevent the gel from shriveling up and to maintain a perfectly horizontal surface during laser ablation. The ablated material was transported by Ar as carrier gas into the ICP. The elements of interest in this study were those with higher probability of being associated to proteins in plankton samples according to bibliography [21]. The isotopes monitored included the following ^{111}Cd, ^{114}Cd, ^{59}Co, ^{63}Cu, ^{65}Cu, ^{55}Mn, ^{60} Ni, ^{62}Ni, ^{206}Pb, ^{207}Pb, ^{208}Pb, ^{64}Zn, ^{66}Zn, and ^{13}C. ^{13}C was used as an internal standard for correcting signal drift. Time dependent current is measured during translation of laser scan. The measured current time profile data of the ICP-MS was exported to Excell software, where current time profile can be calculated by transforming time into a length (cm) scale (using the selected translation velocity) and subsequently transforming cm into a molecular weight scale (using molecular weight standards).

Total protein concentration was determined by Bradford protein assay using a 8452A Diode Array Spectrophotometer (Hewlett Packard, Palo alto, CA, USA) measuring the absorbance at 595 nm.

Materials and methods

Reagents, standards and samples

Tris(hydroxymethyl)aminomethane (Tris), sodium dodecyl sulfate (SDS), glycine, *N*-tris(hydroxymethyl)methylglycine (tricine), ammonium persulfate (APS), *N,N,N´,N´*-tetramethylethylenediamine (TEMED), 30% 29:1 acrylamide/biscrylamide solution (3.3% C), Coomassie® brilliant blue R-250, Quick Start Bradford protein assay and Coomassie® brilliant blue G-250 of electrophoresis purity were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

2-Mercaptoethanol, was provided by Fluka BioChemika (Buchs, Switzerland). Bromophenol blue was from Doesder (Barcelona, Spain). Phenylmethylsulfonyl fluoride (PMSF), Tris (2-carboxietil) fosphine hydrochloride solution (TCEP), Bis(2-hidroxyethyl)amino-tris(hidroxymethyl)methano (Bis-Tris) and Rhodium standard solution (974 mg L^{-1}) were supplied by Sigma-Aldrich Chemie (Stenheim, Germany). Protease inhibitor cocktail (PI), Complete Mini EDTA free, was purchased from Roche, (Mannheim, Germany). Methanol, ethanol, acetic acid glacial for analysis and boric acid crystallized were from Panreac (Barcelona, Spain). Ammonium nitrate and Glycerol was from Probus (Barcelona, Spain). Acetone for analysis, ACS, ISO was from Scharlau (Barcelona, Spain).

HCl (36.5-38% v/v) and HNO_3 (69-70% v/v) (both for trace metal analysis), Trace Metal Standard I solution (100 mg L^{-1}) were purchased from J. T. Baker (Phillipsburg, New York).

Water (18.2 MΩ cm^{-1}) was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

Centrifugal filter units Amicon Ultra-0,5mL-10K membranes were purchased from Millipore (Bedford, USA).

SigmaMarker™ wide range, 6500-200000 Da, Molecular Weight Marker for non-denaturing PAGE, 14000-500000 Da (both from Sigma-Aldrich Chemie, Stenheim, Germany) were used as molecular weight standards.

A plankton certified reference material, BCR-414 from Institute from Reference Materials (IRMM, Geel, Belgium) was used as model sample.

Sample preparation

To evaluate the influence of different extraction methods, about 0,200 g of plankton were homogenised in eppendorf tubes with 0.6 ml of different buffer solutions shown in table 2. The homogenates were magnetically agitated during 48 hr. Then the homogenates were centrifuged at 14000 x g for 30 min. All the preparative steps were performed at 4ºC to minimise the risk of species degradation or transformation. When concentration and purification of proteins is applied, the extracts were introduced in a centrifugal filter unit (with 10 KDa membrane). The extracts were centrifuged at 10000 x g for 30 min. Finally, if proteins precipitation was performed, 4 volumes of acetone to one volume of concentrated extract were added. The mixture was incubated for 1 hr on an ice bath. Next, the mixture was centrifuged at 5000 x g for 30 min at 4ºC and the supernatant was carefully removed and discarded. The pellet was washed with 2 mL of acetone, incubated for 1 hr on an ice bath, centrifuged at 5000 x g for 10 min at 4ºC, and the supernatant was again discarded.

Gel electrophoresis

For protein separation four different 1D-PAGE methods, described in a previous work [18] were applied: two denaturing ones, SDS-Tris-Glycine PAGE method based on a Tris-Glycine system with stacking gel prepared at 4%, and resolving gel at 10%, and SDS-Tris-Tricine PAGE method based on a Tris-Tricine system with stacking gel prepared at 4%, and resolving gel at 12%; and two non-denaturing ones, Anodic-Native-PAGE (AN-PAGE) method based on a Tris/Glycine buffer system with stacking gel prepared at 4%, and resolving gel at 12%, and Blue-Native-PAGE (BN-PAGE) method, based on Tris/Tricine buffer system with stacking gel prepared at 4%, and resolving gel prepared with a gradient of 4-16%.

In all methods, the gels were run duplicate. One gel was stained and the other one was not stained for LA-ICP-MS measurements. Two staining methods were used: one based on Coomassie Blue staining method and the other one using Ag staining method [22]. Gels were finally vacuum dried by the use of a gel dryer. Saran wrap was laid on the top of the gels to protect them from contamination until analysis by LA-ICP-MS.

Protein quantification by the Bradford assay

Total protein concentration in the solutions was determined by the Quick Start Bradford protein assay. The kit supplied Bradford reagent in a solution containing methanol and phosphoric acid and the standard

proteins in NaCl (0.9%) and NaN_3 (0.05%). Bovine serum albumina at seven different concentrations: 0.125, 0.25, 0.5, 0.75, 1, 1.5 and 2 mg mL^{-1} were used as standards for quantification. 5 mL of dye reagent was added to 100 µL of each standard or samples (extracts solutions without or with subsequent protein precipitation), vortexed and incubated at room temperature for at least 5 min. Absorbance was measured at 595 nm in the spectrophotometer. The linear range was estimated to 0.125-1 mg mL^{-1} (y= 1.0200x + 0.5783, R=0.9983).

Analysis of the total metal content in extracts samples (without or with subsequent protein concentration and precipitation)

The total metal content in each extract obtained by the different extraction methods shown in table 2, without or with subsequent protein concentration and precipitation, was analyzed by ICP-MS. Weighted calibration was applied using metal standard solutions from 0 to 1000 µg L^{-1} of Co, Cu,, Mn, Ni, Pb, y Zn, and from 0 to 250 µg L^{-1} of Cd. Extracts solutions samples (without concentration and precipitation) were diluted 10 times, and extracts with subsequent proteins concentration and precipitation were dissolved in the corresponding extraction buffer before introducing to ICP-MS. ^{103}Rhodium was used as the internal standard to compensate for matrix effects.

Results and discussion

Optimization of sample preparation

Different extraction, purification and concentration parameters regarding not only protein concentration and number of bands obtained by PAGE separation, but also metal-protein bonds before electrophoretic separation were evaluated.

Effect of different extraction parameters was studied and proteins were extracted from plankton sample using several extraction buffers which are shown in table 2. Initial studies were made using method 1 based on a Tris/HCl buffer that is a widespread buffer for biological samples [23-24] and a protease inhibitor to avoid protease activities. Using this extraction method 1, the effect of purification and concentration by ultrafiltration using a centrifugal filter unit with 10 KDa membrane on protein extraction was evaluated. The fraction of extract solution with molecular weight (MW) less than 10 KDa was discarded and reserved for later checking. The fraction of extract solution with proteins of MW more than 10 KDa was concentrated around 3 times in volume. No differences were found as regard the number of bands of proteins with or without ultrafiltration, after applying SDS-Tris-Glycine PAGE separation method to the plankton extracts. To check if the fraction of extract solution with MW less than 10 KDa contains some proteins, the Bradford assay was applied and the concentration of protein obtained was below 0.125 mg mL^{-1} (inferior limit of calibration curve) corresponding to 0.14 mg protein g^{-1} plankton. Any band of protein was not observed after applying SDS-Tris-Glycine PAGE separation method. Therefore there is no protein with MW less than 10 KDa in the plankton sample studied.

Some other parameters regarding protein extraction in complex samples, such as the effect of an adsorbent for phenolic compounds (PVPP), SDS (wich exhibits a high capability for hydrophobic membrane proteins not soluble in aqueous solutions) and concentration of Tris/HCl has been studied previously in plant tissues exposed to arsenic during their growth [25]. To study these parameters for our

plankton sample, extraction methods 2a, 2b and 2c (table 2) were applied. In figure 1 results from SDS-Tris-Glycine PAGE separation from plankton protein extract obtained with methods 2a, 2b and 2c are shown. As can be observed the same proteins bands are obtained for the three extraction methods 2a (without SDS), 2b (with SDS) and 2c (with higher Tris/HCl concentration than 2a and 2b) for both staining methods applied (a) Ag and b) Coomassie Blue.

The use of non-denaturing conditions is highly recommended to maintain metal-protein bonds integrity during protein PAGE separation for metal-binding proteins with low metal-protein binding affinity. An extraction buffer containing only ultrapure water was recommended for the determination of Zn-containing proteins in slug tissue using laser ablation ICP-MS after separation by Blue Native-PAGE [14]. For our plankton sample extraction method 3 (table 2) containing only ultrapure water and protease inhibitor cocktail was also applied.

One of the reasons because the determination of metalloproteins in plankton is still a challenge is the low concentration of the species. In our plankton sample the certified values for total concentration of elements which likely form metalloproteins or metalloenzymes, are in somehow very low (0,383 mg Kg^{-1} for Cd, 1,43 mg Kg^{-1} for Co, 29,5 mg Kg^{-1} for Cu, 3,97 for mg Kg^{-1} Pb and 1,75 mg Kg^{-1} for Se). Only Zn and Mn concentrations are a little higher (111.6 mg Kg^{-1} of Zn and 299 mg Kg^{-1} of Mn). Then the expected concentration of metals associated to proteins will be so low. Protein precipitation serves as a very effective preconcentration step for proteins from diluted protein sources. We used acetone as precipitating agent because it has been recommended rather than trichloroacetic acid and ammonium sulphate due to it was proved to be the most-preserving precipitating agent concerning the stability of metal-protein bindings [19].

On one hand, the efficiency of protein extraction using the different extraction methods with or without purification and preconcentration by ultrafiltration and protein precipitation was evaluated by the determination of protein concentration using Bradford assay. The results obtained are shown in table 3. As can be seen, the efficiency of protein extraction is not very different for the extraction methods used without or with subsequent ultrafiltration and precipitation, although with extraction method 1, efficiencies obtained were higher (around 10%). The influence of Tris/HCl concentration in the extraction buffer is checked with method 2a and 2c which have the same other parameters. The protein extraction with 5 mM Tris/HCl (method 2a) (372.8 mg g^{-1}) was higher than the one obtained with 25mM Tris/HCl (method 2c) (365.4 mg g^{-1}) confirming the results of Schmidt *et al* [19]. The effect of SDS in the extraction buffer (method 2b) does not involve an important increase of protein extraction efficiency (399.6 mg g^{-1} for method 2b versus 372.8 mg g^{-1} for method 2a without SDS). In this case the presence of SDS does not improve significantly the protein extraction efficiency conversely as showed by Schmidt *et al* [19] in the case of hydrophobic proteins. This must be due to the fraction of this kind of proteins is not significant in the plankton extract, or maybe because SDS interferes with the protein quantification by Bradford assay [25]. The total proteins concentration for the different extraction modes is almost the same without and with ultrafiltration and precipitation consequently the extracted proteins are the same ones as the proteins in precipitated fraction.

On the other hand, the concentration of elements more probably associated to protein in plankton was determined by ICP-MS for the different protein extraction methods without or with ultrafiltration and protein precipitation. On this way the most efficient extraction method regarding not only protein extraction but also the extraction of metals likely bound to proteins could be selected. Moreover, comparing results from plankton extracts without and with precipitation, the proportion of metals extracted and associated with precipitated proteins could be known. The results of metal extraction percentage (%) for the different extraction methods can be seen in figure 2. In figure 2a the metal extraction percentage in the extracts without ultrafiltration and precipitation is shown and in figure 2b the metal extraction percentage in the extracts with ultrafiltration and precipitation. For elements with more than one measured isotopes, the behaviour was the same for all the isotopes, but only one is shown as example to simplify the figure.

Weighted calibration with ^{103}Rh as internal standard was used for metal quantification obtaining calibration curves with correlation coefficients R>0.9999 for all the isotopes measured except for ^{64}Zn (R=0.9982) and ^{66}Zn (R=0.9825). Quantification limits were in the range of 0.009 mg kg^{-1} for ^{206}Pb up to 0.093 mg kg^{-1} for ^{60}Ni.

Although there were no big differences, the highest extraction percentages in extracts without ultrafiltration and precipitation (figure 3a) were obtained for extraction method 1 in the case of Cd (34.2%), Co (32%), Cu (46.3%) and Zn (24.8%). It is worth to point out that Cu and Zn are some of the most abundant elements in the certified reference sample (29.5 mg Kg^{-1} of Cu and 111.6 mg Kg^{-1} of Zn). For Mn and Pb the extraction percentages were very low and quite similar for the different extraction methods. The main difference between extraction method 1 and 2 is the omission of TCEP (disulfide bridges reducing agent) and PVP (absorbent for phenolic compounds) (PMSF is also a protease inhibitor as IP cocktail in method 1). It seems that the omission of TCEP and PMSF leads to higher metal extraction percentage. The results of TCEP effects found by Schmidt *et al* [19] were not clear. By ESI-MS no effects of TCEP were found and by ICP-MS the omission of TCEP led to a diminished extent of metal-protein binding. For extracts with subsequent ultrafiltration and precipitation, the best metal extraction percentages were also with method 1 in the case of Cu, Mn, Ni and Zn. For Pb the extraction percentage was also so low and similar for the different methods. For Cd and Co the results were slightly higher with method 3 but the concentration of these elements in the extracts is so low (0.144 mg Kg^{-1} for Cd and 0.442 mg Kg^{-1}for Co), so the best results for Cu and Zn were considered.

Regarding the effect of SDS in extraction buffer, the extraction percentage was higher in both cases (with and without ultrafiltration and precipitation) for extraction method 2b (containing 1% SDS) than for method 2a (without SDS). Although Schmidt *et al* [19] found that the binding capacity of Cu and Zn with egg lysozyme was hindered in the presence of 2% SDS, Mena et *et al* [26] found that the presence of SDS at concentrations up to 1% did not affect platinum-protein bonds. In any case the situation is different when spiked metal-proteins and non spiked metal-proteins (as is our case) are extracted. With spiked metal-proteins, protein micelles with SDS are formed previously to metal incubation, however in our case metals are naturally associated to protein before micelles formation.

The effect of Tris/HCl buffer concentration was evaluated with extraction methods 2a y 2c. As can be seen in figure 2a the extraction percentage is higher for method 2c in which Tris/HCl buffer concentration is higher (25 mM in comparison with 5 mM in method 2a) for all the elements. Schmidt *et al* [25] found different results for As because the observed that the extraction efficiency for As from leaves decreased linearly with increasing buffer concentration.

If extraction percentages for each extraction method without and with ultrafiltration and precipitation are compared, they are quite similar. That means metals extracted from plankton by the different extraction methods are associated to proteins because metals concentrations are almost the same in the extracts without ultrafiltration and precipitation and in precipitated proteins fractions.

Taking into account the best results concerning optimization of sample preparation with regard to better proteins and metals extraction, method 1 was selected as extraction method previously to the following experiments.

Electrophoresis methods applied for screening of the metal-binding proteins from plankton

In order to get a deeper insight into the characterization of proteins and the possible metal-binding proteins in the plankton sample studied the following analytical methodology was applied. Firstly, the proteins were separated using two different denaturing PAGE techniques: SDS-Tris-Glycine PAGE based on a Tris-Glycine system and SDS-Tris-Tricine PAGE based on a Tris-Tricine system. Secondly two non-denaturing PAGE techniques were applied: Anodic-Native-PAGE (AN-PAGE) method based on a Tris/Glycine buffer system and Blue-Native-PAGE (BN-PAGE) method, based on Tris/Tricine buffer system. Two gels were then run in tandem, at the same time and under the same conditions. After separation, one gel was used for visualisation of the separated protein bands by use of Coomassie blue or Ag staining method, and the second, unstained gel was used for LA-ICP-MS measurements after gel drying.

SDS-Tris-Glycine PAGE

One of the most common and widely investigated tools used for protein analysis is SDS-PAGE based on a Tris Glycine system. This technique separates proteins according to their molecular weights. Because of this important advantage we have applied this technique and the results obtained for plankton extracts (Method 1) with ultrafiltration and acetone precipitation are presented in Fig. 3a. On the left side of the figure, an image of a Ag stained gel is shown (i) and on the right side an image of Coomassie blue stained gel is shown (iii). In the middle the protein molecular weight (MW) standard is shown (ii). A high number of protein bands can be seen for both staining methods although some of them are more intense with Ag staining method (25-47 and 56 kDa) and some other are more intense with Coomassie Blue (20 kDa). The MW of proteins bands is in the range between 20 and 75 kDa. These values are coincident with Moncheva *et al.* [27, 28], who found proteins bands of 14, 29, 37, 48, 60 and 72 kDa in phytoplankton at Varna Bay (Black Sea), being the most abundant ones the 14 and 75 kDa for samples taken in summer season, and the 45 and 55 kDa for samples taken in spring season. In our case for the certified plankton sample (BCR 414) the most abundant bands are in the range of 20-41 kDa. One of the

most abundant protein in biological samples is carbonic anhidrase (with MW 29 kDa) which plays an important role in the inorganic CO_2 uptake for the photosynthesis in phytoplankton [5, 29]. One of the most intense band we have obtained as can be seen in fig. 3a is the one of 29 kDa which could correspond to the band of carbonic anhidrase.

Conversely, after LA-ICP-MS analysis no band containing any metal could be detected in the gels from SDS-Tris-Glycine PAGE. Only Cu and Zn signals were observed in the zone corresponding to electrophoresis front (figure is not shown). We have demonstrated in previous investigations [17-18] that metals are lost by the protein during electrophoretic process when using denaturing SDS-Tris-Glycine method because metals form complexes with glycine trailing ion, when proteins enter the resolving gel and metals advance with the electrophoresis front.

Because of possible metal losses by use of SDS-Tris-Glycine PAGE, based on our previous experiences, a second non-denaturing separation method based on the use of tricine instead of glycine as trailing ion was applied.

SDS-Tris-Tricine PAGE

Tricine is used as trailing ion instead of glycine for proteins with molecular mass below 14 kDa. At the usual pH values, tricine migrates much faster than glycine and causes less protein movement. Metals could be more retained in the protein than when glycine is used as trailing ion. The results of applying this SDS-Tris-Tricine PAGE method to plankton extracts (Method 1) with ultrafiltration and acetone precipitation are presented in figure 3b. Again a high number of protein bands can be seen for both staining methods with MW in the range of 18-75 kDa quite similar to the ones obtained by SDS-Tris-Glycine PAGE. The only difference is that resolution is worse for the bands between 25 and 42 kDa. Protein bands with MW below 18 kDa were not obtained. We had already checked that there were no proteins with MW below 10 kDa in the ultrafiltration fraction.

The results for Cu and Zn intensity distribution for SDS-Tris-Tricine PAGE-LA-ICP-MS are shown in fig. 4. Only Cu –containing bands can be seen clearly with MW range between 26 and 58 kDa. Some very small signals are observed for Zn-containing bands in the MW range between 26 and 58 kDa also, but there are still some Cu and Zn signals in the zone corresponding to electrophoresis front. That means that because of metal losses by use of denaturing methods, a separation method preventing the native protein structures has to be applied.

Blue-Native-PAGE (BN-PAGE)

Native gel separation is a non-denaturing technique which separates proteins according to their natural charge at a given pH. Although a non denaturing method, Anodic-Native-PAGE (AN-PAGE) based on a Tris/Glycine buffer system and anodal migration at pH 8.9 was also applied, no metal-containing bands could be seen except in the zone corresponding to electrophoresis front again.

BN-PAGE is based on the use of tricine as trailing ion. When BN-PAGE is used for separation a sample with a high number of proteins as is our sample, the use of a gradient gel is recommended and the proteins are separated by BN-PAGE according to their molecular weight [14, 30-31]. In our case the percentage of acrylamide/bisacrylamide for gradient gel was optimized obtaining the best proteins separation resolution results, but not good enough, with 4-16% for plankton extracts (Method 1) with ultrafiltration and acetone precipitation. Figure 5 shows the results of BN-PAGE after LA-ICP-MS measurements. The resolution in protein separation is clearly worse than when a denaturing method (SDS-Tris-Glycine or SDS-Tris-Tricine PAGE) is applied. This is the main disadvantage of native methods. There is a no well resolved band with MW around 15-35 kDa and two more well resolved bands with 45 and 60 kDa.

Nevertheless Cu and Zn intensity distribution can be observed for BN-PAGE-LA-ICP-MS in figure 5. No other metals signals were observed. Concerning Cu intensity distribution, an intense band corresponding to the protein band of 60 kDa is observed for both isotopes measured (^{63}Cu and ^{65}Cu). Another Cu band is also observed in the no well resolved band with MW between 15 and 35 kDa. This finding confirms the results obtained for Cu intensity distribution by SDS-Tris-Tricine PAGE in which Cu signals were obtained in the protein bands between 26 and 58 kDa. Similar results were found for Zn intensity distribution, a Zn band corresponding to protein band of 60 kDa and another band corresponding to protein band with MW between 15 and 35 kDa (blank signals are also shown).

Conclusions

In this study methodology for proteins extraction from a certified plankton sample (BCR 414), proteins separation by electrophoretic methods and detection of metals associated to the proteins by LA-ICP-MS has been developed.

Different extraction methods, protein purification and concentration parameters regarding not only protein concentration and number of bands obtained by PAGE separation, but also metal-protein bonds before electrophoretic separation have been evaluated. The efficiency of proteins extraction with the different methods has been studied using protein quantification by Bradford assay. No big differences between extraction methods were found for proteins extraction efficiencies but method 1 consisting of 25 mM Tris/HCl buffer and a protease inhibitor turns out to be the most efficient means of protein extraction. Regarding Tris/HCl buffer concentration the proteins extraction was slightly higher with lower Tris buffer concentration. However metal extraction efficiencies were higher with higher Tris concentration, although the differences were not bigger than 4.5%. The presence of SDS in the extraction buffer did not have influence either on proteins extraction either on metal extraction efficiencies. The presence of reducing agents as TCEP or absorbents for phenolic compounds as PMSF did not improve the protein and metal extraction efficiencies which are worse in methods containing these compounds. Protein preconcentration by ultrafiltration and acetone precipitation for protein purification are

recommended because its use did not lead to protein or metals losses and same bands profile were obtained after separation by electrophoresis methods.

Applying SDS-PAGE based on a Tris-Glycine system a high number of proteins bands with MW between 20 and 75 kDa were obtained. However the use of SDS-PAGE based on a denaturing glycine system and Anodic-PAGE based on a non-denaturing glycine system were shown to be an unsuitable method for screening of metal-binding protein in plankton by LA-ICP-MS. Metals are lost by the protein during electrophoretic process because metals form complexes with glycine trailing ion, and metals advance with the electrophoresis front. Blue-Native-PAGE was, however successful to identify Cu- and Zn-binding proteins in the plankton extracts after LA-ICP-MS measurements. Two bands corresponding to MW 60 kDa and another one with MW between 15 and 35 kDa were detected confirming the results obtained for Cu intensity distribution by SDS-Tris-Tricine PAGE, in which Cu signals were obtained in the protein bands between 26 and 58 kDa.

The next phase of our research is the ability to achieve quantitative analysis of metals bound to proteins and the use of organic mass spectrometry to obtain structural information about metalloproteins in plankton. Efforts are underway to achieve these goals.

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Table 1. Instrumental operating conditions and measurement parameters for laser ablation inductively coupled plasma mass spectrometry (ICP-MS)

ICP-MS	
Forward power (W)	1,100
Nebulizer Ar gas flow rate	0.95 L min^{-1} (for liquid introduction)
Coolant Ar flow	14 L min^{-1}
Sweeps/reading	1(LA measurements)/20 (solution measurements)
Readings/replicate	According to laser scan line length (LA measurements)
Readings/replicate	1 (solution measurements)
Replicates	1 (LA measurements)/ 3 (solution measurements)
Dwell time	50 ms
Acquisition mode	Peak hopping
Detector mode	Dual/pulses (counting and analog)
Autolens	On
UP-213 laser system	
Laser energy	70% (10 J cm^{-2})
Repetition rate	20 Hz
Spot Size	100 μm
Ablation mode	Continuous (line)
Line scan speed	60 μm s^{-1}
Laser firing deep	5 μm
Ar carrier gas flow rate	1 L min^{-1}

Table 2. Extraction buffers composition

	Tris HCl (mM)	PI cocktail (mg mL^{-1})	PMSF (mM)	TCEP (mM)	PVP (g L^{-1})	SDS (% w/v)
Method 1	25	5	-	-	-	-
Method 2 a	5	-	2	2	1	-
Method 2 b	5	-	2	2	1	1
Method 2 c	25	-	2	2	1	-
Method 3	-	5	-	-	-	-

Table 3: Influence of sample preparation method on the extracted protein concentration from plankton

Extraction method	Without ultrafiltration and precipitation mg protein g^{-1} plankton	With ultrafiltration and precipitation mg protein g^{-1} plankton
Method 1	427.5 ± 4.2	425.3 ± 5.3
Method 2a	372.8 ± 12.1	379.3 ± 7.9
Method 2b	399.6 ± 10.6	396.8 ± 9.4
Method 2c	365.3 ± 4.3	360.4 ± 6.0
Method 3	381.9 ± 7.5	384.9 ± 5.8

Figures of merit

Fig. 1 Influence of extraction method in the plankton proteins bands obtained by SDS-Tris-Glycine separation method using two staining methods (a) Ag staining method and b) Coomassie Blue staining method): **i)** extraction method 1, **ii)** extraction method 2a (without SDS), and **iii)** extraction method 2b (with SDS)

Fig. 2 a) Metal extraction percentage (%) for different extraction methods without ultrafiltration and protein precipitation **b)** Metal extraction percentage (%) for different extraction methods with ultrafiltration and protein precipitation. Average values with standard deviations from three parallel prepared extracts are shown.

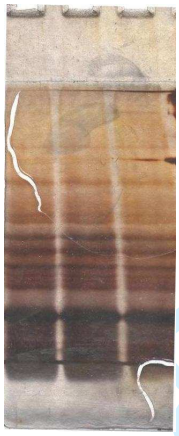
Fig. 3 a) Gel with proteins bands obtained after separation by SDS-Tris-Glycine-PAGE, **b)** Gel with proteins bands obtained after separation by SDS-Tris-Tricine-PAGE: (i) plankton extract with Ag staining method, (ii) MW standard and (iii) plankton extract with Coomassie Blue staining

Fig. 4 Cu and Zn intensity signals for SDS-Tris-Tricine-PAGE-LA-ICP-MS of plankton extracts (extraction method 1).

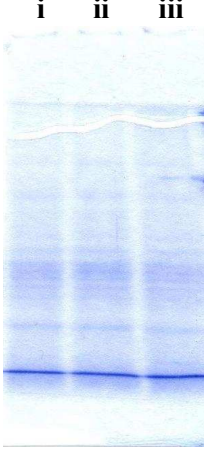
Fig. 5 Cu and Zn intensity signals for Blue-Native-PAGE-LA-ICP-MS of plankton extracts (extraction method 1).

Fig. 1.

a)



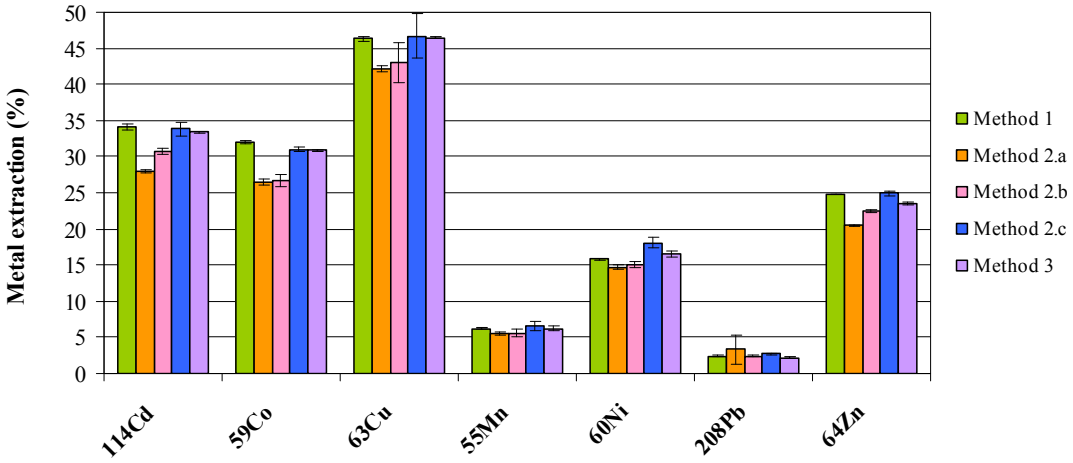
b)



25

Fig. 2.

a)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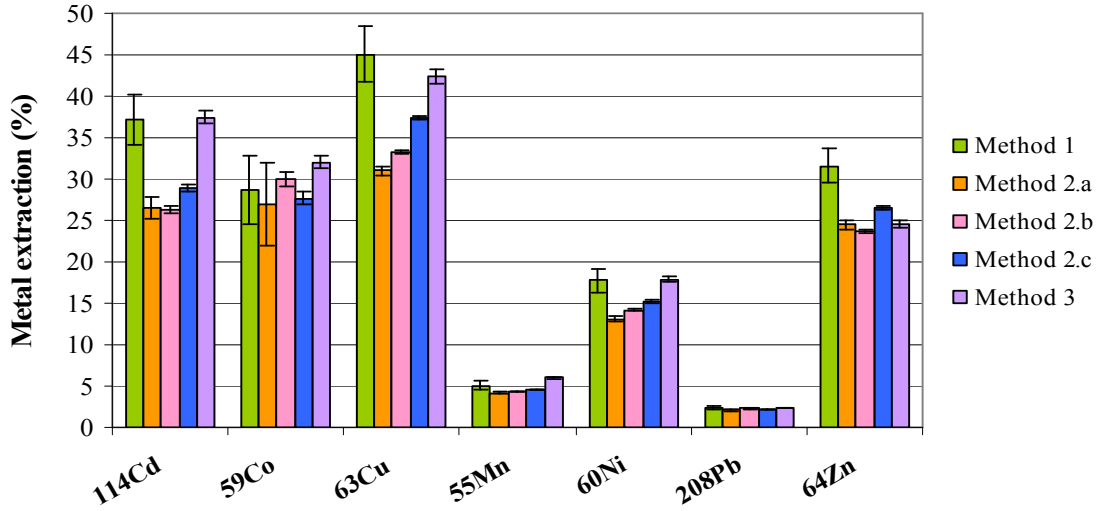
45
40
35
30
25
20
15
10
5
0
114Cd
59Co
63Cu
55Mn
60Ni
208Pb
64Zn
Method 1
Method 2.a
Method 2.b
Method 2.c
Method 3

b)


Metal extraction (%)
50
45
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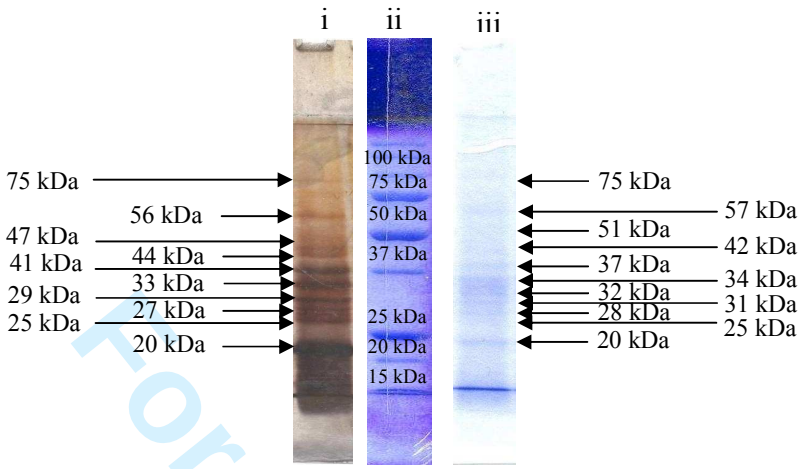
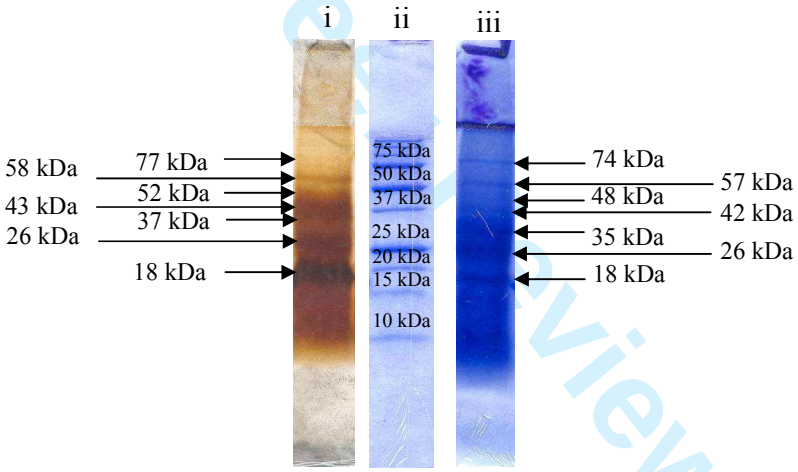
10
5
0
114Cd
59Co
63Cu
55Mn
60Ni
208Pb
64Zn
Method 1
Method 2.a
Method 2.b
Method 2.c
Method 3

Fig. 3.

a)

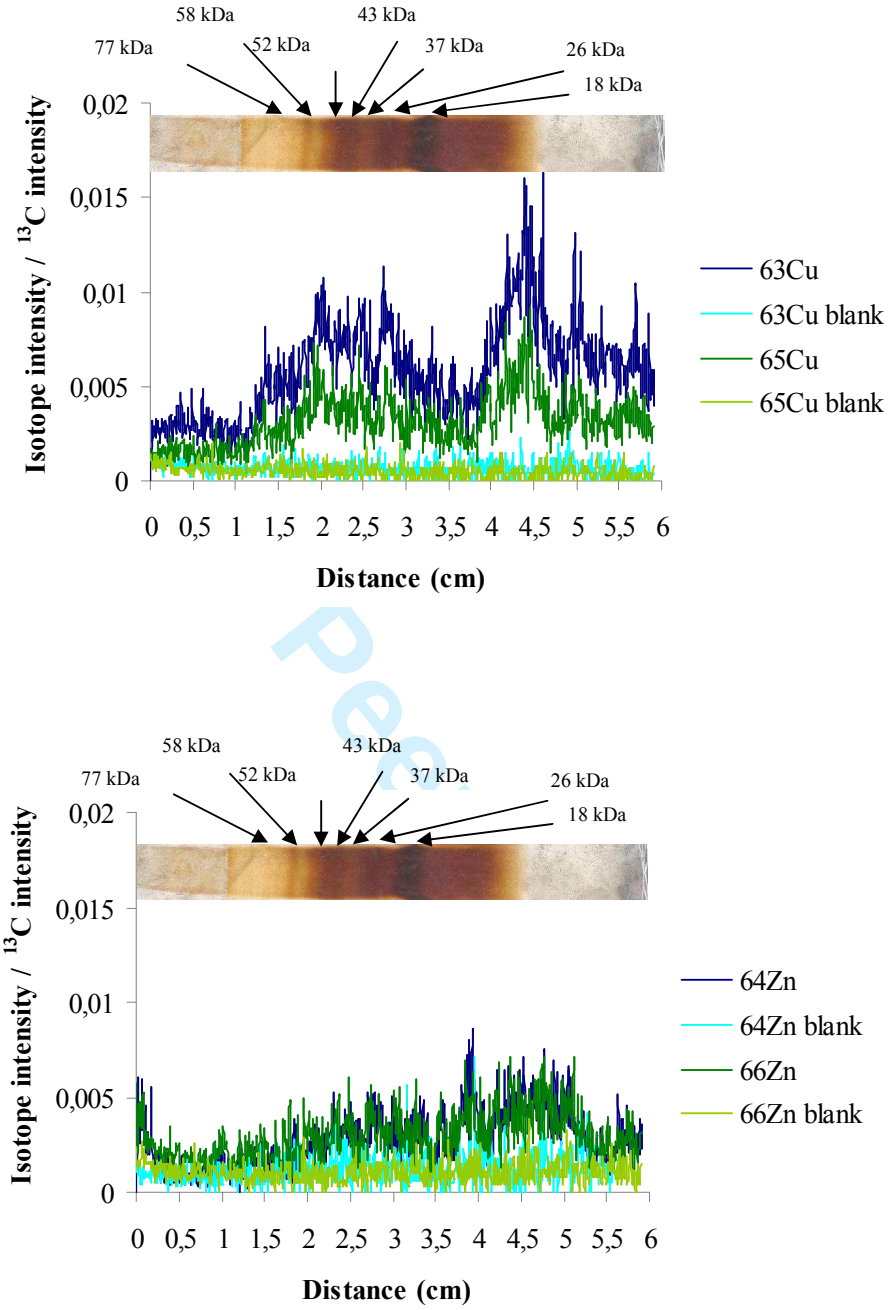


b)



27

Fig. 4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43 kDa
77 kDa
52 kDa
37 kDa
26 kDa
18 kDa
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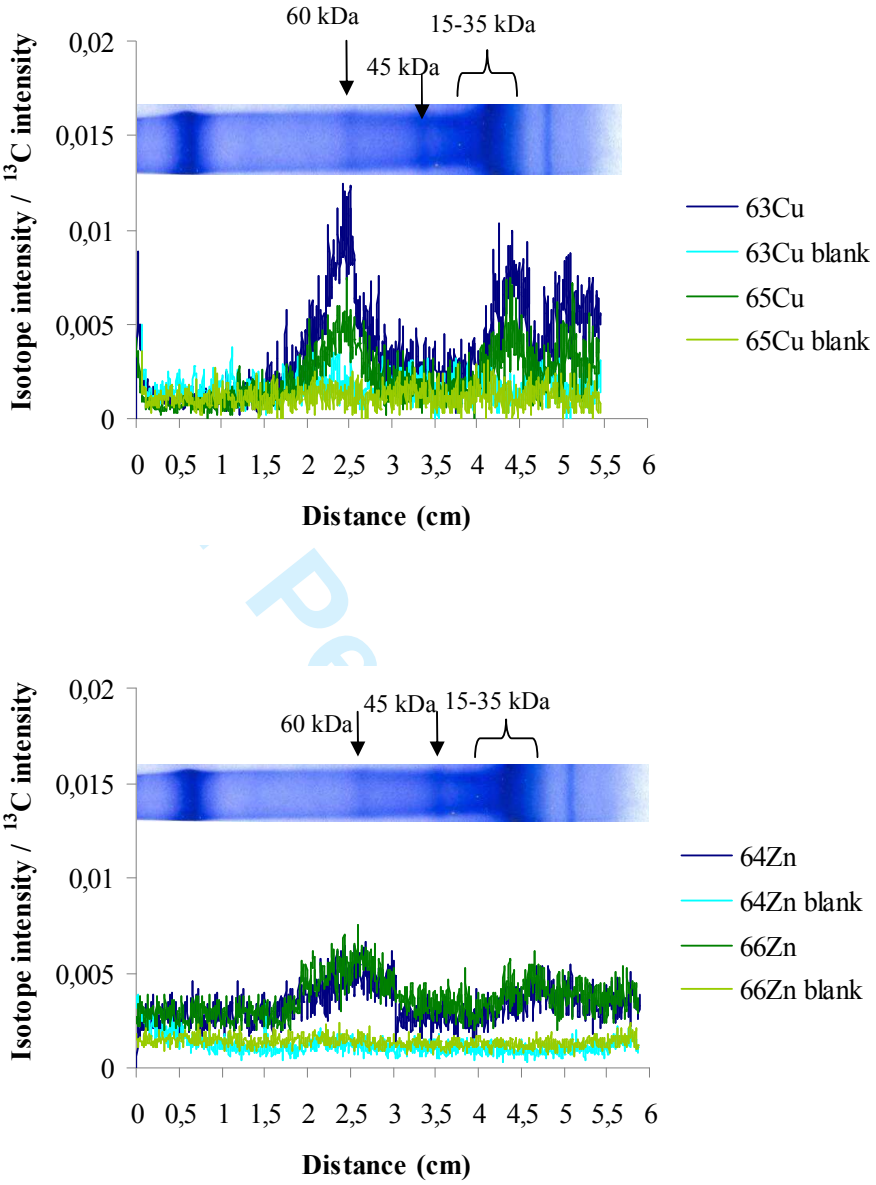
0,015
0,01
0,005
0
Isotope intensity / 13C intensity
0 0,5 1 1,5 2 2,5 3 3,5 4 4,5 5 5,5 6
Distance (cm)
63Cu
63Cu blank
65Cu
65Cu blank
58 kDa
43 kDa
77 kDa
52 kDa
37 kDa
26 kDa
18 kDa
0,02
0,015
0,01
0,005
0
Isotope intensity / 13C intensity
0 0,5 1 1,5 2 2,5 3 3,5 4 4,5 5 5,5 6
Distance (cm)
64Zn
64Zn blank
66Zn
66Zn blank

Fig. 5.


60 kDa
45 kDa
15-35 kDa
Isotope intensity / ^{13}C intensity
0,02
0,015
0,01
0,005
0
0 0,5 1 1,5 2 2,5 3 3,5 4 4,5 5 5,5 6
Distance (cm)
63Cu
63Cu blank
65Cu
65Cu blank
60 kDa
45 kDa
15-35 kDa
Isotope intensity / ^{13}C intensity
0,02
0,015
0,01
0,005
0
0 0,5 1 1,5 2 2,5 3 3,5 4 4,5 5 5,5 6
Distance (cm)
64Zn
64Zn blank
66Zn
66Zn blank