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Received November 18, 2011
Revised December 22, 2011
Accepted December 28, 2011

Research Article

Immunoextraction of zinc proteins from human plasma using chicken yolk antibodies immobilized onto paramagnetic particles and their electrophoretic analysis

Zinc(II) as the only transition metal lacking redox activity is an essential part of approximately 10% proteins as a cofactor of these proteins. Considering the fact that there are numerous zinc(II) containing proteins, proteomics and metallomics studies aimed on them require accurate methods for preparation of real biological samples prior to their subsequent analysis using 2DE and MS. For this purpose, we suggested a new method based on chicken anti-zinc antibodies and magnetizable particles. Antibodies were covalently immobilized to the surface of paramagnetic beads activated with tosyl group. Binding of the antibody to the beads was confirmed by secondary anti-chicken antibody conjugated with horseradish peroxidase. The immunoextraction conditions, such as concentration of the beads (6–18 μg/mL of the sample), time of immunoextraction (6–34 min), pH and composition of the elution buffer, and time of extraction (48–300 s) were optimized. Subsequently, zinc proteins were extracted from human plasma and total concentration of zinc was monitored by electrochemical detection in the extracts. Under optimal conditions it was possible to monitor the proteins and zinc removal from the sample by chip CE, SDS-PAGE, and indirectly using electrochemistry.

Keywords:
Electrochemistry / Magnetic particles / Metallomics / Zincome
DOI 10.1002/elps.201100638

1 Introduction

Various metals including zinc play numerous essential roles in biological pathways as enzyme regulators, catalysts, structural features, etc. [1]. The correct functioning of these pathways can be easily disrupted by a deficiency or excess of these metals or by the presence of other nonessential metals [2, 3]. Therefore, an understanding of the biochemical interaction of such metals and their accurate determination in biological fluids is needed [4]. Zinc is one of the most abundant metals in the organism. Zn(II) ions are key catalytic and structural component of proteins. Beside the regulation function, the free zinc ions have also signaling function [5]. Therefore free zinc ions level is strictly regulated by transport and binding proteins and peptides [6]. The distribution of zinc between these biomolecules has a significant impact on zinc bioavailability. Most of the zinc is bound with proteins in blood plasma, although approximately 1% exists as amino acid complexes [7]. On the other hand, zinc is loosely bound mainly with albumin, and a small amount is firmly bound with α2-macroglobulin [8]. Also, a group of low molecular mass proteins called metallothioneins plays an important role in zinc metabolism [9, 10].

Various techniques such as sucrose density gradient centrifugation [11], polyethylene glycol precipitation [12], electrophoresis [13, 14], chromatography [15, 16] and ultrafiltration [17] have been employed for the separation of metalloproteins from human blood serum or plasma. The chromatographic separation of trace elements including zinc has been investigated by using an HPLC-ICP-MS system [18]. Since zinc bound with albumin is labile, their adsorption on materials often causes serious problems in accuracy and precision [19].

In this study, an immunoextraction method for zinc-containing proteins using zinc-specific antibody-modified magnetic nanoparticles was developed. Immunoextraction conditions including interaction time, elution buffer composition as well as elution time were optimized.

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Abbreviations: KLH, keyhole limpet hemocyanin; **MT**, metallothionein

Colour Online: See the article online to view Figs. 1-4 in colour.

Conventional gel electrophoresis as well as chip-based CE were utilized for determination of extracted proteins. Furthermore, the zinc content has been determined using electrochemical methods and the optimized method was successfully applied to the analysis of zinc-containing proteins in human plasma.

2 Material and methods

2.1 Chemicals and water purification

Unless otherwise indicated, all chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) in ACS purity. Deionized water underwent demineralization by reverse osmosis using the instruments Aqua Osmotic 02 (Aqua Osmotic, Tisnov, Czech Republic) and then it was subsequently purified using Millipore RG (Merck Millipore, Billerica, MA, USA, 18 MΩ)–MiliQ water.

2.2 Preparation of anti-zinc antibodies

Chicken antibodies were prepared by HENA, Prague, Czech Republic. Two hens were immunized by Zn-KLH (keyhole limpet hemocyanin) complex. From the egg yolk, the IgY fraction with reactivity to Zn-KLH was obtained. The antibodies were stabilized with 0.1% sodium azide in PBS (137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH_2PO_4, and 4.3 mM Na_2HPO_4, pH 7.4). The protein concentration was 39.6 mg/mL in immunoglobulin fraction.

2.3 Zn-BSA interaction

To 1 mg/mL of BSA, $ZnCl_2$ (0, 25, and 500 μg/mL) was added. In order to bind Zn(II) BSA was denatured with 6.6 M guanidium hydrochloride for 24 h at 37°C. BSA was refolded and guanidium hydrochloride and free Zn(II) were removed by drop dialysis using the mixed cellulose esters membrane discs with pore diameter 0.05 μm (Merck Millipore, #WSWP 04700). The discs were floated by hydrophilic side up on a surface of 0.1 M phosphate buffer pH 7.6 in volume of 1 L. The sample (100 μL) was pipetted on a membrane and dialysis was performed for 16 h at 4°C with slow magnetic stirring.

2.4 Zn(II) and total proteins determination

Determination of zinc by differential pulse voltammetry were performed with 797 VA Stand instrument connected to 813 Autosampler (Metrohm, Herisau, Switzerland), using a standard cell with three electrodes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3M KCl electrode was the reference and platinum electrode was auxiliary. For data processing, VA Database 2.2 by Metrohm CH was employed. The analyzed samples were deoxygenated prior to measurements by purging with argon (99.999 %). Acetate buffer (0.2 M $CH_3COONa + CH_3COOH$, pH = 5) was used as a supporting electrolyte. The supporting electrolyte was exchanged after each analysis. The parameters of the measurement were as follows: initial potential of −1.3 V, end potential 0.15 V, deoxygenating with argon 90 s, deposition time 120 s, time interval 0.04 s, step potential 4 mV, modulation amplitude 25 mV, adsorption potential −1.15 V, volume of injected sample: 15 μL, volume of measurement cell 2 mL (15 μL of sample + 1985 μL acetate buffer) [20].

Total proteins content in the samples was determined according to Bradford [21]. Absorbance was measured at 595 nm, reagent itself was used as a blank. Obtained values of absorbance (blank, mixture after 10-min incubation) were used for the determination of total proteins. Calibration curve based on BSA within the range of 0.031–0.5 mg/mL was measured and the equation as $y = 1.037\times–0.009$, $R^2 = 0.990$ was obtained. All spectrophotometric measurements were carried using a microplate reader (Original MultiskanEx, Thermo Electron, Waltham, MA, USA).

2.5 Differential pulse voltammetry–Brdicka reaction

Differential pulse voltammetric measurements were performed with 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler (Metrohm), using a standard cell with three electrodes and cooled sample holder (4°C). An HMDE was the working electrode. An Ag/AgCl/3M KCl electrode was the reference and glassy carbon electrode was auxiliary. For data processing, GPES 4.9 supplied by EcoChemie was employed. Brdicka supporting electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer ($NH_3(aq) + NH_4Cl$, pH = 9.6) was used. The parameters of the measurement were as follows: initial potential of −0.7 V, end potential of −1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude 250 mV. All experiments were carried out at temperature 4°C employing thermostat Julabo F25 (JULABO, Labortechnik, Seelbach, Germany) [22].

2.6 SDS-PAGE

The electrophoresis was performed using a Mini Protean Tetra apparatus with gel dimension of 8.3 × 7.3 cm (Bio-Rad Laboratories, Hercules, CA, USA). First 15 or 12.5% (*m*/*V*) running, then 5% (*m*/*V*) stacking gel was poured. The gels were prepared from 30% (*m*/*V*) acrylamide stock solution with 1% (*m*/*V*) bisacrylamide. The polymerization of the running or stacking gels was carried out at room temperature for 45 min or 30 min, respectively. Prior to analysis, the samples were mixed with nonreduction

sample buffer in a 2:1 ratio. The samples were incubated at 93°C for 3 min, and the sample was loaded onto a gel. For determination of the molecular mass, the protein ladder "Precision plus protein standards" from Bio-Rad was used. The electrophoresis was run at 150 V for 1 h at laboratory temperature (23°C) (Power Basic, Bio-Rad) in tris-glycine buffer (0.025 M Trizma-base, 0.19 M glycine, and 3.5 mM SDS, pH = 8.3). Then, the gels were stained Coomassie blue and consequently with silver. The procedure of rapid Coomassie blue staining was adopted from Wong et al. [23], silver staining was performed according to Krizkova et al. [24] with omitting the fixation (1.1% (*v/v*) acetic acid, 6.4% (*v/v*) methanol, and 0.37% (*v/v*) formaldehyde) and first two washing steps (50% (*v/v*) methanol).

2.7 Western blot

After the electrophoretic separation, the proteins were transferred on a PVDF membrane by using Biometra Fastblot apparatus (Biometra, Gottingen, Germany). PVDF membranes were activated by soaking in methanol for 30 s prior to blotting. Further, the membrane was equilibrated for 5 min in blotting buffer (12.5 mM Tris-base, 75 mM glycine, and 15 % (*v/v*) methanol). The blotting sandwich was composed of three layers of filter paper soaked in blotting buffer, membrane, polyacrylamide gel and additional three layers of soaked filter paper. The blotting was carried out at room temperature for 1 h at constant current of 0.9 mA for 1 cm^2 of the membrane. After the transfer, the membrane was blocked in 1 % BSA in PBS for 30 min. The incubation either with chicken anti-MT or chicken anti-Zn primary antibody in dilutions of 1:1000 in PBS with 0.1 % of BSA was carried out for 12 h at 4°C. After three times of repeated washing with PBS containing 0.05 % (*v/v*) Tween-20 (PBS-T) for 5 min, the membrane was incubated in the presence of secondary antibody (rabbit anti-chicken labeled with horseradish peroxidase, Sigma-Aldrich in dilution 1:6000) for 1 h at room temperature. Then, the membrane was washed three times with PBS-T for 5 min and incubated with chromogenic substrate (0.4 mg/mL^{-1} AEC - 3-aminoethyl-9-carbazole in 0.5 M acetate buffer with 0.1% H_2O_2, pH 5.5), after adequate development, the reaction was stopped by rinsing with water.

2.8 Capillary chip electrophoresis

Analyses on an automated microfluidic Experion electrophoresis system (Bio-Rad) were carried out according to the manufacturer's instructions with supplied chemicals (Experion Pro260 analysis kit, Bio-Rad). A sample (4 μL) was mixed with 2 μL of reducing sample buffer (3.3% β-mercaptoethanol), and after 3 min boiling, 84 μL of water was added. After the priming of the chip with the gel and gel-staining solution in the diluted priming station sample, the mixture (6 μL) was loaded into the sample wells. The Pro260 Ladder included in the kit was used as a standard. For operation and standard data analysis, Experion software v. 3.10 (Bio-Rad) was used.

2.9 Immobilization of antibodies to the paramagnetic beads

For covalent immobilization of the antibodies, p-toluenesulphonyl chloride-activated superparamagnetic polystyrene beads coated with polyurethane layer were used (Dynabeads® MyOne™ Tosylactivated, #655.01, Dynal Biotech ASA, Oslo, Norway). The procedure of antibodies preparation and immobilization was adopted from suppliers manual (Invitrogen, Oslo, Norway). For immobilization, 1000 μg of the antibodies per 25 mg of the beads were used. Prior to immobilization, the preservative (sodium azide) was removed and antibodies were acidified to pH 2.5 by addition of HCl. After 15 min the antibodies were brought into physiological pH (7.4). For all buffer exchanges, Amicon Ultra 0.5 columns with membrane cut off 50 K (Merck Millipore) were used. Covalent immobilization was carried out in total volume of 625 μL in the presence of 0.1 M borate buffer pH 9.5 with 0.1 M $(NH_4)_2SO_4$ for 24 h under mild rotation. The free surface of the particles was then blocked with 0.5% BSA in PBS (*w/v*) with 0.05% Tween-20 (*v/v*) for 10 h. After blocking, the beads were washed three times with 1 mL of 0.1% BSA in PBS (*w/v*) with 0.05% Tween-20 (*v/v*) and resuspended in 625 μL of storage buffer (washing buffer with 0.02% NaN_3 (*w/v*)).

2.10 Immunoextraction

Immunoextraction of the zinc-binding proteins was carried out using paramagnetic beads with covalently immobilized polyclonal chicken anti-Zinc-KLH antibodies and magnetic stand Dynal Magnetic Particle Concentrator-S both supplied by Invitrogen. All experiments with paramagnetic particles were performed in RNA/DNA UV cleaner box UVT - S - AR (Biosan). For centrifuging and vortexing of a sample, multispin MSC-3000 centrifuge (Biosan) placed in UV cleaner box was used. The buffers used in our experiments were as follows – (i) PBS, pH 7.4; (ii) elution buffer: 0.1 M sodium citrate, pH was adjusted by adding NaOH; (iii) washing buffer: 0.1% BSA in PBS with 0.05% Tween-20.

Beads concentration, extraction and elution times, and composition and pH of the elution buffer were optimized. The optimized condition, which were used for further experiments, were as follows: 18 μg/mL beads per one sample were washed in 1 mL of PBS. Then the beads were resuspended in original volume of PBS and aliquoted. After the removal of PBS, 30 μL of 100 × diluted sample was added. The extraction was carried out for 50 cycles (22.5 min) in a multispin centrifuge at laboratory temperature (1 cycle: spin at 1500 rpm for 1 s, vortex soft for 20 s, one cycle takes 27 s). After extraction, the beads were left on a magnetic stand

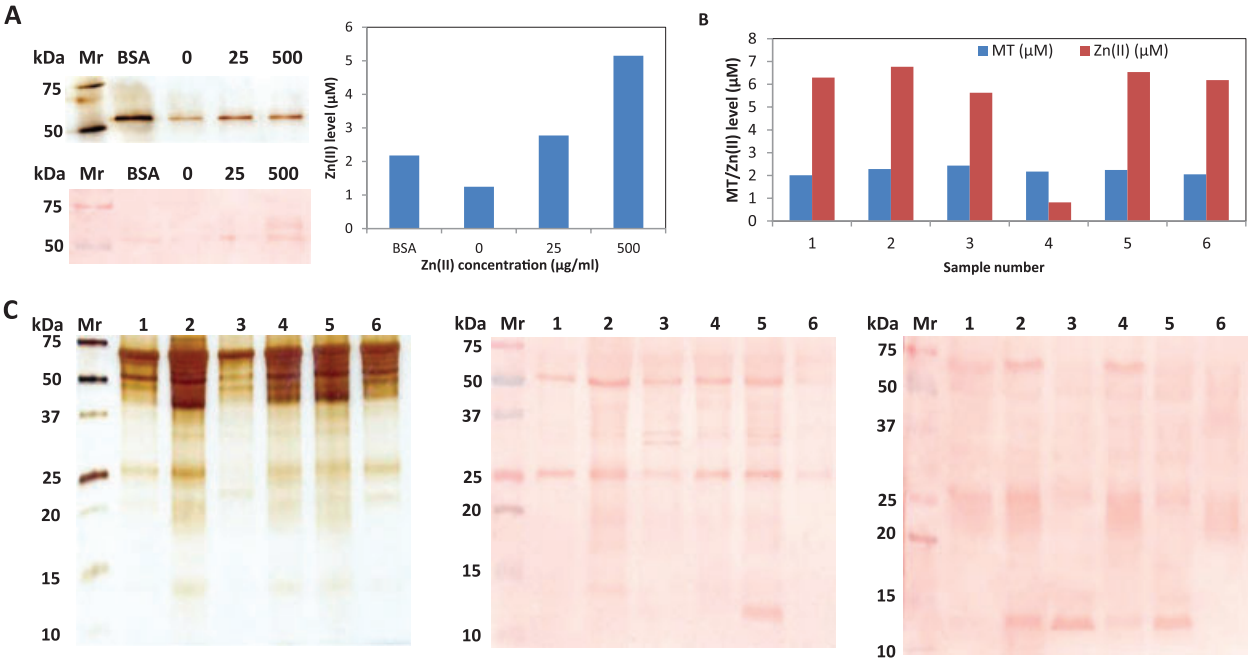

Figure 1. (A) Zn(II)-BSA interaction. SDS-PAGE and Western blot of 50 μg/mL BSA enriched with Zn(II), Zn(II) content in the samples. (B) MT and Zn(II) levels in thermally denatured plasma. (C) SDS-PAGE of the thermally denatured blood plasma, Western blots of the same samples with anti-MT and anti-Zn antibodies.

until the beads settled down and then the beads were left on a magnet and washed with 500 μL of PBS. The captured proteins were eluted by adding 30 μL of the elution buffer (0.1 M citrate pH 2.5) and spun-vortexed for 15 cycles or 2 min 25 s. Elution was repeated once more and the eluates were combined.

2.11 Biological samples

Blood samples were obtained from six healthy volunteers (three males, three females, average age 32.5 years). Plasma was separated by centrifugation at 4000 × *g* for 10 min in plasma preparation tubes with heparin (Dialab, Prague, Czech Republic). The samples were stored in −80°C until assayed. For immunoextraction, the samples were 100 × diluted in ultrapure water (Sigma-Aldrich). For electrochemical determination of MT and Western blot, the samples were denatured at 99°C in a thermomixer Eppendorf 5430 (Eppendorf, Hamburg, Germany) for 15 min with shaking and centrifuged at 4°C, 15 000 × *g* for 30 min (Eppendorf 5402) [25].

3 Results and discussion

3.1 Zn-BSA interaction

As we mentioned in Introduction, zinc is loosely bound mainly with albumin, and a small amount is firmly bound with α2-macroglobulin [8]. Interaction of proteins with zinc is strongly dependent on pH and zinc can be redistributed or released during the analytical process. For this reason as a model system for Zn proteins immunoanalysis, BSA was chosen. Firstly, we unfolded BSA using guanidium-thiocyanate in the presence of 0, 25, and 500 mg/mL of $ZnCl_2$. Relatively high Zn(II) concentrations were used in order to ensure saturation of BSA with Zn(II). BSA was refolded and unbound $ZnCl_2$ was removed by drop dialysis. SDS-PAGE gel of 200 ng BSA before and after dialysis is shown in Fig. 1A, right. Zn(II) content determined by differential pulse voltammetry was increased compared to BSA without additions of zinc (Fig. 1A, left). After blotting with anti-Zn antibody, we detected a weak band in size of approximately 60 kDa. The intensity of the band increased with applied Zn(II) concentration. The presence of additional bands with higher molecular mass can be explained by unspecific refolding of BSA due to excess of Zn(II) (Fig. 1A).

3.2 Western blotting analysis of Zn-proteins in plasma

After the sufficient detection of Zn(II) binding into BSA, we analysed thermally denatured samples of plasma, which were primarily intended for electrochemical metallothionein analysis. Zn(II) level oscillated from 1 to 7 μM (Fig. 1B). Physiological Zn(II) level in plasma is from 10 to 20 μM [26].

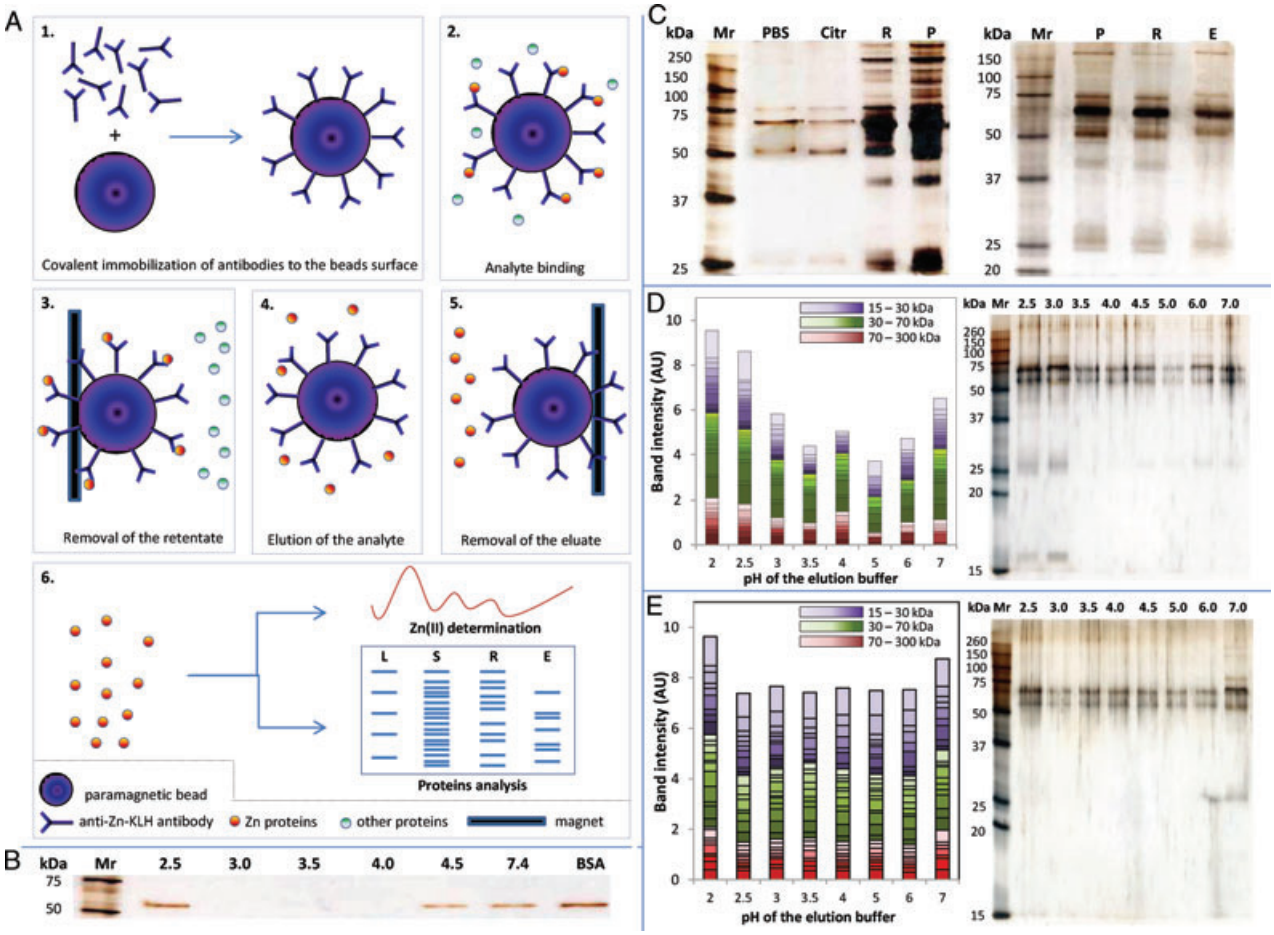

Figure 2. (A) Scheme of the proposed process of Zn proteins immunoextraction with paramagnetic beads. (B) Elution of Zn(II) enriched 50 μg/mL BSA with PBS of different pH. (C) Comparison of protein profiles of original sample, retentate, and eluates obtained with PBS and 0.1 M citrate buffer, pH 7.0 (left) and protein profile of the original sample, retentate, and eluates obtained with optimized protocol (right). Optimization of elution buffer pH, 0.1 M sodium citrate. (D) Densitometric protein profiles and silver-stained SDS-PAGE gel. (E) Fractionation of the proteins by subsequent elution with 0.1 M sodium citrate of different pH.

This indicates that Zn(II) was partially removed by denaturation process. After blotting with anti-MT antibody, three major bands in size of approximately 12.5, 25, and 50 kDa were detected. This corresponds to single MT isoforms and oligomers, as we confirmed in our previous papers [24, 27]. After blotting with anti-Zn antibody, also numerous bands in similar size were detected. However, blotting patterns were different from those obtained with anti-MT antibody (Fig. 1C). It is known that thermal denaturation is sufficient to remove excess of thermolabile proteins, which could interfere with electrochemical determination of MT. Sensitivity of the electrochemical determination for MTs is achieved due to its high content of electroactive cysteine residues (more than 30%), thus the signal of other thermostable proteins is negligible [28]. It follows from the results obtained that other proteins, which remained in thermally denatured plasma, contained Zn(II) in their structure. This confirms the hypothesis that there were other proteins in denatured plasma, other than metallothioneins.

3.3 Immunoextraction of Zn proteins

After that, we confirmed that there are numerous zinc-containing proteins in denatured sample, we followed with suggesting an easy way how to isolate these proteins. It is known that electrophoresis conditions can have an influence on metals binding in protein complexes and loss of proteins was observed during electrophoresis [29]. Moreover, further analysis of the blotted proteins is laborious and is conflicted with commonly used methods. Immunoextraction of Zn-proteins offers more possibilities for consequent proteins analysis. Therefore, we primarily aimed at this approach, of which schematic illustration is shown in Fig. 2A. The process is composed of analyte binding from the sample matrix and its elution. SDS-PAGE, chip CE, and electrochemical Zn(II) determination was sufficient to test the effectiveness and usability of the method.

Firstly, immunoextraction of BSA standard was tested. Zn-protein interaction is dependent strongly on pH of the

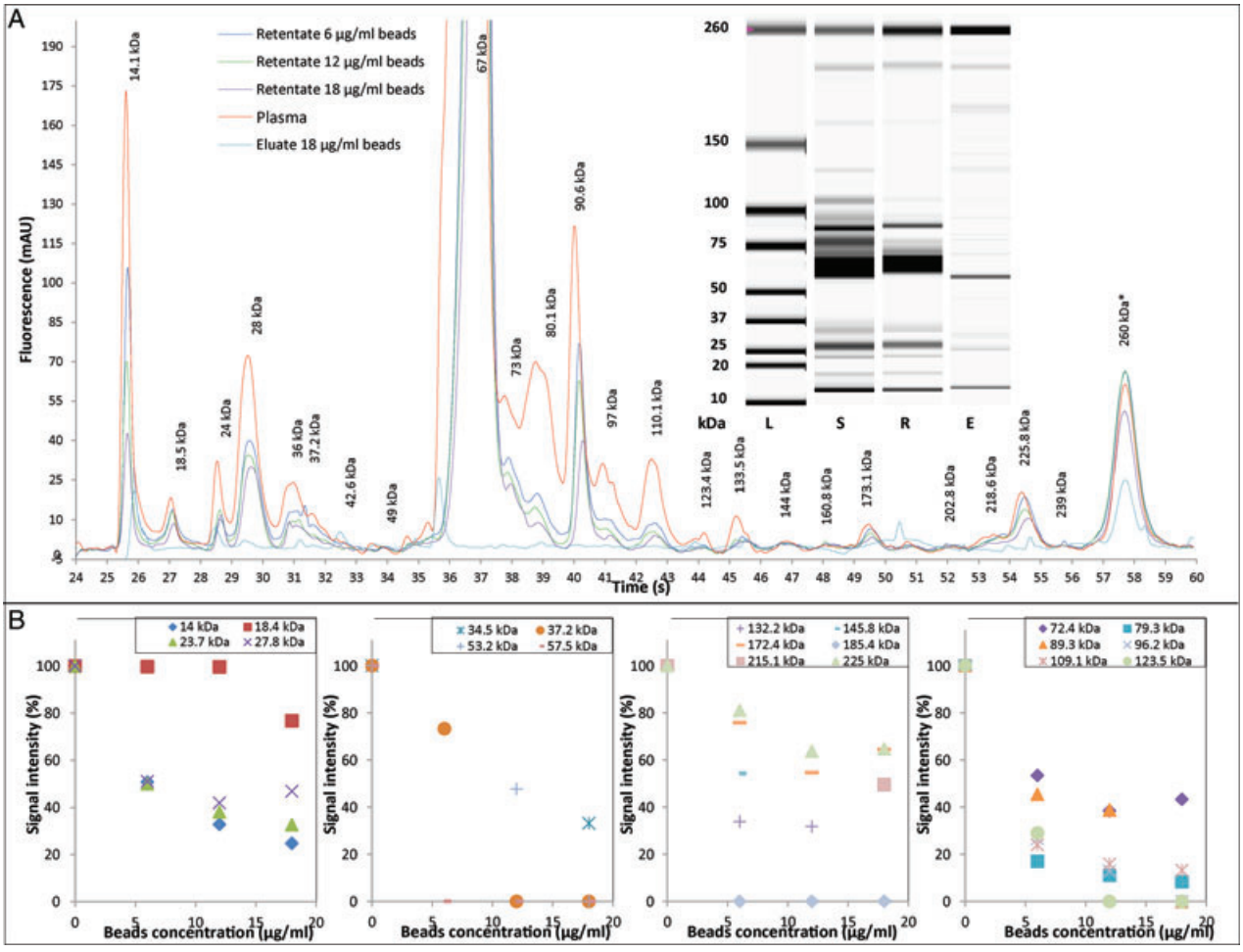

Figure 3. (A) Chip CE electrophoreogram of 100 × diluted plasma, retentates after extraction with 6, 12, and 18 µg/mL of beads and eluate from 18 µg/mL beads. In inset: virtual gel output of plasma, retentate, and eluate obtained with optimized protocol. (B) Decrease of single protein peaks after extraction with 6, 12, and 18 µg/mL of the beads.

extraction solution. The highest yield was achieved after using elution buffer of pH 2.5 or higher than 4.5. This may be caused by dual interaction of Zn(II) either with protein or with antibody (Fig. 2B). Elution condition from extraction of Zn proteins from plasma had to be optimized due to initial low yield caused probably by different affinity of captured proteins to antibody or zinc. The difference between initial and optimized conditions is shown in Fig. 2C. The yield was greatly enhanced by optimizing beads concentration and elution buffer composition. While BSA was easily eluted using PBS, only part of the extracted proteins from plasma was eluted.

The influence of elution buffer pH was similar as in the case of BSA. Most proteins were eluted using elution buffer pH lower than 3.0 or higher than 4.5 (Fig. 2D). The pattern of the eluates was not uniform. This led us to the attempt to fractionate the proteins by gradual decrease of pH of the elution buffer from 2.5 to 7.0 (Fig. 2E). After the densitometric analysis of silver-stained gel, it was obvious that the lanes corresponding to used elution buffer pH contained proteins in different molecular mass (Fig. 2E). The total yield was highest for pH 2.5, where also the highest percentage of low-molecular proteins was contained.

In order to quantify better the yield of proteins extraction, chip CE was used. Chip CE allows to complete proteins electrophoresis in less than 45 min and easy comparison of the electrophoreograms, which is especially beneficial for optimization of separation procedures [30, 31] or for monitoring and screening purposes [31, 32]. However, the sensitivity is comparable to Coomassie-blue staining and sample volume is strictly limited.

First, different concentrations of beads were tested. Three concentrations (6, 12, and 18 µg/mL) of beads were added to 30 µL of 100 × diluted plasma. After elution, the original sample, eluate and retentate were run on a chip. Compared to original sample, decrease of proteins signal of was observed (Fig. 3A). The decrease varied from 10% to 100% and the peak position in retentate was shifted compared to original sample (Fig. 3A and B). This indicates that some proteins were extracted readily from the sample. The

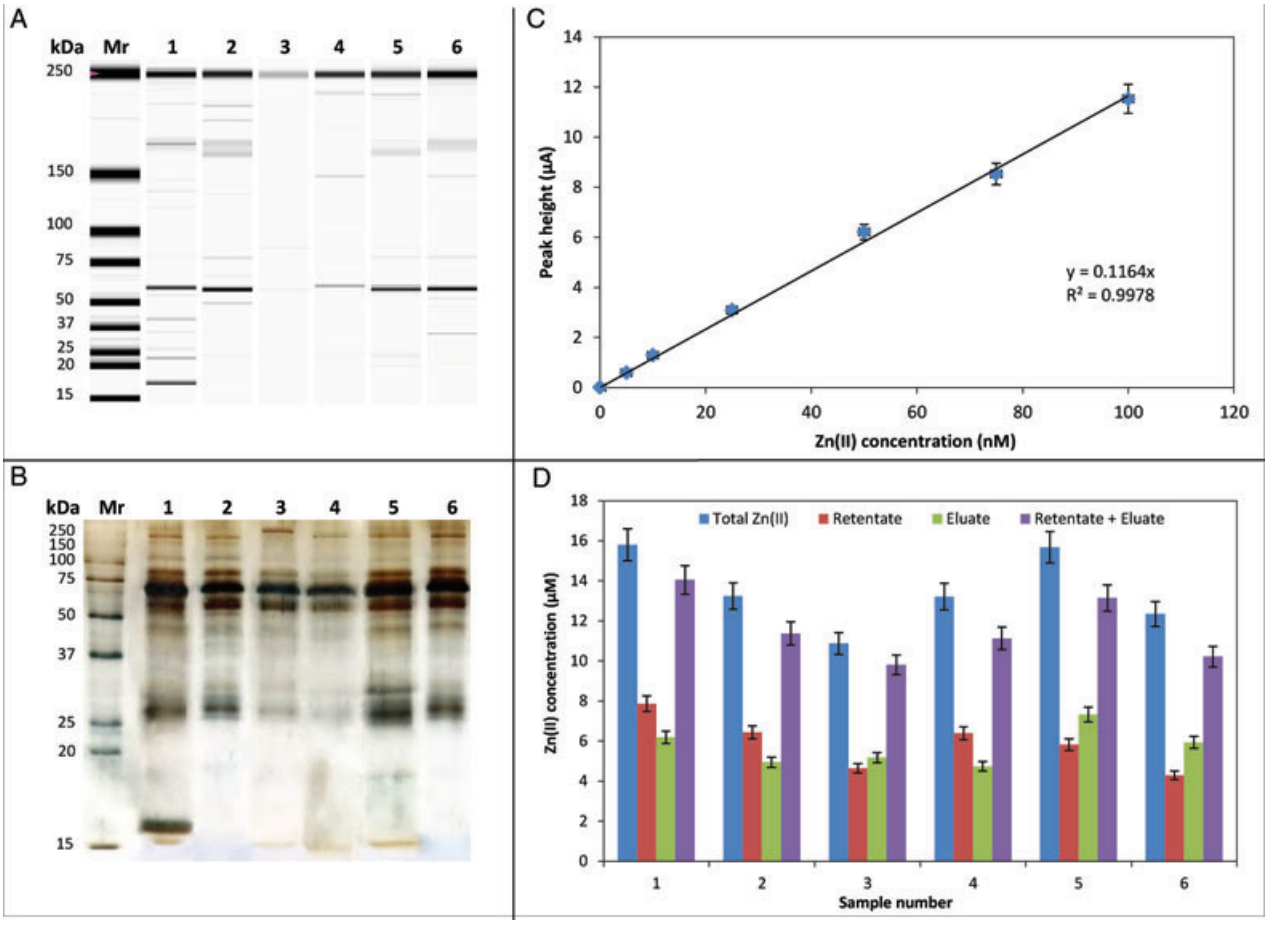

Figure 4. (A) Virtual gel output of eluted Zn-proteins. (B) Silver-stained SDS-PAGE gel of the eluted Zn-proteins. (C) Calibration dependence of Zn(II) concentration and signal height in differential pulse voltammetry. (D) Zn(II) levels in total plasma, retentate, and eluate and sum of Zn(II) concentrations in retentate and eluate.

recommended beads concentration (6 μg/mL) was insufficient to capture all proteins. The best results were achieved with three time higher beads concentration (18 μg/mL) than recommended by the manufacturer. This can be explained by the fact that the sample concentration and beads volume had to be compromised with the detection method used. With further increase of beads concentration, no decrease of the signal was observed. However, reliable quantification of proteins in the eluates was not possible due to low proteins concentration. The yield was further improved by repeated elution of captured proteins. Virtual gel outputs of original sample, retentate and eluate are shown in inset of Fig. 3A. It is obvious, that protein concentration in original sample and separated fractions are disproportional and that eluate and retentate contain proteins of different size. In Fig. 4A and B, the virtual gel outputs from chip CE and silver-stained SDS-PAGE gel of the eluted proteins are shown. Only approximately 9% (9 ± 0.3%) of total proteins were eluted from the beads.

It follows from bioinformatic proteomes analysis that there are almost 300 proteins present in human plasma proteome [33]. At least 12 high-abundant proteins are known to bind zinc [34]. From total plasma proteome, it is assumed that 10% of the proteins contain zinc. Zn is also a common cofactor of enzymes, especially hydrolases, ligases, and transferases [35]. The proteins bind Zn(II) with different affinity and Zn(II) ion can be present either at the surface of the protein or in the enzymes active sites, it can be bound by single aminoacid or in special binding sites, domains, or aminoacids motifs (like metallothioneins or Zn-fingers) [7]. Based on the mentioned fact, one may speculate that approximately 30% of blood plasma proteome are potentially Zn binding proteins. However, concentration of total Zn binding proteins in plasma has not been published yet. In order to evaluate the efficiency of Zn proteins extraction, Zn(II) concentration on the samples and separated fraction was determined by ultrasensitive electrochemical method (Fig. 4C). In all analysed samples, Zn(II) concentration varied from 10.9 to 15.8 μM, which corresponds to physiological Zn(II) level in plasma (10–20 μM) [26]. Zn content in retentate was approximately 50% of Zn(II) content in original sample. When Zn(II) amount in retentate and eluate was added up, the resulting Zn(II) concentration was slightly lower (2 × than SD). This indicates that elution of the proteins from beads was quantitative and that approximately 50% of total Zn(II)

was extracted (Fig. 4D). However, with prolonged extraction, and elution time and with increased beads volume, no further decrease in proteins signals was detected. This indicates that Zn(II), which was not extracted, was not bound by proteins or was bound only very loosely, thus it was insufficient for proteins extraction. Even some steps of the procedure need further optimization, using Zn proteins immunoextraction from complex biological matrix was illustrated. This procedure has a potential especially for advanced proteomic methods, like 2D-SDS-PAGE or MS and it is verisimilar that adjustment of the immunoextraction procedure with respect to further applications will be needed.

Immunoextraction of the proteins from plasma via paramagnetic beads is very promising for complex samples analysis [36, 37]. Extracted proteins can be further analyzed by wide spectrum of sophisticated proteomic methods, like 2D-SDS-PAGE or MALDI-MS. Especially for those methods, fractionation of the sample and removal of high-abundant proteins greatly enhances the sensitivity. Therefore, using chicken anti-Zn antibody for immunoextraction of Zn proteins offers great possibilities. Classically, high abundant proteins from plasma/serum are depleted. Many commercial kits are obtainable for this purpose; however, it was found that numerous nontarget proteins are codepleted as a consequence of nonspecific protein–protein interactions or binding with target high-abundant proteins. Label-free shotgun mass spectroscopy revealed that in this fraction, which was named as depletome, 137 nontarget low-abundant proteins were present. Their functions were predominantly in transport and binding of nucleotides, metal ions, carbohydrates, and lipids. Twelve proteins in this usually ignored fraction were identified as to be involved in zinc ion binding [38]. Advantages of depletion-free approach for peptidomic analysis in biomarkers discovery are shown in the paper of Araki et al. [39]. The authors have shown that the concentration of low-abundant peptides, from which some contained Zn binding sequence, is affected by albumin depletion.

4 Conclusions

The importance of metalloproteins and other metal binding species is unexceptionable; however the accurate and precise determination of these compounds has to overcome certain obstacles. Spectroscopic methods including inductively coupled plasma mass spectrometry and others are widely used for determination of the content and distribution of numerous micro and macroelements including zinc in biological samples [40, 41], but they do not allow separation of the protein-bound analyte from the sample and their use for preparation purposes is not possible. Imaging of zinc proteins in biological materials via fluorescent probes [42], quantum dots [43], laser-induced breakdown spectroscopy (LIBS) [44, 45], or secondary ion mass spectrometry at a spatial resolution of approximately 50 nm (NanoSIMS) [46], allow to study zinc localization in cells or tissues, but they don't allow separation of Zn proteins or their further analysis. Except chromatographic techniques mentioned above [47], electrophoresis would be also usable for separation of Zn-proteins after their radioactive labeling with excellent detection [48]. However, the necessity of radioactive labeling limits practical usability of this method or its using in separation manner. Other possibility is combination of 1DE and laser ablation ICP-MS as shown in [49] or 2DE and synchrotron radiation X-ray fluorescence (SR-XRF) and graphite furnace atomic absorption spectrometry (GFAAS) [50] for metals identification in bands or spots and consequent proteins identification with MALDI-TOF. This approach offers possibility of identification of Zn-proteins in the sample, but combination of at least three sophisticated methods makes it complicated and both separation and detection results may be affected by the presence of high-abundant proteins and metal losses during electrophoretic separation [29].

To improve this process, an elegant and effective method utilizing paramagnetic particles has been suggested in this study. The employed anti-zinc antibodies proved to be an effective tool for immunoseparation of zinc containing species and even though further optimization may be required for particular application, this method has the potential for use in practice.

Financial support from Liga proti rakovine Praha 2012, NANOSEMED GA AV KAN208130801, NanoBioTECell GACR P102/11/1068, and CEITEC CZ.1.05/1.1.00/02.0068 is highly acknowledged. The authors wish to express their thanks to Natalie Matijescukova for excellent technical assistance.

The authors have declared no conflict of interest.

5 References

[1] Maret, W., *Biometals* 2011, *24*, 411–418.

[2] Gumulec, J., Masarik, M., Krizkova, S., Adam, V., Hubalek, J., Hrabeta, J., Eckschlager, T., Stiborova, M., Kizek, R., *Curr. Med. Chem.* 2011, *18*, 5041–5051.

[3] Haase, H., Mazzatti, D. J., White, A., Ibs, K. H., Engelhardt, G., Hebel, S., Powell, J. R., Rink, L., *Mol. Med.* 2007, *13*, 362–370.

[4] Scott, B. J., Bradwell, A. R., *Clin. Chem.* 1983, *29*, 629–633.

[5] Beyersmann, D., Haase, H., *Biometals* 2001, *14*, 331–341.

[6] Wellenreuther, G., Cianci, M., Tucoulou, R., Meyer-Klaucke, W., Haase, H., *Biochem. Biophys. Res. Commun.* 2009, *380*, 198–203.

[7] Maret, W., Li, Y., *Chem. Rev.* 2009, *109*, 4682–4707.

[8] Foote, J. W., Delves, H. T., *J. Clin. Pathol.* 1984, *37*, 1050–1054.

[9] Ryvolova, M., Krizkova, S., Adam, V., Beklova, M., Trnkova, L., Hubalek, J., Kizek, R., *Curr. Anal. Chem.* 2011, *7*, 243–261.

[10] Zitka, O., Ryvolova, M., Hubalek, J., Eckschlager, T., Adam, V., Kizek, R., *Curr. Drug Metab.* 2012, *13*, 306–320.

[11] Song, M. K., Adham, N. F., *Clin. Chim. Acta* 1979, *99*, 13–21.

[12] Giroux, E. L., Durieux, M., Schechter, P. J., *Bioinorg. Chem.* 1976, *5*, 211–218.

[13] Boyett, J. D., Sullivan, J. F., *Metabolism* 1970, *19*, 148–157.

[14] Ryvolova, M., Adam, V., Kizek, R., *J. Chromatogr. A* 2012, *1326*, 31–42.

[15] Foote, J. W., Delves, H. T., *Analyst* 1984, *109*, 709–711.

[16] Adam, V., Fabrik, I., Eckschlager, T., Stiborova, M., Trnkova, L., Kizek, R., *TRAC-Trends Anal. Chem.* 2010, *29*, 409–418.

[17] Lin, T. H., Cheng, S. Y., *Biol. Trace Elem. Res.* 1996, *51*, 267–276.

[18] Bayon, M. M., Cabezuelo, A. B. S., Gonzalez, E. B., Alonso, J. I. G., Sanz-Medel, A., *J. Anal. At. Spectrom.* 1999, *14*, 947–951.

[19] Inagaki, K., Mikuriya, N., Morita, S., Haraguchi, H., Nakahara, Y., Hattori, M., Kinosita, T., Saito, H., *Analyst* 1999, *125*, 197–203.

[20] Majzlik, P., Strasky, A., Adam, V., Nemec, M., Trnkova, L., Zehnalek, J., Hubalek, J., Provaznik, I., Kizek, R., *Int. J. Electrochem. Sci.* 2011, *6*, 2171–2191.

[21] Bradford, M. M., *Anal. Biochem.* 1976, *72*, 248–254.

[22] Krizkova, S., Masarik, M., Majzlik, P., Kukacka, J., Kruseova, J., Adam, V., Prusa, R., Eckschlager, T., Stiborova, M., Kizek, R., *Acta Biochim. Pol.* 2010, *57*, 561–566.

[23] Wong, C., Sridhara, S., Bardwell, J. C. A., Jakob, U., *Biotechniques* 2000, *28*, 426–428.

[24] Krizkova, S., Adam, V., Eckschlager, T., Kizek, R., *Electrophoresis* 2009, *30*, 3726–3735.

[25] Krizkova, S., Blahova, P., Nakielna, J., Fabrik, I., Adam, V., Eckschlager, T., Beklova, M., Svobodova, Z., Horak, V., Kizek, R., *Electroanalysis* 2009, *21*, 2575–2583.

[26] Davies, I. J. T., Musa, M., Dormandy, T. L., *J. Clin. Pathol.* 1968, *21*, 359–365.

[27] Krizkova, S., Adam, V., Kizek, R., *Electrophoresis* 2009, *30*, 4029–4033.

[28] Krizkova, S., Fabrik, I., Huska, D., Adam, V., Babula, P., Hrabeta, J., Eckschlager, T., Pochop, P., Darsova, D., Kukacka, J., Prusa, R., Trnkova, L., Kizek, R., *Int. J. Mol. Sci.* 2010, *11*, 4826–4842.

[29] Jimenez, M. S., Rodriguez, L., Gomez, M. T., Castillo, J. R., *Talanta* 2010, *81*, 241–247.

[30] Adam, V., Zitka, O., Dolezal, P., Zeman, L., Horna, A., Hubalek, J., Sileny, J., Krizkova, S., Trnkova, L., Kizek, R., *Sensors* 2008, *8*, 464–487.

[31] Gingrich, J., Freeby, S., Paulus, A., *Mol. Cell. Proteomics* 2005, *4*, S364.

[32] He, X., Strong, W., *Mol. Cell. Proteomics* 2005, *4*, S365–S365.

[33] Anderson, N. L., Anderson, N. G., *Mol. Cell. Proteomics* 2002, *1*, 845–867.

[34] Scott, B. J., Bradwell, A. R., *Clin. Chem.* 1983, *29*, 629–633.

[35] Bertini, I., Decaria, L., Rosato, A., *J. Biol. Inorg. Chem.* 2010, *15*, 1071–1078.

[36] Zitka, O., Krizkova, S., Krejcova, L., Hynek, D., Gumulec, J., Masarik, M., Sochor, J., Adam, V., Hubalek, J., Trnkova, L., Kizek, R., *Electrophoresis* 2011, *32*, 3207–3220.

[37] Trnkova, L., Fabrik, I., Huska, D., Skutkova, H., Beklova, M., Hubalek, J., Adam, V., Provaznik, I., Kizek, R., *J. Environ. Monit.* 2011, *13*, 2763–2769.

[38] Koutroukides, T. A., Guest, P. C., Leweke, F. M., Bailey, D. M. D., Rahmoune, H., Bahn, S., Martins-de-Souza, D., *J. Sep. Sci.* 2011, *34*, 1621–1626.

[39] Araki, Y., Nonaka, D., Tajima, A., Maruyama, M., Nitto, T., Ishikawa, H., Yoshitake, H., Yoshida, E., Kuronaka, N., Asada, K., Yanagida, M., Nojima, M., Yoshida, K., Takamori, K., Hashiguchi, T., Maruyama, I., Lee, L. J., Tanaka, K., *Proteomics* 2011, *11*, 2727–2737.

[40] Hanc, A., Komorowicz, I., Iskra, M., Majewski, W., Baralkiewicz, D., *Anal. Bioanal. Chem.* 2011, *399*, 3221–3231.

[41] Kobayashi, K., Katsuya, Y., Abdulah, R., Koyama, H., *Biol. Trace Elem. Res.* 2007, *115*, 87–93.

[42] Ueno, T., Nagano, T., *Nat. Methods* 2011, *8*, 642–645.

[43] Ren, H. B., Wu, B. Y., Chen, J. T., Yan, X. P., *Anal. Chem.* 2011, *83*, 8239–8244.

[44] Sun, Q., Tran, M., Smith, B. W., Winefordner, J. D., *Talanta* 2000, *52*, 293–300.

[45] Singh, V. K., Rai, A. K., *Lasers Med. Sci.* 2011, *26*, 673–687.

[46] Moore, K., Lombi, E., Zhao, F. J., Grovenor, C., *Anal. Bioanal. Chem.* 2011, *402*, 3263–3273.

[47] Gutierrez, R., del Valle, E. M. M., Galan, M. A., *Sep. Purif. Rev.* 2007, *36*, 71–111.

[48] Vyoral, D., Babusiak, M., Fuchs, O., Petrak, J., *J. Biochem. Biophys. Methods* 2003, *57*, 177–182.

[49] Becker, J. S., Pozebon, D., Matusch, A., Dressler, V. L., *Int. J. Mass Spectrom.* 2011, *307*, 66–69.

[50] Santos, F. A., Lima, P. M., Neves, R. C. F., Moraes, P. M., Perez, C. A., Silva, M. O. A., Arruda, M. A. Z., Castro, G. R., Padilha, P. D., *Microchim. Acta* 2011, *173*, 43–49.

