Full Paper

Comparison of Metallothionein Detection by Using Brdicka Reaction and Enzyme-Linked Immunosorbent Assay Employing Chicken Yolk Antibodies

Sona Krizkova,[a] *Pavlina Blahova*,[a] *Johana Nakielna*,[a] *Ivo Fabrik*,[a] *Vojtech Adam*,[a,b] *Tomas Eckschlager*,[c] *Miroslava Beklova*,[d] *Zdenka Svobodova*,[e,f] *Vratislav Horak*,[g] *Rene Kizek*[a]*

[a] Department of Chemistry and Biochemistry, Mendel University of Agriculture and Forestry, Zemedelska 1, CZ-613 00 Brno, Czech Republic
*e-mail: kizek@sci.muni.cz
[b] Department of Animal Nutrition and Forage Production, Faculty of Agronomy, Mendel University of Agriculture and Forestry, Zemedelska 1, CZ-61300 Brno, Czech Republic
[c] Department of Paediatric Haematology and Oncology, 2nd Faculty of Medicine, Charles University, V Uvalu 84, CZ-15006 Prague 5, Czech Republic
[d] Department of Veterinary Ecology and Environmental Protection, University of Veterinary and Pharmaceutical Sciences, Palackeho 1–3, CZ-61242 Brno, Czech Republic
[e] Department of Veterinary Public Health and Toxicology, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences, Palackeho 1-3, CZ-61242 Brno, Czech Republic
[f] Research Institute of Fish Culture and Hydrobiology, University of South Bohemia Ceske Budejovice, Zatisi 728/II, CZ-38925 Vodnany, Czech Republic
[g] Laboratory of Tumour Biology, Department of Animal Embryology, Cell and Tissue Differentitation, Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, v.v.i., CZ-27721 Libechov, Czech Republic

Received: May 8, 2009
Accepted: August 1, 2009

Abstract
In this study, two methods for metallothioneins (MT) determination in a biological sample were compared. Particularly, twenty five human and nine pig blood serum samples and liver and kidney samples from thirty five carps (*Cyprinus carpio*) were analyzed by enzyme-linked immunosorbent assay and differential pulse voltammetry Brdicka's reaction. The results obtained by these two methods were in good agreement. For commercially available MT standard the correlation coefficient between the single concentrations signal height was 0.99. In biological samples the correlation coefficients were 0.90 for fish liver and kidney samples, 0.91 for pig blood serum and 0.93 for human blood serum.

Keywords: Metallothionein, Differential pulse voltammetry, ELISA, Chicken yolk antibodies, Human blood serum, Immunoassays, Antibodies

DOI: 10.1002/elan.200900243

1. Introduction

Metallothioneins (MT) are low molecular mass intracellular proteins rich in cysteine (30% from all amino acids). Aromatic amino acids are not present in its primary structure, but recently it was found that some metallothioneins can contain histidine [1]. In 1957, the first work describing the properties of a new protein isolated from horse kidney was published [2]. The relative molecular mass of MT ranges from 6000 to 10000. This protein is present in animals, fungi, plants and bacteria [3–8]. The number and position of cysteine residues in metallothioneins structure is highly conservative and the structural motifs cys-X-cys, cys-cys and cys-X-X-cys are formed, where X and Y are amino acid residues different from cysteine. From these motifs the binding α and β domains are able to chelate heavy metal ions [9, 10]. In numerous published papers, the MT level and stage and/or grade of malignant tumors were given into the relationship. Moreover, MT is likely to be involved in resistance formation against platinum-based cytostatics [9, 11–19] .It was shown that MT level in blood serum or in full blood can give us valuable information about the presence, type and stage of a tumor disease [3, 20–24].

For metallothionein's analysis many methods which are different in the analytical approach and character of information obtained can be used [25–29]. The most frequently used techniques for MT determination in biological samples are the saturation ones, e.g. Cd(II) saturation assay [30, 31], Ag(I) saturation assay [32, 33] and Hg(II) saturation assay [34, 35], followed by immunochemical [36–41], separation [27, 28, 42–45], electromigration [46–51] and electrochemical techniques [52–60]. Except the proteomic approach the information about the expression and function of single MT isoforms can be found by mRNA

analysis using RT-polymerase chain reaction or expression microarray [61–64]. Those methods do not inform about MT levels, but about mRNA which doesn't need to correlate with protein expression [65, 66].

Enzyme-Linked Immunosorbent Assay (ELISA) is a routine method for the determination of a wide spectrum of analytes both in clinical diagnostics and basic research. For the metallothionein determination many protocols using polyclonal [17, 37, 39, 40, 67–71] and monoclonal [36, 38, 72–74] antibodies were developed, whereas single protocols vary in arrangement, duration and sensitivity. Most frequently used polyclonal antibodies are rat and rabbit IgG fraction isolated from the blood serum of immunized animals. Considering a time-variable antibodies formation and limited amount of blood serum obtained, problems with antiserum characterization arise. An advantageous source of the polyclonal antibodies are sheep and goats, where the immunoglobulins are obtained from the milk, thus their multiple obtaining in large volumes is possible [75]. For antibodies preparation also birds, especially chickens can be used [76]. Birds as a source of polyclonal antibodies are in some respects more preferable than mammals. Higher immunoreactivity of mammalian antigens in bird organism simplifies the preparation of antibodies against mammalian conserved antigen like MT or RNA-polymerase II, which are poorly immunogenic in mammals [17, 77]. The IgY corresponding to mammalian IgG are isolated from egg yolks, which enables their obtaining in high amount. Other benefits are several fold higher immunoglobulins production and lower crossreactivity compared to the mammalian antisera.

Brdicka's reaction is one from the most frequently used electrochemical method for MT determination in biological samples [3, 14, 22, 53, 57, 78–81]. This method is based on measurement of the hydrogen evolution from the supporting electrolyte catalyzed by complex of ($[Co(NH_3)_6]Cl_3$) with SH groups of the protein at the potential from -1.3 to -1.5 V [82]. Moreover, the automated electrochemical analyzer was recently utilized for detection of metallothionein [79, 83]. The mechanism of Brdicka's reaction, however, remains still unclear [84].

As it was mentioned above, in real samples the height of single Brdicka's peaks and their positions is a resultant from the signals of all proteins present, so the contribution of MT to the total signal of all sulfur containing compounds present in the sample might be critical [58]. The proportion of MT in the sample can be increased by technique of its preparation, when some its specific properties (e.g. thermostability) are used [82, 85]. MT is not the only thermostable protein remaining in the solution after heat treatment, but it is usually the only species having significant electrochemical activity [58].

To our knowledge, Brdicka's reaction was compared to immunoanalysis only in one paper, where differential pulse polarographic MT determination in kidney and liver of the fish *Perca fluviatilis* exposed to $CdCl_2$ and $ZnSO_4$ was compared to RIA [86]. Both immunoanalysis and electroanalysis got modernized and substantial progressions particularly in instrumentation and signal processing were done. The aim of this study is to compare Brdicka's reaction in automated arrangement and direct enzyme-linked immunosorbent assay employing chicken yolk antibodies on the determination of MT in blood serum and animal tissues.

2. Experimental

2.1. Chemicals and Water Purification

Unless otherwise indicated, all chemicals were purchased from Sigma Aldrich (USA) in ACS purity. Chicken antibodies were prepared by HENA, s. r. o., Prague, Czech Republic. Two hens were immunized by the commercially available metallothionein (1 mg of the mixture of horse MT 1 and MT 2, Sigma Aldrich, USA), which was diluted in water and incubated for seven days at room temperature in order to polymerize. From the egg yolk the IgY fraction with reactivity to MT was obtained. The antibodies in phosphate buffered saline (PBS) were stabilized with 0.1% sodium azide. The protein concentration was 54.7 mg/mL in immunoglobulin fraction. 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 (Millipore Corp., USA, 18 MΩ) – MiliQ water.

2.2. Electrochemical Measurements

Differential pulse voltammetric Brdicka's reaction measurements were performed with 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and cooled sample holder (4 °C). The instrument is shown in Figure 1A. A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3 M KCl electrode was the reference and glassy carbon electrode was the auxiliary electrode. The software GPES 4.9 supplied by EcoChemie was employed for smoothing and baseline correction of raw data. The analyzed samples were deoxygenated prior to measurements by purging with argon (99.999%), saturated with water for 120 s. The Brdicka's 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 and after each measurement the supporting electrolyte was exchanged. 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, $E_{ads} = 0$ V. All experiments were carried out at 4 °C employing thermostat Julabo F12 (Labortechnik GmbH, Germany). One hundred times diluted sample in 0.1 M phosphate buffer pH 7.0 (20 μL) was used.

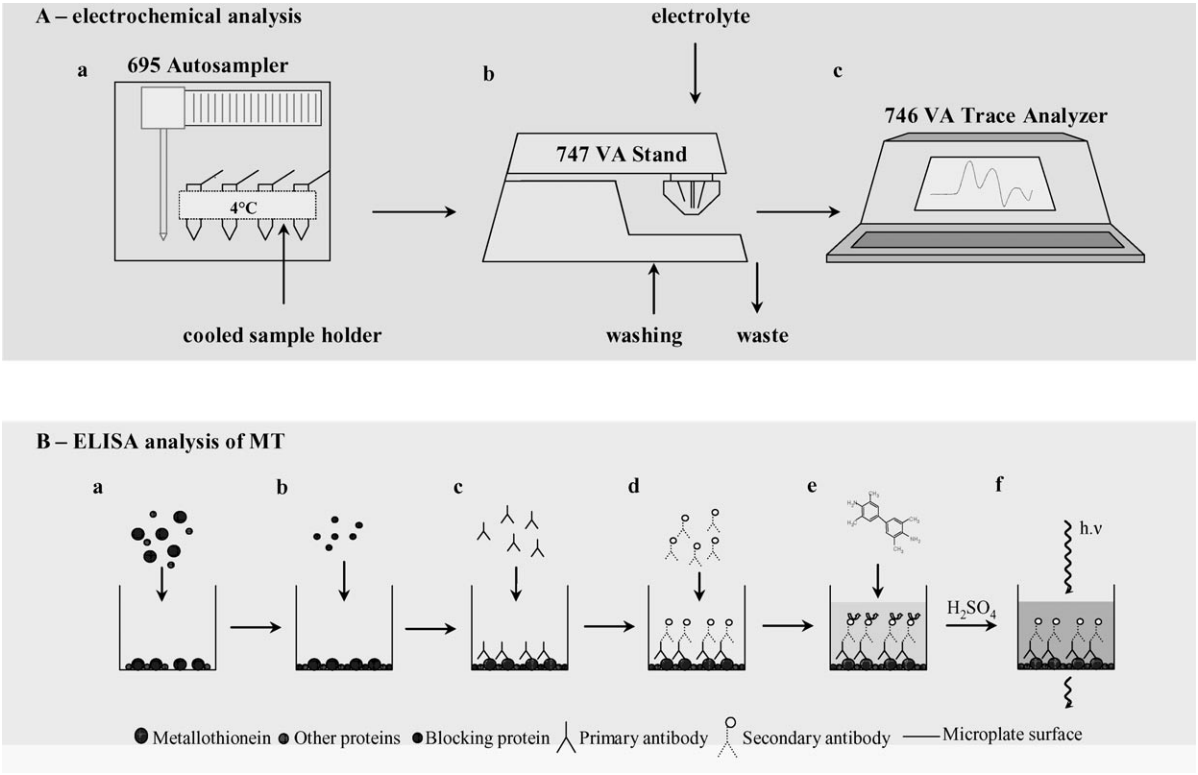

Fig. 1. (A) Scheme of automated metallothionein detection by using Brdicka's reaction. Electrochemical instrument includes (a) autosampler with the cooled sample holder; (b) VA Stand instrument using a standard cell with three electrodes; (c) 746 VA Trace Analyzer. (B) Scheme of direct ELISA. (a) Proteins including metallothioneins in a real sample were immobilized to microplate surface. (b) Then the free surface of the well was blocked with 2% BSA in PBS to prevent the unspecific binding of antibodies to its surface. (c) After the blocking the incubation with chicken primary antibody against MT followed. (d) The interaction of MT with the antibody was visualized by incubation with rabbit secondary antibody against chicken immonoglobulins labelled with horseradish peroxidase, (e) consequent enzymatic reaction with the chromogenic substrate (TMB) and absorbance (f) measurement at 450 nm.

2.3. Dot-Immunobinding Assay

For immunobinding assay the PVDF membrane (Bio-Rad, USA) was used. The sample (1 μL) was applied and dried. Further the membrane was blocked in 2% bovine serum albumin (BSA) in PBS (137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH_2PO_4, 4.3 mM Na_2HPO_4, pH 7.4) for 0.5 h with constant shaking. The incubation with primary antibody (1:500 diluted) was carried out for 1 h at 37 °C. After the three times repeated washing in PBS containing 0.05% (v/v) Tween-20 (0.05% PBS-T) for 5 min, the membrane was incubated in the presence of secondary antibody (rabbit anti-chicken labeled with horseradish peroxidase, Sigma Aldrich, USA) in dilution 1:5000 for 1 h at 37 °C. Then the membrane was washed three times in 0.05% PBS-T for 5 min and incubated in 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 the sufficient coloring the reaction was stopped by rinsing in water.

2.4. Enzyme-Linked ImmunoSorbent Assay (ELISA)

MT standard (100 μL) or 1000 × diluted sample in 0.05 M carbonate buffer (0.032 M Na_2CO_3 and 0.068 M $NaHCO_3$, pH 9.6) was adsorbed on polystyrene 96 well microplate (Schoeller, Czech Republic) for 1 h at 37 °C. After the removing of the samples and five-times repeated washing of the wells with PBS buffer with 0.05% (v/v) Tween-20 (PBS-T) for 5 min, the free surface of the well was blocked with 2% (m/v) BSA in PBS for 2 h at 37 °C to prevent the unspecific binding of antibodies to the microplate surface. After the five-times washing of the wells with PBS-T, which was repeated after each step of the procedure, the plate was incubated with 100 μL of the primary antibody (1:500 diluted) in 1% BSA-PBS for 14 h at 4 °C. Then, the plate was incubated with 100 μL of rabbit anti-chicken secondary antibody (rabbit anti-chicken labeled with horseradish peroxidase, Sigma Aldrich, USA) in dilution of 1:30 000 in 1% BSA-PBS for 1 h at 37 °C to visualize the interaction of MT with the primary antibody. The amount of primary and secondary antibodies and incubation times were optimized (not shown). Prior to application of the chromogenic substrate the plate was incubated for 15 min with PBS. The mixture (100 μL) from the following substances (0.001% (w/v) of 3,3′,5,5′-tetramethylbenzidine (TMB) as substrate for horseradish peroxidase, 10 μL of hydrogen peroxide (30%, v/v), 0.5 mL of 2 M sodium acetate adjusted to pH 5.8 with 1 M citric acid and 10 mL of Mili-Q water) was applied to single well on the microplate. After the

sufficient coloring (30 min at 37 °C) the reaction was stopped with 50 μL of 0.5 M H_2SO_4. The absorbance of the yellow product was measured at 450 nm using TECAN Sunrise microplate reader (Switzerland). Measurements of the samples and standards was carried out in triplicates, in each plate the calibration curve (MT standard diluted in carbonate buffer) and blind wells (with carbonate buffer instead sample and diluting solution for antibodies instead primary antibody) was included. The scheme of the procedure is shown in Figure 1B.

2.5. Sodium-Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis was performed according to Laemmli [87] using a Biometra maxigel apparatus with gel dimension of 17 × 18 cm (Biometra, Germany). First 15% (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 concentration; the polymerization of the running gel was carried out at room temperature for 1 h and 30 min for the stacking gel. Prior to analysis the samples were mixed with sample buffer containing 5% (v/v) 2-mercaptoethanol in a 1:1 ratio. The samples were boiled for 2 min, and then loaded onto a gel in 20 μL aliquots. For the determination of the molecular mass, the protein ladder 'Precision plus protein standards' from Biorad was used. The electrophoresis was run at 150 V with cooling by tap water at 7 °C until the front dye reached the bottom of the gel. Silver staining of the gels was performed according to Oakley et al. [88].

2.6. Biological Samples

Human blood serum. Blood serum samples for twenty five patients with head and neck cancer were obtained from the Department of Otolaryngology and Maxillofacial Surgery, University Hospital, Brno, Czech Republic. The average age of the patients was 46 ± 9. *MeLiM pigs*. The miniature pigs are housed in the Institute of Animal Physiology and Genetics of the Academy of Sciences of the Czech Republic, Libechov. The MeLiM strain with hereditary melanoma was established using selective breeding. Three blood serum samples from animals with melanomas and six from clinically healthy animals were used in our experiments. *Carp samples*. The samples of liver and kidney tissues from 35 carp (*Cyprinus carpio*) of average weight 84 ± 6 g and one year old were obtained from Fisheries Pohořelice C.o. Czech Republic.

2.7. Sample Preparation

The samples of fish tissues in weight of approximately 0.1 g were frozen with liquid nitrogen and spread in mortar, and then exactly 1000 μL of 0.2 M phosphate buffer pH 7.2 was added to the homogenized sample. The obtained homogenate was transferred into test-tube and vortexed for 15 min at 4 °C (Vortex Genie, USA). The supernatant was subsequently heat treated.

The samples of blood serum and disintegrated fish tissues were kept at 99 °C in a thermomixer (Eppendorf 5430, USA) for 15 min with occasional stirring, and then cooled to 4 °C. The denatured homogenates were centrifuged at 4 °C, 15000 × g for 30 min. (Eppendorf 5402, USA). Heat treatment effectively denatures and removes thermolabile and high molecular weight proteins out from samples [55]. The prepared samples were analyzed by automated Brdicka's reaction and by ELISA.

2.8. Accuracy, Precision and Recovery

Accuracy, precision and recovery of metallothionein were evaluated with homogenates (pig blood serum) spiked with MT standard. Homogenates were assayed blindly and MT concentration was derived from the calibration curves. The spiking of metallothionein was determined as a standard measured without presence of real sample. Accuracy was evaluated by comparing estimated concentrations with known concentrations of MT. Calculation of accuracy (%Bias), precision (%CV) and recovery was carried out as indicated by Causon [89] and Bugianesi et al. [90].

3. Results and Discussion

3.1. Sodium-Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) belongs to the most commonly and routinely used methods for determination of proteins [91]. The typical denaturing SDS-PAGE electrophoreograms of fish tissue samples (lines 1 and 2), human (lines 3 and 4) and pig (lines 5 and 6) blood serum samples are shown in Figure 2. The samples were heat treated prior to SDS-PAGE according to procedure mentioned in Section 2. We were able to detect MT in the samples, however, in spite of this purification step denaturing numerous proteins, relatively big amount of proteins different from metallothioneins potentially interfering with its electrochemical determination was present in samples. Moreover, low-molecular mass thiols (cysteine, glutathione and SH-rich peptides), which can be hardly denatured by heat treatment, can interfere with electrochemical MT determination too [92]. Nevertheless, we proved that both low molecular thiols and non-heated-degradable proteins did not interfere the Brdicka's measurements [24]. Surface active compounds present in biological samples can influence the adsorption of the analytes onto the electrode surface, however it was shown that the influence is minimal in this type of a solution [24].

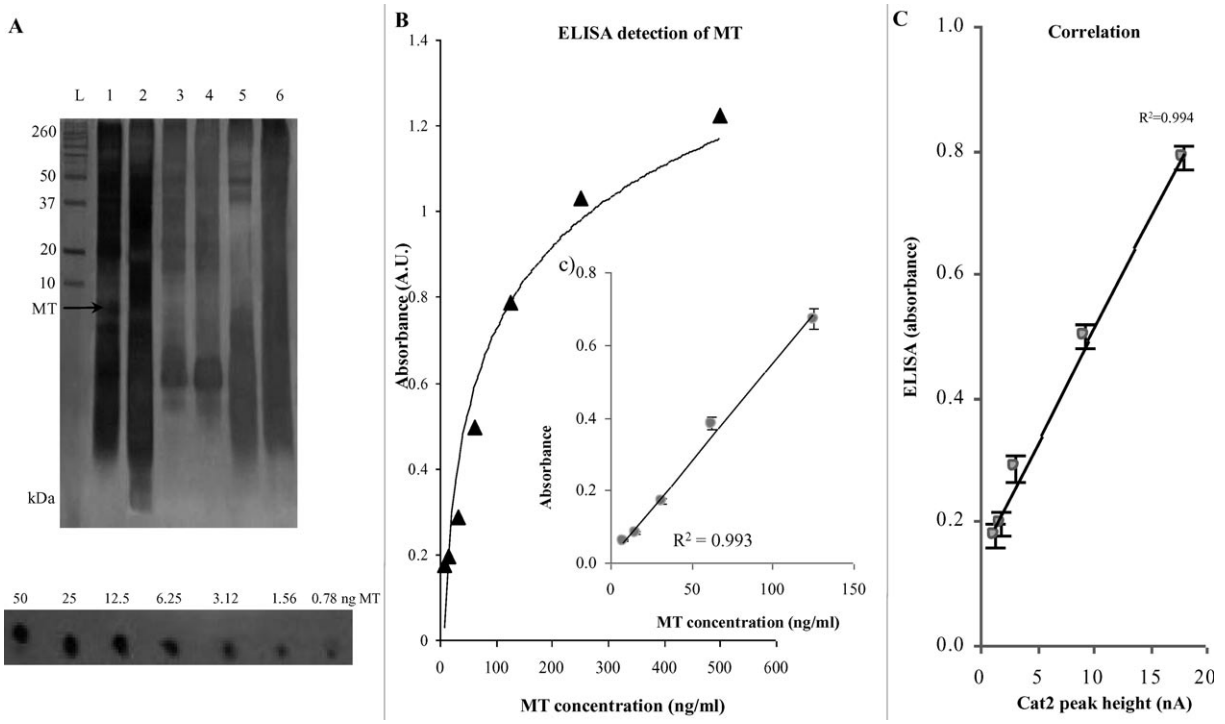

Fig. 2. (A) SDS-PAGE. Line L: molecular mass standards, the sizes of the standards: 10, 20, 37, 50, 75, 100, 150, 260 kDa, lines 1 and 2: kidney and liver homogenate from perch, lines 3 and 4: human blood serum, lines 5 and 6: pig blood serum, in inset: detection of MT by dotting immunoassay. (B) The dependence of absorbance on MT concentration; in inset: the linear part of this dependence within the range from 7.8 to 125 ng/mL. (C) Correlation between the signal heights obtained by ELISA and Brdicka's reaction determined for MT standard within the range from 7.8 to 62.5 ng/mL.

3.2. Electrochemical Determination of MT

Autosampler allowing injection of low sample volumes (units of μL) coupled with stationary electrochemical analyzer was used for MT determination in the samples. The used instrumentation allows minimizing sample volumes compared to classical arrangement, where tens or even hundreds μL sample volumes are needed (Fig. 1). A measurement lasts less than five minutes. The calibration curve (dependence of Cat2 peak height on MT concentration, $y = 1.4961 + 0.0799$, $R^2 = 0.9928$) within the range from 0.25 to 5 μM was measured. Relative standard deviation of a measurement was 3.6%. The detection limit for MT estimated as 3 signal/noise (3 S/N) was 5 nM. The detection limit (3 *S*/*N*) were calculated according to Long and Winefordner [93], whereas *N* was expressed as standard deviation of noise determined in the signal domain. The proposed methodology was utilized for determination of MT levels samples of interest. MT concentration was from 1.5 to 4.2 μM in blood serum of the patients with head and neck cancer, 1.80 to 6.2 μM in pig blood serum samples and 10 to 440 μg/g in fish tissues.

3.3. Immunochemical Determination of Metallothionein

The functionality and reactivity of the antibodies with metallothionein standard (mixture of horse MT1 and MT2, Sigma-Aldrich, St. Louis, USA) was confirmed by dot immunobinding analysis (Fig. 2A). We were able to detect 0.78 ng MT in one μL, which refers to the concentration of 0.78 μg/mL (0.15 μM) by using chicken antibodies. After the confirmation of antibodies reactivity, the ELISA method was optimized. The dilutions of primary (1:50; 1:100; 1:200; 1:300; 1:500; 1:700 and 1:1000) and secondary (1:5000; 1:10 000; 1:20 000; 1:30 000 and 1:50 000) antibody were tested. The dilutions of the antibodies were proposed on basis of manufacturers manual. In addition, the blocking time of the free well surface (1 to 14 h), time of incubation with primary (1 to 14 h) and secondary antibody (1 to 3 h) were optimized too (not shown). Under the optimal conditions (dilution of the primary antibody 1:500, dilution of the secondary antibody 1:30 000, blocking time 2 h, incubation with primary antibody 14 h, incubation with secondary antibody 1 h, for other details see Experimental section) the dependence of the absorbance on MT concentration was determined (Fig. 2B). This dependence was linear within the range from 7.8 to 125 ng/mL (1.1 nM to 18.4 nM) with R^2 higher than 0.99 (relative standard deviation 8.8%) and is shown in inset in Figure 2B. The detection limit was estimated as 2 ng/mL (0.3 nM). To find suitable dilution of a sample we tested 100×; 500×; 1000×; 2000× and 10 000× diluted human blood serum samples with coating buffer. Samples diluted 100× and 500× gave only minimal decrease of the absorbance, which was not proportional to dilution. At the last three samples dilutions

Table 1. Recovery of MT for analysis of the pig blood serum sample ($n = 3$).

	Homogenate (ng/mL) [a]	Spiking (ng/mL) [a]	Homogenate + spiking (μM)[a]	Recovery (%)
MT addition 1	25.3 ± 1.2 (4.7)	12.5 ± 0.5 (4.0)	36.7 ± 2.8 (7.7)	97
MT addition 2	25.3 ± 1.2 (4.7)	25.0 ± 1.0 (4.0)	57.5 ± 4.9 (8.5)	114
MT addition 3	25.3 ± 1.2 (4.7)	50.0 ± 0.4 (1.0)	78.2 ± 4.5 (5.8)	104

[a] MT concentration; expressed as mean ± *SD* (CV%)

(e.g. 1000 × ; 2000× and 10 000 ×) the linear decrease of the absorbance was observed. Dilution 1000× was chosen for the following experiments. Recovery of the method was evaluated by addition of MT standard to the 1000× diluted sample into resulting concentrations of 12.5, 25 and 50 ng/mL (Table 1). The recovery varied from 97 to 114%. ELISA was used also for detection of the same biological samples as Brdicka's reaction. MT concentration was from 2.1 to 3.4 μM in blood serum of the patients with head and neck cancer, 2.4 to 5.6 μM in pig blood serum samples and 2 to 357 μg/g per fish tissue.

3.4. Correlation Between ELISA and Differential Pulse Voltammetry Brdicka's Reaction

The correlation coefficient between the signal height of MT standards measured by ELISA and by Brdicka's reaction was higher than 0.99 within concentration range from 7.8 to 62.5 ng/mL (Fig. 2C). In the case of human blood serum samples the correlation coefficient between the MT concentration determined by both methods was higher than 0.935 (Fig. 3A). The relation between ELISA and Brdicka's reaction results can be expressed by the following equation: $MT(ELISA) = 0.504 \times MT(Brdicka's\ reaction) + 1.211$. The average difference between Brdicka's reaction and ELISA was 12.2%. The correlation coefficient for pig blood serum samples analysis was 0.918 with the equation: $MT(ELISA) = 0.730 \times MT(Brdicka's\ reaction) + 1.136$ (Fig. 3B). The average difference between the results obtained by these two methods was 10%. In the case of liver and kidney tissues from carp the correlation coefficient was 0.903 with the equation: $MT(ELISA) = 0.751 \times MT(Brdicka's\ reaction) + 9.968$ (Fig. 3C). The average difference between the results was 23%.

We proposed and optimized ELISA method for MT determination using chicken yolk antibodies. This method was utilized for analysis of blood serum from pigs and patients with tumor disease and extracts from carp tissues. The results were compared to those obtained by routinely used electrochemical method differential pulse voltammetry Brdicka's reaction. The values of the correlation coefficients and average differences between the results indicate that the ballast proteins and low-molecular mass thiols did not affect the determination of MT by both techniques. The slight differences between techniques could be caused by high MT content in the samples and its aggregation and multimerization as a consequence of its reoxidation [71]. This aggregation can be prevented by addition of detergents or reducing agents into the sample prior its heat treatment. Moreover, higher differences between Brdicka's reaction and ELISA and lower correlation coefficient in the fish tissues, can be probably associated with lower reactivity of the antibodies for fish MT.

MT determination in animal tissues by immunoanalysis was previously compared to other methods, mostly to saturation assays. The comparison of ELISA with Ag saturation assay and thiomolybdate assay was performed by Bienengraber et al. [94]. In spite of the fact that MT can be quantified by all three mentioned methods, ELISA, in contrast to saturation methods, didn't give false negative or false positive MT levels in real samples (rat liver cytosol) and exhibited three-times higher sensitivity. Chan et al. compared ELISA to Ag-hem binding assay [40]. ELISA routinely estimated the total MT in samples of rat, mouse, and human liver and kidney at 88%, of the value obtained by the silver-hem binding assay. Estimation of MT in samples using purified MT-2 antibodies provided slightly lower values (72%) for MT in tissues as compared to the Ag-hem method [40]. The differences correspond to the proportion of MT-2 in total content of all MT isoforms. Waalkes et al. compared three methods, particularly cadmium radioassay, mercury radioassay, and radioimmunoassay (RIA), for MT detection in livers of rats treated with $CdCl_2$ and $ZnCl_2$ [95]. In livers of rats treated with $CdCl_2$ RIA gave lower results in comparison to metal radioassays. This phenomenon can be related to the fact that an organism protects itself by a complex process of heavy metals chelatation and detoxification, thus all proteins with heavy metal content can influence the analysis results by using the saturation technique. Our data indicate that although Brdicka's reaction is based on unspecific interaction of protein SH groups with Co^{3+} ion, the results obtained are comparable to ELISA (Fig. 3). To our knowledge, chicken antibodies against MT have been used only once as coating antibody in sandwich ELISA [17]. Moreover differential pulse voltammetry Brdicka's reaction for MT determination has not been compared to other techniques yet.

Hogstrand and Haux compared an electrochemical method, differential pulse polarography to RIA [86]. They determined that MT contents in tissues of fish (*Perca fluviatilis*, perch) exposed to $CdCl_2$ and $ZnSO_4$ measured by differential pulse polarography and RIA were in very good agreement with the correlation coefficient 0.92. The relationship between MT concentration determined by these two methods was expressed by the following equation: $MT(RIA) = MT(DPP) \times 0.99 - 0.048$ [86]. The higher correlation between the results obtained might be caused by

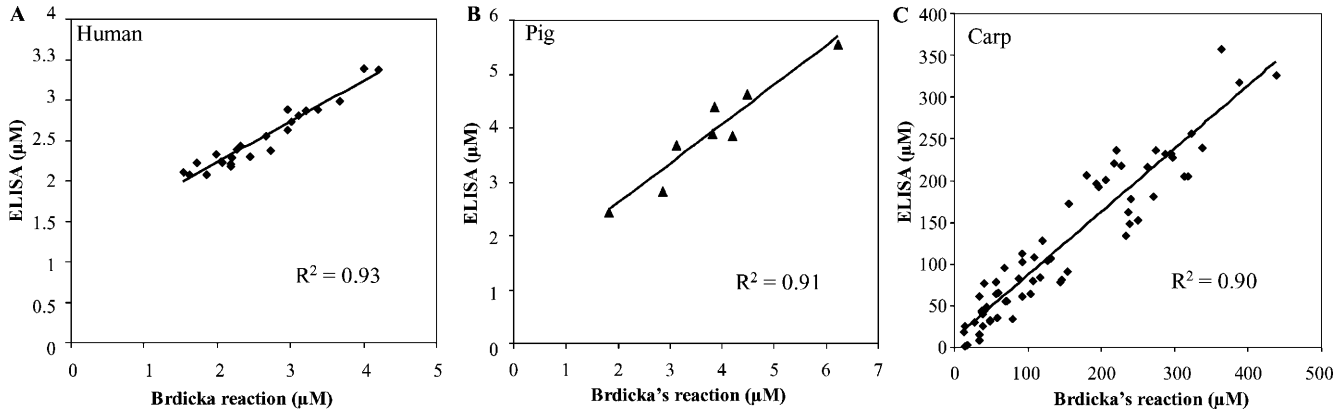

Fig. 3. The correlation between MT concentration measured by ELISA and Brdicka's reaction for (a) samples of human blood serum from patients with tumor disease, (b) samples of pig blood serum and (c) samples of fish livers and kidneys.

using metallothionein from *Perca fluviatilis* as an antigen for antibodies preparation, which ensured a higher reactivity of the antibodies for fish metallothionein. Since time of the publishing of the mentioned paper, the detection limit of Brdicka's reaction has been lowered by seven orders. This great development was achieved primarily by adsorptive transfer technique (AdTS), in which the target protein are adsorbed onto the surface of the hanging mercury drop electrode (HMDE) and transferred into the supporting electrolyte, where the measurement is carried out. Other improvement is using differential pulse voltammetry instead polarography and foremost mathematical processing of the electroanalytical signal, which allows its automation. Moreover, we shown that ELISA under optimal conditions and ultrasensitive Brdicka's reaction gave very similar results as shown Hogstrand and Haux [86].

4. Conclusions

Two methods (differential pulse voltammetry Brdicka's reaction and ELISA) for metallothionein determination in biological samples were compared. In case of MT standards analysis the correlation coefficient higher than 0.99 was achieved and in the case of typical samples analyzed by automated electrochemical analysis – fish tissues, blood serum samples from patients with tumor disease and pig blood serum the correlation coefficients were as follows: 0.90, 0.93 and 0.91, respectively. It can be concluded that both methods give very similar results. In addition, both methods are suitable for routine analysis of larger set of samples, but they are different in the type of the result obtained.

5. Acknowledgements

Financial support from INCHEMBIOL MSM 0021622412, MSM 6215712402, MSM 0021620813, GAAV IAA401990701 and Ligra proti rakovine Prague is highly acknowledged. Moreover, the authors express their thanks to Veronika Kohoutkova for English correction.

6. Abbreviations

AdTS	Adsorptive transfer technique
AEC	3-Aminoethyl-9-carbazole
BSA	Bovine serum albumin
DPV	Differential pulse voltammetry
ELISA	Enzyme-linked immunosorbent assay
HMDE	Hanging mercury drop electrode
IgG	Immunoglobulin G
IgY	Immunoglobulin Y
MT	Metallothionein
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween-20
PVDF	Polyvinylidenfluoride
RIA	RadioImmunoAssay
RT-PCR	Reverse transcription polymerase chain reaction
S.D.	Standard deviation
SDS-PAGE	Sodium-dodecyl sulfate polyacrylamide gel electrophoresis
SH	Thiol group
TMB	3,3′,5,5′-Tetramethylbenzidine

7. References

[1] C. A. Blindauer, *J. Inorg. Biochem.* **2007**, *102*, 507.
[2] M. Margoshes, B. L. Vallee, *J. Am. Chem. Soc.* **1957**, *79*, 4813.
[3] S. Krizkova, I. Fabrik, V. Adam, J. Kukacka, R. Prusa, G. J. Chavis, L. Trnkova, J. Strnadel, V. Horak, R. Kizek, *Sensors* **2008**, *8*, 3106.
[4] J. H. R. Kägi, A. Schäffer, *Biochemistry* **1988**, *27*, 8509.
[5] D. H. Hamer, *Annu. Rev. Biochem.* **1986**, *55*, 913.
[6] R. J. Cousins, *Physiol. Rev.* **1985**, *65*, 238.
[7] J. H. R. Kagi, *Meth. Enzymol.* **1991**, *205*, 613.
[8] G. Roesijadi, *Aquat. Toxicol.* **1992**, *22*, 81.
[9] P. Moffatt, F. Denizeau, *Drug Metab. Rev.* **1997**, *29*, 261.
[10] P. Coyle, J. C. Philcox, L. C. Carey, A. M. Rofe, *Cell. Mol. Life Sci.* **2002**, *59*, 627.

[11] P. Surowiak, V. Materna, I. Kaplenko, M. Spaczynski, M. Dietel, H. Lage, M. Zabel, *Virchows Arch.* **2005**, *447*, 626.
[12] C. Wulfing, H. van Ahlen, E. Eltze, H. Piechota, L. Hertle, K. W. Schmid, *World J. Urol.* **2007**, *25*, 199.
[13] P. Surowiak, V. Materna, A. Maciejczyk, M. Pudelko, E. Markwitz, M. Spaczynski, M. Dietel, M. Zabel, H. Lage, *Virchows Arch.* **2007**, *450*, 279.
[14] J. Kukacka, H. Tesfaye, J. Malis, V. Adam, I. Fabrik, K. Kotaska, J. Stary, R. Kizek, R. Prusa, *Clin. Chem.* **2008**, *54*, A11.
[15] S. Krizkova, I. Fabrik, V. Adam, P. Hrabeta, T. Eckschlager, R. Kizek, *Bratislava Med. J. – Bratislavske Lekarske Listy* **2009**, *110*, 93.
[16] I. Fabrik, S. Krizkova, D. Huska, V. Adam, J. Hubalek, L. Trnkova, T. Eckschlager, J. Kukacka, R. Prusa, R. Kizek, *Electroanalysis* **2008**, *20*, 1521.
[17] V. K. Sullivan, F. R. Burnett, R. J. Cousins, *J. Nutr.* **1997**, *128*, 707.
[18] V. Adam, O. Blastik, S. Krizkova, P. Lubal, J. Kukacka, R. Prusa, R. Kizek, *Chem. Listy.* **2008**, *102*, 51.
[19] S. Krizkova, I. Fabrik, V. Adam, J. Hrabeta, T. Eckschlager, R. Kizek, *Bratislava Med. J. – Bratislavske Lekarske Listy* **2009**, *110*, 93.
[20] G. Weinlich, K. Eisendle, E. Hassler, M. Baltaci, P. O. Fritsch, B. Zelger, *Br. J. Cancer*. **2006**, *94*, 835.
[21] V. Adam, S. Krizkova, I. Fabrik, O. Zitka, Z. Horakova, H. Binkova, J. Hrabeta, T. Eckschlager, J. Kukacka, R. Prusa, E. Sykorova, R. Kizek, *Tumor Biol.* **2007**, *28*, 43.
[22] V. Adam, I. Fabrik, J. Nakielna, V. Hrdinova, P. Blahova, S. Krizkova, J. Kukacka, R. Prusa, R. Kizek, *Tumor Biol.* **2007**, *28*, 79.
[23] J. Petrlova, O. Blastik, R. Prusa, J. Kukacka, R. Mikelova, M. Stiborova, V. Adam, O. Zitka, T. Eckschlager, R. Kizek, *Klinicka Onkologie* **2006**, *19*, 138.
[24] S. Krizkova, I. Fabrik, V. Adam, J. Kukacka, R. Prusa, L. Trnkova, J. Strnadel, V. Horak, R. Kizek, *Electroanalysis* **2009**, *21*, 640.
[25] M. Dabrio, A. R. Rodriguez, G. Bordin, M. J. Bebianno, M. De Ley, I. Sestakova, M. Vasak, M. Nordberg, *J. Inorg. Biochem.* **2002**, *88*, 123.
[26] J. Szpunar, *Analyst* **2005**, *130*, 442.
[27] R. Lobinski, H. Chassaigne, J. Szpunar, *Talanta* **1998**, *46*, 271.
[28] A. Prange, D. Schaumloffel, *Anal. Bioanal. Chem.* **2002**, *373*, 441.
[29] K. H. Summer, D. Klein, *Meth. Enzymol.* **1991**, *205*, 57.
[30] D. Klein, S. Sato, K. H. Summer, *Anal. Biochem.* **1994**, *221*, 405.
[31] D. Klein, R. Bartsch, K. H. Summer, *Anal. Biochem.* **1990**, *189*, 35.
[32] J. Delramo, A. Torreblanca, M. Martinez, A. Pastor, J. Diazmayans, *Mar. Environ. Res.* **1995**, *39*, 121.
[33] A. M. Scheuhammer, M. G. Cherian, *Meth. Enzymol.* **1991**, *205*, 78.
[34] M. D. Dutton, M. Stephenson, J. F. Klaverkamp, *Environ. Toxicol. Chem.* **1993**, *12*, 1193.
[35] J. F. Klaverkamp, K. Wautier, C. L. Baron, *Aquat. Toxicol.* **2000**, *50*, 13.
[36] T. Hirauchi, M. Okabe, T. Nagashima, T. Niioka, *Trace Element Electrolytes* **1999**, *16*, 177.
[37] A. Tanguy, I. Boutet, R. Riso, P. Boudry, M. Auffret, D. Moraga, *Mar. Ecol. – Prog. Ser.* **2003**, *257*, 87.
[38] H. Butcher, W. Kennette, O. Collins, J. Demoor, J. Koropatnick, *J. Immunol. Meth*. **2003**, *272*, 247.
[39] I. Boutet, A. Tanguy, M. Auffret, R. Riso, D. Moraga, *Environ. Toxicol. Chem.* **2002**, *21*, 1009.
[40] H. M. Chan, M. G. Cherian, I. Bremner, *Toxicol. Appl. Pharmacol.* **1992**, *116*, 267.
[41] W. F. Tang, T. Kido, W. A. Gross, K. Nogawa, E. Sabbioni, Z. A. Shaikh, *J. Anal. Toxicol.* **1999**, *23*, 153.
[42] K. Nostelbacher, M. Kirchgessner, G. I. Stangl, *J. Chromatogr. B* **2000**, *744*, 273.
[43] H. Chassaigne, R. Lobinski, *Fresenius J. Anal. Chem.* **1998**, *361*, 267.
[44] J. Jebali, M. Banni, H. Gerbej, H. Boussetta, J. Lopez-Barea, J. Alhama, *Mar. Environ. Res.* **2008**, *65*, 358.
[45] J. Szpunar, R. Lobinski, *Anal. Bioanal. Chem.* **2002**, *373*, 404.
[46] J. H. Beattie, *Talanta* **1998**, *46*, 255.
[47] V. Virtanen, G. Bordin, A. R. Rodriguez, *J. Chromatogr. A*. **1996**, *734*, 391.
[48] D. Schaumloffel, A. Prange, G. Marx, K. G. Heumann, P. Bratter, *Anal. Bioanal. Chem.* **2002**, *372*, 155.
[49] M. P. Richards, J. H. Beattie, *J. Chromatogr. B.* **1995**, *669*, 27.
[50] A. Prange, D. Schaumloffel, P. Bratter, A. N. Richarz, C. Wolf, *Fresenius J. Anal. Chem.* **2001**, *371*, 764.
[51] D. Profrock, P. Leonhard, A. Prange, *Anal. Bioanal. Chem.* **2003**, *377*, 132.
[52] J. Petrlova, S. Krizkova, O. Zitka, J. Hubalek, R. Prusa, V. Adam, J. Wang, M. Beklova, B. Sures, R. Kizek, *Sens. Actuators B, Chem.* **2007**, *127*, 112.
[53] S. Krizkova, O. Zitka, V. Adam, M. Beklova, A. Horna, Z. Svobodova, B. Sures, L. Trnkova, L. Zeman, R. Kizek, *Czech J. Animal Sci*. **2007**, *52*, 143.
[54] M. L. Yang, Z. J. Zhang, Z. B. Hu, J. H. Li, *Talanta* **2006**, *69*, 1162.
[55] M. Erk, D. Ivankovic, B. Raspor, J. Pavicic, *Talanta* **2002**, *57*, 1211.
[56] M. Canli, R. M. Stagg, G. Rodger, *Environ. Pollut.* **1997**, *96*, 343.
[57] M. Strouhal, R. Kizek, J. Vecek, L. Trnkova, M. Nemec, *Bioelectrochemistry* **2003**, *60*, 29.
[58] R. W. Olafson, P. E. Olsson, *Meth. Enzymol.* **1991**, *205*, 205.
[59] J. Pavicic, B. Raspor, D. Martincic, *Mar. Biol.* **1993**, *115*, 435.
[60] B. Raspor, J. Pavicic, *Fresenius J. Anal. Chem.* **1996**, *354*, 529.
[61] R. A. Butler, G. Roesijadi, *Aquat. Toxicol.* **2001**, *54*, 59.
[62] M. Chabicovsky, H. Niederstatter, R. Thaler, E. Hodl, W. Parson, W. Rossmanith, R. Dallinger, *Toxicol. Appl. Pharmacol.* **2003**, *190*, 25.
[63] K. F. Lee, K. M. Lau, S. M. Ho, *Toxicol. Appl. Pharmacol.* **1999**, *154*, 20.
[64] M. W. Pfaffl, B. Gerstmayer, A. Bosio, W. Windlisch, *J. Nutr. Biochem.* **2003**, *14*, 691.
[65] T. Eckschlager, V. Adam, J. Hrabeta, K. Figova, R. Kizek, *Curr. Protein Pept. Sci.* **2009**, *10*, 360.
[66] S. Krizkova, I. Fabrik, V. Adam, J. Hrabeta, T. Eckschlager, R. Kizek, *Bratislava Med. J. – Bratislavske Lekarske Listy* **2009**, *110*, 93.
[67] T. Gasull, J. Hidalgo, *Neurochem. Res.* **1996**, *21*, 969.
[68] A. M. Tokheim, I. M. Armitage, B. L. Martin, *J. Biochem. Biophys. Meth*. **2005**, *63*, 43.
[69] S. Swierzcek, R. A. Abuknesha, I. Chivers, I. Baranovska, P. Cunningham, R. G. Price, *Biomarkers* **2004**, *9*, 331.
[70] Y. Liu, J. H. Zheng, W. W. Dang, H. W. Ren, M. M. Yu, B. G. Ru, *Analusis* **2000**, *28*, 361.
[71] H. M. Chan, G. A. Pringle, M. G. Cherian, *J. Biochem. Toxicol.* **1992**, *7*, 219.
[72] K. Vanhoudt, I. Nicasi, E. Vanmechelen, B. Veulemans, M. Deley, *Immunol. Lett*. **1992**, *32*, 21.
[73] M. M. Chu, Z. Q. Guo, N. Muto, N. Itoh, K. Tanaka, H. W. Ren, *Front. Biosci.* **2006**, *11*, 2113.
[74] J. Y. Cao, R. J. Cousins, *J. Nutr.* **2000**, *130*, 2180.
[75] J. P. Gosling, *Clin. Chem.* **1990**, *36*, 1408.
[76] A. Polson, M. B. Vonwechmar, G. Fazakerley, *Immun. Commun.* **1980**, *9*, 495.

[77] M. Gassmann, P. Thommes, T. Weiser, U. Hubscher, *Faseb J.* **1990**, *4*, 2528.
[78] D. Huska, S. Krizkova, M. Beklova, L. Havel, J. Zehnalek, V. Diopan, V. Adam, L. Zeman, P. Babula, R. Kizek, *Sensors* **2008**, *8*, 1039.
[79] I. Fabrik, Z. Svobodova, V. Adam, S. Krizkova, L. Trnkova, M. Beklova, M. Rodina, R. Kizek, *J. Appl. Ichthyol.* **2008**, *24*, 522.
[80] J. Petrlova, D. Potesil, R. Mikelova, O. Blastik, V. Adam, L. Trnkova, F. Jelen, R. Prusa, J. Kukacka, R. Kizek, *Electrochim. Acta* **2006**, *51*, 5112.
[81] V. Adam, J. Baloun, I. Fabrik, L. Trnkova, R. Kizek, *Sensors* **2008**, *8*, 2293.
[82] R. Kizek, J. Vacek, L. Trnkova, B. Klejdus, L. Havel, *Chem. Listy* **2004**, *98*, 166.
[83] I. Fabrik, V. Adam, R. Kizek, *CHEMagazín* **2008**, *18*, 13.
[84] B. Raspor, *J. Electroanal. Chem.* **2001**, *503*, 159.
[85] F. Geret, F. Rainglet, R. P. Cosson, *Mar. Environ. Res.* **1998**, *46*, 545.
[86] C. Hogstrand, C. Haux, *Anal. Biochem.* **1992**, *200*, 388.
[87] U. K. Laemmli, *Nature* **1970**, *227*, 680.
[88] B. R. Oakley, D. R. Kirsch, N. R. Morris, *Anal. Biochem.* **1980**, *105*, 361.
[89] R. Causon, *J. Chromatogr. B.* **1996**, *689*, 175.
[90] R. Bugianesi, M. Serafini, F. Simone, D. Y. Wu, S. Meydani, A. Ferro-Luzzi, E. Azzini, G. Maiani, *Anal. Biochem.* **2000**, *284*, 296.
[91] Y. B. Aoki, K. T. Suzuki, *Meth. Enzymol.* **1991**, *205*, 108.
[92] R. Selesovska-Fadrna, M. Fojta, T. Navratil, J. Chylkova, *Anal. Chim. Acta* **2007**, *582*, 344.
[93] G. L. Long, J. D. Winefordner, *Anal. Chem.* **1983**, *55*, A712.
[94] M. Bienengraber, S. Forderkunz, D. Klein, K. H. Summer, *Anal. Biochem.* **1995**, *228*, 69.
[95] M. P. Waalkes, J. S. Garvey, C. D. Klaassen, *Toxicol. Appl. Pharmacol.* **1985**, *79*, 524.