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Distribution of selected essential (Co, Cu, Fe, Mn, Mo, Se, Zn) and nonessential (Cd, Pb) trace elements among protein fractions from hepatic cytosol of European chub (*Squalius cephalus* L.)

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Abstract

Association of selected essential (Co, Cu, Fe, Mn, Mo, Se, Zn) and nonessential (Cd, Pb) trace elements with cytosolic proteins of different molecular masses was described for the liver of European chub (*Squalius cephalus*) from weakly contaminated Sutla River in Croatia. Principal aim was to establish basic trace element distributions among protein fractions characteristic for the fish living in the conditions of low metal exposure in the water. The fractionation of chub hepatic cytosols was carried out by size exclusion high performance liquid chromatography (SE-HPLC; Superdex™ 200 10/300 GL column), and measurements were performed by high resolution inductively coupled plasma mass spectrometry (HR ICP-MS). Elution profiles of essential elements were mostly characterized by broad peaks covering wide range of molecular masses, as a sign of incorporation of essential elements in various proteins within hepatic cytosol. Exceptions were Cu and Fe, with elution profiles characterized by sharp, narrow peaks indicating their probable association with specific proteins, metallothionein (MT) and ferritin, respectively. The main feature of the elution profile of nonessential metal Cd was also single sharp, narrow peak, coinciding with MT elution time, and indicating almost complete Cd detoxification by MT under the conditions of weak metal exposure in the water (dissolved Cd concentration ≤ 0.3 μg L^{-1}). Contrary, nonessential metal Pb was observed to bind to wide spectrum of proteins, mostly of medium molecular masses (30-100 kDa), after exposure to dissolved Pb concentration of ~1 μg L^{-1}. The obtained information within this study presents the starting point for identification and characterization of specific metal/metalloid-binding proteins in chub hepatic cytosol, which could be further on used as markers of metal/metalloid exposure or effect on fish.

Key words: European chub, hepatic cytosol, trace elements, proteins, SE-HPLC, HR ICP-MS

1. Introduction

Many trace elements play important biological roles, notably as integral parts of enzymes or protein structures (Smith et al. 1997). For example, metalloproteins are involved in electron transport, oxygen storage, metal transport, chemical bond hydrolysis, redox processes, and synthesis of biological compounds (Gellein et al. 2007). However, even essential metals (e.g. Cu, Fe, Zn), and especially those that have no known physiological functions (e.g. Cd, Pb), could also be toxic. Their toxicity is often postulated to arise from reactions in the cytosol, through non-specific binding of metals to physiologically important molecules and their consequent inactivation (Mason and Jenkins 1995). For many trace elements, biological functions and mechanisms of toxicity in different organisms are still not thoroughly investigated, and the proteins to which they bind are only partially identified and characterized (e.g. Cd: McGeer et al. 2012; Mo: Reid 2012; Se: Janz 2012).

To obtain the information on the bioavailability and toxicity of metals in the aquatic environment, it is, therefore, not sufficient to determine total or cytosolic metal concentrations in the tissues of aquatic organisms (de la Calle Guntinas et al. 2002). The knowledge on metal subcellular partitioning is also needed, which can serve as a potential indicator of metal toxicity, as reported for Cd (Wang and Rainbow 2006). Fractionation and a first screening of the complex samples, such as fish tissue cytosols, which contain so far unknown element species, could be performed by the use of size-exclusion chromatography in combination with measurement by inductively coupled plasma mass spectrometry (ICP-MS; Vacchina et al. 1999). By this approach, specific metal-binding proteins in fish tissue cytosols that participate in normal metabolism or in mechanisms of toxicity could be eventually identified and potentially used for detection of the consequences of metal contamination in the aquatic environment.

In our studies on metal induced disturbances within freshwater ecosystems, we have frequently used European chub (*Squalius cephalus*) as a bioindicator organism. So far we have investigated in detail physiological variability and levels of cytosolic concentrations of several trace elements in different chub tissues (Podrug et al. 2009; Filipović Marijić and Raspor 2010; Dragun et al. 2012a-b; Filipović Marijić and Raspor 2012). However, to our best knowledge, there is no available information on metal distribution among cytosolic proteins in organs of this important bioindicator species. As a target organ in this study we have chosen the liver, because it performs many metabolic functions and serves as a detoxification center. In addition, liver is a major producer of metal-binding proteins and therefore contains high concentrations of most metals (Roesijadi and Robinson

1994). Our primary aim was to define the basic distribution profiles of seven essential (Co, Cu, Fe, Mn, Mo, Se, Zn) and two nonessential (Cd, Pb) trace elements in the hepatic cytosol of chub living in the aquatic environment not severely contaminated with metals/metalloids, i.e. to establish to which protein group, based on their molecular masses, each trace element was associated. To achieve this aim, we have used chub from the Sutla River in Croatia, which was selected as a study area since dissolved metal concentrations in its water have been classified as either comparable to natural levels or moderately increased (Cd: 0.01-0.31 μg L^{-1}; Co: 0.06-0.42 μg L^{-1}; Cu: 0.17-3.74 μg L^{-1}; Fe: 3.1-80.5 μg L^{-1} Mn: 0.4-261.1 μg L^{-1}; Mo: 0.5-20.1 μg L^{-1}; Pb: ≤1.18 μg L^{-1}; Zn: <5.0 μg L^{-1}; Dragun et al. 2011). The information obtained by the current study will present the first step towards defining the specific metal-binding proteins in the chub hepatic cytosol which could be eventually used as biomarkers of metal exposure and/or effects.

2. Materials and methods

2.1 Fish sampling

Trace element distribution among protein fractions from hepatic cytosol was studied on fish caught during the water quality survey on the Sutla River in the late summer of 2009 (September 14th to 16th) at five selected locations from the river source to its mouth (Dragun et al. 2011 and 2012b). The selected fish species was European chub (*S. cephalus* L.), as an omnivorous fish species, wide spread in European freshwaters, and therefore suitable for monitoring purposes. The sampling was performed by electro fishing. The captured fish (75 specimens) were kept alive in aerated water tank till further processing in the laboratory. After the fish were anesthetized with Clove oil (Sigma) and sacrificed, the liver were isolated, weighed and stored at -80°C until further analyses. All captured fish were characterized by length of 15-35 cm, mass of 33-400 g and age of 1-4 years. Smaller and younger chub specimens were not included in this study due to small liver mass and consequently lack of sample for analyses. As a result, HPLC-analyzed group of chub comprised 28 larger specimens with length in the range from 18 to 35 cm, mass from 54 to 400 g and age from 2 to 4 years. Sex composition of all captured chub and of HPLC-analyzed group was comparable, with approximately 80% of females and 20% of males.

2.2 Isolation of cytosolic fraction from European chub liver

The samples of liver tissue were cut into small pieces, diluted 6 times with cooled homogenization buffer (20 mM Tris-HCl/Base, Sigma, pH 8.6 at 4°C) supplemented with reducing agent (2 mM dithiotreitol, Sigma), and

then homogenized by 10 strokes of Potter-Elvehjem homogenizer (Glas-Col) in ice cooled tube at 6,000 rpm. For better separation, the homogenates were centrifuged subsequently two times in the Avanti J-E centrifuge (Beckman Coulter) at 50,000×g for 2 h at 4°C. Supernatant (S50) obtained after second centrifugation, which represents water soluble cytosolic tissue fraction containing lyzosomes and microsomes (Bonneris et al. 2005), was separated. Aliquots of S50 were stored at -20°C for metal analyses in cytosol and at -80°C for separation by size exclusion high performance liquid chromatography (SE-HPLC).

2.3 SE-HPLC separation of chub hepatic cytosol

For the separation of chub hepatic cytosol into fractions containing different molecular mass (MM) proteins we have used size exclusion column Tricorn™ Superdex™ 200 10/300 GL (GE Healthcare Biosciences) and HPLC system (Perkin Elmer, series 200) equipped with high pressure pump, on line degasser, column oven, cooled auto sampler with injector (100 µL sample loop) and a diode array UV/VIS detector. The Superdex™ 200 10/300 GL column exclusion limit was defined as MM of 1300 kDa for globular proteins, whereas the optimal separation range was given as MM of 10-600 kDa. For determination of void volume, blue dextran was applied, with MM defined as 2000 kDa. It was eluted from 14.5 to 18.6 minutes, which corresponded to MM in the range from 1000-350 kDa. For column calibration, six standard proteins were used (thyroglobulin, apoferritin, ß-amylase, alcohol dehydrogenase, bovine serum albumin and carbonic anhydrase, Sigma), dissolved in homogenization buffer (20 mM Tris-HCl/Base, Sigma, pH 8.1 at 22°C), which was also used as mobile phase at a flow rate of 0.5 mL min^{-1} (isocratic mode). Non-denaturating mobile phase at physiological pH, such as the Tris-buffer, stabilizes the original metalloprotein complexes and is easily tolerated by HR ICP-MS (Prange and Schaumlöffel 2002; Wang et al. 2001). For each standard protein, separate chromatographic run was performed, and chromatograms were obtained using UV detection at 280 nm (Fig. 1a). Calibration straight line was created based on known MM of standard proteins and their respective elution times (t_e, Fig. 1a-b). In addition, metallothionein (MT) standard Zn-MT95 (Ikzus) was also applied, and chromatogram was obtained using UV detection at 254 nm, characteristic for metal-thiolate bond absorption (Fig. 1a). Narrow and well defined double peak, which was obtained for MTs at t_e from 29 to 32 minutes, could be a consequence of a partial overlap of MT monomer and dimer: more intense MT peak at longer retention time is characteristic for the monomers, whereas the peak at shorter retention time is characteristic for dimers or other complexes (Wang et al. 2001). The injection volume for samples (untreated hepatic cytosols) was 50 µL. The fractions were collected at one minute intervals in the plastic tubes using a fraction collector (FC 203B, Gilson). The resolution of these fractions with

the respect to molecular mass is given by the equation of the calibration straight line (Fig. 1b). For each sample, four consecutive chromatographic runs were performed, i.e. collected fractions were obtained after chromatographic separation of 200 μL of hepatic cytosol.

2.4 Determination of trace element concentrations

Trace element concentrations were determined in hepatic cytosols and in SE-HPLC separated cytosolic fractions. Hepatic cytosols were 10 times diluted with Milli-Q water and acidified (0.65% HNO_3, *Suprapur*, Merck) prior to measurements, whereas SE-HPLC collected cytosolic fractions were only acidified (0.16% HNO_3, *Suprapur*, Merck). Indium (Fluka) was added to all samples as an internal standard (1 μg L^{-1}). The measurements were performed on HR ICP-MS (Element 2, Thermo Finnigan), equipped with a double focusing mass analyzer using reverse Nier-Johnson geometry. An autosampler (ASX 510, Cetac Technologies) and sample introduction kit consisting of SeaSpray nebulizer and cyclonic spray chamber Twister were employed to transport the analytes into the plasma of HR ICP-MS. Measurements of ^{82}Se, ^{98}Mo, ^{111}Cd and ^{208}Pb were operated in low resolution mode, whereas ^{55}Mn, ^{56}Fe, ^{59}Co, ^{63}Cu and ^{66}Zn were measured in medium resolution mode. External calibration was performed using standards prepared in 2% HNO_3 (S*uprapur*, Merck) by appropriate dilutions of 100 mg L^{-1} multielement stock standard solution (Analytika). Quality control sample (QC for trace metals, UNEP GEMS/Water PE Study No. 7) was used for checking the accuracy of trace element measurements by HR ICP-MS. A generally good agreement was observed between our data and the certified values. Limits of detection (LOD) were determined based on three standard deviations of ten consecutively determined trace element concentrations in the blank sample (2 mM Tris-HCl/Base, 0.2 mM dithiotreitol, 0.65% HNO_3). LODs for trace elements measured within this study were as follows (μg L^{-1}): Cd - 0.005, Co - 0.002, Cu - 0.037, Fe - 0.084, Mn - 0.002, Mo - 0.004, Pb - 0.010, Se - 0.138, Zn - 2.40.

2.5 Data processing and statistical analyses

Chromatographic results were processed using Totalchrom Version 6.3.1 software (Perkin-Elmer). Descriptive statistical analysis and graphs were created using the statistical program SigmaPlot 11.0 for Windows. Only few representative distribution profiles for each trace element are presented in the figures (Fig. 3-5), while the remaining data are given as supplementary information.

3. Results and discussion

The chromatographic column applied in this study (Superdex™ 200 10/300 GL) enabled only rough categorization of proteins from chub hepatic cytosols according to their MM. We have designated four main protein categories (Table 1): HMM (high MM proteins, >100 kDa), MMM (medium MM proteins, 30-100 kDa), LMM (low MM proteins, 10-30 kDa), and VLMM (very low MM proteins, <10 kDa). Finer separation was not enabled by applied column, as can be seen from the exemplary chromatogram obtained for chub hepatic cytosol, with UV detection at two wavelengths, 280 nm characteristic for aromatic aminoacids (Fig. 2a) and 254 nm characteristic for metal-thiolate bond absorption (Fig. 2b). Such rough separation of protein fractions from hepatic cytosol, and subsequent estimation of protein category within which specific elements were mainly eluted, presents a first step towards defining specific metal-binding proteins in the chub hepatic cytosol.

3.1 Distribution profiles of essential elements with the narrowest cytosolic concentration range

Among studied elements, essential elements Co, Mo, Mn and Zn had the narrowest ranges of cytosolic concentrations in the chub liver, with maximum to minimum ratio amounting to 2-4. Their concentrations in the hepatic cytosol of the studied chub specimens (Co: 2.1-5.1 ng mL^{-1}; Mo: 8.7-38.5 ng mL^{-1}; Mn: 73.5-252.8 ng mL^{-1}; Zn: 3.0-11.1 µg mL^{-1}) fell mainly within their previously defined basal ranges (Co: 4.1-5.1 ng mL^{-1}; Mo: 22.8-30.6 ng mL^{-1}; Dragun et al. 2012a; Mn: 110-190 ng mL^{-1}; Zn: 3.5-6.5 µg mL^{-1}; Podrug et al. 2009). Low cytosolic Co could be explained by low Co affinity to accumulate in the liver, since it was observed in different fish species that under normal Co exposure Co strongly accumulates in the gut and kidneys (Baudin and Fritsch 1989), whereas the role of the liver becomes more important only after increasing Co exposure (Mukherjee and Kaviraj 2009). Contrary to Co, Mo was shown to accumulate in the fish liver in a dose-dependent and saturable manner (Reid 2002), and, despite the fact that this is an essential micronutrient, there is no known homeostatic control system for Mo in fish (Reid 2012). Low cytosolic Mo concentration therefore could be a consequence of specific Mo accumulation within the nuclei and mitochondria, as revealed by the studies on turtles (Anan et al. 2002). Similar to Mo, two out of three primary Mn metalloenzymes in mammals, Mn superoxide dismutase (MnSOD), which is considered to be one of the most important intracellular antioxidant enzymes (88 kDa; Fridovich and Freeman 1986), and pyruvate carboxylase (480-600 kDa; Libor et al. 1979), are localized in mitochondria, whereas only hepatic arginase is cytosolic enzyme (87 kDa; Singh and Singh 1990). And finally, fish are capable of regulating Zn accumulation over a wide range of ambient Zn levels, as shown for perch (*Perca fluviatilis*): a 100-fold increase in total water Zn concentration resulted in only a modest 1.2-fold increase of Zn concentration in the perch liver (Hogstrand et al. 1991). Dissolved Zn concentrations in the Sutla River

water were lower than 5 μg L^{-1} (Dragun et al. 2011), and therefore it could not be expected to find wide range of cytosolic Zn concentrations in the liver of chub from this river. Therefore, the principal aim for Co, Mo, Mn and Zn was to establish their basic distributions among protein fractions, characteristic either for the fish living in the conditions of low metal exposure in the water, or for very good cellular regulation which was still not exceeded.

3.1.1 Cobalt

Co distribution profile included three separate Co-containing peaks (Fig. 3a), with the predominant peak corresponding to HMM protein category (Table 1). The remaining two smaller peaks corresponded to VLMM protein category (Table 1). The VLMM peak with maximum obtained at the elution time of 40 minutes corresponded to proteins of MM in the range from 0.8-2.4 kDa. Cobalt containing compound cobalamin has MM of 1.3 kDa (Kirschbaum 1981), and therefore could be eluted together with VLMM proteins. Although main role of Co as an essential element in the fish organism is associated with its constitutive role in cobalamin, i.e. the vitamin B12 (Blust 2012), only minor part of Co present in the chub hepatic cytosol was eluted within the fraction presumably containing cobalamin. Taking in consideration that studies concerning the molecular aspects of Co uptake, its internal processing, and mechanisms of toxicity are largely lacking (Blust 2012), it would be useful to further define the characteristics and functions of HMM cytosolic proteins that bind major proportion of cytosolic Co.

3.1.2 Molybdenum

Molybdenum was mainly eluted with HMM proteins (Fig. 3b). A smaller HMM peak was eluted within the void volume, corresponding to proteins with MM above 600 kDa (Table 1), which could not be distinguished by applied column. Molybdenum serves as a cofactor of at least seven enzymes (Beers and Berkow 1998), and a major HMM peak obtained in our study (Table 1) encompassed their MM (e.g. aldehyde oxidase, ~130 kDa, Uchida et al. 2003; sulfite oxidase, ~120 kDa, Johnson and Rajagopalan 1976; Fe-Mo flavoprotein xanthine oxidase, 275 kDa, Truglio et al. 2001). Minor part of Mo was eluted after 30 minutes, within VLMM fraction (Fig. 3b, Table 1). In addition, our study confirmed the absence of Mo binding to MT in the liver of chub exposed to low or moderate Mo concentrations in the river water (up to 20 μg L^{-1}), since Mo peak was not obtained at t_e of MT (Fig. 1a). This is consistent with the report of Ricketts (2009), that MT is probably not involved in the internal detoxification of Mo. After short-term exposure of rainbow trout to Mo in concentrations

as high as 1000 mg L^{-1}, Mo failed to induce the synthesis of MT in liver, despite its obvious accumulation (Reid 2012).

3.1.3 Manganese

Manganese was distributed in three peaks (Fig. 3c). The predominant Mn peak was associated to HMM proteins (Table 1), and nearly coincided with t_e of albumin (Fig. 1), which is involved in Mn transport from the intestine to the liver (Schäfer 2004). In the liver, Mn binds to transferrin (80 kDa; Martin-Antonio et al. 2009) and in that form presents a source of Mn for delivery to other tissues (Schäfer 2004). HMM peak encompassed MM of both of these transport proteins (Table 1). Smaller second and third peak could be associated to MMM and LMM proteins with MM around 50 kDa and 20 kDa, respectively.

3.1.4 Zinc

Distribution profile of Zn was characterized by poorly resolved peaks covering wide range of MM, approximately from 10 to >600 kDa (Fig. 3d). This was not surprising, since it is well known that Zn has constitutive and catalytic roles in many proteins and enzymes. More than 3000 proteins in humans, representing 10% of the entire human genome, as well as about 10% of all genes in sequenced fish genomes carry the annotation of Zn binding (Andreini et al. 2005; Passerini et al. 2007). The first Zn peak appeared within t_e of void volume, same as in the case of Mo (Fig. 3b), and could be associated with HMM proteins of MM above 600 kDa (Table 1). The predominant peak was rather wide and asymmetrical covering both HMM and MMM protein categories, and with maximum in MMM area (Table 1). For example, t_e of standard protein alcohol dehydrogenase (Fig. 1), which is known as Zn-containing protein (Szpunar and Lobinski 1999), coincided with the HMM part of this peak. The third peak was narrow and sharp, and appeared within LMM protein category (Table 1), with maximum coinciding with t_e of MT (Fig. 1a). Since MTs were reported to play important roles in detoxification of toxic metals and maintenance of homeostasis of essential metals like Zn and Cu (Huang et al. 2004), it was expected to find Zn in chub liver in the binding form such as Zn/Cu MT (Huang et al. 2007). This peak, however, also encompassed t_e of carbonic anhydrase, which is also Zn metallo-enzyme (Szpunar and Lobinski 1999).

3.2 Distribution profiles of essential elements with wider cytosolic concentration ranges

Further on, the distribution profiles of three additional essential trace elements are presented, Cu, Fe and Se. Copper is an essential element for all aerobic organisms since its redox potential is utilized by mitochondrial cytochrome c oxidase; Cu also acts as a cofactor for a large number of other enzymes (Solomon and Lowery 1993). Iron is essential for life as an integral part of the oxygen binding metalloprotein hemoglobin and of cytochrome c oxidases in respiratory chain acting as an electron donor or acceptor (Bury et al. 2012). It also plays a role in DNA synthesis (Bury et al. 2012) and in the defense against bacterial infection (Vidal et al. 1993). Selenium is an essential element to living organisms, with a very narrow range between essentiality and toxicity (Jukola et al. 1996). Despite their essentiality, Cu, Fe and Se occurred in somewhat wider concentration ranges in the chub hepatic cytosol compared to Co, Mo, Mn and Zn (maximum to minimum ratio: 7-9) indicating enhanced trace element accumulation in the liver of some chub specimens possibly due to increased exposure, and less strict cellular regulation. Cytosolic concentrations of Cu, Fe and Se in the liver of the studied chub specimens were the following, respectively: 0.4-3.9 μg mL^{-1}; 2.3-16.8 μg mL^{-1}; 25.6-229.2 ng mL^{-1}. Copper and especially Fe levels somewhat exceeded previously defined basal ranges (Cu: 0.7-2.3 μg mL^{-1}; Fe: 3.4-6.6 μg mL^{-1}; Podrug et al. 2009). For Cu, Fe and Se, therefore, not only the basic distribution profiles could be described, but also the possible changes in their distribution within chub hepatic cytosol due to increased accumulation.

3.2.1 Copper

Copper was mainly eluted within LMM protein category (Fig. 4a) with maximum at elution time of 30 minutes (Table 1), coinciding with t_e of MT (Fig. 1a). It was an indication that the major part of Cu was probably associated with MT fraction. Apart from MT, many proteins are known to contain Cu (Szpunar and Lobinski 1999), such as transcuprein (270 kDa; Liu et al. 2007), β-amylase (200 kDa; Fig. 1), ceruloplasmin (132 kDa; Boivin et al. 2001), albumin (66 kDa; Fig. 1), superoxide dismutase (32 kDa; Richardson et al. 1975) and carbonic anhydrase (29 kDa; Fig. 1). However, additional smaller Cu peak which appeared within MMM protein region implicated Cu binding only to proteins of MM from 7-60 kDa (Table 1), such as carbonic anhydrase (LMM peak) or superoxide dismutase (SOD; MMM peak). Cu-SOD association, for example, could point both to Cu essential role in the SOD activity as a protection against oxidative stress (Sanchez et al. 2005) or to risk of Cu inhibitory effect on this antioxidant enzyme (Vutukuru et al. 2006).

Increase of cytosolic Cu concentrations was principally reflected as the increase of Cu peak height in LMM region as much as 10 times in the specimens with the highest cytosolic Cu (Fig. 4a). MT contribution to binding of total cell Cu content is relatively minor (Hogstrand et al. 1991), and can account for no more than 30-40% of total cellular Cu pool. However, our results indicate that in the hepatic cytosol MT has predominant role in the binding of Cu.

3.2.2 Iron

In chub hepatic cytosol, the basic distribution profile of Fe was characterized by two clear Fe-containing peaks of comparable height (Fig. 4b). The first peak was eluted within HMM region with maximum associated to proteins of MM around 400 kDa (Table 1), and corresponded well with t_e and MM of apoferritin (Fig. 1). Ferritin (450 kDa) is a protein mainly present in the liver tissue which serves as Fe storage protein and keeps Fe in a soluble bioavailable non-toxic form in the cytoplasm (Szpunar and Lobinski 1999; Martin-Antonio et al. 2009; Bury et al. 2012). The second Fe-peak appeared within MMM region with maximum corresponding to proteins with MM of 36 kDa. It, however, covered the range of MM from 20-60 kDa (Table 1), which could possibly involve minor binding to known Fe-containing proteins of different functions, such as catalase (60 kDa) or myoglobin (17 kDa, Martin-Antonio et al. 2009). Similar to absence of Cu HMM-peak which could be associated to ceruloplasmin, Fe peak was not registered within the region which would correspond to hemoglobin (65 kDa; Martin-Antonio et al. 2009), indicating absence of blood proteins in the samples of hepatic cytosol (Martin-Antonio et al. 2009).

Slight Fe cytosolic concentration increase was reflected in the proportional double increase of both peaks (Fig. 4b). Additional increase of cytosolic Fe concentration above 10 μg mL^{-1}, however, resulted almost completely in binding to storage protein ferritin. It could be presumed based on clear increase of the height of HMM peak, up to five times in the specimens with the highest cytosolic Fe (Fig. 4b).

3.2.3 Selenium

In chub liver, basic Se distribution among cytosolic protein fractions included three peaks (Fig. 4c). The first peak was the smallest with a maximum in HMM region, whereas the second peak, connected to the first one, was predominant and had a maximum within LMM protein category (Table 1). Both peaks together covered the wide range of MM from approximately 10 to 400 kDa, encompassing MM of several well characterized fish

selenoproteins (Janz 2012), such as enzymes involved in antioxidant defense (glutathione peroxidase, 85 kDa, Shulgin et al. 2008; thioredoxin reductase, 66 kDa, Larsson 1973), in thyroid hormone metabolism (iodothyronine deiodinase, 65 kDa, Fekkes et al. 1980), as well as MTs (12.5-16.6 kDa, Fig. 1). Paliwal et al. (1986) established that a particular isoform of MT showed a unique binding of Se, whereas Ferrarello et al. (2002) suggested that association of Se with MT plays a synergistic protective role against heavy metal toxicity. Iwai et al. (1988), however, established that radiolabelled Se was mostly eluted in the fraction corresponding to MM larger than that of MT. In our study, although t_e of MT was encompassed within the second Se peak, it was placed at the peak's right tail, thus implying only the possibility of minor Se binding to MT. The majority of Se was eluted with proteins of higher MM, same as described by Iwai et al. (1988). The third peak was eluted within VLMM category, and its maximum corresponded to MM of 1.1 kDa, pointing to the presence of some small Se-containing compound.

Slight increase of Se cytosolic concentrations in chub liver was reflected in Se sequestration within VLMM category (Fig. 4c). More substantial Se excess in the hepatic cytosol, however, resulted in its association with LMM proteins (Fig. 4c).

3.3 Distribution profiles of nonessential elements

Nonessential elements Cd and Pb had the widest cytosolic concentration ranges in the chub liver of all studied elements (maximum to minimum ratio: ~20), confirming well known fact that regulation of nonessential elements is not characteristic for fish (Heath 1987). Cytosolic concentrations in the chub liver in this study were 3.4-59.4 ng mL^{-1} for Cd and from LOD to 44.2 ng mL^{-1} for Pb. Since both Cd and Pb are known to be toxic in very low concentrations, it is very important to establish to which proteins in the soluble tissue fraction is excess quantity of these metals bound. Potential toxicity will relate to metal proportion bound to detoxifying versus non-detoxifying cellular constituents, and metal-binding proteins should be therefore well characterized (Mager 2012).

3.3.1 Cadmium

Basic Cd distribution among cytosolic protein fractions of chub liver included narrow Cd peak within LMM protein region with maximum obtained at t_e of 30 minutes (Table 1 and Fig. 5a), corresponding to MT peak (Fig. 1a). After prominent increase in cytosolic Cd concentrations, the majority of Cd was still eluted within the same

protein fraction, indicating predominant association of Cd with MTs. The height of presumable Cd-MT peak increased eight times in the samples with the highest compared to the samples with the lowest cytosolic Cd concentrations (Fig. 5a). Furthermore, we have observed the additional small Cd peak within MMM protein category (approximately 35 to 100 kDa; Table 1; Fig. 5a). It became more obvious in the samples with higher cytosolic Cd concentrations, above the range defined for the liver of chub (3-13 ng mL^{-1}; Podrug et al. 2009) living in relatively noncontaminated river water. Cadmium elution in MMM protein category, as low as it was, still was an indication that increased Cd accumulation in the hepatic cytosol could result with incomplete Cd detoxification and binding to proteins of higher MM than MTs.

The pathways of Cd metabolism in different fish organs are complex, and many of Cd-responsive proteins are yet to be identified (McGeer et al. 2012). The intracellular detoxification of Cd is primarily mediated by GSH and MTs. Insufficient binding to these ligands may lead to potential Cd competition with the essential metals for binding sites on non-MT proteins, which could induce cellular damage (McGeer et al. 2012). However, in addition to GSH and MTs, recent studies suggest that the induction of heat shock proteins (e.g. HSP70, HSP90) expression also plays an important role in the physiological changes related to metabolism and cell protection that occur in Cd-exposed aquatic animals including fish (Kwong et al. 2011; Matz and Krone 2007). As seen from Table 1 and Fig. 5a, MM of HSPs was encompassed by Cd-MMM peak. Further studies, preferably with higher Cd exposure, are therefore needed, to determine more precisely to which non-MT proteins excessive quantity of cytosolic Cd binds: does the observed MMM peak reflect an additional mode of detoxification or a potential for toxic effects.

3.3.2 Lead

Although dissolved Pb concentrations in the Sutla River water were within recommended levels for natural waters, they were noticeably increased at one sampling site (~1 μg L^{-1}; Dragun et al. 2011), which was reflected in the increased Pb concentrations in the chub hepatic cytosols (11.8-44.2 ng mL^{-1}; Dragun et al. 2012b). At four "uncontaminated" sites with dissolved Pb in river water ≤0.1 μg L^{-1} (Dragun et al. 2011) Pb cytosolic concentrations were rather low (bellow 5 μg L^{-1}, with average value amounting to 1.9 μg L^{-1}; Dragun et al. 2012b) and comparable with previously reported cytosolic Pb concentrations (0.97-5.93 ng mL^{-1}) in the liver of chub from weakly contaminated Sava River water (Dragun et al. 2012a). Due to exceeding dilution during the HPLC separation it was not possible to measure Pb in SE-HPLC separated fractions from uncontaminated sites.

Lead distribution was, therefore, presented only for the one "contaminated" site, with the aim to define the protein category within which surplus Pb was sequestered.

As shown in Fig. 5b, Pb was eluted mainly in the fractions corresponding to MMM proteins, with maximal quantity at elution time of 25 minutes which could be associated to proteins with MM of about 60 kDa. The remaining Pb was distributed among several LMM and VLMM fractions, among which the most recognizable was the fraction corresponding to MT peak (Fig. 1a and Table 1). Pavičić et al. (1993) reported appearance of small, but well-defined Pb maximum at MT position on the elution profile obtained from mussels exposed to metal mixture (Cd, Cu, and Pb). On the other hand, several authors reported a lack of evidence on the induction of Pb-binding proteins related to MTs in fish tissues (Reichert et al. 1979; Roesijadi and Robinson 1994). However, a classical characteristic feature of Pb exposure in fish is Pb sequestration within the metal-rich granules and cellular debris (e.g. in the whole body of killifish; Goto and Wallace 2010). Similarly, in mammals Pb could be found within insoluble Pb-protein aggregates known as inclusion bodies (Goyer 1983). Evidence has indicated that MT, α-synuclein (Zuo et al. 2009), and a cleavage product of α_2-microglobulin (Fowler 1998) are critical to inclusion body formation. Since Pb is a poor inducer of MT compared to other metals such as Cd and Zn (Waalkes and Klaassen 1985), the role of MT, and possible Pb-MT association observed in this study, may be also associated to inclusion body formation (Mager 2012).

4. Conclusions

Basic distributions of essential elements Co, Cu, Fe, Mn, Mo, Se and Zn and nonessential elements Cd and Pb among cytosolic proteins of different molecular masses in the liver of European chub were defined for the first time based on the study performed on feral chub specimens from aquatic environment weakly contaminated by metals/metalloids (Cd: 0.01-0.31 μg L^{-1}; Co: 0.06-0.42 μg L^{-1}; Cu: 0.17-3.74 μg L^{-1}; Fe: 3.1-80.5 μg L^{-1} Mn: 0.4-261.1 μg L^{-1}; Mo: 0.5-20.1 μg L^{-1}; Pb: ≤1.18 μg L^{-1}; Zn: <5.0 μg L^{-1}; Dragun et al. 2011). Several essential elements (Cu, Fe and Se) and nonessentail elements Cd and Pb were present in the hepatic cytosol in wide concentration ranges (maximum to minimum ratio 7-20), thus allowing additional observation of the changes of their distributions among protein fractions as a consequence of increased accumulation of these elements in the chub liver. Increased quantity of cytosolic Cu and Cd was almost completely sequestered by MT, whereas Fe was associated to Fe-storage protein ferritin. In the case of Se and Pb, it was not possible to define single protein fraction to which their additional amounts in the hepatic cytosol associate, but rather a spectrum of proteins,

mostly of low (10-60 kDa) or medium molecular masses (30-100 kDa), respectively. Based on our results, the changes of cytosolic trace element distributions could serve as a sensitive tool for identification of metal/metalloid induced stress in chronically exposed fish.

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References

Anan Y, Kunito T, Sakai H, Tanabe S (2002) Subcellular distribution of trace elements in the liver of sea turtles. Mar Pollut Bull 45:224-229.

Andreini C, Banci L, Bertini I, Rosato A (2005) Counting the zinc-proteins encoded in the human genome. J Proteome Res 5:196-201.

Baudin JP, Fritsch AF (1989) Relative contributions of food and water in the accumulation of ^{60}Co by a freshwater fish. Water Res 23:817-823.

Beers MH, Berkow R (eds) (1998) The Merck manual of diagnosis and therapy, 17th edition. Merck & Co., Whitehouse Station.

Blust R (2012) Cobalt. In: Wood CM, Farrell AP, Brauner CJ (eds) Fish physiology: homeostasis and toxicology of essential metals, volume 31A. Academic Press, London, pp 291-326.

Boivin S, Aouffen M, Fournier A, Mateescu M (2001) Molecular characterization of human and bovine ceruloplasmin using MALDI-TOF mass spectrometry. Biochem Bioph Res Co 288:1006-1010.

Bonneris E, Giguère A, Perceval O, Buronfosse T, Masson S, Hare L, Campbell PGC (2005) Sub-cellular partitioning of metals (Cd, Cu, Zn) in the gills of a freshwater bivalve, *Pyganodon grandis*: role of calcium concretions in metal sequestration. Aquat toxicol 71:319-334.

Bury NR, Boyle D, Cooper CA (2012) Iron. In: Wood CM, Farrell AP, Brauner CJ (eds) Fish physiology: homeostasis and toxicology of essential metals, volume 31A. Academic Press, London, pp 201-251.

de la Calle Guntińas MB, Bordin G, Rodriguez AR (2002) Identification, characterization and determination of metal-binding proteins by liquid chromatography. A review. Anal Bioanal Chem 374:369-378.

Dragun Z, Kapetanović D, Raspor B, Teskeredžić E (2011) Water quality of medium size watercourse under baseflow conditions: the case study of river Sutla in Croatia. AMBIO 40:391-407.

Dragun Z, Fiket Ž, Vuković M, Raspor B (2012a) Multielement analysis in fish hepatic cytosol as a screening tool in the monitoring of natural waters. Environ Monit Assess doi: 10.1007/s10661-012-2734-6.

Dragun Z, Krasnići N, Stržak Ž, Raspor B (2012b) Lead concentration increase in the hepatic and gill soluble fractions of European chub - an early indicator of increased Pb exposure from the river water. Environ Sci Pollut R doi: 10.1007/s11356-011-0706-y

Fekkes D, van Overmeeren E, Hennemann G, Visser TJ (1980) Solubilization and partial characterization of rat liver iodothyronine deiodinases. Biochim Biophys Acta 613:41-51.

Ferrarello CN, Fernandez de la Campa MR, Carrasco JF, Sanz-Medel A (2002) Speciation of metallothionein-like proteins of the mussel *Mytilus edulis* by orthogonal separation mechanisms with inductively coupled plasma-mass spectrometry detection: effect of selenium administration. Spectrochim Acta B 57:439-449.

Filipović Marijić V, Raspor B (2010) The impact of fish spawning on metal and protein levels in gastrointestinal cytosol of indigenous European chub. Comp Biochem Phys C 152:133-138.

Filipović Marijić V, Raspor B (2012) Site-specific gastrointestinal metal variability in relation to the gut content and fish age of indigenous European chub from the Sava River. Water Air Soil Pollut doi: 10.1007/s11270-012-1233-2.

Fowler BA (1998) Roles of lead-binding proteins in mediating lead bioavailability. Environ Health Perspect 106(Suppl. 6):1585-1587.

Fridovich I, Freeman B (1986) Antioxidant defenses in the lung. Annu Rev Physiol 48:693-702.

Gellein K, Roos PM, Evje L, Vesterberg O, Flatena TP, Nordberg M, Syversen T (2007) Separation of proteins including metallothionein in cerebrospinal fluid by size exclusion HPLC and determination of trace elements by HR-ICP-MS. Brain Res 1174:136-142.

Goto D, Wallace WG (2010) Metal intracellular partitioning as a detoxification mechanism for mummichogs (*Fundulus heteroclitus*) living in metal-polluted salt marshes. Mar Environ Res 69:163-171.

Goyer RA (1983) Intracellular sites of toxic metals. Neurotoxicology 4:147-156.

Heath AG (1987) Water Pollution and Fish Physiology. CRC Press, USA.

Hogstrand C, Lithner G, Haux C (1991) The importance of metallothionein for the accumulation of copper, zinc and cadmium in environmentally exposed perch, *Perca fluviatilis*. Pharmacol Toxicol 68:492-501.

Huang ZY, Shen JC, Zhuang ZX, Wang XR, Lee FSC (2004) Metallothionein as a biomarker for mercury in tissues of rat fed orally with cinnabar. Appl Organometal Chem 18:255-261.

Huang ZY, Zhang Q, Chen J, Zhuang ZX, Wang XR (2007) Bioaccumulation of metals and induction of metallothioneins in selected tissues of common carp (*Cyprinus carpio* L.) co-exposed to cadmium, mercury and lead. Appl Organomet Chem 21:101-107.

Iwai N, Watanabe C, Suzuki T, Suzuki KT, Tohyama C (1988) Metallothionein induction by sodium selenite at two different ambient temperature in mice. Arch Toxicol 62:447-451.

Janz DM (2012) Selenium. In: Wood CM, Farrell AP, Brauner CJ (eds) Fish physiology: homeostasis and toxicology of essential metals, volume 31A. Academic Press, London, pp 327-374.

Johnson JL, Rajagopalan KV (1976) Purification and properties of sulphite oxidase from human liver. J Clin Invest 58:543-550.

Jukola E, Hakkarainen J, Saloniemi H, Sankari S (1996) Blood selenium, vitamin E, vitamin A, and β-carotene concentrations and udder health, fertility treatments, and fertility. J Dairy Sci 79:838-845.

Kirschbaum J (1981) Cyanocobalamin. In: Florey K (ed) Analytical profiles of drug substances, volume 10. Academic Press, New York, pp 183-288.

Kwong RWM, Andre´s JA, Niyogi S (2011) Effects of dietary cadmium exposure on tissue-specific cadmium accumulation, iron status and expression of iron-handling and stress-inducible genes in rainbow trout: influence of elevated dietary iron. Aquat Toxicol 102:1-9.

Larsson A (1973) Thioredoxin reductase from rat liver. Eur J Biochem 35:346-349.

Libor S, Sundaram TK, Warwick R, Chapman JA, Grundy SMW (1979) Pyruvate carboxylase from a thermophilic *Bacillus*: some molecular characteristics. Biochemistry 18:3647-3653.

Liu N, Lo LS, Askary SH, Jones L, Kidane TZ, Trang T, Nguyen M, Goforth J, Chu Y-H, Vivas E, Tsai M, Westbrook T, Linder MC (2007) Transcuprein is a macroglobulin regulated by copper and iron availability. J Nutr Biochem 18:597-608.

Mager EM (2012) Lead. In: Wood CM, Farrell AP, Brauner CJ (eds) Fish physiology: homeostasis and toxicology of non-essential metals, volume 31B. Elsevier Academic Press, London, pp 185-236.

Martin-Antonio B, Jimenez-Cantizano RM, Salas-Leiton E, Infante C, Manchado M (2009) Genomic characterization and gene expression analysis of four hepcidin genes in the redbanded seabream (*Pagrus auriga*). Fish Shellfish Immun 26:483-491.

Mason AZ, Jenkins KD (1995) Metal detoxification in aquatic organisms. In: Tessier A, Turner DR (eds) Metal speciation and bioavailability in aquatic systems. IUPAC, John Wiley, New York, pp 479-512.

Matz CJ, KronePH (2007) Cell death, stress-responsive transgene activation, and deficits in the olfactory system of larval zebrafish following cadmium exposure. Environ Sci Technol 41:5143-5148.

McGeer JC, Niyogi S, Smith DS (2012) Cadmium. In: Wood CM, Farrell AP, Brauner CJ (eds) Fish physiology: homeostasis and toxicology of non-essential metals, volume 31B. Elsevier Academic Press, London, pp 125-184.

Mukherjee S, Kaviraj A (2009) Evaluation of growth and bioaccumulation of cobalt in different tissues of common carp, *Cyprinus carpio* (Actinopterygii: Cypriniformes: Cyprinidae), fed cobalt-supplemented diets. Acta Ichthyol Pisc 39:87-93.

Paliwal VK, Kholi KK, Sharma M, Nath R (1986) Purification and characterization of metallothionein from liver of cadmium exposed rhesus monkeys (*Macaca mulatta*). Mol Cell Biochem 71:139-147.

Passerini A, Andreini C, Menchetti S, Rosato A, Frasconi P (2007) Predicting zinc binding at the proteome level. BMC Bioinform 8:39.

Pavičić J, Raspor B, Martinčić D (1993) Quantitative determination of metallothionein-like proteins in mussels. Methodological approach and field evaluation. Mar Biol 115:435-444.

Podrug M, Raspor B, Erk M, Dragun Z (2009) Protein and metal concentrations in two fractions of hepatic cytosol of the European chub (*Squalius cephalus* L.). Chemosphere 75:843-849.

Prange A, Schaumloffel D (2002) Hyphenated techniques for the characterization and quantification of metallothionein isoforms. Anal Bioanal Chem 373:441-453.

Reichert JF, Jarosik N, Herrick R, Andersen J (1979) Observation of electron spin resonance of negative ions in liquid ^{3}He. Phys Rev Lett 42:1359-1361.

Reid SD (2002) Physiological impact of acute molybdenum exposure in juvenile kokanee salmon (*Oncorhynchus nerka*). Comp Biochem Physiol C 133:355-367.

Reid SD (2012) Molybdenum and chromium. In: Wood CM, Farrell AP, Brauner CJ (eds) Fish physiology: homeostasis and toxicology of essential metals, volume 31A. Academic Press, London, pp 375-415.

Richardson JS, Thomas KA, Rubin BH, Richardson DC (1975) Crystal structure of bovine Cu,Zn superoxide dismutase at 3Å resolution: chain tracing and metal ligands. Proc Nat Acad Sci USA 72:1349-1353.

Ricketts CD (2009) The effect of acute waterborne exposure of sub-lethal concentrations of molybdenum on the stress response in rainbow trout (*Oncorhynchus mykiss*). MSc thesis, University of British Columbia, Okanagan.

Roesijadi G, Robinson WE (1994) Metal regulation in aquatic animals: mechanisms of uptake, accumulation, and release. In: Malins DC, Ostrander GK (eds) Aquatic toxicology: molecular, biochemical, and cellular perspectives. CRC Press, Boca Raton, pp 387-420.

Sanchez W, Palluel O, Meunier L, Coquery M, Porcher J-M, Aît-Aïssa S (2005) Copper-induced oxidative stress in the three-spined stickleback: relationship with hepatic metal levels. Environ Toxicol Pharmacol 19:177-183.

Schäfer U (2004) Manganese. In: Merian E, Anke M, Ihnat M, Stoeppler M (eds) Elements and their compounds in the environment: occurrence, analysis and biological relevance, volume 2: Metals and their compounds. Wiley-VCH, Weinheim, pp 901-930.

Shulgin KK, Popova TN, Rakhmanova TI (2008) Isolation and purification of glutathione peroxidase. Appl Bioch Micro 44:247-250.

Singh RA, Singh SN (1990) Purification and properties of liver arginase from teleostean fish *Clarias batrachus* (L.). Arch Physiol Biochem 98:411-419.

Smith QR, Rabin O, Chikhale EG (1997) Delivery of metals to brain and the role of the blood-brain barrier. In: Connor JR (ed) Metals and oxidative damage in neurological disorders. Plenum Press, New York, pp 113-130.

Solomon EI, Lowery MD (1993) Electronic structure contributions to function in bioinorganic chemistry. Science 259:1575-1581.

Szpunar J, Lobinski R (1999) Species-selective analysis for metal-biomacromolecular complexes using hyphenated techniques. Pure Appl Chem 71:899-918.

Truglio JJ, Theis K, Leimkühler S, Rappa R, Rajagopalan KV, Kisker C (2002) Crystal structures of the active and alloxanthine inhibited forms of xanthine dehydrogenase from *Rhodobacter capsulatus*. Structure 10:115-125.

Uchida H, Kondo D, Yamashita A, Nagaosa Y, Sakurai T, Fujii Y, Fujishiro K, Aisaka K, Uwajima T (2003) Purification and characterization of an aldehyde oxidase from *Pseudomonas sp*. KY 4690. FEMS Microbiol Lett 229:31-36.

Vacchina V, Polec K, Szpunar J (1999) Speciation of cadmium in plant tissues by size-exclusion chromatography with ICP-MS detection. J Anal At Spectrom 14:1557-1566.

Vidal SM, Malo D, Vogan K, Skamene E, Gros P (1993) Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg. Cell 73:469-485.

Vutukuru SS, Chintada S, Madhavi KR, Rao JV, Anjaneyulu Y (2006) Acute effects of copper on superoxide dismutase, catalase and lipid peroxidation in the freshwater teleost fish, *Esomus danricus*. Fish Physiol Biochem 32:221-229.

Waalkes MP, Klaassen CD (1985) Concentration of metallothionein in major organs of rats after administration of various metals. Fundam Appl Toxicol 5:473-477.

Wang J, Dreessen D, Wiederin DR, Houk RS (2001) Measurement of trace elements in proteins extracted from liver by size exclusion chromatography-inductively coupled plasma-mass spectrometry with a magnetic sector mass spectrometer. Anal Biochem 288:89-96.

Wang WX, Rainbow PS (2006) Subcellular partitioning and the prediction of cadmium toxicity to aquatic organisms. Environ Chem 3:395-399.

Zuo P, Qu W, Cooper RN, Goyer RA, Diwan BA, Waalkes MP (2009) Potential role of α-synuclein and metallothionein in lead-induced inclusion body formation. Toxicol Sci 111:100-108.

Figure captions

Fig 1 a) Separately obtained size exclusion chromatograms of six standard proteins with UV detection at λ = 280 nm, and of rabbit metallothionein (MT) standard (Ikzus Zn-MT95) with UV detection at λ = 254 nm: 1 – thyroglobulin (8 mg mL^{-1}; t_e, 16.65 min; MM, 669 kDa), 2 – apoferritin (10 mg mL^{-1}; t_e, 18.04 min; MM, 443 kDa), 3 - β-amylase (4 mg mL^{-1}; t_e, 20.74 min; MM, 200 kDa), 4 - alcohol dehydrogenase (5 mg mL^{-1}; t_e, 21.82 min; MM, 150 kDa), 5 - bovine serum albumin (10 mg mL^{-1}; t_e, 22.86 min; MM, 66 kDa), 6 - carbonic anhydrase (3 mg mL^{-1}; t_e, 28.66 min; MM, 29 kDa), 7 – MT (5 mg mL^{-1}; first peak: t_e, 29.85 min; MM, 16.6 kDa; second peak: t_e, 30.90 min; MM, 12.5 kDa); b) The calibration straight line for Superdex™ 200 10/300 GL size exclusion column, with linear regression equation presented in the figure; t_e – elution time; MM – molecular mass (MM of MT was calculated from calibration equation)

Fig 2 An example of SE-HPLC chromatogram profile of chub hepatic cytosol (50 μL) after separation on Superdex™ 200 10/300 GL column, with UV detection at two wavelengths: a) λ = 280 nm; b) λ = 254 nm

Fig 3 Distribution profiles of essential trace elements: a) Co; b) Mo; c) Mn; and d) Zn, among cytosolic fractions of chub liver containing proteins of different molecular masses, separated by SE-HPLC with Superdex™ 200 10/300 GL column; the results are presented as ng of trace element eluted at specific elution times, after passing 200 μL of hepatic cytosol through the chromatographic column

Fig 4 Distribution profiles of essential trace elements: a) Cu; b) Fe; and c) Se, among cytosolic fractions of chub liver containing proteins of different molecular masses, separated by SE-HPLC with Superdex™ 200 10/300 GL column; the results are presented in the same way as described in the caption of Fig. 3

Fig 5 Distribution profiles of nonessential trace elements: a) Cd; and b) Pb, among cytosolic fractions of chub liver containing proteins of different molecular masses, separated by SE-HPLC with Superdex™ 200 10/300 GL column; the results are presented in the same way as described in the caption of Fig. 3

Figure 1.

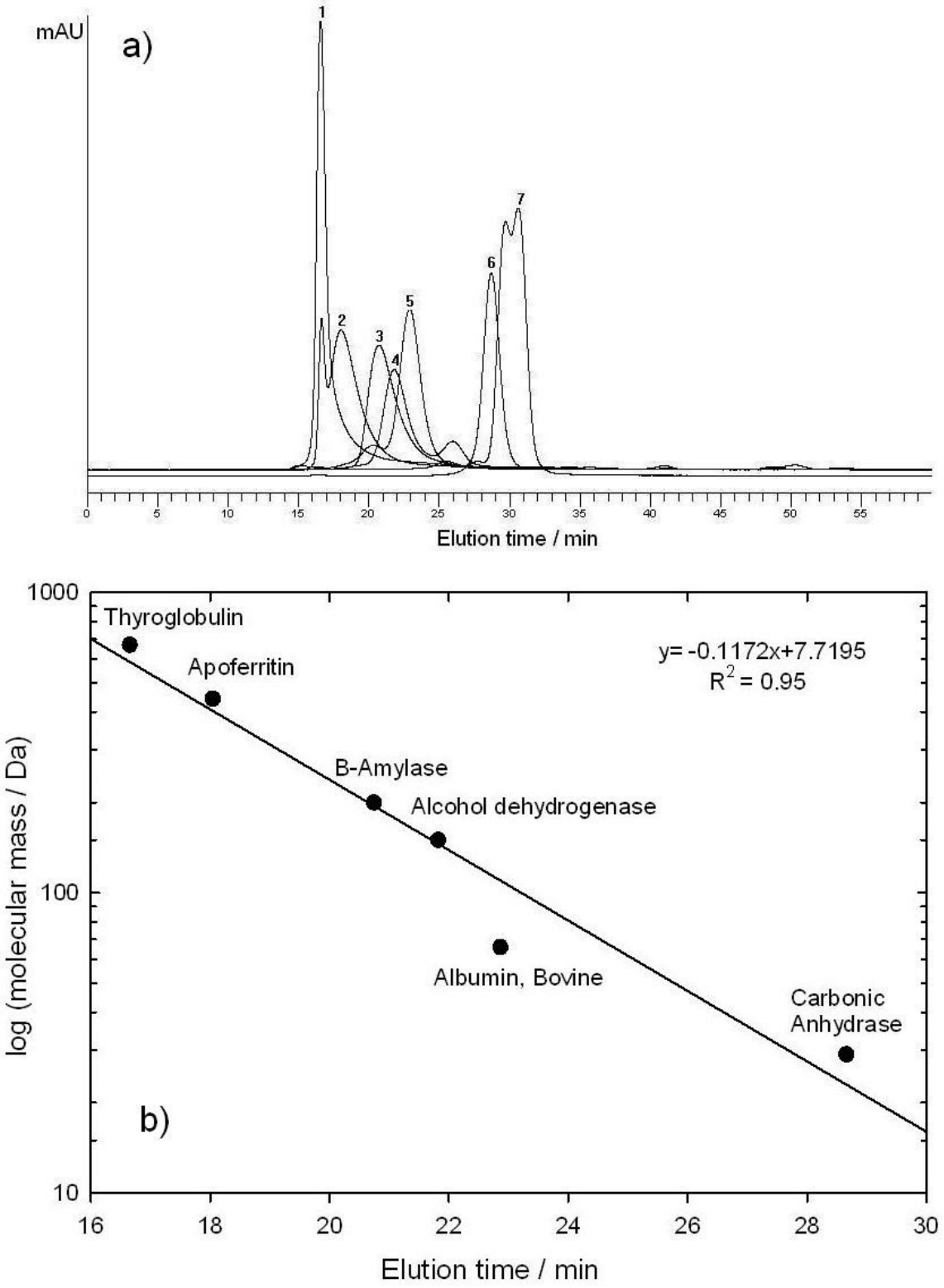
mAU
a)
1
2
3
4
5
6
7
0
5
10
15
20
25
30
35
40
45
50
55
Elution time / min
1000
100
10
Thyroglobulin
Apoferritin
B-Amylase
Alcohol dehydrogenase
Albumin, Bovine
Carbonic Anhydrase
y= -0.1172x+7.7195
R2 = 0.95
log (molecular mass / Da)
b)
16
18
20
22
24
26
28
30
Elution time / min

Figure 2.

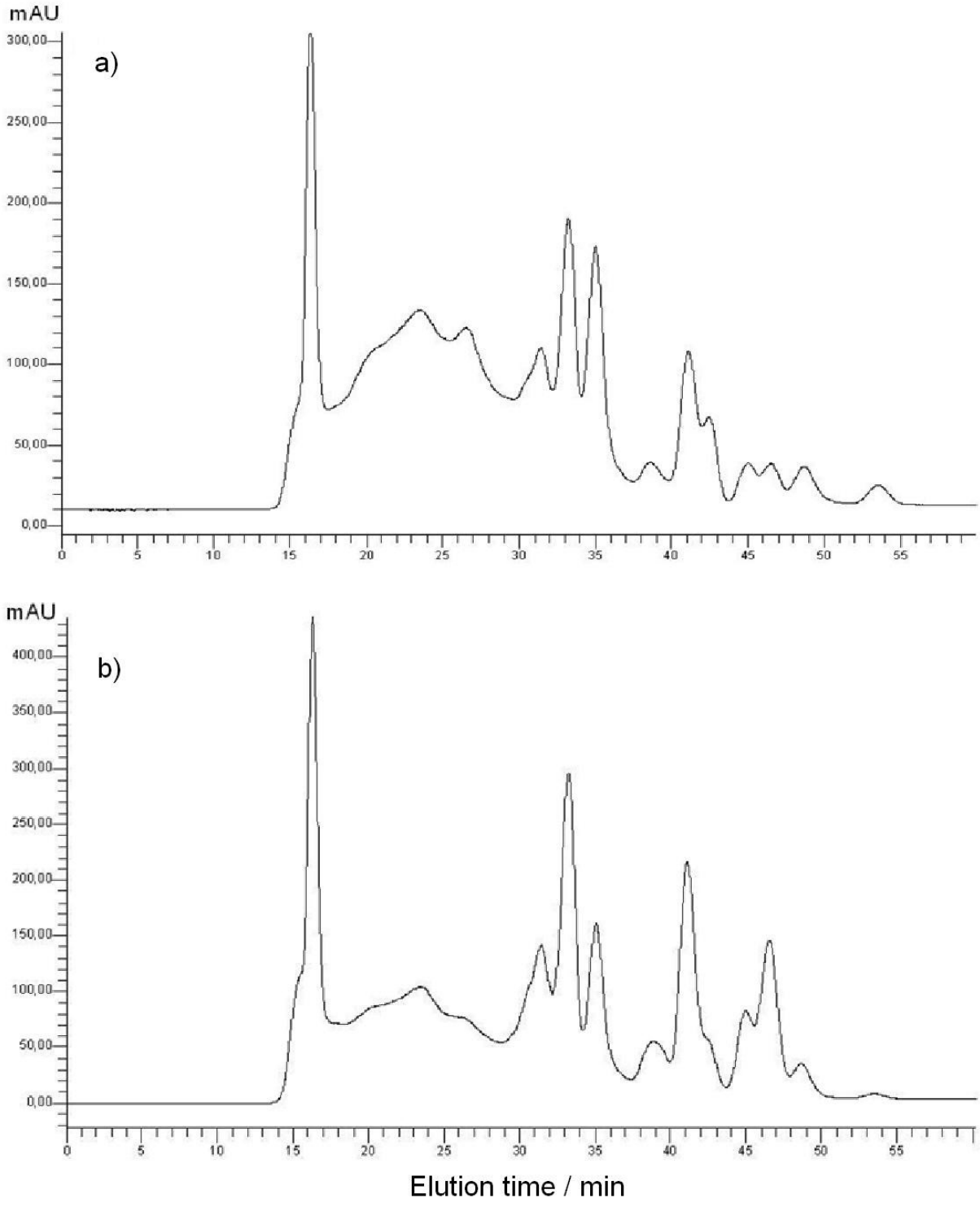
mAU
a)
300,00
250,00
200,00
150,00
100,00
50,00
0,00
0
5
10
15
20
25
30
35
40
45
50
55
mAU
b)
400,00
350,00
300,00
250,00
200,00
150,00
100,00
50,00
0,00
Elution time / min

Figure 3.

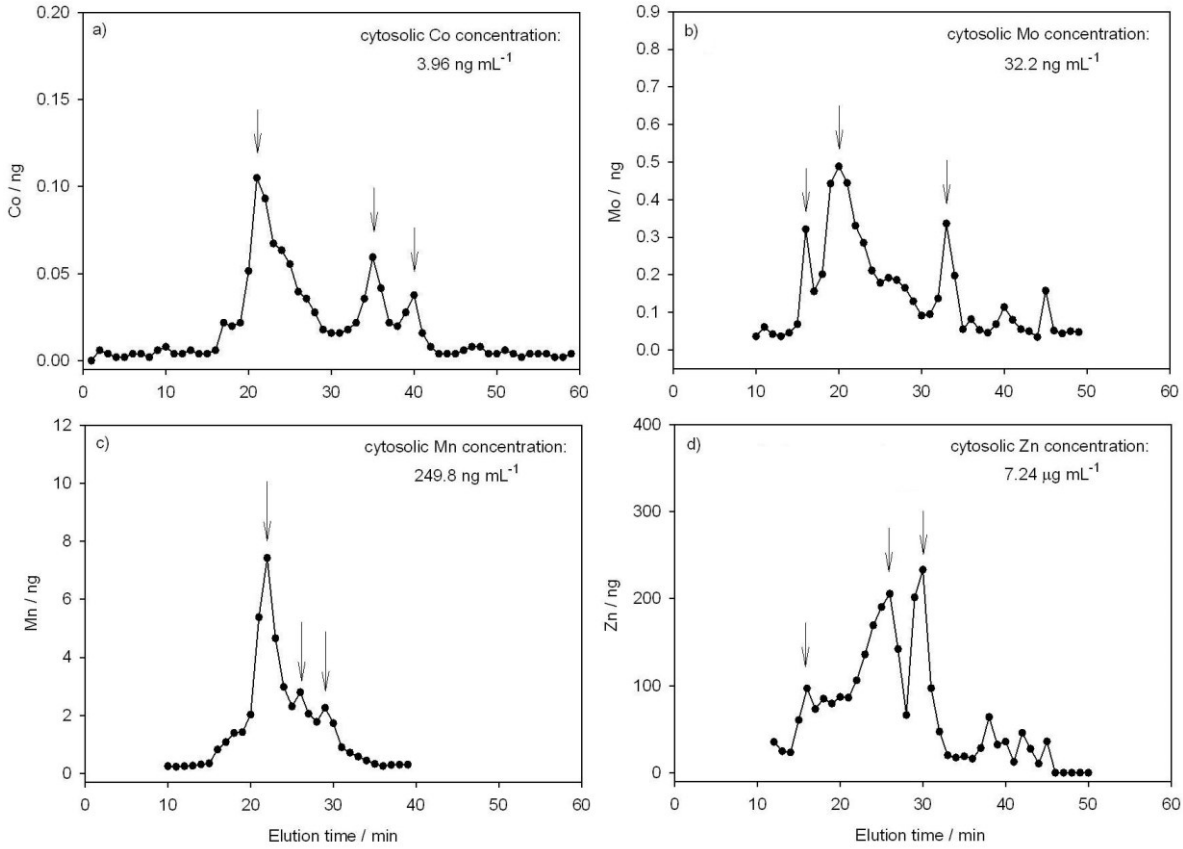
a)
cytosolic Co concentration:
3.96 ng mL-1
Co / ng
b)
cytosolic Mo concentration:
32.2 ng mL-1
Mo / ng
c)
cytosolic Mn concentration:
249.8 ng mL-1
Mn / ng
d)
cytosolic Zn concentration:
7.24 µg mL-1
Zn / ng
Elution time / min
Elution time / min

Figure 4.

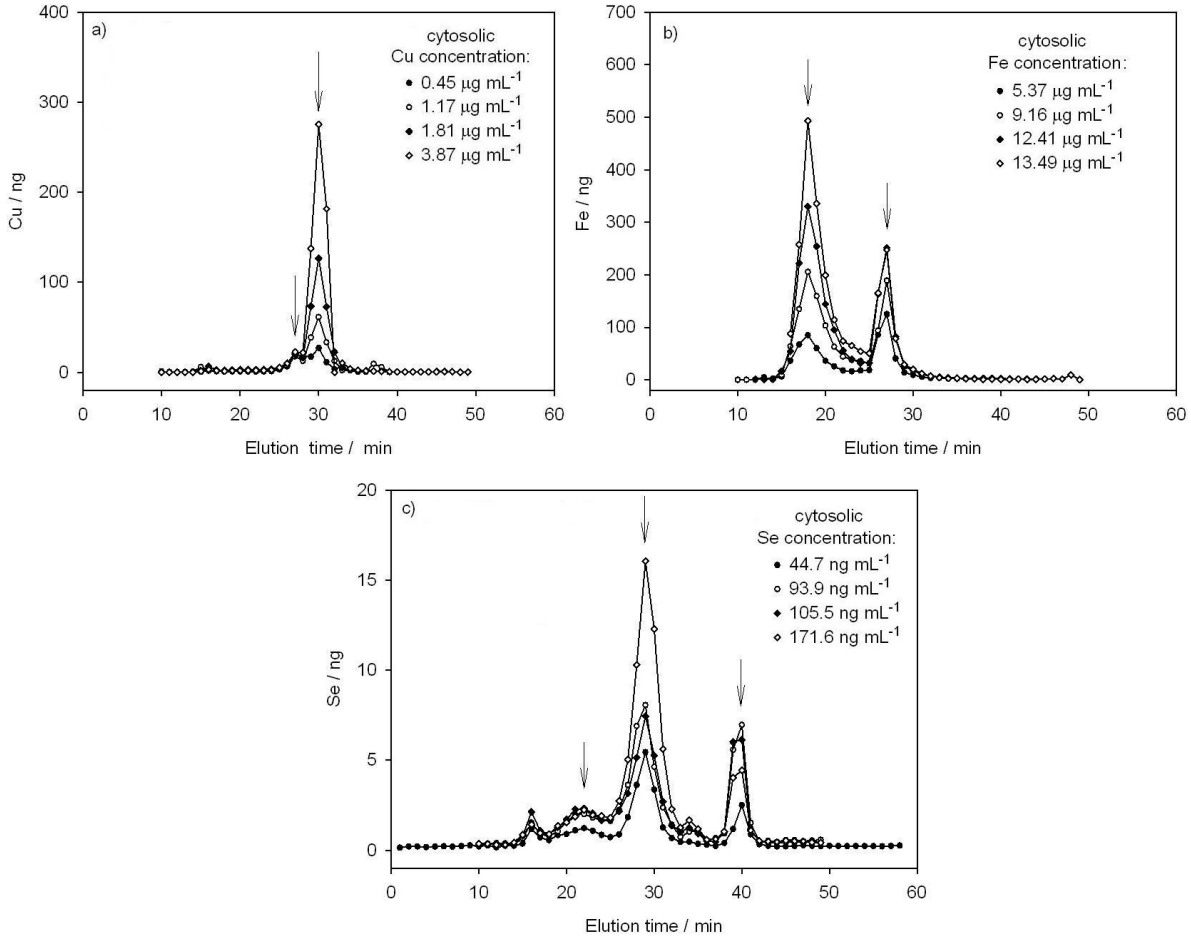
a)
cytosolic
Cu concentration:
0.45 μg mL-1
1.17 μg mL-1
1.81 μg mL-1
3.87 μg mL-1
Cu / ng
Elution time / min
b)
cytosolic
Fe concentration:
5.37 μg mL-1
9.16 μg mL-1
12.41 μg mL-1
13.49 μg mL-1
Fe / ng
Elution time / min
c)
cytosolic
Se concentration:
44.7 ng mL-1
93.9 ng mL-1
105.5 ng mL-1
171.6 ng mL-1
Se / ng
Elution time / min

Figure 5.

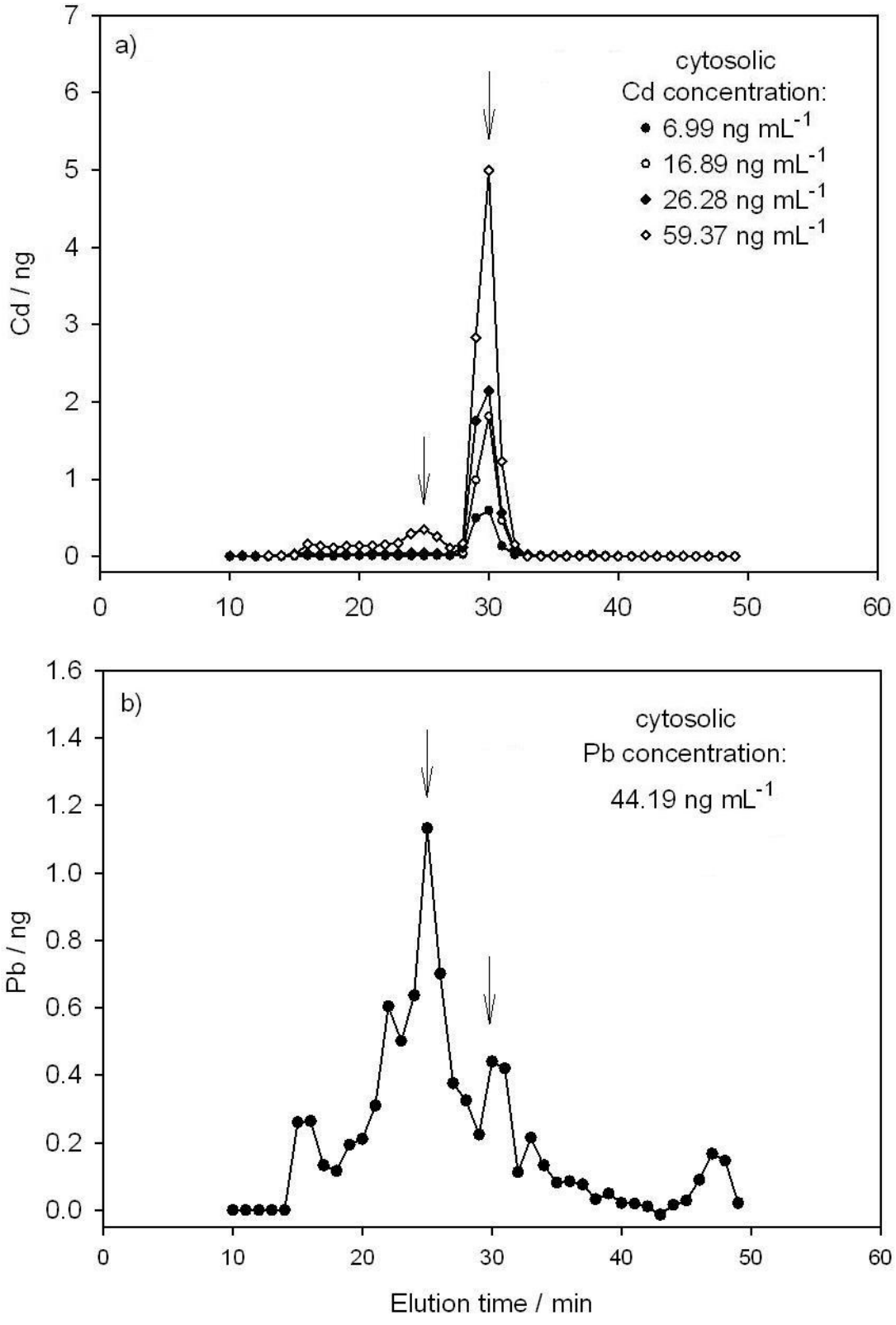
a)
cytosolic
Cd concentration:
6.99 ng mL-1
16.89 ng mL-1
26.28 ng mL-1
59.37 ng mL-1
Cd / ng
b)
cytosolic
Pb concentration:
44.19 ng mL-1
Pb / ng
Elution time / min

Table 1. Distribution of trace elements among cytosolic fractions of chub liver containing proteins of different molecular masses, separated by size exclusion HPLC with Superdex 200 10/300 GL column. Elution times (t_e) and molecular masses (MM) of proteins contained in the fractions in which respective elements were eluted are given in the table. Presented numbers refer to maximums of trace element peaks (i.e. the fractions with the highest trace element concentrations), whereas the numbers within the brackets refer to the beginnings and the ends of trace element peaks.

	Element	[a]HMM peak 1		[a]HMM peak 2		[b]MMM peak		[c]LMM peak		[d]VLMM peak 1		[d]VLMM peak 2	
		t_e / min	MM / kDa	t_e / min	MM / kDa	t_e / min	MM / kDa	t_e / min	MM / kDa	t_e / min	MM / kDa	t_e / min	MM / kDa
Essential elements	Co	-	-	21 (18-25)	181.3 (407.3-61.6)	-	-	-	-	35 (32-37)	4.1 (9.3-2.4)	40 (37-41)	1.1 (2.4-0.82)
	Mo	16 (15-17)	698.7 (915.2-533.5)	20 (18-25)	237.4 (407.3-61.6)	-	-	-	-	33 (31-35)	7.11 (12.2-4.1)	-	-
	Mn	-	-	22 (18-25)	138.4 (407.3-61.6)	26 (25-27)	47.0 (61.6-35.9)	29 (27-33)	20.9 (35.9-7.1)	-	-	-	-
	Zn	16 (15-18)	698.7 (915.2-407.3)	-	-	26 (16-27)	47.0 (698.7-35.9)	30 (28-32)	16.0 (27.4-9.3)	-	-	-	-
	Cu	-	-	-	-	27 (25-28)	35.9 (61.6-27.4)	30 (28-33)	16.0 (27.4-7.1)	-	-	-	-
	Fe	18 (16-21)	407.3 (698.7-181.3)	-	-	27 (25-29)	35.9 (61.6-20.9)	-	-	-	-	-	-
	Se	-	-	22 (18-25)	138.4 (407.3-61.6)	-	-	29 (25-33)	20.9 (61.6-7.1)	-	-	40 (38-42)	1.1 (1.8-0.63)
Non-essential elements	Cd	-	-	-	-	25 (23-27)	61.6 (105.6-35.9)	30 (28-32)	16.0 (27.4-9.3)	-	-	-	-
	Pb	-	-	-	-	25 (20-27)	61.6 (237.4-35.9)	30 (29-32)	16.0 (20.9-9.3)	-	-	-	-

[a]HMM peak – a peak of trace element concentration in the cytosolic fractions with a maximum in high molecular mass protein region (>100 kDa)
[b]MMM peak – a peak of trace element concentration in the cytosolic fractions with a maximum in medium molecular mass protein region (30-100 kDa)
[c]LMM peak – a peak of trace element concentration in the cytosolic fractions with a maximum in low molecular mass protein region (10-30 kDa)
[d]VLMM peak – a peak of trace element concentration in the cytosolic fractions with a maximum in very low molecular mass protein region (<10 kDa)