The study of acidic/basic nature of metallothioneins and other metal-binding biomolecules in the soluble hepatic fraction of the northern pike (*Esox lucius*)

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Highlights

- important new contribution to better understanding of metal behaviour/fate in fish
- predominant acidic nature of cytosolic metalloproteins in the northern pike liver
- Tl and Cs notably bound to basic biomolecules
- Ag, Cd, Cu, and Zn presumably bound to two MT-isoforms
- Cd/Zn preferentially bound to MT1, Ag to MT2, Cu evenly distributed between the two

Abstract

Since fish metalloproteins are still not thoroughly characterized, the aim of this study was to investigate the acidic/basic nature of biomolecules involved in the sequestration of twelve selected metals in the soluble hepatic fraction of an important aquatic bioindicator organism, namely the fish species northern pike (*Esox lucius*). For this purpose, the hyphenated system HPLC-ICP-MS was applied, with chromatographic separation based on anion/cation-exchange principle at physiological pH (7.4). The results indicated predominant acidic nature of metal-binding peptides/proteins in the studied hepatic fraction. More than 90% of Ag, Cd, Co, Cu, Fe, Mo, and Pb were eluted with negatively charged biomolecules, and more than 70% of Bi, Mn, and Zn. Thallium was revealed to bind equally to negatively and positively charged biomolecules, and Cs predominantly to positively charged ones. The majority of acidic (negatively charged) metalloproteins/peptides were coeluted within the elution time range of applied standard proteins, having pIs clustered around 4-6. Furthermore, binding of several metals (Ag, Cd, Cu, Zn) to two MT-isoforms was assumed, with Cd and Zn preferentially bound to MT1 and Ag to MT2, and Cu evenly distributed between the two. The results presented here are the first of their kind for the important bioindicator species, the northern pike, as well as one of the rare comprehensive studies on the acidic/basic nature of metal-binding biomolecules in fish, which can contribute significantly to a better understanding of the behaviour and fate of metals in the fish organism, specifically in liver as main metabolic and detoxification organ.

Key words: cytosol, fish, isoelectric point, liver, metalloproteins, superoxide-dismutase

1. Introduction

Metals that are introduced in the freshwater environment from various sources, such as industries, mining, agriculture, traffic, urbanization [1], can pose a serious threat to ecosystem sustainability and compromise the health of aquatic biota, ultimately leading to a reduction in biodiversity [2,3,4]. The problem with metals is their persistent nature, the fact that they cannot be degraded, and their tendency to bioaccumulate in living organisms, which can have toxic effects [1]. Although many metals (e.g. Fe, Cu, Zn) have essential functions in aquatic organisms such as fish, they can be toxic at high concentrations; on the other hand, non-essential metals (e.g. Ag, Cd, Tl) tend to be highly toxic even at very low concentrations [5]. Toxicity is often achieved by metals binding to or interacting with cytosolic biomolecules, thereby impairing cellular functions [5,6]. Various aspects of metal-binding biomolecules (i.e., metalloproteins) that relate to (1) metal involvement in metabolic processes (as essential components of enzymes, transporters, and signal transducers), (2) metal binding to transport or storage proteins as a form of homeostasis or detoxification, or even (3) metals as a cause of metabolic dysfunction [7], thus represent an important area of investigation encompassed by the field of metallomics [8].

One of the most important and defining characteristics of metalloproteins is their isoelectric point (pI) [9]. It is the pH at which the protein net charge is equal to zero, i.e., they are positively charged when the pH of their environment is below their pI and negatively charged when it is above their pI [9]. Protein pI can vary widely from extremely acidic to highly alkaline pH values (i.e., about 4.0-12.0) and is primarily defined by amino acid composition/sequence [9]. The distribution of protein pI of the entire proteome is bimodal in most organisms (e.g., bacteria [9]; humans [10]), with the two major protein sets clustered around pI 5.0-6.0 and pI 8.0-9.0 (acidic and basic proteins) [9,10,11]. However, different species may exhibit differences in the abundance of acidic and basic proteins that ultimately determine their metabolic and physiological properties, and that may be related to, for example, taxonomy, the ecological niche of the organisms, or subcellular localization [11]. Accordingly, cytoplasmic proteins as well as proteins of the Golgi apparatus, vacuoles, lysosomes, and cytoskeleton

are generally more acidic (clustered at a pI of 5.0-6.0), whereas mitochondrial and integral membrane proteins are generally more alkaline (clustered at a pI of 8.5-9.0) [11,12,13,14]. On the other hand, the nuclear proteome is equally composed of acidic and basic proteins (wide pI range: 4.5-10.0), and some authors have even described a small third peak between two main peaks with a pI maximum near 7.4 [11,12,14]. Moreover, acidic proteins have been reported to have a higher average number of interactions in both prokaryotic and eukaryotic proteomes and to be significantly longer compared to basic proteins [9,11]. In contrast, basic proteins show a greater variance in their pIs compared to acidic proteins, indicating a greater diversity of basic proteins [11].

Knowledge of metalloproteins and other metal-handling strategies in aquatic organisms, specifically in fish, is far from well understood. Analyses are often focused on a few isolated metalloproteins, such as metallothionein [15,16,17], ferritin [18], haemoglobin [19,20], superoxide-dismutase [21,22], while comprehensive studies on fish proteomes are still lacking. In our previous research, we have mainly focused on the size characterization of fish metalloproteins, by applying the size-exclusion (SEC)-HPLC-ICP-MS approach, in several species (e.g., brown trout, *Salmo trutta* [23]; Prussian carp, *Carassius gibelio* [24]), including the important bioindicator species northern pike *Esox lucius* [25], which is also the subject of the current study. Here, we aimed to complement our previous findings on the size distribution of northern pike cytosolic metalloproteins by determining their distribution based on total net charges, i.e. pI values, using anion-exchange (AEX) and cation-exchange (CEX) HPLC separations followed by ICP-MS metal detection. This approach was chosen with the goal of gaining greater insight into the acidic and basic nature of the metal-binding proteins associated with twelve essential and nonessential metals with the special emphasis on some well-known metalloproteins, such as metallothioneins.

2. Materials and methods

2.1. The sampling of the northern pike and the dissection of the liver

The sampling of the northern pike (*E. lucius* Linnaeus, 1758) was carried out in April/May 2021 in the Mrežnica River (Croatia) in front of the former textile factory in the town of Duga Resa (N 45° 27' 4.38" E 15° 30' 18.96") (the map of the location was provided in Dragun et al., 2022b). Electrofishing device Hans Grassl (EL63 II GI, 5.0 KW, 137 Honda GX270, 300/600V max, 27/15A max) was used for sampling according to the recommendations of the Croatian standard HRN EN 14011 [26]. Fish were euthanised at the sampling location using unbuffered tricaine methanesulphonate (MS 222, Sigma Aldrich; ~50 mg L^{-1}, duration less than 10 min [24]), complying with the requirements of the Ordinance on the Protection of Animals used for Scientific Purposes [27]. The liver of each fish was removed and stored first in liquid nitrogen and then in the freezer at -80°C. A total of 17 fish were caught at this location and three female specimens were selected for this study (lengths: EL-1, 41.5 cm; EL-2, 32.5 cm; EL-3, 48.0 cm).

2.2. Isolation and digestion of soluble hepatic fractions

Isolation of cytosolic fractions from the hepatic samples of the northern pike involved the following steps: (1) liver was cut into small pieces in ice-cold glass containers; (2) cooled homogenisation buffer [100 mM Tris-HCl/Base (Sigma Aldrich, USA; pH 7.5 at 4 °C) supplemented with a reducing agent (1 mM dithiothreitol, Sigma Aldrich, USA)] was added to each container (m_{liver}/v_{buffer} 1:5); (3) the resulting suspensions were homogenised in an ice-cooled tube with 10 strokes of the Potter-Elvehjem homogeniser (Glas-Col, USA) at 6000 rpm; and (4) the homogenates were then centrifuged (2 h, 50,000×g, +4 °C, Avanti J-E centrifuge, Beckman Coulter, USA). The supernatants (S50) obtained from this procedure represented soluble hepatic fractions, which mainly refer to the cytosol, but further contain microsomes and extracellular fluids (including traces of blood). The samples obtained were immediately stored at -80 °C, and thus kept until further analyses [25].

Prior to measuring metal concentrations in hepatic cytosols, they were digested in an acid/peroxide digestion mixture (concentrated HNO_3 (*Normatom® 67–69% for trace element analysis*, VWR Chemicals, UK) and 30% H_2O_2 (*Suprapur®*, Merck, Germany); v_{HNO3}/v_{H2O2} 3:1), with a volume ratio

of cytosol to digestion mixture of 1:1. Digestion was carried out in duplicate at 85 °C in the laboratory drying oven for 3.5 hours. The digested samples were then diluted five times with Milli-Q water [25].

2.3. Measurement of metal concentrations in hepatic cytosols using HR ICP-MS

The concentrations of twelve trace elements (Ag, Bi, Cd, Co, Cs, Cu, Fe, Mn, Mo, Pb, Tl, and Zn) were measured in the digested hepatic cytosols of northern pike using a high-resolution inductively coupled plasma mass spectrometer (HR ICP-MS; Element 2, Thermo Finnigan, Germany). Measurements were made in low resolution mode for ^{98}Mo, ^{109}Ag, ^{111}Cd, ^{133}Cs, ^{205}Tl, ^{208}Pb and ^{209}Bi; and in medium resolution mode for ^{55}Mn, ^{56}Fe, ^{59}Co, ^{63}Cu, and ^{66}Zn. Metal concentrations were calculated based on the external calibrations performed using appropriate dilutions of multi-element standard solution for trace elements (Analytika, Czech Republic) prepared in 2 % (vol.) HNO_3 (*Normatom® 67*-69 % *for trace element analysis*, VWR Chemicals, UK) and supplemented with caesium standard solution (Fluka, Germany). For Ag, a separate calibration was performed with silver standard solution (Fluka, Germany). Indium (Fluka, Germany) was added to all samples and calibration standards as an internal standard (1 μg L^{-1}).

Limits of detection (LOD) for metals in digested hepatic cytosols were calculated based on three standard deviations of ten successively measured metal concentrations in the blank samples (homogenisation buffer, HNO_3, H_2O_2), processed by the same procedure as for hepatic cytosol digestion. The LODs were the following (in ng g^{-1}): Bi, 0.241; Cd, 0.080; Co, 0.220; Cs, 0.099; Cu, 16.3; Fe, 75.2; Mn, 1.70; Mo, 0.205; Pb, 0.486; Tl, 0.001; and Zn, 22.9. The accuracy of the HR ICP-MS measurements was verified using certified quality control samples (UNEP GEMS, Canada) on two separate occasions. The following recoveries (average ± standard deviation) were obtained (%): Cd, 102.6±1.1; Co, 101.9±5.6; Cu, 102.2±0.5; Fe, 95.8±0.1; Mn, 103.0±1.0; Mo, 101.6±2.5; Pb, 99.4±1.5; and Zn, 107.1±2.4. The results of these analyses are shown in Table 1, expressed on a wet mass basis.

2.4. AEX/CEX-HPLC separation of cytosolic metal-binding biomolecules with subsequent ICP-MS metal detection

Separation of metal-binding biomolecules (MBBs) of different net charges from northern pike hepatic cytosols and subsequent metal detection was performed by hyphenated HPLC-ICP-MS system (HPLC, Agilent 1260 Infinity II with a diode array UV/VIS detector; ICP-MS, Agilent 7900; Agilent Technologies, USA). For AEX-separation of acidic MBBs, the MonoQ™ 5/50 GL column (GE Healthcare Biosciences, USA) was used, while for CEX-separation of basic MBBs, the MonoS™ 5/50 GL column (GE Healthcare Biosciences, USA) was used. Separations were performed in duplicate for three samples of northern pike hepatic cytosol (Figs. 1-3) and for eight protein standards (transferrin, alcohol dehydrogenase, superoxide dismutase, carbonic anhydrase, cytochrome C, lysozyme (Sigma Aldrich, USA), and metallothionein 1 and 2 (Enzo Life Sciences, USA)) (Table 2; Figs. SI.1-7) applying the same procedure and with subsequent ICP-MS detection of the intensities for the twelve metals listed above. The sample injection volume was 100 μL for both the AEX and CEX separations. Only before CEX separation, the cytosolic samples were pre-concentrated twice with Amicon Ultra centrifugal filters (cut off 3 kDa, Merck, Millipore, Ireland). The flow rate was 1.0 mL min^{-1}, and the method consisted of three steps in both cases: (1) 5 min 100 % starting buffer A; (2) 20 min linear gradient 0-100 % elution buffer B; (3) 5 min 100 % elution buffer B. AEX mobile phases were: (A) starting buffer – 4 mM Tris-HCl/Base (Sigma Aldrich, USA; pH 7.4, 22 °C); (B) elution buffer – 10 mM Tris-HCl/Base + 500 mM ammonium acetate (NH_4OAc; Merck KgaA, Germany) (pH 7.4, 22 °C). CEX mobile phases were: (A) starting buffer – 15 mM phosphate buffer (Merck KgaA, Germany; pH 7.4, 22 °C); (B) elution buffer – 15 mM phosphate buffer + 500 mM ammonium acetate (NH_4OAc) (pH 7.4, 22 °C). The protocol for AEX-separation was adapted from procedures previously described by Krasnići et al. [5] and Rodríguez-Cea et al. [17], and for CEX-separation from procedures described by Joshi et al. [28] and Zheng et al. [29]. All necessary measures were taken to avoid dissociation of metals from metal-binding biomolecules, including the use of ice-cold

conditions and a constant pH of 7.4 throughout the HPLC-separations to maintain physiological conditions (after de la Calle Guntiñas et al. [30]).

2.5. Statistical analyses and graphical representation of the data

All calculations were performed in Microsoft Office Excel (version 16). The metal intensity of each peak was calculated as the sum of all measured intensities within that peak (measurements were performed approximately every 3.3 seconds). Some authors have reported that the sum of peak intensities even outperforms peak area integration in mass spectrometry analysis [31]. The percentage contribution of each peak to the metal distribution profile was calculated as the ratio of the peak metal intensity to the total metal intensity within the given elution time of the column, multiplied by 100. The plots were made with the statistical programme SigmaPlot 11.0 for Windows, which was also used for the statistical analyses, i.e. the calculation of Pearson correlation coefficients. The results of the metal distributions among the biomolecules of different net charges in the hepatic cytosol of northern pike are presented in Tables 3-6 and Figures 1-3.

3. Results and discussion

3.1. General metal distribution between AEX and CEX column

Separation of MBBs from northern pike hepatic cytosol according to their net surface charges was carried out under neutral physiological pH conditions (pH 7.4) to maintain an environment similar to that of hepatic cells/tissue. Under such conditions, the MBBs with acidic nature, i.e. with a pI roughly below 7, were eluted within the separation range of the AEX-column, and those with basic nature, i.e. with pI roughly above 8, were eluted within the separation range of the CEX-column. Dithiothreitol, which was added to all samples to prevent oxidation, was eluted within the void volumes of both columns and thus did not contribute to the observed metal distributions within the working ranges of the columns applied (Table 2). The results obtained, i.e. the almost complete elution of the majority of the analysed metals within the separation range of the AEX-column (Figs. 1-3; Tables 3-5), indicated that the biomolecules binding these metals in the northern pike hepatic cytosol are predominantly

acidic in nature, which is consistent with previous reports on cytosolic proteins [11]. Kiraga et al. [11] further stated that Eukaryota have proteomes that generally tend to be of acidic pH values. In our study, the decreasing order of percentages of metals predominantly bound to acidic MBBs, i.e. to the AEX-column, was as follows: Pb (99.2-99.4) > Ag (99.0-99.2) > Cd (97.9-99.1) > Cu (98.5-99.0) > Mo (98.7-98.9) > Fe (96.7-98.5) > Co (92.5-96.8) > Bi (79.6-94.2) > Zn (77.5-84.7) > Mn (68.0-75.1). Thus, the proportion of their elution within the void volume of the AEX-column, or the proportion of MBBs with either neutral or basic nature, was very low for Pb, Ag, Cd, Cu, Mo, Fe and Co (0.6-7.5%) and slightly higher for Bi, Zn and Mn (5.8-32.0%). These results were confirmed by subsequent separation on the CEX-column, where visible elution within the column separation range was obtained only for Bi, Cu, Fe, Mn and Zn (Figs. 2-3; Tables 3-5), with the peaks of Bi (Fig. 2b; Table 3) and Mn (Fig. 3b; Table 4) being best resolved.

In contrast to the ten listed metals, two other metals (Cs and Tl) showed remarkable additional binding to basic MBBs. Namely, Tl was evenly distributed between two columns (Table 5), i.e. separation on the AEX-column indicated that 52.6-57.3% of Tl was bound to acidic MBBs (Fig. 3c), while separation on the CEX-column indicated that 47.5-57.2% of Tl was bound to basic MBBs (Fig. 3d). Caesium, on the other hand, was predominantly bound to the CEX-column (98.4-98.7%), indicating almost complete binding by basic MBBs (Table 4; Fig. 2c-d).

AEX-separation of acidic standard proteins (pI 3.9-5.8, a representative range for the majority of cytosolic proteins, which were previously described to cluster around pIs of 5.0 to 6.0 [9]) resulted in metal-containing peaks with elution times ranging from 8.2-12.2 min (Table 2; Figs. SI.1-5). The majority of Ag, Cd, Cu, Fe, Mn, Tl and Zn were eluted within this range, as were some Co and Pb, indicating that the pIs of their MBBs are within the most common cytosolic protein pI range of 4-6. Later elution times were recorded for the majority of Co, Mo, Pb, Bi, and only part of Zn, indicating MBBs with lower, i.e. more acidic pI values.

CEX-separation of basic standard proteins (mitochondrial enzyme cytochrome C and serum enzyme lysozyme; pI 9.6-11.1) revealed metal/non-metal peaks with elution times in the range of 19.3-24.1 min (Table 2; Figs. SI.6-7). Only Fe was eluted from the CEX-column within this range, while the

other metals bound to basic MBBs (Bi, Cs, Cu, Mn, Tl, Zn) were eluted earlier, indicating the lower pI values closer to neutral pH.

Kiraga et al. [11] reported a significant correlation between the pI value and the size of the proteins for many proteomes, which was positive for acidic proteins and negative for basic proteins. Consequently, in both cases, the proteins with pI values closer to neutral pH (the majority of the peaks obtained in our study) seem to indicate longer protein chains compared to the proteins with the most extreme (acidic and basic) pI values (observed in our study for the majority of acidic MBBs of Co, Mo, Pb, Bi, and part of Zn, and for basic Fe-MBBs), which probably indicate shorter protein chains.

3.2. AEX/CEX distribution profiles of the individual metals

3.2.1. Silver

Most of the Ag (~99%) was bound to the AEX column, and only discernible Ag-peaks were obtained by AEX-HPLC separation (Table 3; Fig. 1a). The silver distribution on the AEX column encompassed seven clearly resolved peaks of different height and width. The most predominant peaks were Ag3 (maximum at 10.1 min; ~30-40% of AEX-bound Ag), followed by Ag2 (maximum at 9.8 min; ~10-15% of AEX-bound Ag), presumably two metallothionein (MT) isoforms. Our hypothesis is in agreement with our previous report on the cytosolic distribution of Ag in northern pike liver based on size-exclusion chromatography (SEC), where the major amount of Ag was eluted at the same time as the MT standard [25]. However, the AEX-elution times of the MT standard isoforms, presented in Table 2 and Fig. SI.4, differed somewhat from the results for Ag-AEX-peak of the northern pike, but this can be attributed to different source materials, i.e. rabbit liver as opposed to pike liver in our case. MT isoforms from the northern pike appear to be more acidic than rabbit MTs, which can be concluded from the stronger ionic strength conditions required for their elution, similar to what Rodríguez-Cea et al. [17] observed for MTs of white sea catfish (*Netuma barba*). MTs are proteins containing ~60 amino acids, of which ~30% are highly conserved cysteines, and are characterised by the absence of aromatic amino acids [32]. It has been reported that their isoforms have very similar

molecular masses but differ somewhat in net charges [5]. Therefore, based on what we know about Ag behaviour in cells, we assume that the earlier eluted Ag-peak (Ag2) refers to MT1, while the later eluted predominant Ag-peak (Ag3) refers to MT2. However, with increasing cytosolic Ag concentration, the percentage of Ag within these two peaks either remained the same (Ag2; r = -0.06) or even decreased (Ag3; r = -0.58), while increased elution was observed within peaks Ag5 and Ag7 (r = 0.76-0.90). Caron et al. [33] also reported that although Ag is bound to MTs in juvenile yellow perch (*Perca flavescens*) cells, the increase in Ag bioaccumulation is not accompanied by an increase in the Ag-MT SEC-HPLC peak. They suggested that the reason for this might be found in the modest metal concentration gradient, as is the case in our study (Table 1). It would therefore be advisable to investigate the consequences of even higher Ag bioaccumulation, as these preliminary data suggest that excess Ag is not completely detoxified by MTs.

3.2.2. Bismuth

Although most of the Bi (~80-94%) was bound to the AEX column, clearly discernible Bi-peaks were obtained by both AEX-HPLC and CEX-HPLC separations (Table 3; Fig. 2a,b). As much as seven peaks were recorded after AEX-separation (Fig. 2a), but only the last three contained notable percentages of AEX-bound Bi (~5-25% each; Table 3). The predominant peaks were Bi5 (maximum at 16.6 min; ~16-25% of AEX-bound Bi), followed by Bi7 (maximum at 20.9 min; ~13-19% of AEX-bound Bi), and Bi6 (maximum at 20.0 min; ~5-10% of AEX-bound Bi). As discussed above, comparison with standard proteins (Table 2; Figs. SI.1-5) indicates that the pI values of the predominant Bi-MBBs are lower, i.e. more acidic than pH 4. With increasing cytosolic Bi concentration, the percentage of Bi increased in all three major peaks (r = 0.701-0.995), especially in Bi7. Previous SEC-analyses of Bi-MBBs in northern pike liver suggested a possibility of Bi binding to MTs [25]; however, AEX-separation did not clearly confirm this hypothesis, although a small Bi-peak was observed at an elution time of 9.9 min (Bi3), close to the suspected MT1 isoform (at 9.8 min). Previous analyses of low molecular mass Bi-binding cytosolic proteins from rat kidneys have

shown that they are most likely not identical to MTs, based on the differences in amino acid composition (much lower content of cysteine, and higher content of leucine, isoleucine, and glutamic acid) and differences in isoelectric points, among others [34]. Furthermore, analysis of human serum proteins revealed strong binding of Bi to transferrin [35], but this was not confirmed in our study either, as no matching or close AEX-peaks of standard transferrin (Table 2; Fig. SI.1) and Bi (Table 3) were registered. After CEX-separation (Fig. 2b), two clear Bi-peaks were obtained, with elution times already at 8.7 min (~3-11% of CEX-bound Bi) and 9.2 min (~9-19% of CEX-bound Bi), indicating pI values well below 9.6, i.e. closer to neutral pH values, when compared to standard proteins (Table 2; Figs. SI.6-7).

3.2.3. Cadmium

Most of the Cd (~98-99%) was bound to the AEX column, and only discernible Cd-peaks were obtained by AEX-HPLC separation (Table 3; Fig. 1b). The cadmium distribution on the AEX column included three clearly resolved peaks of different heights, all within the elution range of the standard proteins (i.e., with presumed pIs between 4-6; Table 2; Figs. SI.1-5). The predominant peaks were Cd2 (maximum at 9.8 min; ~4-50% of AEX-bound Cd) and Cd3 (maximum at 10.1 min; ~3-24% of AEX-bound Cd). This wide range of Cd percentages within the individual peaks may be attributed to the high contribution of background noise to the total intensity in the case of very low metal concentrations. As in the above presented case of Ag, and knowing the Cd behaviour in fish cells, we suspected that these two peaks correspond to two MT isoforms (Table 2), with the predominant peak, Cd2, assigned to MT1, and Cd3 to MT2. Moreover, the SEC-distribution of cytosolic Cd in northern pike liver also showed a predominant elution at the elution time of the MT standard [25]. In contrast to Ag, the percentage of Cd within both MT peaks increased with increasing cytosolic Cd concentration (Table 3; $r = 0.97$-0.98). A similar finding of predominant Cd-MT binding and its increase with increasing Cd bioaccumulation was previously reported by Caron et al. [33] following SEC-HPLC analysis of hepatic cytosol from juvenile yellow perch. Nevertheless, in our study, the

increase of the presumably non-MT peak, i.e. the first Cd-peak, was also observed (Cd1; r = 0.88). Although efficient detoxification of Cd by MTs is well known, the fact that an increase was also observed at the probable non-MT peak, pointed to a need to additionally investigate the intracellular distribution of higher Cd concentrations and to define the point at which MT-detoxification ceases to be efficient, as well as to identify the non-MT biomolecules that bind Cd.

3.2.4. Caesium

The main difference of Cs compared to the other elements was that ~90% of it was eluted within the void volume of the AEX column, with only a small AEX-peak (Fig. 2c) observed with a maximum at 8.0 min (~43-49% of AEX-bound Cs), which did not show a high correlation with Cs concentrations in the hepatic cytosol (Table 4). This finding, which indicated the basic nature of the Cs-binding biomolecules, was further confirmed by the high percentage of Cs bound to the CEX-column (~98-99%; Table 4), with two distinct Cs-peaks (Fig. 2d). The predominant peak, Cs1, had its maximum at 8.0 min (~48-58% of CEX-bound Cs), and the smaller one, Cs2, at 10.6 min (~21-26% of CEX-bound Cs). As cytosolic Cs concentration increased in northern pike liver, the percentage of Cs increased within the first peak (Cs1, r = 0.999) and decreased within the second peak (Cs2, r = -0.999). Comparison with standard proteins (Table 2; Figs. SI.6-7) indicated that the pI values of basic Cs-MBBs are lower than the pI of cytochrome C (pH 9.6), and, as in the case of Bi-MBBs, tend towards neutral values.

3.2.5. Cobalt

Most of the Co (~93-97%) was bound to the AEX column, and only discernible Co-peaks were obtained by AEX-HPLC separation (Table 3; Fig. 1c). The cobalt MBBs separated on the AEX-column were distributed among numerous peaks, five of which were the most recognisable. The first four minor peaks (Co1-Co4) were eluted within a period of 7-10 min, which, compared to the standard protein elution times (Table 2; Figs. SI.1-5), indicated a pI higher than 4 with a tendency towards neutral pH. Cobalt-binding proteins in human serum and plasma were characterised by a pI

value of 5, and in whole blood additionally by a pI value of 7 [36]. As previously observed, the acidic proteins with higher pI near neutral values tend to have longer protein chains, which is consistent with the results for Co SEC-distribution reported for northern pike liver, where a large proportion of Co was bound to proteins with high molecular mass (~170-230 kDa) [25]. Schumacher-Wittkopf [36] further pointed out the possibility of Co binding to haemoglobin (pI closed to neutral pH range, molecular mass ~64 kDa [5,37]), but our results do not show any Co-overlap with an adequate Fe-peak that would confirm this assumption. However, the predominant AEX-peak was Co5 (maximum at 16.2 min; ~44-73% of AEX-bound Co), with a pI presumably in more acidic range. The Co5 peak could refer to essential Co-binding in cyanocobalamin (vitamin B12) [36], which has a pI of 1.9 [38], and is a small molecule (1.3 kDa). This is further consistent with the Co-SEC-distribution in northern pike liver, i.e. its partial elution with biomolecules with molecular masses below 10 kDa [25]. With increasing cytosolic Co concentration, the percentage of Co increased in all peaks, but it was more pronounced in MBB-peaks with higher pI values (Co1-Co4; r = 0.60-0.91) than in MBB-peak with more acidic pI (Co5; r = 0.35) (Table 3).

3.2.6. Copper

Most of the Cu (~99%) was bound to the AEX column; nevertheless, discernible Cu-peaks were obtained by both AEX-HPLC and CEX-HPLC separations (Table 4; Fig. 2e,f). Copper was distributed among eight clearly resolved AEX-peaks (Fig. 2e), and the five predominant ones were in the elution range of the standard proteins (i.e., in the common pI range of 4-6; Table 2; Figs. SI.1-5), while the three minor peaks (accounting for less than 5% of AEX-bound Cu) had later elution times, indicating their more acidic protein nature. The two predominant Cu-peaks of comparable height, as in the case of Ag and Cd, had their maxima at 9.8 min (Cu2, ~20-29% of AEX-bound Cu) and at 10.1 min (Cu3, ~22-26% of AEX-bound Cu) and probably referred to MT isoforms, MT1 and MT2 (Table 2). This was also consistent with the report of Cu-SEC distribution in northern pike liver, i.e. with Cu elution at the elution time of the standard protein MT [25]. The fourth peak (Cu4, maximum at 11.0 min; ~15-

20% of AEX-bound Cu) could possibly refer to superoxide-dismutase (SOD), although the elution time of the standard protein (at 9.7 min; Table 2; Fig. SI.3) did not exactly match our results, possibly due to different source material (bovine erythrocytes), as also observed above for MTs. Even fish SODs were reported to show a large pI variability [39]. For comparison, the pI of the used SOD standard is 4.95 (bovine erythrocytes, Table 2), while it was reported to be 4.5-5.5 for carp (*Cyprinus carpio*) [40], 5.65 for sea cucumber (*Apostichopus japonicus*) [41] and 5.9-6.2 for Japanese flounder (*Paralichthys olivaceus*) [21]. Thus, Osatomi et al. [21] also reported differences in the electrophoretic mobility of bovine and flounder SOD. We also performed a preliminary analysis of the Cu4-fraction at MALDI-TOF MS, which indicated the possibility of Cu-SOD association within the Cu4 peak [42], but further investigation is required. Following the increase in cytosolic Cu concentrations in northern pike liver, increases in Cu percentages were recorded only in two MT peaks (r = 0.86-0.89), which is consistent with the report by Caron et al. [33] for juvenile yellow perch.

Although only ~1% of the Cu was bound on the CEX-column (Table 4), two distinct Cu-peaks were obtained (Fig. 2f), with elution times as early as 8.8 min (~10-14% of the CEX-bound Cu) and 9.7 min (~8-10% of the CEX-bound Cu), indicating basic pIs tending towards neutral pH values, when compared to standard proteins (Table 2; Figs. SI.6-7). The predominant peak was the first one, but both decreased with increasing Cu concentration (Table 4). The presence of basic Cu-binding biomolecules has been reported for several organisms, e.g. hepcidin-25, a cysteine-rich peptide that plays a role in iron homeostasis and has a pI of 8.5 [43].

3.2.7. Iron

Most of the Fe (~97-99%) was bound to the AEX column, but discernible Fe-peaks were obtained by both AEX-HPLC and CEX-HPLC separations (Table 4; Fig. 2g,h). On the AEX-column, Fe was distributed among five peaks (Fig. 2g), and the predominant Fe-peak had its maximum at 10.6 min (Fe3, ~72-77% of the AEX-bound Fe). The elution time of this peak was in the range of standard proteins, i.e. pI was probably within pH range of 4-6 (Table 2; Figs. SI.1-5). Based on the knowledge

of Fe metabolism, and the results of SEC-analysis of Fe-MBBs in northern pike liver (major Fe elution associated with proteins with a molecular mass of about 400 kDa) [25], we assumed that the Fe3 peak referred to the storage protein ferritin (450 kDa [18]; fish *Dasyatis akajei*: 400 kDa [44]), whose pI in fish liver was reported to range from 4.1 to 7.0, depending on the fish species [18]. Geetha and Deshpande [18] also reported notable microheterogeneity, with multiple ferritin isoforms with different pI values observed within each fish species (e.g. in freshwater fishes: murrel (*Chana punctatus*), five pIs: 5.2-6.8; and rohu (*Labeo rohita*), seven pIs: 5.9-7.0; brackish water fish, perch (*Lates calcalifer*), five pIs: 4.1-5.2). The ferritin isoforms consist of 24 subunits comprising various combinations of two subunits of different sizes (rat liver: heavy, 21 kDa, and light, 19 kDa [45]; in the liver of the fish *D. akajei:* heavy, 18 kDa, and light, 13 kDa [44]), and their isoelectric points differ according to the proportions of the two types of subunits [45]. Thus, some of the remaining Fe-AEX-peaks could also refer to additional, less represented ferritin isoforms. And it is also interesting to note that according to some studies, the acidic isoferritins release Fe more rapidly than the basic forms [45]. Further SEC-HPLC and mass spectrometric analyses of the isolated Fe3-fraction confirmed the hypothesis that the Fe elution at 10.6 min referred to ferritin, while the minor Fe-peak with the maximum at 9.3 min (Fe2) probably contains haemoglobin [46], but additional investigation is required. The finding of haemoglobin is consistent with our previous report on Fe-SEC distribution in northern pike liver, where minor fraction of Fe was also eluted with MBBs of ~30-80 kDa [25]. Vertebrate haemoglobin is a globular protein composed of four globin chains (mainly 2 α and 2 β) that differ from species to species, as well as among isoforms of the same species, and a prosthetic group called haem bound to each [47]. So far, a number of haemoglobin isoforms have been reported to have either asymmetric chain arrangements or variable α and β chains, which are classified according to their electrophoretic behaviour as cathodic haemoglobins (with high isoelectric points, pI $\geq$ 8.0) or anodic haemoglobins (with low isoelectric points, pI < 8.0), which differ significantly in their functional properties [20,47]. It has been reported that cathodic haemoglobins have high oxygen affinity and are only weakly affected by pH, while anodic haemoglobins have low oxygen affinity and

considerable sensitivity to pH [19,20]. Krasnići et al. [5] previously reported the detection of haemoglobin subunits (predominantly β) in the hepatic cytosol of the Vardar chub (*Squalius vardarensis*). In addition, the haemoglobins of some fish were reported to have pI values close to neutral pH (e.g. *Fundulus heteroclitus*: four isohaemoglobins with isoelectric points between pH 5.8-8.2, with the most represented haemoglobins having a a pI value of 6.5-7.5) [37], which is consistent with the rather early elution time of the Fe2 peak (Table 4). Following the increase in cytosolic Fe concentrations in northern pike liver, the increase in Fe percentages was recorded only in probable ferritin peak (Fe3; r = 0.55).

Only ~1.5% of Fe was bound on the CEX-column (Table 4), but two small Fe-peaks were recorded at elution times of 22.2 min (~6-10% of CEX-bound Fe) and 23.5 min (~3-5% of CEX-bound Fe) (Fig. 2h). Comparison with the elution times of standard proteins (Table 2; Figs. SI.6-7) indicates highly basic pIs of these Fe-binding biomolecules. The increase in Fe-CEX-peaks was not observed as a result of increased cytosolic Fe concentrations (Table 4).

3.2.8. Manganese

More than half of the Mn (~68-75%) was bound to the AEX-column, but a considerable amount was also eluted in the void volume of this column, suggesting that some of the Mn-MBBs have either a neutral or basic pI (Table 4). On the AEX-column, the Mn was distributed among five major peaks (Fig. 3a), and the two predominant Mn-peaks had maxima at 8.2 min (Mn1, ~11-19% of AEX-bound Mn) and at 10.2 min (Mn2, ~11-17% of AEX-bound Mn). The elution time of these peaks indicated that the pI range of the Mn-MBBs was comparable to standard proteins (4-6; Table 2; Figs. SI.1-5). The increase in Mn-peaks was not observed after the increase in cytosolic Mn concentrations in northern pike liver.

Separation of Mn on the CEX-column (Table 4, Fig. 3b) resulted with 55-60% of CEX-bound Mn (some discrepancy between columns could be due to the influence of higher background noise on the AEX column). A clear, well-resolved CEX-peak of Mn was obtained with a maximum at the elution

time of 14.4 min (~67-69% of CEX-bound Mn) (Fig. 3b). Comparison with standard proteins (Table 2; Figs. SI.6-7) indicated lower pI values closer to neutral pH. There is a possibility that the Mn-CEX peak refers to the cytosolic form of Mn-SOD, which has been reported to have a basic isoelectric point in some organisms (pI = 8.98 in the crab *Eriocheir hepuensis* [48]). The increase in the Mn-CEX-peak was not observed as a result of increased cytosolic concentrations of Mn, i.e. the variabilities in peak height and width were very small (Table 4; Fig. 3b).

3.2.9. Molybdenum

Most of the Mo (~99%) was bound to the AEX column, and only discernible Mo-peaks were obtained by the AEX-HPLC separation (Table 5; Fig. 1d). The molybdenum was distributed among several AEX-peaks; three of these were the most discernible, and all were eluted after the 15th minute indicating rather acidic pI values. Most of the Mo was divided between the second peak, for which we have marked three distinct maxima (Mo2-Mo4; 15.9-17.3 min; 32-35% of the AEX-bound Mo), and the predominant third peak with a prominent maximum (Mo5; 23.8 min; 52-57% of the AEX-bound Mo). With increasing cytosolic Mo concentration, the percentage of Mo somewhat increased in the Mo4 peak ($r = 0.98$) (Table 5). Known Mo-enzymes include, for example, aldehyde-oxidases (a subfamily of cytosolic molybdo-flavoenzymes), with a pI of 5.58 in the silkworm [49]; however, our results indicate Mo-MBBs with probably more acidic pI values.

3.2.10. Lead

In the case of Pb, the major amount (~99%) was also bound to the AEX column, and only discernible Pb-peaks were obtained by AEX-HPLC separation (Table 5; Fig. 1e). The lead was distributed among four main AEX-peaks, the first three of which were eluted in the elution range of the standard proteins, indicating a common protein pI of 4-6 (Table 2; Figs. SI.1-5). The fourth peak had a later elution time (maximum at 14.1 min) and thus probably revealed more acidic pI value. The predominant Pb-peaks were Pb3 (maximum at 11.3 min; 26-62% of AEX-bound Pb) and Pb4 (maximum at 14.1 min; 15-34% of AEX-bound Pb). The cytosolic Pb concentrations were below the

detection limit of the used HR ICP-MS instrument, so that a correlation with the Pb-percentages within the AEX-peaks could not be calculated. Previous analyses of cytosolic Pb distribution in fish liver (European chub, *Squalius cephalus*), based on the size exclusion principle, indicated possible binding of Pb to MTs [50]. Although in this study the elution time of one of the Pb-peaks (Pb2; maximum at 9.64 min) was close to the elution time of the presumable MT1 peak (maximum at 9.75 min), further analyses should be performed before a final conclusion is reached. Furthermore, due to the high toxicity of Pb, the possibility of Pb-binding to a vital cellular protein within the Pb3 peak (which is placed within the common pI range for cytoplasmic proteins, usually clustered around pI of 5.0 to 6.0 [9]) should be additionally investigated.

3.2.11. Thallium

Thallium was almost evenly distributed between AEX and CEX columns (Table 5; Fig. 3c,d). On the AEX-column, Tl was distributed among three peaks (Fig. 3c), and the two predominant Tl-peaks had maxima at 7.4 min (Tl1, ~26-35% of AEX-bound Tl) and at 11.0 min (Tl3, ~20-30% of AEX-bound Tl). The elution time of the first peak was quite close to the void volume, indicating labile binding to the AEX-column, and close to the neutral pI value, while the other two peaks fell within the pI range of the standard proteins (4-6; Table 2; Figs. 1-5). Following the increase in the cytosolic Tl concentrations in northern pike liver, the increase in the first two Tl-peaks was observed ($r = 0.985$-0.995).

On the CEX-column (Table 5, Fig. 3d), a clear, well-resolved Tl-peak was obtained with a maximum at an elution time of 14.6 min (~69-75% of CEX-bound Tl) (Fig. 3d). As in the case of Mn, comparison with standard proteins (Table 2; Figs. SI.6-7) indicated lower pI values closer to neutral pH. The increase in the Tl-CEX-peak was observed as a result of the increased cytosolic concentrations of Tl ($r = 0.997$). Considering the high toxicity of Tl, the proteins that bind it should be further investigated; the elution time close to that of Mn-CEX-MBBs (14.4 min) might indicate binding to the same proteins that are vital for the cell.

3.2.12. Zinc

More than three-quarters of the Zn (~78-85%) was bound to the AEX column, but as in the case of Mn, a considerable amount was also eluted in the void volume, indicating that a significant proportion of the Zn-MBBs had either a neutral or basic pI (Table 5; Fig. 3e,f). The zinc was distributed among seven major peaks (Fig. 3e), the first five of which were within the elution time range of standard proteins (including the known Zn-proteins alcohol-dehydrogenase, superoxide-dismutase, metallothioneins, and carbonic anhydrase), indicating their likely pIs in the range of 4-6 (Table 2; Figs. SI.1-5). Only the last two peaks indicated Zn-MBBs with more acidic pI values (Table 5). The two highest Zn-AEX-peaks (Fig. 3e) had maxima at 9.8 min (Zn2, ~13-17% of AEX-bound Zn) and at 10.1 min (Zn3, ~6-7% of AEX-bound Zn), which coincided with the elution times of the suspected MT isoforms, as in the case of Ag, Cd and Cu; and the predominant one was the suspected MT1 isoform (Zn2). The SEC-distribution of Zn in northern pike liver also suggested a large proportion of Zn bound to MTs [25], and MTs are well-known to play an important regulatory role in zinc metabolism [7]. The fourth AEX-peak (Zn4, maximum at 11.0 min; ~7-10% of AEX-bound Zn) coincided with the elution time of the Cu4 peak, which we assumed above to refer to the enzyme SOD, known to contain both Cu and Zn [51]. Following the increase in cytosolic Zn concentrations in northern pike liver, the increase was observed within the Zn1 and Zn7 peaks; however, an increase within the MT-peaks was not observed, suggesting that MTs do not serve for long-term storage of Zn or that Zn-binding to MTs is more labile than that of the other elements and thus possibly some redistribution occurs during processing.

Separation of Zn on the CEX-column (Table 5, Fig. 3f) revealed up to 40% CEX-bound Zn (however, some increase in background signal was observed during separation, which contributed to the overall estimate). We labelled the maxima of seven Zn-CEX-peaks in the range from 9.5 to 19.5 min, but they were not clearly distinguishable, so further processing and interpretation was not performed. The currently collected data undoubtedly show that Zn is part of a large number of intracellular proteins with both acidic and basic pI values, and that the acidic proteins predominate.

3.3. Metal coelutions

3.2.1. Metal coelution within the time frame from 8th to 12th minute

As discussed above, it is well-known that most cytosolic proteins involved in intracellular processes tend to be of acidic nature [11]. Acidic proteins are also characterized by a lower variance of their pIs than basic ones [11], and are therefore commonly clustered in the pH range of 4 to 6 [9]. Therefore, coelution of cytosolic biomolecules that bind metals and are essential for cell functioning was expected to occur within this pI range, i.e. within an elution time frame from 8th to 12th minute (Table 2). Accordingly, a high percentage of the essential elements Co, Cu, Fe, Mn and Zn were eluted within the aforementioned time frame, which only confirms their important functions in cellular metabolism (Tables 3-5). On the other hand, the high percentage of Ag and Cd eluted in the same time frame could be reasonably associated to their high tendency to bind to MTs and to their consequent detoxification, which is further discussed below. However, the binding of Cs, Pb and Tl to MBBs within this elution time frame could indicate some toxic potential of these non-essential and highly toxic metals through their binding to biologically relevant molecules and should be further investigated.

3.2.2. Metallothioneins

Four metals, Ag, Cd, Cu and Zn, were observed to coelute within the same two peaks (maxima at 9.75 and 10.12 min) when the AEX-column was applied (Fig. 1a,b; Fig. 2e; Fig. 3e), most likely referring to the MT isoforms, MT1 and MT2 (Table 2; Fig. SI.4). This is consistent with the general knowledge about metals that bind to MTs, i.e. with MT function in the homeostasis of the essential elements Cu and Zn, and detoxification of the non-essential elements Ag and Cd [15,52]. The presence of two MT isoforms with the same mass (6.0 kDa) but different total charge was previously confirmed by SEC-AEX-HPLC separation and subsequent MALDI-TOF-MS analyses in the hepatic cytosol of Vardar chub [5]. The presence of protein isoforms with different isoelectric point but similar/equal mass may arise from variations in the balance between acidic and basic amino acid residues within the

polypeptide chain or from chemical modifications of the conserved residues [53]. However, it is interesting to note that the distribution of all four metals between two isoforms is not equal, i.e. certain metals show a preference for a particular MT-isoform, as previously indicated by Werner et al. [52]. Table 6 shows the approximate molar concentrations of the four metals within each MT-isoform peak, as well as the ratios between different metals within each isoform, and the same metal between two different isoforms. The obtained results revealed that higher quantity of Ag is bound to the MT2 isoform, which contains 2.5-3.4 times more Ag compared to the MT1 isoform. In contrast, about twice higher quantities of Cd and Zn were bound to the MT1 isoform compared to the MT2 isoform. For Zn, this ratio was relatively constant (2.1-2.4), whereas for Cd it increased with increasing cytosolic Cd concentration (1.2-2.1), suggesting that higher Cd bioaccumulation is associated with higher relative sequestration of Cd by MT1-isoform. Copper, on the other hand, was evenly distributed between the two MT-isoforms, and showed no dependence on cytosolic Cu concentration. Preferential Cd binding to the MT1 isoform has previously been reported for the liver of gibel carp (*Carassius auratus gibelio*), Vardar chub and bighead carp (*Aristichthys nobilis*), among others [5,16,54]. It was also observed in salmonid fish that MT1 preferentially binds both Zn and Cd, while MT2 preferentially binds Cu [52]. The observed differences in metal-binding by specific MT-isoforms may reflect their different intracellular functions [5,17], and may indicate different metal affinities for specific MT isoforms [52] and/or even different amounts of each MT isoform within cells [54].

Furthermore, the ratio between the two essential elements, Cu and Zn, within each MT-isoform showed comparable amounts of both metals in the MT2-isoform, whereas the quantity of Zn was about 2.5 times higher than Cu in MT1-isoform. These ratios were also fairly constant regardless of cytosolic Cu and Zn concentrations. In contrast, the ratios between non-essential metals, Ag and Cd, depended strongly on their cytosolic concentrations. Since Ag was predominantly bound to MT2, it was higher than Cd in this particular isoform in the two samples with lower cytosolic Cd concentrations (EL1-2; Table 1), while in sample EL3, with the highest cytosolic Cd concentration, the amount of Cd bound to MT2 was even higher compared to Ag. In the Cd-preferred MT1 isoform,

the ratio of Cd to Ag also increased with increasing cytosolic Cd concentration, from almost equal amounts of both metals in the sample with the lowest cytosolic Cd concentration (EL2; Table 1) to a Cd content about seven times higher than Ag in the sample with the highest cytosolic Cd concentration (EL3; Table 1). It is also interesting to note that the ratios between essential and non-essential elements within each isoform, i.e. the amounts of Cu and Zn compared to the amounts of Ag and Cd, were quite high (Cu:Ag in MT1 = 684-994; Cu:Ag in MT2 = 237-365; Zn:Ag in MT1 = 1805-2227; Zn:Ag in MT2 = 256-376; Cu:Cd in MT1 = 144-716; Cu:Cd in MT2 = 272-938; Zn:Cd in MT1 = 333-1888; Zn:Cd in MT2 = 280-999) even in the samples with the highest bioaccumulated Cd/Ag concentrations. This was an indication that the capacity for further Cd/Ag detoxification was still far from saturated, as Ag and Cd can displace Cu and Zn from their binding sites on MTs [15,54].

3.2.3. Superoxide-dismutase (SOD)

Ninety percent of eukaryotic superoxide-dismutases are classified as Cu/Zn-SODs, and the rest are mostly Mn-SODs [41]. SODs are antioxidant metallo-enzymes that are widely distributed in many tissues and cell types of different species [41], with Cu/Zn-enzymes predominantly located in the cytosol [21] and Mn-enzymes in both mitochondria and cytosol [22,55]. They are specialised in eliminating reactive oxygen species (ROS) and are thus the first line of defence against oxidative stress [21,41]. They catalyse the dismutation of superoxide anion radicals in a two-step reaction that generates molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) [41]. Cytosolic Cu,Zn-SODs are mostly homodimers with a molecular mass of ~32 kDa and a subunit molecular mass of ~16 kDa [21]. They consist of one Cu and one Zn ion per subunit, with Cu being essential for activity, while Zn plays no direct role in catalysis, but rather stabilises the protein [51,56]. The ratios of Cu to Zn determined for the presumed Cu/Zn-SOD-peak (AEX column: maximum at 11 min; Fig. 2e and 3e; Tables 4-5) in our study showed 50-100% higher levels of Zn bound to SOD than of Cu (Table 6), which may indicate that some Cu-binding sites were not saturated. A pool of apoSOD, i.e. enzyme without bound Cu (~35% of total enzyme amount), was previously reported for human lymphoblasts

and it has been suggested that it may serve as a mechanism for providing increased SOD activity on demand [57].

On the other hand, mitochondrial Mn-SOD is synthesised as a precursor protein in the cytosol and imported into mitochondria after cleavage of its signal peptide; it is a homotetrameric protein with subunits of ~22-25 kDa, whereas cytosolic Mn-SOD has been described as a homodimeric protein with altered signal peptide and with monomers of 24-25 kDa and one Mn ion per subunit [55,58,59]. On the AEX-column, elution of Mn was observed at the elution time of the presumable Cu/Zn-SOD-peak (Fig. 3a; Table 4, Mn3: 11 min), but a clear, sharp Mn-peak was also obtained after separation on the CEX-column (Fig. 3b; Table 4, Mn1: 14.4 min). This finding is consistent with the report on the crab *Eriocheir hepuensis*, where three superoxidase dismutases were detected in the cytosol, Cu/Zn-SOD and two Mn-SODs; Cu/Zn-SOD and Mn-SOD1 had a similar slightly acidic pI (6.48 and 6.64, respectively), while Mn-SOD2 had a basic pI (8.98) [48]. Therefore, it can be assumed that the obtained Mn-elution profiles indicate the possibility of the presence of two forms of cytosolic Mn-SOD in the hepatic cytosol of northern pike, namely cytosolic Mn-SOD overlapping with Cu/Zn-SOD on the AEX-column (at 11 min), and another form of Mn-SOD eluting from the CEX-column (at 14.4 min) (Tables 4-5). One of the cytosolic Mn-SODs may even be the cytosolic precursor of mitochondrial Mn-SOD, as mitochondrial Mn-SODs have been reported to have a wide range of pI values, from mildly acidic to basic (6.75, bivalve, *Laternula elliptica* [58]; 5.96, sipuncula, *Phascolosoma esculenta* [59]; 8.3, striped murrel, *Channa striatus* [22]). In addition, Tl was also eluted from both columns at the coinciding elution times as Mn, indicating its possible binding to biologically important molecules. This is consistent with previous reports of Tl binding to high molecular mass cytosolic biomolecules in the liver of various fish (juvenile yellow perch [33]; brown trout, *Salmo trutta*, [23]. However, the discussion on the intracellular behaviour of Mn/Tl is purely hypothetical, and requires further investigation to better elucidate the mechanisms of Tl toxicity.

4. Conclusions

Investigation of the distribution of 12 metals among cytosolic biomolecules with different net charges in northern pike liver by anion- and cation-exchange high performance liquid chromatography revealed that the cytosolic metal-binding peptides and proteins were predominantly of acidic nature. The majority of biomolecules binding Ag, Cd, Co, Cu, Fe, Mo, and Pb (>90%), and a large proportion of biomolecules binding Bi, Mn and Zn (>70%) were bound to the anion-exchange column, indicating a pI value below 7.4. On the other hand, Tl was evenly distributed between anion- and cation-exchange columns, indicating the presence of both acidic (pI < 7.4) and basic (pI > 7.4) Tl-binding biomolecules in the hepatic cytosol of northern pike, while Cs-binding biomolecules were shown to have predominantly basic pI. Furthermore, the majority of acidic metal-binding biomolecules were observed to coelute within the elution time range characteristic for cytosolic proteins (with pI values roughly clustered around 4-6). The performed study also enabled a detailed description of the cytosolic distributions of each of the 12 metals among biomolecules with different pI values, from strongly acidic to strongly basic, consequently leading to several interesting observations. Binding of several metals (Ag, Cd, Cu, and Zn) to MTs was demonstrated, with probable recognition of two MT isoforms (MT1 and MT2). Further calculations revealed preferential binding of Cd and Zn to MT1, and of Ag to MT2, while Cu was evenly distributed between the two. The ratio between the essential metals Cu and Zn within each MT isoform also showed that the amount of Zn bound to MT1 was twice that of Cu, while the amount of both metals in MT2 was comparable. The amounts of Cu and Zn were notably higher than the amounts of non-essential metals Ag and Cd bound to MTs, suggesting that the capacity of MTs to detoxify metals is far from saturated. In addition, the ratio of Cu to Zn presumably bound to superoxide-dismutase suggests the presence of certain proportion of the enzyme that is not saturated with Cu, and contains only Zn. The results presented here are the first of their kind for an important bioindicator species, the northern pike, and one of the rare comprehensive studies on the acidic/basic nature of metal-binding biomolecules in fish, which can significantly contribute to a better understanding of the behaviour and fate of metals in the fish organism, specifically in the liver as the main metabolic and detoxification organ.

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Figure captions

Figure 1. The distribution profiles of the metals analysed by the online coupled HPLC-ICP-MS, using the ion-exchange approach. The metals presented in this figure were characterised by binding only to the anion-exchange (AEX) column: a) Ag; b) Cd; c) Co; d) Mo; and e) Pb. Intensity measurements were taken approximately every 3.2 seconds from the 6^{th} to the 31^{st} minute of separation protocol. The results for each measurement point are presented as a percentage of the total intensity summed for the entire duration of the separation.

Figure 2. The distribution profiles of the metals analysed by the online coupled HPLC-ICP-MS, using the ion-exchange approach. The elements presented in this figure were characterized by binding to both anion-exchange (AEX) and cation-exchange (CEX) columns: a) Bi-AEX; b) Bi-CEX; c) Cs-AEX; d) Cs-CEX; e) Cu-AEX; f) Cu-CEX; g) Fe-AEX; and h) Fe-CEX. The results are presented as in the Fig. 1.

Figure 3. The distribution profiles of the metals analysed by the online coupled HPLC-ICP-MS, using the ion-exchange approach. The elements presented in this figure were characterized by binding to both anion-exchange (AEX) and cation-exchange (CEX) columns: a) Mn-AEX; b) Mn-CEX; c) Tl-AEX; d) Tl-CEX; e) Zn-AEX; and f) Zn-CEX. The results are presented as in the Fig. 1.

Figure 1.

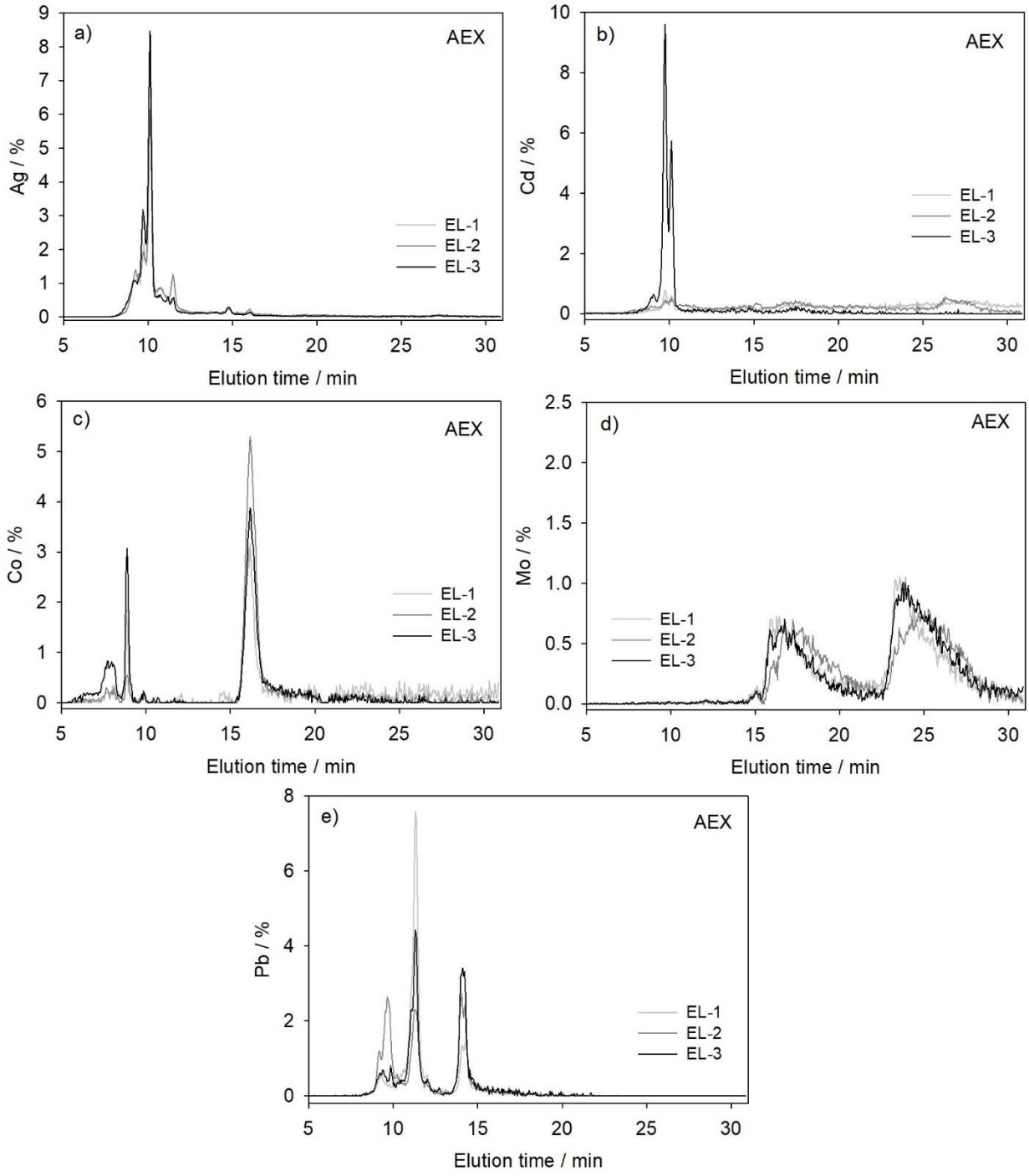
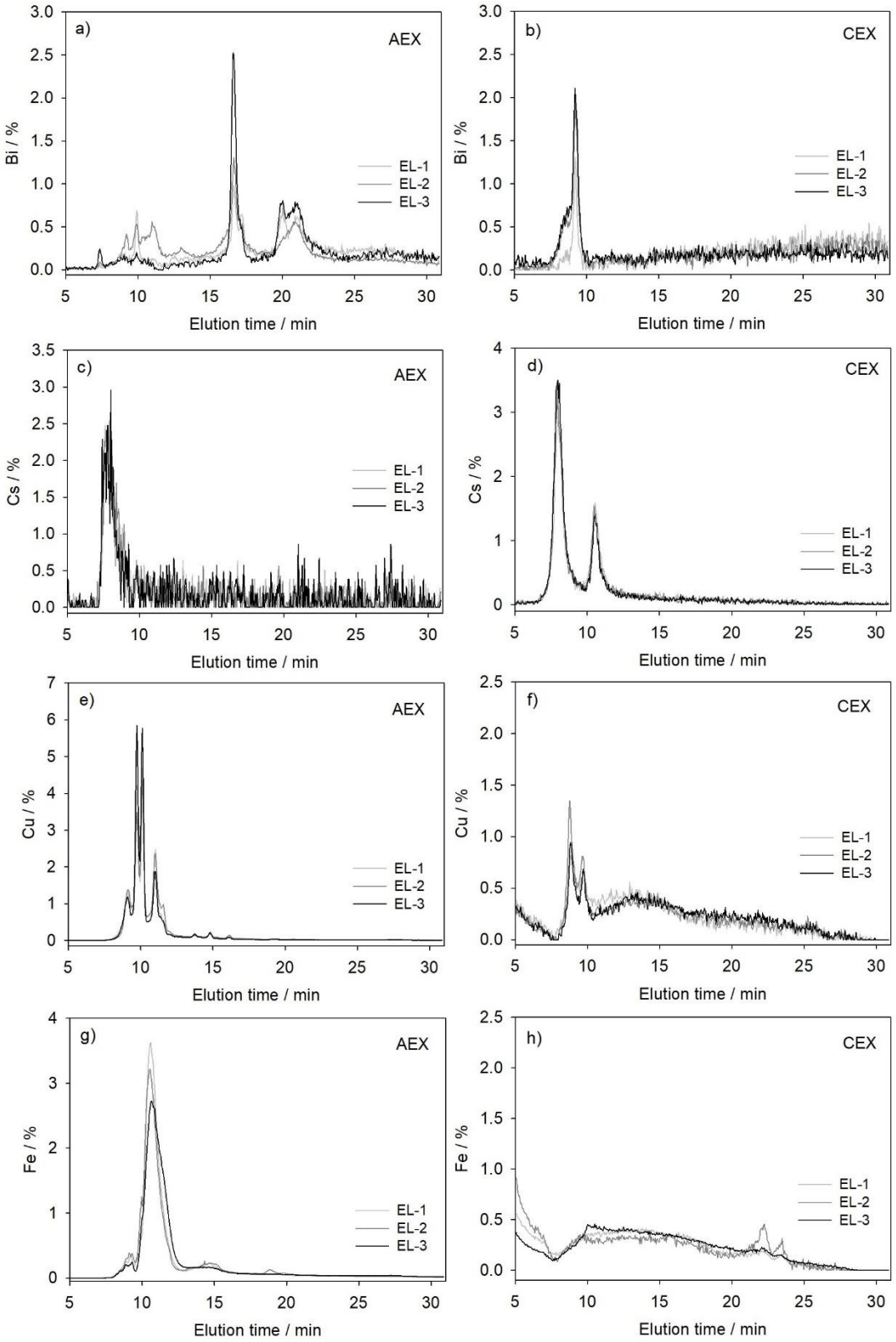

a)
AEX
Ag / %
EL-1
EL-2
EL-3
Elution time / min
b)
AEX
Cd / %
EL-1
EL-2
EL-3
Elution time / min
c)
AEX
Co / %
EL-1
EL-2
EL-3
Elution time / min
d)
AEX
Mo / %
EL-1
EL-2
EL-3
Elution time / min
e)
AEX
Pb / %
EL-1
EL-2
EL-3
Elution time / min

Figure 2.


a)
AEX
Bi / %
EL-1
EL-2
EL-3
Elution time / min
b)
CEX
Bi / %
EL-1
EL-2
EL-3
Elution time / min
c)
AEX
Cs / %
EL-1
EL-2
EL-3
Elution time / min
d)
CEX
Cs / %
EL-1
EL-2
EL-3
Elution time / min
e)
AEX
Cu / %
EL-1
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Elution time / min
f)
CEX
Cu / %
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Elution time / min
g)
AEX
Fe / %
EL-1
EL-2
EL-3
Elution time / min
h)
CEX
Fe / %
EL-1
EL-2
EL-3
Elution time / min

Figure 3.

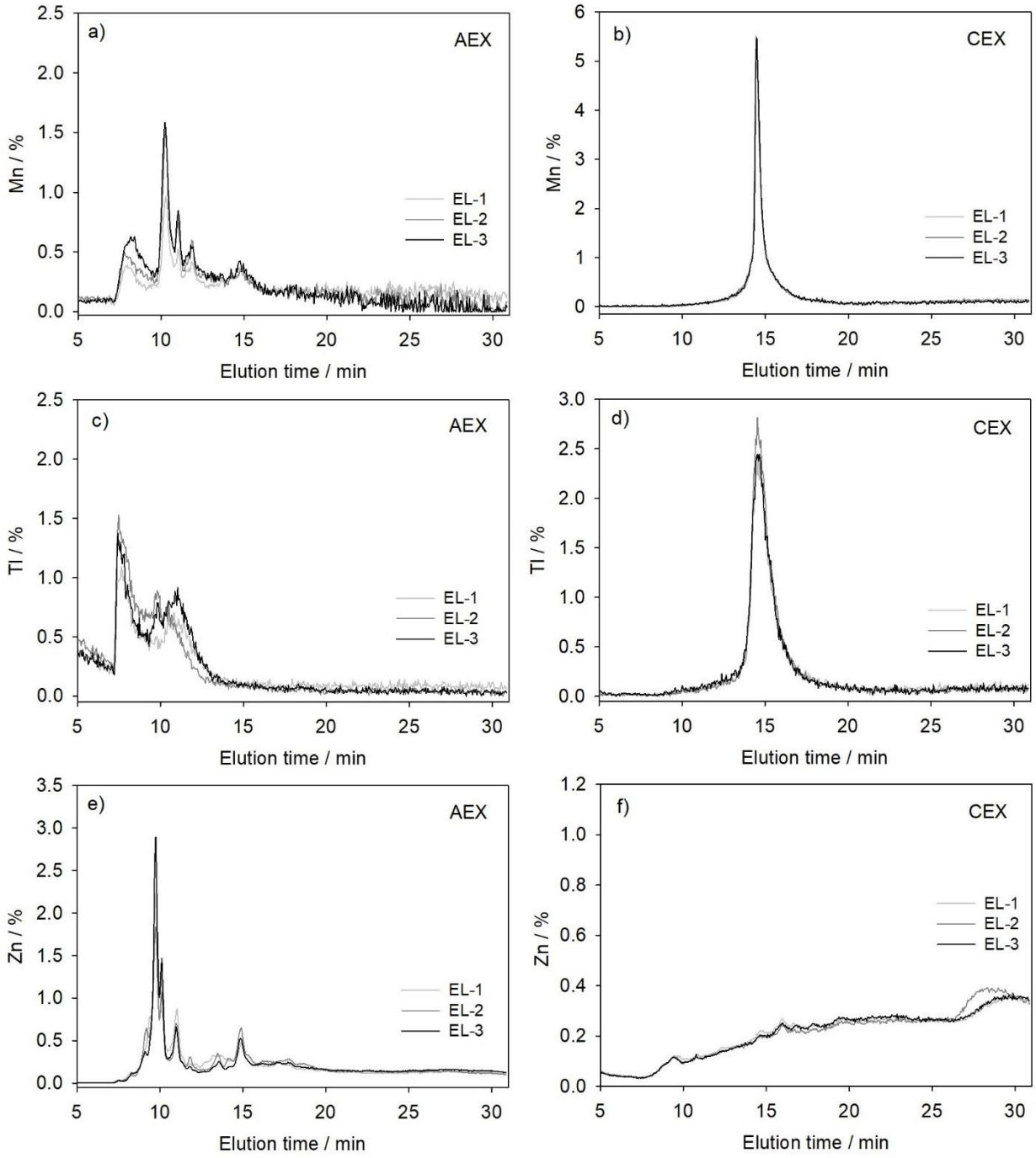
a)
AEX
Mn / %
EL-1
EL-2
EL-3
Elution time / min
b)
CEX
Mn / %
EL-1
EL-2
EL-3
Elution time / min
c)
AEX
Tl / %
EL-1
EL-2
EL-3
Elution time / min
d)
CEX
Tl / %
EL-1
EL-2
EL-3
Elution time / min
e)
AEX
Zn / %
EL-1
EL-2
EL-3
Elution time / min
f)
CEX
Zn / %
EL-1
EL-2
EL-3
Elution time / min

Table 1. The concentrations of studied metals measured in the hepatic soluble fraction of the northern pike (*Esox lucius*) from the Mrežnica River (expressed as ng or µg of soluble metal per gram of hepatic tissue).

	EL-1	**EL-2**	**EL-3**
Ag / ng g^{-1}	14.2	20.1	17.0
Bi / ng g^{-1}	8.73	7.07	12.7
Cd / ng g^{-1}	5.12	0.68	19.8
Co / ng g^{-1}	12.7	14.5	16.3
Cs / ng g^{-1}	3.65	4.72	5.44
Cu / µg g^{-1}	3.64	3.99	4.85
Fe / µg g^{-1}	56.2	44.7	102
Mn / ng g^{-1}	519	419	433
Mo / ng g^{-1}	52.6	67.4	55.0
Pb / ng g^{-1}	ND	ND	ND
Tl / ng g^{-1}	4.70	6.32	5.47
Zn / µg g^{-1}	19.8	22.7	21.1

ND – nondetectable (below detection limit of 0.486 ng g^{-1})

Table 2. The analysis of standard proteins: source materials, molecular masses (MM), isoelectric points (pI), concentrations (c), columns applied (anion-exchange, AEX; or cation-exchange, CEX), and elution times (E_t).

	Source material	**MM**	**pI**	**c** / mg mL^{-1}	**Column**	**E_t / min**
Transferrin	Human	80 kDa	5.8	1.0	AEX	12.17
Alcohol-dehydrogenase	*Saccharomyces cerevisiae*	150 kDa	5.3	5.0	AEX	11.42
Superoxide-dismutase	Bovine erythrocytes	32.5 kDa	4.95	5.0	AEX	9.69
Metallothionein 2	Rabbit liver	6.145 kDa	4.5	0.5	AEX	9.64
Metallothionein 1	Rabbit liver	6.145 kDa	3.9	0.5	AEX	8.35
Carbonic-anhydrase	Bovine erythrocytes	29 kDa	4.9	3.0	AEX	8.19
Cytochrome C	Bovine heart	12.384 kDa	9.6	5.0	CEX	19.28
Lysozyme	Chicken egg white	14.3 kDa	11.1	1.0	CEX	24.12
Dithiothreitol	-	154 Da	-	1.0 mM	AEX	1.72
					CEX	3.61

Table 3. Analysis of distribution of Ag, Bi, Cd, and Co in the northern pike (*Esox lucius*) liver from the Mrežnica River among cytosolic biomolecules of acidic and basic nature using online coupled system AEX- and CEX-HPLC-ICP-MS. For each metal on each column, the portions (%) eluted in the void volume and those bound to the column are presented, as well as the overview of the major peaks of the bound metals (the predominant peaks are shaded in grey). The correlations (r) between metal portions within each peak (%) and cytosolic metal concentrations (c_c) (n=3) are also given.

	AEX-column				**CEX column**		
	E_t / min Range (max)	**%**	**r [%; c_c]**		**E_t / min Range (max)**	**%**	**r [%; c_c]**
[a]**Ag_{void}**	**0.05-5.01 (0.86-0.92)**	**0.84-1.05**	-0.103		**0.05-5.01**	**98.51-98.67**	0.344
Ag_{bound}:	**5.06-30.86:**	**98.95-99.16**	0.103		**5.06-30.86:**	**1.33-1.49**	-0.344
[b]Ag1	8.40-9.53 (9.42)	14.94-15.44	-0.445		-	-	-
Ag2	9.59-9.86 (9.75)	10.17-15.47	-0.061		-	-	-
Ag3	9.91-10.39 (10.12)	31.01-39.01	-0.577		-	-	-
Ag4	10.45-11.09 (10.72)	7.24-9.97	0.168		-	-	-
Ag5	11.15-11.69 (11.47)	4.76-8.69	0.898		-	-	-
Ag6	14.43-14.97 (14.81)	2.03-2.30	-0.997*		-	-	-
Ag7	15.83-16.37 (16.05)	1.04-1.71	0.759		-	-	-
Bi_{void}	**0.05-5.01 (0.81-0.86)**	**5.85-20.36**	0.732		**0.05-5.01**	**73.84-81.66**	1.000*
Bi_{bound}:	**5.06-30.86:**	**79.64-94.15**	-0.732		**5.06-30.86:**	**18.34-26.16**	-1.000*
Bi1	7.06-7.76 (7.38)	0.44-1.43	0.860		7.54-8.89 (8.67)	2.56-10.95	0.408
Bi2	8.56-9.48 (9.05)	2.13-4.40	-0.867		8.94-10.23 (9.21)	8.48-18.72	0.421
Bi3	9.53-10.29 (9.91)	1.81-5.59	-1.000*		-	-	-
Bi4	10.34-11.69 (10.72)	1.27-9.80	-0.814		-	-	-
Bi5	15.73-17.66 (16.59)	15.93-25.33	0.701		-	-	-
Bi6	19.49-20.36 (20.03)	5.43-10.01	0.922		-	-	-
Bi7	20.41-22.29 (20.89)	13.39-19.01	0.995*		-	-	-
Cd_{void}	**0.05-5.01 (0.86-0.92)**	**0.88-2.14**	-0.310		**0.05-5.01**	**59.21-97.42**	0.724
Cd_{bound}:	**5.06-30.86:**	**97.86-99.12**	0.310		**5.06-30.86:**	**2.58-40.79**	-0.724
Cd1	8.56-9.26 (9.05)	1.59-5.78	0.875		-	-	-
Cd2	9.32-9.96 (9.75)	4.01-49.28	0.979		-	-	-
Cd3	10.02-10.45 (10.12)	3.17-24.06	0.974		-	-	-
Co_{void}	**0.05-5.01 (0.92)**	**3.21-7.54**	0.669		**0.05-5.01**	**99.98-100.0**	-0.866
Co_{bound}:	**5.06-30.86:**	**92.46-96.79**	-0.669		**5.06-30.86:**	**0.00-0.02**	0.866
Co1	7.22-7.86 (7.76)	1.29-6.85	0.914		-	-	-
Co2	7.92-8.40 (7.97)	1.53-5.26	0.793		-	-	-
Co3	8.62-9.32 (8.89)	2.98-13.05	0.604		-	-	-
Co4	9.59-10.02 (9.86)	0.57-0.87	0.894		-	-	-
Co5	15.40-17.77 (16.16)	44.06-72.74	0.352		-	-	-

[a]for metals eluted in the void volume (the first five minutes of the separation protocol) and metals bound to the column (6^{th} to 31^{st} minute of the separation protocol) calculated percentages refer to total intensity; [b]for each peak, calculated percentages refer to portion of the bound intensity (considered now as 100%; i.e. excluding the intensity of void volume); *p<0.10; E_t – elution time

Table 4. Analysis of distribution of Cs, Cu, Fe, and Mn in the northern pike (*Esox lucius*) liver from the Mrežnica River among cytosolic biomolecules of acidic and basic nature using online coupled system AEX- and CEX-HPLC-ICP-MS. For each metal on each column, the portions (%) eluted in the void volume and those bound to the column are presented, as well as the overview of the major peaks of the bound metals (the predominant peaks are shaded in grey). The correlations (r) between metal portions within each peak (%) and cytosolic metal concentrations (n=3) are also given.

	AEX-column				**CEX column**		
	E_t / min Range (max)	**%**	**r [%; c_c]**		**E_t / min Range (max)**	**%**	**r [%; c_c]**
[a]**Cs_{void}**	**0.05-5.01 (0.86-0.92)**	**84.10-92.25**	1.000*		**0.05-5.01**	**1.33-1.59**	-0.982
Cs_{bound}:	**5.06-30.86:**	**7.75-15.90**	-1.000*		**5.06-30.86:**	**98.41-98.67**	0.982
[b]Cs1	7.11-9.30 (8.02)	43.42-49.12	-0.396		6.62-9.69 (8.02)	47.82-57.91	0.999*
Cs2	-	-	-		9.75-11.79 (10.56)	21.01-25.78	-0.999*
Cu_{void}	**0.05-5.01 (0.86)**	**0.99-1.48**	-0.843		**0.05-5.01**	**99.04-99.15**	-0.028
Cu_{bound}:	**5.06-30.86:**	**98.52-99.01**	0.843		**5.06-30.86:**	**0.85-0.96**	0.028
Cu1	8.24-9.37 (9.10)	12.50-14.17	-0.967		7.97-9.32 (8.78)	10.04-14.33	-0.432
Cu2	9.42-9.91 (9.75)	20.35-28.66	0.892		9.37-10.34 (9.69)	7.74-9.55	-0.936
Cu3	9.96-10.39 (10.12)	21.47-26.18	0.863		-	-	-
Cu4	10.61-11.31 (11.04)	14.74-19.53	-0.890		-	-	-
Cu5	11.36-11.79 (11.58)	4.17-6.87	-0.265		-	-	-
Cu6	13.52-14.00 (13.79)	1.20-1.45	-0.545		-	-	-
Cu7	14.49-15.02 (14.86)	1.41-1.54	-0.323		-	-	-
Cu8	15.94-16.43 (16.16)	0.64-0.99	-0.497		-	-	-
Fe_{void}	**0.05-5.01 (0.92)**	**1.46-3.34**	-0.753		**0.05-5.01**	**98.46-99.28**	-0.663
Fe_{bound}:	**5.06-30.86:**	**96.66-98.54**	0.753		**5.06-30.86:**	**0.72-1.54**	0.663
Fe1	8.02-9.10 (9.10)	2.40-3.30	-0.998*		20.89-22.73 (22.24)	5.63-9.85	-0.451
Fe2	9.16-9.48 (9.32)	1.36-2.29	-0.965		22.78-24.23 (23.48)	3.01-4.59	-0.402
Fe3	9.53-12.82 (10.56)	72.32-76.57	0.551		-	-	-
Fe4	13.03-16.59 (14.65)	8.97-10.54	-0.923		-	-	-
Fe5	18.31-20.30 (18.79)	1.88-3.17	-0.520		-	-	-
Mn_{void}	**0.05-5.01 (0.86-0.92)**	**24.92-31.98**	-0.937		**0.05-5.01**	**40.04-45.17**	-0.881
Mn_{bound}:	**5.06-30.86:**	**68.02-75.08**	0.937		**5.06-30.86:**	**54.83-59.96**	0.881
Mn1	7.22-9.53 (8.19)	11.13-18.91	-0.772		12.82-16.80 (14.43)	67.01-69.29	-0.999*
Mn2	9.80-10.77 (10.23)	10.77-16.95	-0.974		-	-	-
Mn3	10.82-11.30 (11.04)	4.04-6.24	-0.928		-	-	-
Mn4	11.36-12.55 (11.79)	7.22-9.77	-0.911		-	-	-
Mn5	14.06-16.32 (14.76)	10.98-12.33	-0.378		-	-	-

[a]for metals eluted in the void volume (the first five minutes of the separation protocol) and metals bound to the column (6^{th} to 31^{st} minute of the separation protocol) calculated percentages refer to total intensity; [b]for each peak, calculated percentages refer to portion of the bound intensity (considered now as 100%; i.e. excluding the intensity of void volume); *p<0.10; E_t – elution time

Table 5. Analysis of distribution of Mo, Pb, Tl, and Zn in the northern pike (*Esox lucius*) liver from the Mrežnica River among cytosolic biomolecules of acidic and basic nature using online coupled system AEX- and CEX-HPLC-ICP-MS. For each metal on each column, the portions (%) eluted in the void volume and those bound to the column are presented, as well as the overview of the major peaks of the bound metals (the predominant peaks are shaded in grey). The correlations (r) between metal portions within each peak (%) and cytosolic metal concentrations (n=3) are also given.

	AEX-column				**CEX column**		
	E_t / min Range (max)	**%**	**r [%; c_c]**		**E_t / min Range (max)**	**%**	**r [%; c_c]**
[a]**Mo_{void}**	**0.05-5.01 (0.86)**	**1.09-1.26**	0.314		**0.05-5.01**	**94.42-97.00**	0.683
Mo_{bound}:	**5.06-30.86:**	**98.74-98.91**	-0.314		**5.06-30.86:**	**3.00-5.58**	-0.683
[b]Mo1	14.49-15.35 (15.08)	0.86-1.37	-0.746		-	-	-
Mo2	15.40-16.05 (15.89)	1.83-5.46	-0.994*		-	-	-
Mo3	16.10-16.91 (16.75)	7.21-10.01	-0.988*		-	-	-
Mo4	16.96-20.09 (17.29)	18.22-26.29	0.981		-	-	-
Mo5	22.62-28.38 (23.75)	51.66-57.07	-0.238		-	-	-
Pb_{void}	**0.05-5.01 (0.86-0.92)**	**0.56-0.76**	-		**0.05-5.01**	**98.91-99.41**	-
Pb_{bound}:	**5.06-30.86:**	**99.24-99.44**	-		**5.06-30.86:**	**0.59-1.09**	-
Pb1	8.94-9.43 (9.16)	3.82-9.33	-		-	-	-
Pb2	9.48-10.12 (9.64)	3.67-21.35	-		-	-	-
Pb3	10.72-12.22 (11.26)	25.82-61.57	-		-	-	-
Pb4	13.41-14.86 (14.06)	15.41-33.55	-		-	-	-
Tl_{void}	**0.05-5.01 (1.08-1.19)**	**42.68-47.44**	0.279		**0.05-5.01**	**42.76-52.55**	0.047
Tl_{bound}:	**5.06-30.86:**	**52.56-57.32**	-0.279		**5.06-30.86:**	**47.45-57.24**	-0.047
Tl1	7.16-9.16 (7.43)	26.00-35.18	0.985		12.98-17.45 (14.59)	68.58-75.45	0.997*
Tl2	9.21-10.18 (9.86)	8.60-13.99	0.995*		-	-	-
Tl3	10.23-13.03 (11.04)	19.98-29.51	-0.452		-	-	-
Zn_{void}	**0.05-5.01 (0.86)**	**15.34-22.48**	0.861		**0.05-5.01**	**61.25-64.93**	0.401
Zn_{bound}:	**5.06-30.86:**	**77.52-84.66**	-0.861		**5.06-30.86:**	**35.07-38.75**	-0.401
Zn1	8.62-9.32 (9.21)	3.74-5.20	0.504		8.26-9.99 (9.45)	-	-
Zn2	9.37-9.96 (9.75)	13.26-17.29	-0.441		10.53-11.13 (10.80)	-	-
Zn3	10.02-10.45 (10.12)	5.66-7.26	-0.850		14.27-15.19 (14.65)	-	-
Zn4	10.50-11.52 (10.99)	7.28-9.77	-0.802		15.30-16.43 (16.00)	-	-
Zn5	11.58-12.28 (11.85)	2.30-3.13	0.135		16.50-17.40 (16.80)	-	-
Zn6	12.92-13.84 (13.46)	3.52-5.53	-0.515		17.57-18.33 (17.84)	-	-
Zn7	14.42-15.83 (14.92)	8.09-10.04	0.929		19.03-20.28 (19.47)	-	-

[a]for metals eluted in the void volume (the first five minutes of the separation protocol) and metals bound to the column (6^{th} to 31^{st} minute of the separation protocol) calculated percentages refer to total intensity; [b]for each peak, calculated percentages refer to portion of the bound intensity (considered now as 100%; i.e. excluding the intensity of void volume); *p<0.10; E_t – elution time

Table 6. Approximate molar concentrations (mol L^{-1}) of Ag, Cd, Cu, and Zn within AEX-fractions of metallothionein (MT) isoforms, MT1 and MT2, and superoxide dismutase (SOD) from hepatic cytosol of three northern pike specimens (EL1-EL3) from the Mrežnica River. As there is no possibility to obtain metal concentrations, but only metal intensities, when applying online HPLC-ICP-MS system, we have used calibration equations for these four metals obtained during concentration measurements on ICP-MS; since intensities could somewhat vary from day to day, we consider these values to be only approximations of apsolute metal quantities in analyzed protein fractions.

		MT1	**MT2**	**MT1 : MT2**	**SOD**
Ag					
	EL1	3.57×10^{-9}	12.0×10^{-9}	1 : 3.4	-
	EL2	4.73×10^{-9}	14.2×10^{-9}	1 : 3.0	-
	EL3	5.31×10^{-9}	13.2×10^{-9}	1 : 2.5	-
Cd					
	EL1	6.49×10^{-9}	4.13×10^{-9}	1.6 : 1	-
	EL2	4.52×10^{-9}	3.64×10^{-9}	1.2 : 1	-
	EL3	36.7×10^{-9}	17.7×10^{-9}	2.1 : 1	-
Cu					
	EL1	2757×10^{-9}	2846×10^{-9}	1 : 1.03	2333×10^{-9}
	EL2	3237×10^{-9}	3416×10^{-9}	1 : 1.06	3108×10^{-9}
	EL3	5277×10^{-9}	4820×10^{-9}	1.09 : 1	2715×10^{-9}
Zn					
	EL1	7171×10^{-9}	3429×10^{-9}	2.1 : 1	4722×10^{-9}
	EL2	8536×10^{-9}	3636×10^{-9}	2.4 : 1	4788×10^{-9}
	EL3	11828×10^{-9}	4960×10^{-9}	2.4 : 1	4976×10^{-9}
Ag : Cd					
	EL1	1 : 1.8	2.9 : 1	-	-
	EL2	1.1 : 1	3.9 : 1	-	-
	EL3	1 : 6.9	1 : 1.3	-	-
Cu : Zn					
	EL1	1 : 2.6	1 : 1.2	-	1 : 2.0
	EL2	1 : 2.6	1 : 1.1	-	1 : 1.5
	EL3	1 : 2.2	1 : 1.0	-	1 : 1.8