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Cytosolic distributions of highly toxic metals Cd and Tl and several essential elements in the liver of brown trout (*Salmo trutta* L.) analyzed by size exclusion chromatography and inductively coupled plasma mass spectrometry

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Abstract

Cytosolic distributions of nonessential metals Cd and Tl and seven essential elements among compounds of different molecular masses were studied in the liver of brown trout (*Salmo trutta*) from the karstic Krka River in Croatia. Analyses were done by size exclusion high performance liquid chromatography and high resolution inductively coupled plasma mass spectrometry. Common feature of Cd and Tl, as highly toxic elements, was their distribution within only two narrow peaks. The increase of cytosolic Cd concentrations was reflected in marked increase of Cd elution within low molecular mass peak (maximum at ~15 kDa), presumably containing metallothioneins (MTs), which indicated successful Cd detoxification in brown trout liver under studied exposure conditions. Contrary, the increase of cytosolic Tl concentrations was reflected in marked increase of Tl elution within high molecular mass peak (maximum at 140 kDa), which probably indicated incomplete Tl detoxification. Common feature of the majority of studied essential elements was their distribution within more peaks, often broad and not well resolved, which is consistent with their numerous physiological functions. Among observed associations of essential metals/nonmetal to proteins, the following could be singled out: Cu and Zn association to MTs, Fe association to storage protein ferritin, and Se association to compounds of very low molecular masses (<5 kDa). The obtained results present the first step towards identification of metal-binding compounds in hepatic cytosol of brown trout, and thus a significant contribution to better understanding of metal fate in the liver of that important bioindicator species.

Key words: fish, hepatic biomolecules, ICP-MS, Krka River, metallothioneins, SEC-HPLC

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1. Introduction

The metal pollution can affect every stage of the aquatic food chain, leading to the disturbance of the whole ecosystem (Van Campenhout et al., 2010). In highly contaminated aquatic environment, fish can accumulate metals both from surrounding water and from food (Dragun et al., 2012, 2016; Filipović Marijić and Raspor, 2012; Rajeshkumar et al., 2018). It can result with high level of metal bioaccumulation in their organs and possible development of toxic effects (Dragun et al., 2013; Qu et al., 2014). However, although the concentrations of trace metals in organs of aquatic animals are often used to assess their exposure to metals in aquatic systems (Luoma and Rainbow, 2008), such information is not sufficient to estimate if bioaccumulated quantity of metal would cause damage to exposed fish. Metal toxicity arises predominantly from the binding of metals to essential biomolecules such as enzymes and transporters and the involvement of certain metals in the formation of radicals (Mason and Jenkins, 1995), but part of metal bioaccumulated within the organism can also be detoxified. Therefore, next to basic studies on the concentrations of metals bioaccumulated in fish organs, it is essential to further investigate the fate of those metals in the cells, and to determine whether they are more likely to cause harm to the fish or to be detoxified and excreted from fish organism. In the scientific field which deals with the comprehensive analysis of the entirety of metal and metalloid species within a cell or tissue (Szpunar, 2005), it is a common first step to use the combination of different techniques of high performance liquid chromatography (HPLC) and inductively coupled plasma mass spectrometry (ICP-MS) as a powerful tool for recognizing the cytosolic ligands that bind specific elements (Goenaga Infante et al., 2006; Santiago-Rivas et al., 2007).

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So far, there were only few studies of such kind performed on fish. Several investigations were, for example, directed to study of metal detoxification by metallothioneins (MTs) in different fish organs by application of size exclusion HPLC (SEC-HPLC) and ICP-MS in field populations of the European eel (*Anguilla anguilla*) (Van Campenhout et al., 2008) and gibel carp (*Carassius auratus gibelio*) (Van Campenhout et al., 2010). Caron et al. (2018) applied similar methodology to perform somewhat more extensive study on liver of juvenile yellow perch (*Perca flavescens*) regarding binding of several trace elements (Ag, Cd, Co, Cu, Ni, and Tl) to various cytosolic biomolecules. Our previous studies in this field included comprehensive investigation of cytosolic distributions of Cd, Co, Cu, Fe, Mn, Mo, Pb, Se, and Zn in the liver and gills of European chub (*Squalius cephalus*) from the moderately contaminated Sutla River in Croatia (Krasnići et al., 2013, 2014) and of Vardar chub (*Squalius vardarensis*) from highly contaminated Macedonian rivers (Krasnići et al., 2018).

In this study, our main aim was to identify the molecular masses of cytosolic biomolecules that bind specific trace elements in the liver of brown trout (*Salmo trutta* Linnaeus, 1758), as a representative fish species and important bioindicator of karstic rivers. We have previously characterized the water quality of the Krka River, at the locations where brown trout were sampled (Filipović Marijić et al., 2018), as well as total metal bioaccumulation in the liver of the same brown trout specimens that were used in this study (Dragun et al., 2018). The specific goals of the investigation presented in this paper included application of SEC-HPLC in combination with offline metal measurement on high resolution ICP-MS (HR ICP-MS) to separate hepatic cytosols of brown trout into fractions that contain various metal-binding biomolecules,

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and to define the distribution profiles among cytosolic biomolecules of different molecular masses for nine selected elements, two highly toxic metals (Cd and Tl) and seven essential elements (Co, Cu, Fe, Mn, Mo, Se, and Zn). As it is very likely that various fish species have different metal handling strategies, the additional aim of this study was to compare metal distribution profiles characteristic for brown trout liver with previously published profiles for liver of European chub (Krasnići et al., 2013) and Vardar chub (Krasnići et al., 2018), and to recognize and describe the differences between them, which could indicate different susceptibility to metal exposure in these distinct fish species.

2. Materials and methods

2.1. Study period and area

This study was a part of the comprehensive pollution study on the Croatian karstic river Krka, performed within two sampling campaigns in October 2015 and May 2016. The first results of that study, regarding water quality (Filipović Marijić et al., 2018) and metal bioaccumulation in the fish liver (Dragun et al., 2018) have been already published. The map of the sampling area, comprising of two sampling sites, was also previously published by Dragun et al. (2018). As a reference site we have chosen the Krka River source, whereas a location downstream of Knin town was chosen as a potentially contaminated site, due to known pollution sources in Knin area (e.g., industrial wastewater of screw factory and untreated municipal wastewater discharge; Filipović Marijić et al., 2018). The analyses of dissolved metals in the river water conducted in the course of this study, simultaneously with fish sampling, confirmed a slight concentration increase of several trace elements (e.g. Al, As, Co, Fe, Mn, Mo, Rb, Sr, V, and Zn) downstream of Knin town (Dragun et al., 2018; Filipović Marijić et al., 2018). The other physico-chemical parameters of the river-water, such as pH and total dissolved solids (TDS), were comparable at both sites in both seasons (Krka River source: pH 7.6-7.7, TDS ~180 mg L^{-1}; Krka Knin: pH 7.8-8.1, TDS 201-208 mg L^{-1}), and were not further considered as the significant factors in this study. The values of pH and TDS were determined *in situ* using a multiparameter water quality monitoring instruments SevenGo pro (Mettler Toledo).

2.2. Fish sampling and organ dissection

The selected bioindicator for this study was fish species brown trout (*Salmo trutta* Linnaeus, 1758). Fish samplings were carried out by electrofishing, in accordance with the Croatian standard HRN EN 14011 (2005), as described by Dragun et al. (2018). The captured fish were kept alive in aerated water tank during transportation, and in the laboratory they were anesthetized with tricaine methane sulphonate (MS 222, Sigma Aldrich) before they were sacrificed. Thereafter, we have recorded fish total masses and lengths, then isolated and weighed the liver and the gonads, and stored the liver at -80°C before further analyses. We have calculated gonadosomatic indices (GSI) based on the ratios of gonad masses to total trout masses, and Fulton condition indices (FCI) according to Rätz and Lloret (2003), as the ratios of total masses to total lengths cubed, and multiplied with 100. Sex was determined by examination of fish gonads, both macroscopic and microscopic, under magnification of 40× and 100×, using optical microscope BH-2 (Olympus). Out of 65 fish sampled for the assessment of metal bioaccumulation, 15 to 18 at each site in each sampling campaign (Dragun et al., 2018),

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we have selected twelve specimens for the analyses of cytosolic metal distributions performed within the current study, three per each site in each season. Biometric characteristics of these twelve fish are presented in Table 1.

2.3. Hepatic tissue homogenization and isolation of soluble hepatic fractions

The samples of hepatic tissue were cut into small pieces within glass crystallizing dishes set on ice. Then cooled homogenization buffer [100 mM Tris-HCl/Base (Sigma, pH 8.1 at 4°C) supplemented with reducing agent (1 mM dithiotreitol, Sigma)] was added into each dish to dilute hepatic tissue (w/v 1:5). Thereafter followed homogenization by application of 10 strokes of Potter-Elvehjem homogenizer (Glas-Col, USA) at 6,000 rpm in an ice cooled tube. The homogenates were then centrifuged at 50,000×g for 2 h at 4°C in the Avanti J-E centrifuge (Beckman Coulter, USA). Supernatants (S50) obtained after centrifugation represented water soluble cytosolic tissue fractions containing lysosomes and microsomes (Bonneris et al., 2005). S50 fractions were separated from centrifuge tubes, transferred to clean tubes and stored in the freezer at -80°C.

2.4. SEC-HPLC fractionation of brown trout hepatic cytosols

Distributions of selected trace elements among cytosolic biomolecules of various molecular masses in brown trout liver were studied using SEC-HPLC (Perkin Elmer HPLC system, series 200, USA) with prepacked Tricorn™ Superdex 200 10/300 GL size exclusion column (GE Healthcare Biosciences, USA) with a separation range of 10-600 kDa, for globular proteins (Krasnići et al., 2013, 2014, 2018). Prior to application on the column, samples of hepatic cytosols were centrifuged at 10,000×*g* for 10 min at 4°C (Heraeus Biofuge Fresco, Kendro, USA) to remove the possible clots that could clog the system. For each sample, two consecutive chromatographic runs were performed, each with application of 100 µL of the sample on the column, i.e. 200 µL of each cytosolic sample was run through the column. Mobile phase for the separation was 20 mM Tris-HCl/Base (Sigma, pH 8.1 at 22°C), with a flow rate of 0.5 mL min^{-1} using isocratic mode. Chromatographic fractions were collected at one minute intervals from 13th to 52nd minute in the plastic tubes using a fraction collector (FC 203B, Gilson, USA). For column calibration, several protein standards (thyroglobulin, apoferritin, β-amylase, alcohol dehydrogenase, bovine albumin, and carbonic anhydrase, Sigma, USA) were run through the column under the same conditions as the samples and the equation of the calibration straight line is given in Table 2. In addition, elution times were also determined for MT standards, MT-1 and MT-2 (Enzo Life Sciences, USA), whereas the void volume of the column was determined by use of blue dextran (Table 2).

2.5. Determination of trace element concentrations in the SEC-HPLC fractions of hepatic cytosol

In this study we have measured the concentrations of two highly toxic metals (Cd and Tl) and seven essential elements (Co, Cu, Fe, Mn, Mo, Se, and Zn) in each one-minute fraction obtained by SEC-HPLC separation. These fractions were acidified with HNO_3 (*Suprapur*, Merck, Germany, final acid concentration in the samples 0.16%), and prior to measurement In (Fluka, Germany) was added as an internal standard (1 µg L^{-1}). The measurements were performed on HR ICP-MS (Element 2, Thermo Finnigan, Germany), equipped with an autosampler SC-2 DX FAST (Elemental Scientific, USA)

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and sample introduction kit consisting of a SeaSpray nebulizer and cyclonic spray chamber Twister. Typical instrumental conditions and measurement parameters were reported previously (Fiket et al., 2007). Measurements of ^{82}Se, ^{98}Mo, ^{111}Cd, and ^{205}Tl were performed in low-resolution mode, whereas ^{55}Mn, ^{56}Fe, ^{59}Co, ^{63}Cu, and ^{66}Zn were measured in medium resolution mode. Standards for external calibration were prepared in 1.3% HNO_3 (*Suprapur*; Merck, Germany) using multielement standard solution for trace elements (Analitika, Czech Republic), and supplemented with In (1 $\mu g\ L^{-1}$; Fluka, Germany). Limits of detection (LOD) were determined based on three standard deviations of ten consecutively determined trace element concentrations in the blank sample (Tris-HCl/Base, dithiothreitol, HNO_3). LODs for trace elements measured within this study were as follows (in $\mu g\ L^{-1}$): Cd, 0.005; Co, 0.002, Cu, 0.037; Fe, 0.084; Mn, 0.002; Mo, 0.004; Se, 0.138; Tl, 0.001; and Zn, 2.40 (Krasnići et al., 2013, 2014). The accuracy of trace element measurements by HR ICP-MS was checked by analysis of quality control sample (QC for trace metals, catalog no. 8072, lot no. 146142-146143, Burlington, Canada). A generally good agreement was observed between our data and the certified values, as seen from the following recovery values: Cd, 99.7±3.3; Co, 99.8±2.5, Cu, 98.7±3.2; Fe, 103.9±9.5; Mn, 99.3±2.0; Se, 103.0±5.4; Tl, 101.6±4.8; and Zn, 97.7±5.9.

2.6. Data processing and statistical analyses

All basic calculations were done in Microsoft Excel 2007. Based on the column calibration (Table 2), elution times of specific peaks were associated to corresponding molecular masses, with the aim to define the molecular masses of biomolecules that bind each studied element (Table 3). In this study and for the purpose of easier discussion, we have categorized the biomolecules in four classes according to their molecular masses (Table 3), as already described in our previous publications (Krasnići et al., 2013, 2014, 2018): 1) HMM or biomolecules of high molecular mass (>100 kDa); 2) MMM or biomolecules of medium molecular mass (30-100 kDa); 3) LMM or biomolecules of low molecular mass (10-30 kDa); and 4) VLMM or biomolecules of very low molecular mass (<10 kDa).

Graphs were created using the statistical program SigmaPlot 11.0 for Windows. In Figs. 1-5, we have graphically presented distribution profiles with obtained peaks for each one of nine analyzed elements, separately for each site in each season. Total cytosolic concentrations of studied elements in the liver of twelve analyzed fish specimens that are presented within the figures were taken from concurrent study on hepatic metal bioaccumulation in brown trout (Dragun et al., 2018).

3. Results and discussion

The study presented in this paper was a constitutive part of a comprehensive study on metal exposure and hepatic bioaccumulation in brown trout (*S. trutta*), which was conducted on the Krka River in autumn 2015/spring 2016 (Dragun et al., 2018). It revealed increased cytosolic concentrations of several metals in brown trout liver at the location downstream of Knin town, which is influenced by untreated municipal and industrial wastewaters (Dragun et al., 2018). However, it also revealed increased cytosolic hepatic concentrations of few metals in brown trout at the source of the Krka River, which is considered as a pristine area (Dragun et al., 2018). Among nine elements analyzed in the current study, highly toxic metals Cd and Tl, as well as essential metal Mo, were present in somewhat higher concentrations in brown trout liver at presumably clean site, the Krka River source (Dragun et al., 2018). It was possibly the consequence of increased exposure from sediments as a result of some natural cause, such as rock weathering, since concentrations of dissolved Cd and Tl in the river water were comparable and rather low at both sites and amounted to 0.005-0.010 μg L^{-1} for Cd and 0.004-0.005 μg L^{-1} for Tl (Dragun et al., 2018). Contrary, essential elements Co and Se were increased in brown trout liver at the expectedly contaminated site downstream of Knin town; hepatic concentrations of Cu, Fe, Mn and Zn in brown trout were mainly comparable at both sampling sites (Dragun et al., 2018). Accordingly, 12 samples analyzed in the current study (three per each site and each season) were chosen in such a way, so that they reflect the observations of bioaccumulation study as closely as possible, especially concerning the most toxic metals, Cd and Tl. In the other words, for the purpose of metal distribution analyses, we have selected those specimens, from the whole set of sampled fish, that had comparatively higher Cd and Tl concentrations at the Krka River source (Cd: 118-296 ng g^{-1}; Tl: 87.5-456 ng g^{-1}) than at the Krka River downstream of Knin town (Cd: 3.45-20.1 ng g^{-1}; Tl: 4.1-199 ng g^{-1}).

Twelve fish selected for distribution analyses varied in size from 18.3 to 27.5 cm, and they differed in GSI and sex (Table 1). GSIs were generally higher in autumn than in spring, indicating active reproductive period (Dragun et al., 2018), but comparison of metal distribution profiles obtained in two seasons, as well as comparisons between males and females, have not indicated any variations in metal cytosolic distributions that could be attributed to fish physiology. It could be assumed that all the observed differences, mainly in peak heights, were the results of differences in exposure levels and in the levels of consequent hepatic accumulation. Thus, the obtained results will be further on discussed precisely from that point of view for each one of the studied elements.

3.1. Highly toxic elements

Common feature of Cd and Tl, as nonessential and highly toxic elements, was that they were distributed within small number of narrow and well resolved peaks (Fig. 1a and b). This was an indication of their association to limited number of compounds in the cytosols, which is in accordance with the fact that they do not possess a known biological function in the fish organism.

3.1.1. Cadmium

Cadmium is a nonessential element and its toxicity could be partly associated to competitive inhibition of calcium pumps (Verbost et al., 1989). Cadmium was eluted in two narrow peaks (Fig. 1a, Table 3). The first Cd peak was located in HMM biomolecule region, at elution time from the 14th to 17th minute which corresponded to biomolecules of molecular masses in the range from ~500 to 1000 kDa. The second Cd peak was located in the LMM biomolecule region, at elution times from the 29th to 34th minute which corresponded to biomolecules of molecular masses in the range from 7 to 24 kDa.

At low Cd concentrations in the cytosol of brown trout liver (≤20 ng g^{-1}), the presence of Cd in both peaks was either comparable or even more pronounced in the HMM peak, as seen in the fish from the sampling site Krka-Knin (Fig. 1a, lower row). Cadmium elution in HMM region observed in this study on brown trout liver was not observed in the previous studies on the liver of European and Vardar chub (Krasnići et al., 2013, 2018), indicating possible higher susceptibility of brown trout to Cd toxicity. However, with the increasing cytosolic Cd concentrations (>100 ng g^{-1}), a marked increase of Cd quantity in LMM peak was observed, with a maximum of molecular mass equal to ~15 kDa, as seen in the fish from the Krka River source (Fig. 1a, upper row). The maximum elution time of that peak, which was equal to 31 minute (Table 3), was the same as the elution time of MT standard (Table 2). This result obtained for brown trout liver was comparable to previously published information on Cd binding to MT in the liver of European and Vardar chub (Krasnići et al., 2013, 2018), of juvenile yellow perch (Caron et al., 2018), of European eel (Van Campenhout et al., 2008), and of gibel carp (Van Campenhout et al., 2010), which was interpreted as evidence of successful Cd detoxification (Caron et al., 2018). It was also consistent with the known fact that Cd detoxification primarily takes place through binding to glutathione (GSH) and MT (McGeer et al., 2012), and indicated that Cd was mainly detoxified in brown trout liver under studied exposure conditions. Recently it has been suggested that, next to GSH and MT, heat shock proteins (HSP70 and HSP90) play an important role in physiological changes associated to cell protection in fish exposed to Cd (Kwong et al., 2011) and an indication of such association of Cd was reported for European chub liver (Krasnići et al., 2013). However, in this study Cd association to biomolecules of molecular masses that would correspond to heat shock proteins was not observed.

3.1.2. Thallium

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Thallium is a nonessential metal, which is toxic already in very low concentrations due to its interference with K-dependent biological processes (Jaiswal et al., 2012). It is well known that Tl^+ can replace K^+, which is physiologically required for activation of several enzymes, for example aldehyde dehydrogenase and ATPase enzyme in Na^+-K^+ exchange pump (Jaiswal et al., 2012). Same as Cd, Tl was also eluted in two narrow peaks (Fig. 1b, Table 3). The first and more pronounced Tl peak was located in HMM biomolecule region, at elution time from the 19^{th} to 24^{th} minute. That elution time corresponded to biomolecules of molecular masses in the range from ~85 to 300 kDa and encompassed, for example, molecular masses of aldehyde dehydrogenase (187 kDa, von Tigerstrom and Razzell, 1968) and (Na^++K^+)-ATP-ase (tetramer with molecular mass in the range of 274-280 kDa; Peterson and Hokin, 1981), enzymes that are known to be activated by Tl. Comparable Tl peak in the range of molecular masses from ~50-450 kDa was reported for the liver of juvenile yellow perch (Caron et al., 2018). The second Tl peak, which was not always clearly visible, was located in the VLMM biomolecule region, at elution time from the 32^{nd} to 36^{th} minute (Fig. 1b), which corresponded to biomolecules of molecular masses in the range from ~4 to 11 kDa. Caron et al. (2018) obtained the second Tl peak in the yellow perch liver in the region of even lower molecular masses, below 1.3 kDa.

With the increasing cytosolic Tl concentrations in brown trout liver, a marked increase of Tl quantity was observed in HMM peak, with a maximum of molecular mass equal to 140 kDa, which was more observable in the fish from the Krka River source, and which, according to Caron et al. (2018), is an indication of incomplete Tl detoxification.

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3.2. Essential elements

Common feature of the majority of studied essential elements was that they were distributed within larger number of peaks, often broad and not well resolved. Contrary to nonessential elements, this was an indication of their association to numerous compounds in the hepatic cytosol, which is consistent with their important roles in the fish metabolism, and their catalytic or structural functions within the cell. This was especially obvious for Cu, Fe, Mn, and Zn (Figs. 2b, 3a, 3b and 5).

3.2.1. Cobalt

Cobalt was eluted in three peaks (Fig. 2a, Table 3). The first and most pronounced Co peak was located in HMM biomolecule region, at elution time from 18^{th} to 24^{th} minute which covered biomolecules in the range from ~85-400 kDa. The predominant Co peak in HMM region was previously also found in the liver of European and Vardar chub (Krasnići et al., 2013, 2018) and in the liver of juvenile yellow perch (Caron et al., 2018). The second Co peak was much lower and located in the MMM biomolecule region. It appeared at elution time from the 24^{th} to 29^{th} minute, and corresponded to biomolecules of molecular masses in the range from ~20 to 85 kDa. The third Co peak was not always clearly visible and it was located in the VLMM biomolecule region, at elution time from the 33^{rd} to 38^{th} minute, and corresponded to biomolecules of molecular masses in the range from 2.5 to 9 kDa, which was similar to Co peak in the range of molecular masses from 1.3-6.8 kDa reported for liver of juvenile yellow perch (Caron et al., 2018).

With the increasing cytosolic Co concentrations, a marked increase of Co quantity was observed in the HMM peak, with a maximum of molecular mass equal to 180 kDa, as best seen in the fish from the Krka Knin location in the spring sampling (Fig. 2a).

Important role of Co, as an essential element, in fish organism is associated with its contribution to cobalamine (vitamin B12) structure (Blust, 2012), which molecular mass equals to 1.3 kDa (Kirschbaum, 1981). In contrast to previous findings for European and Vardar chub liver, where Co elution was observed in the region of biomolecules of VLMM, below 2.5 kDa (Krasnići et al., 2013, 2018), in brown trout Co elution in that molecular mass region, which would confirm Co association to cobalamin in hepatic cytosol, was not seen.

3.2.2. Copper

Copper is essential metal and it has a function as cofactor in many enzymes (Mogobe et al., 2015). Copper had similar distribution profile to Cd (Fig. 1a), but it was eluted in one additional peak between two peaks observed for Cd (Fig. 2b, Table 3). The first Cu peak was located in HMM biomolecule region, at elution time from the 14^{th} to 17^{th} minute which corresponded to biomolecules of molecular masses in the range from ~500 to 1000 kDa. The second, shoulder-shaped, Cu peak was located in HMM biomolecule region, at elution time from the 18^{th} to 24^{th} minute, and covered biomolecules of molecular masses in the range from ~85 to 400 kDa. Contrary to brown trout liver, Cu elution was not observed in HMM region in the previous studies on European and Vardar chub liver (Krasnići et al., 2013, 2018). The third Cu peak was located in the LMM biomolecule region, at elution time from the 29^{th} to 34^{th} minute, which corresponded to biomolecules of molecular masses in the range from 7 to 24 kDa.

The elution of Cu was most pronounced in the LMM peak, with a maximum of molecular mass equal to 15 kDa, same as reported for the liver of European and Vardar chub (Krasnići et al., 2013, 2018), of juvenile yellow perch (Caron et al., 2018), of European eel (Van Campenhout et al., 2008), and of gibel carp (Van Campenhout et al., 2010). Elution time of Cu-LMM peak coincided with the elution time of MT standard (Table 2), so it can be presumed that Cu in the hepatic cytosol was predominantly bound to MTs. However, comparison of Cu distribution profiles at lower and higher levels of Cu bioaccumulation revealed that, although increase of eluted Cu quantity was more obvious in LMM region, as also reported for European and Vardar chub liver (Krasnići et al., 2013, 2018), it was also present in HMM region in brown trout liver with higher cytosolic Cu concentrations (Fig. 2b).

3.2.3. Iron

Iron is an essential metal and has numerous roles in physiological functions of fish and other organisms, and one of its most important functions is participation in oxygen transport (Mogobe et al., 2015). Iron was eluted in three peaks (Fig. 3a, Table 3). The first broad Fe peak was located in HMM biomolecule region, at elution time from the 14^{th} to 21^{st} minute which corresponded to biomolecules of molecular masses in the range from ~200 to 1000 kDa. It can be presumed that this first peak in HMM region, with a maximum at ~380 kDa included iron storage protein ferritin (450 kDa), which is predominantly present in hepatic tissue and which additionally serves for keeping Fe in soluble, nontoxic form within the cells (Bury et al., 2012; Martin-Antonio et al., 2009;

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Szpunar and Lobinski, 1999). The second Fe peak was located in MMM biomolecule region, at elution time from the 24th to 28th minute, and covered biomolecules of molecular masses in the range from ~30 to 85 kDa, which could reflect Fe binding to various proteins of different functions, such as blood protein haemoglobin (65 kDa) and enzyme catalase (60 kDa) (Martin-Antonio et al., 2009). The third Fe peak was located in the VLMM biomolecule region, at elution time from the 32nd to 35th minute which corresponded to biomolecules of molecular masses in the range from ~5 to 10 kDa. The first two peaks were also previously observed in the liver of European and Vardar chub (Krasnići et al., 2013, 2018). The difference between species was revealed in the fact that, unlike brown trout, in the European and Vardar chub liver Fe binding to VLMM biomolecules was not noticed (Krasnići et al., 2013, 2018).

An increase in the quantity of eluted Fe as a consequence of higher cytosolic Fe concentrations in the brown trout liver was observed in all three peaks, but was somewhat higher in HMM region, with a maximum of molecular mass equal to ~380 kDa, as best seen in the fish from the Krka Knin location in the autumn sampling (Fig. 3a). The same Fe increase in HMM peak was also reported for European and Vardar chub liver (Krasnići et al., 2013, 2018), which is consistent with the function of ferritin in Fe storage in hepatic tissue.

3.2.4. Manganese

Manganese is a metal that has essential function in activities of various enzymes (Mogobe et al., 2015). Manganese was seemingly eluted in only one rather broad peak (Fig. 3b, Table 3), covering biomolecules from high to low molecular masses. However, by more careful insight it can be seen that this peak could be divided into

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four narrower peaks. The first two Mn peaks were located in HMM biomolecule region, the first one at elution time from the 14^{th} to 17^{th} minute, and the second one at elution time from the 18^{th} to 23^{rd} minute. These two peaks corresponded to biomolecules of molecular masses in the range from ~500 to 1000 kDa and from ~100-400 kDa, respectively. The third Mn peak was located in MMM biomolecule region, at elution time from the 23^{rd} to 26^{th} minute, and covered biomolecules of molecular masses in the range from ~50 to 110 kDa. This range included molecular masses of well known transport proteins: albumin (66 kDa, Table 2), which participates in Mn transport from intestine to liver (Schäfer, 2004), and transferrin (80 kDa, Martin-Antonio et al., 2009), which binds Mn in liver and transfers it to the other organs (Schäfer, 2004). The fourth Mn peak was located in the LMM biomolecule region, at elution time from the 26^{th} to 31^{st} minute which corresponded to biomolecules of molecular masses in the range from ~15 to 50 kDa. The distribution profile of Mn previously reported for the liver of European and Vardar chub was very similar to that of brown trout presented here, with the exception that it did not contain the first HMM peak, covering biomolecules of 500-1000 kDa (Krasnići et al., 2013, 2018).

The presence of Mn was mostly comparable in all four peaks, same as the increase of eluted Mn quantity due to higher Mn bioaccumulation in brown trout liver (Fig. 3b).

3.2.5. Molybdenum

Molybdenum is an important micronutrient because it acts as a catalytic centre for more than 50 enzymes (Ricketts et al., 2015). Molybdenum was eluted in two clear peaks (Fig. 4a, Table 3). The first, much higher Mo peak was located in HMM biomolecule region, at elution time from the 17^{th} to 23^{rd} minute, which corresponded to biomolecules of molecular masses in the range from ~100 to 500 kDa, with a maximum at ~230 kDa. It was consistent with previous reports for European and Vardar chub liver (Krasnići et al., 2013, 2018), which suggested that this Mo peak included molecular masses of enzymes that contain Mo as cofactor, for example aldehyde oxidase (130 kDa, Uchida et al., 2003), sulphite oxidase (120 kDa, Johnson and Rajagopalan, 1976) and Fe-Mo flavoprotein xanthine oxidase (275 kDa, Truglio et al., 2002). The second and much lower Mo peak was located in the VLMM biomolecule region, at elution time from the 34th to 37th minute which corresponded to biomolecules of molecular masses in the range from ~3 to 7 kDa with maximum at 5 kDa, which was also previously reported for European and Vardar chub liver (Krasnići et al., 2013, 2018).

The increase of eluted Mo quantity due to higher Mo bioaccumulation in brown trout liver was observed in both peaks, but was somewhat more pronounced in HMM peak (Fig. 4a).

3.2.6. Selenium

Selenium is an essential nonmetal and a constitutive part of glutathione peroxidase, whereas in a conjunction with vitamin E it contributes to avoidance of nutritional muscular dystrophy (Watanabe et al., 1997). Selenium was eluted in four peaks (Fig. 4b, Table 3). The first two poorly resolved Se peaks were located in HMM biomolecule region, at elution times from the 14th to 17th minute and from the 20th to 24th minute, respectively, and were barely visible. The elution times of these two peaks covered biomolecules in the range from ~500 to 1000 kDa and from ~85-230 kDa, respectively. The third and fourth Se peaks were much better resolved and higher, and located in the

VLMM biomolecule region at elution times from the 35th to 38th minute and from the 40th to 44th minute, respectively. These peaks covered biomolecules of molecular masses in the range from 2.5 to 5 kDa and from 0.5 to 1.5 kDa, respectively.

The most evident feature of hepatic Se in the cytosolic fraction was association with biomolecules of molecular masses lower than 5 kDa, and with increasing cytosolic Se concentrations, a marked increase of Se quantity was observed precisely in these two VLMM peaks, with maxima of molecular masses equal to ~1 kDa and ~3 kDa. It can be presumed that these peaks contained seleno-compounds of rather low molecular masses, such as those efficient in a defence against oxidative stress, like selenomethionine (~0.2 kDa; Klotz et al., 2003) or recently identified organic Se compound in tuna (*Thunnus orientalis*), selenoneine (~0.5 kDa; Yamashita and Yamashita, 2010; Yamashita et al., 2012). Such Se association was much more noticeable in the previous studies on gills than on the liver of European and Vardar chub (Krasnići et al., 2013, 2014, and 2018). In European and Vardar chub liver, the large portions of Se were eluted in the region of molecular masses from 10 to 60 kDa, possibly associated to some Se-containing enzymes (Krasnići et al., 2013, 2018), whereas in the brown trout liver there was no clear indication of such Se association.

3.2.7. Zinc

Zinc is an essential metal which has structural and catalytic roles in many proteins and enzymes. It is very important for gene expression and cell growth (Mogobe et al., 2015). Zinc was eluted in three peaks (Fig. 5, Table 3). The first two Zn peaks were located mainly within HMM biomolecule region, at elution times from the 14th to 17th minute and from the 18th to 29th minute, respectively. The elution times of these two

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peaks covered biomolecules in the wide ranges of molecular masses, from ~500 to 1000 kDa and from ~20-400 kDa, respectively. The third Zn peak was located in the LMM biomolecule region at elution times from the 30th to 33rd minute and covered biomolecules of molecular masses in the range from 9 to 19 kDa. Comparable to Cd and Cu, the elution time of LMM peak maximum coincided with the elution time of MT standard (Table 2). Similar peak was also observed in the liver of European and Vardar chub (Krasnići et al., 2013, 2018), of European eel (Van Campenhout et al., 2008), and of gibel carp (Van Campenhout et al., 2010), which is consistent with important role that MTs have in homeostasis of essential metals, such as Zn and Cu, and in detoxification of toxic metals, such as Cd (Huang et al., 2004).

Among three peaks, Zn presence was most pronounced in the middle peak in the HMM region (~20-400 kDa), with the maximum of molecular mass around ~100 kDa, similar as reported for Zn in European chub liver (Krasnići et al., 2013). Some well known Zn-containing proteins are encompassed by that molecular mass region, such as alcohol dehydrogenase (150 kDa, Table 2, Szpunar and Lobinski, 1999). Furthermore, increased Zn bioaccumulation in the liver of brown trout resulted with increased quantity of Zn precisely in that molecular mass region. It was best seen in the fish from the Krka River source in the autumn sampling.

4. Conclusions

Based on HR ICP-MS analyses of trace elements in the fractions of hepatic cytosol of brown trout, which were obtained by separation using SEC-HPLC, we were able to define the molecular masses of cytosolic compounds that bind highly toxic metals Cd and Tl, as well as seven essential elements (Co, Cu, Fe, Mn, Mo, Se and Zn). Association of Cd, Cu and Zn to MTs, which was previously well described for some other fish species, was now also confirmed for brown trout liver. Comparison of the results obtained at different sites and in different seasons, as well as considerations of some physiological factors, such as sex, indicated that cytosolic distributions of analyzed trace elements among biomolecules of different molecular masses probably depended mainly on the level of trace element bioaccumulation in the liver. Variations that could be associated to the other factors were not observed. Additionally, several differences were observed in trace element distributions within hepatic cytosol of brown trout in comparison to previously published information for European and Vardar chub liver. Features that were observed only in brown trout liver included Cd and Cu elution in the region of high molecular mass biomolecules (above 500 and 100 kDa, respectively), absence of Co association with biomolecules of molecular masses below 2.5 kDa, Fe elution in the region of compounds of molecular masses below 10 kDa, and almost exclusive Se association with biomolecules of molecular masses below 5 kDa. Such differences indicated possible existence of different detoxification strategies and consequently different susceptibility to metal exposure in various fish species.

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Table 1. Biometric characteristics of twelve specimens of brown trout (*Salmo trutta* Linnaeus, 1758) used in this study for analyses of distributions of trace elements in hepatic cytosol.

		Sample No.	Total length / cm	Total mass / g	GSI / %	FCI / %	Sex*
Autumn 2015	**Krka Source**	**1**	27.0	201.7	5.09	1.02	M
		2	20.0	87.0	5.82	1.09	M
		3	26.1	182.1	4.46	1.02	M
	Krka Knin	**4**	22.5	114.6	5.25	1.01	M
		5	26.4	203.6	0.02	1.11	M
		6	27.5	225.0	4.30	1.08	M
Spring 2016	**Krka Source**	**7**	20.5	89.7	0.16	1.04	M
		8	19.2	70.3	0.83	0.99	F
		9	18.3	66.8	0.55	1.09	F
	Krka Knin	**10**	23.3	159.8	0.08	1.26	M
		11	18.5	86.7	0.18	1.37	F
		12	18.4	78.7	0.08	1.26	M

*M – male; F – female

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Table 2. Molecular masses (MM), applied concentrations and elution times (t_e) of blue dextran, metallothionein standards and six proteins used as calibration standards for Superdex™ 200 10/300 GL size exclusion column. Equation of calibration straight line was: Kav=-0.281×log MM+1.647.

	MM / kDa	Concentration / mg mL^{-1}	t_e / min
Blue dextran	2000	2	15.47
Metallothionein standards			
Metallothionein 1	6.15	1	32.32
Metallothionein 2	6.15	1	31.22
Protein standards for column calibration			
Carbonic anhydrase	29	3	29.72
Bovine albumin	66	10	23.04
Alcohol dehydrogenase	150	5	21.78
β-amilase	200	4	20.38
Apoferritin	443	10	17.84
Thyroglobulin	669	8	16.08

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Table 3. Elution times (t_e) and molecular masses (MM) of biomolecules contained in cytosolic fractions of liver of brown trout (*Salmo trutta* Linnaeus, 1758) in which respective elements were eluted after separation by SEC-HPLC (Superdex 200 10/300 GL column). Table contains peak maxima of each analyzed element (i.e., the information on fractions with the highest trace element concentrations), as well as peak widths presented within the brackets.

Element		[a]HMM 1		[a]HMM 2		[b]MMM		[c]LMM		[d]VLMM 1		[d]VLMM 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
Highly toxic elements	Cd	15 (14-17)	818 (1052-494)					31 (29-34)	15 (24-7)				
	Tl			22 (19-24)	140 (299-85)					34 (32-36)	7 (11-4.1)		
Essential elements	Co			21 (18-24)	180 (384-85)	26 (24-29)	51 (85-24)			35 (33-38)	5 (9-2.5)		
	Cu	15 (14-17)	818 (1052-494)	19 (18-24)	299 (384-85)			31 (29-34)	15 (24-7)				
	Fe			18 (14-21)	384 (1052-180)	26 (24-28)	51 (85-31)			34 (32-35)	7 (11-5)		
	Mn	16 (14-17)	636 (1052-494)	21 (18-23)	180 (384-109)	24 (23-26)	85 (109-51)	29 (26-31)	24 (51-15)				
	Mo			20 (17-23)	232 (494-109)					35 (34-37)	5 (7-3.2)		
	Se	15 (14-17)	818 (1052-494)	22 (20-24)	140 (232-85)					37 (35-38)	3.2 (5-2.5)	42 (40-44)	0.9 (1.5-0.5)
	Zn	15 (14-17)	818 (1052-494)	23 (18-29)	109 (384-24)			31 (30-33)	15 (19-9)				

[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)

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Figure captions

Figure 1. Distribution profiles of two highly toxic metals (a - Cd, b - Tl) among cytosolic biomolecules of different molecular masses in the liver of brown trout (*Salmo trutta*) from two sites at the Krka River (Krka River source and Krka River downstream of Knin town) in two sampling campaigns (autumn: October 2015; spring: May 2016). The results are presented as nanograms of metals eluted at the specific elution times, which can be associated to corresponding molecular masses based on the column calibration (Table 2, Table 3). Twelve fish in total were used for these analyses, three per each site in each season. Total cytosolic concentrations of analyzed elements in the liver of brown trout are presented within each figure (taken from Dragun et al., 2018).

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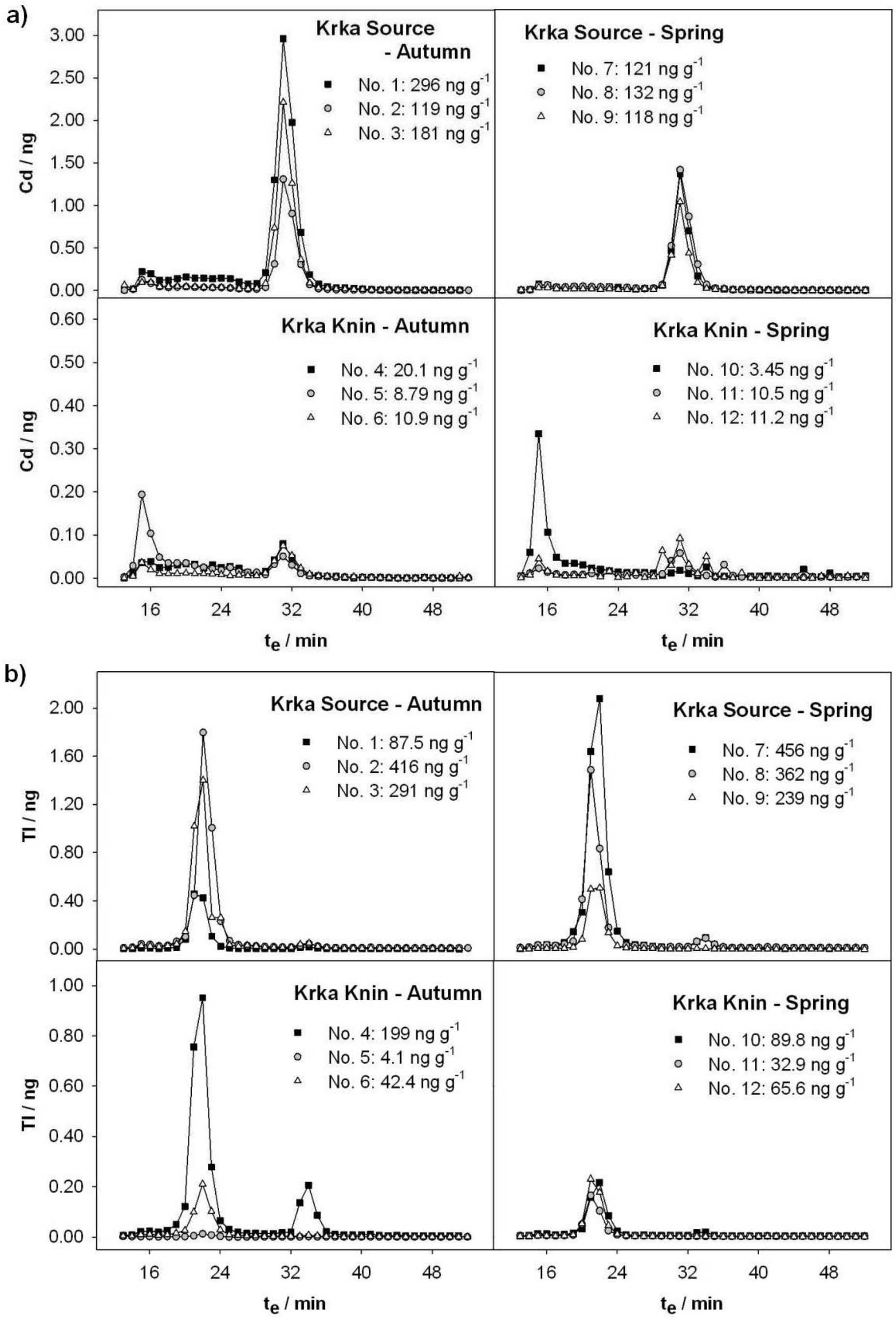

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Figure 2. Distribution profiles of two essential metals (a - Co, b - Cu) among cytosolic biomolecules of different molecular masses in the liver of brown trout (*Salmo trutta*) from two sites at the Krka River (Krka River source and Krka River downstream of Knin town) in two sampling campaigns (autumn: October 2015; spring: May 2016). The results are presented in the same way as in Figure 1.

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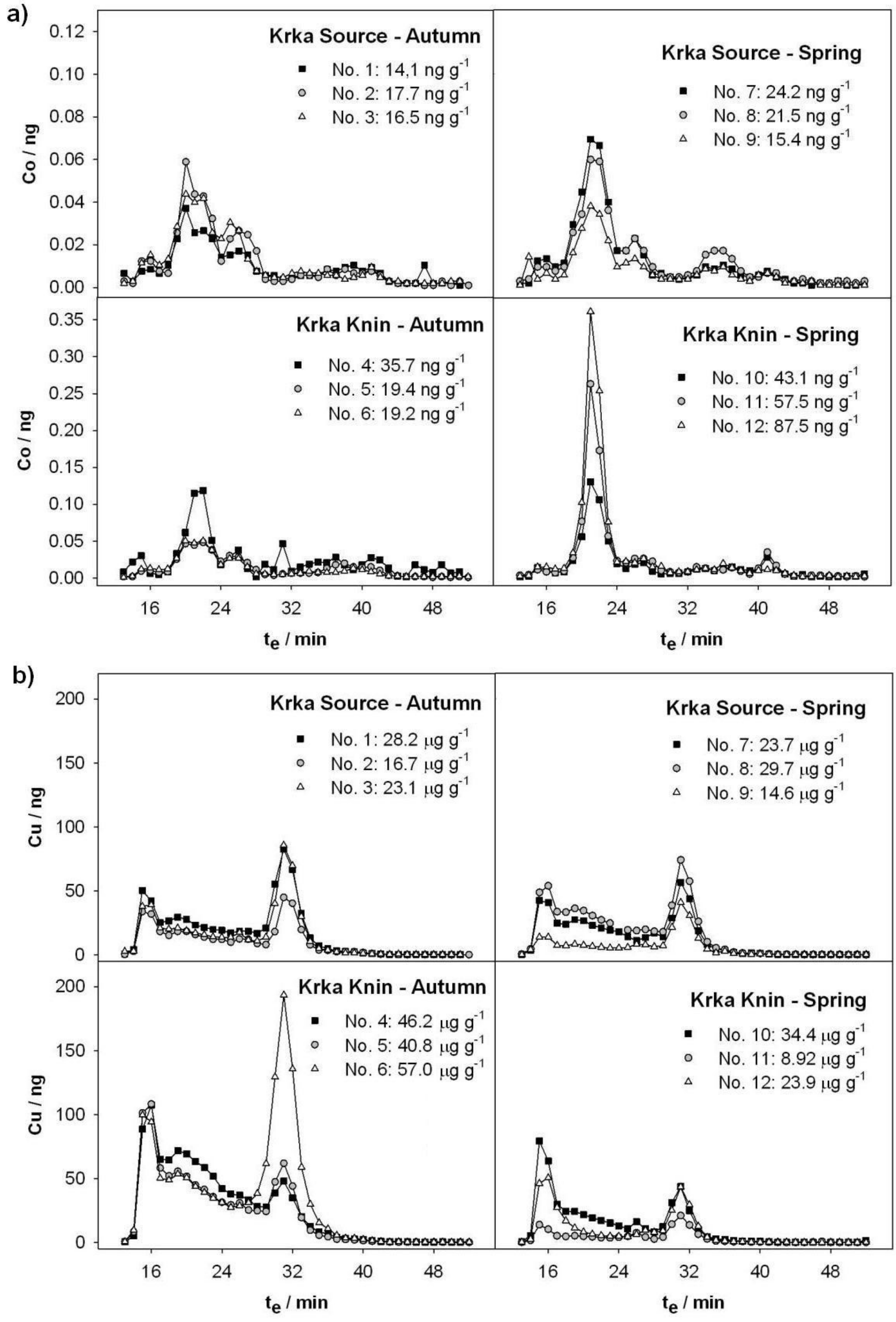

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Figure 3. Distribution profiles of two essential metals (a - Fe, b - Mn) among cytosolic biomolecules of different molecular masses in the liver of brown trout (*Salmo trutta*) from two sites at the Krka River (Krka River source and Krka River downstream of Knin town) in two sampling campaigns (autumn: October 2015; spring: May 2016). The results are presented in the same way as in Figure 1.

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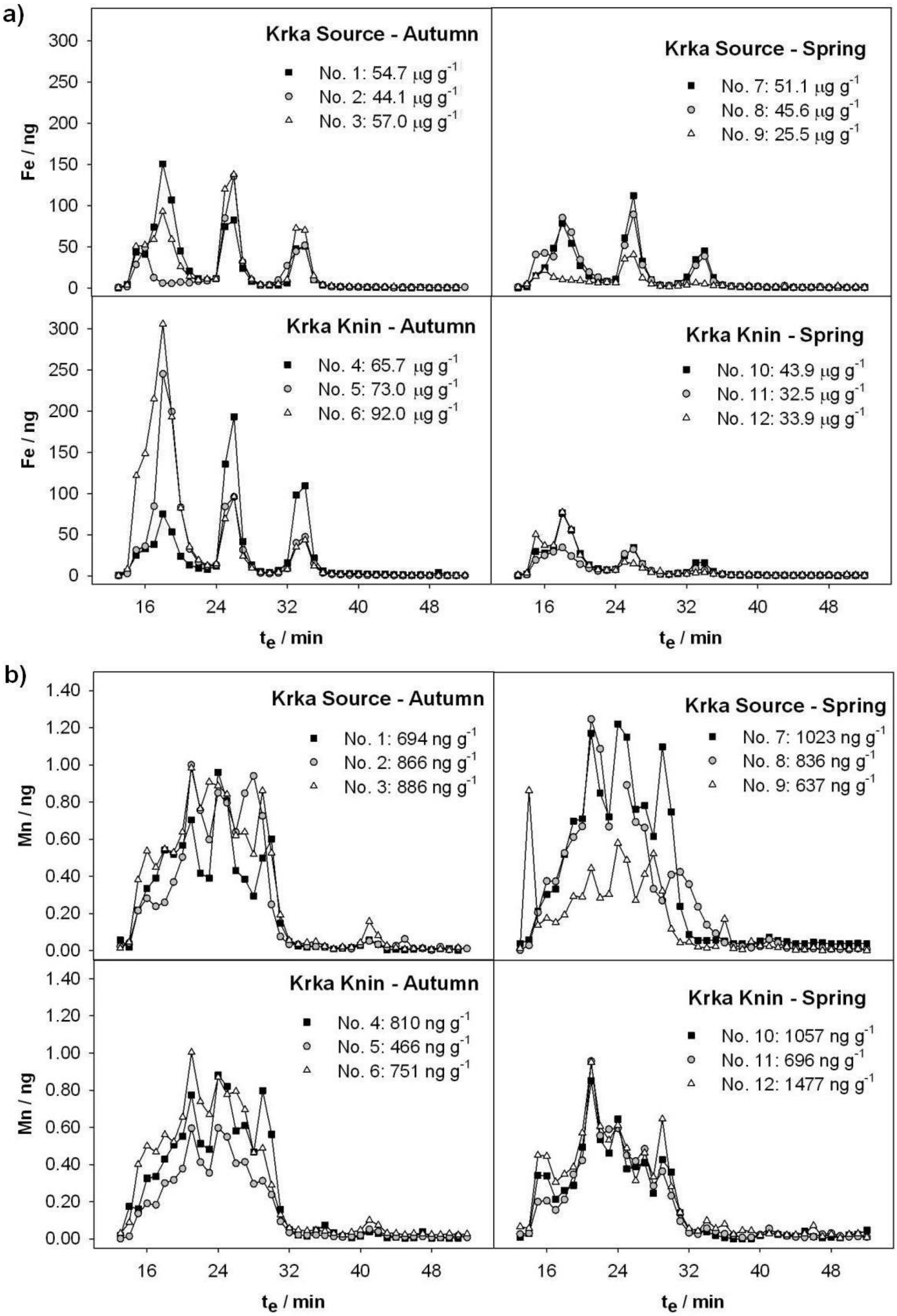

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Figure 4. Distribution profiles of two essential elements (a - Mo, b - Se) among cytosolic biomolecules of different molecular masses in the liver of brown trout (*Salmo trutta*) from two sites at the Krka River (Krka River source and Krka River downstream of Knin town) in two sampling campaigns (autumn: October 2015; spring: May 2016). The results are presented in the same way as in Figure 1.

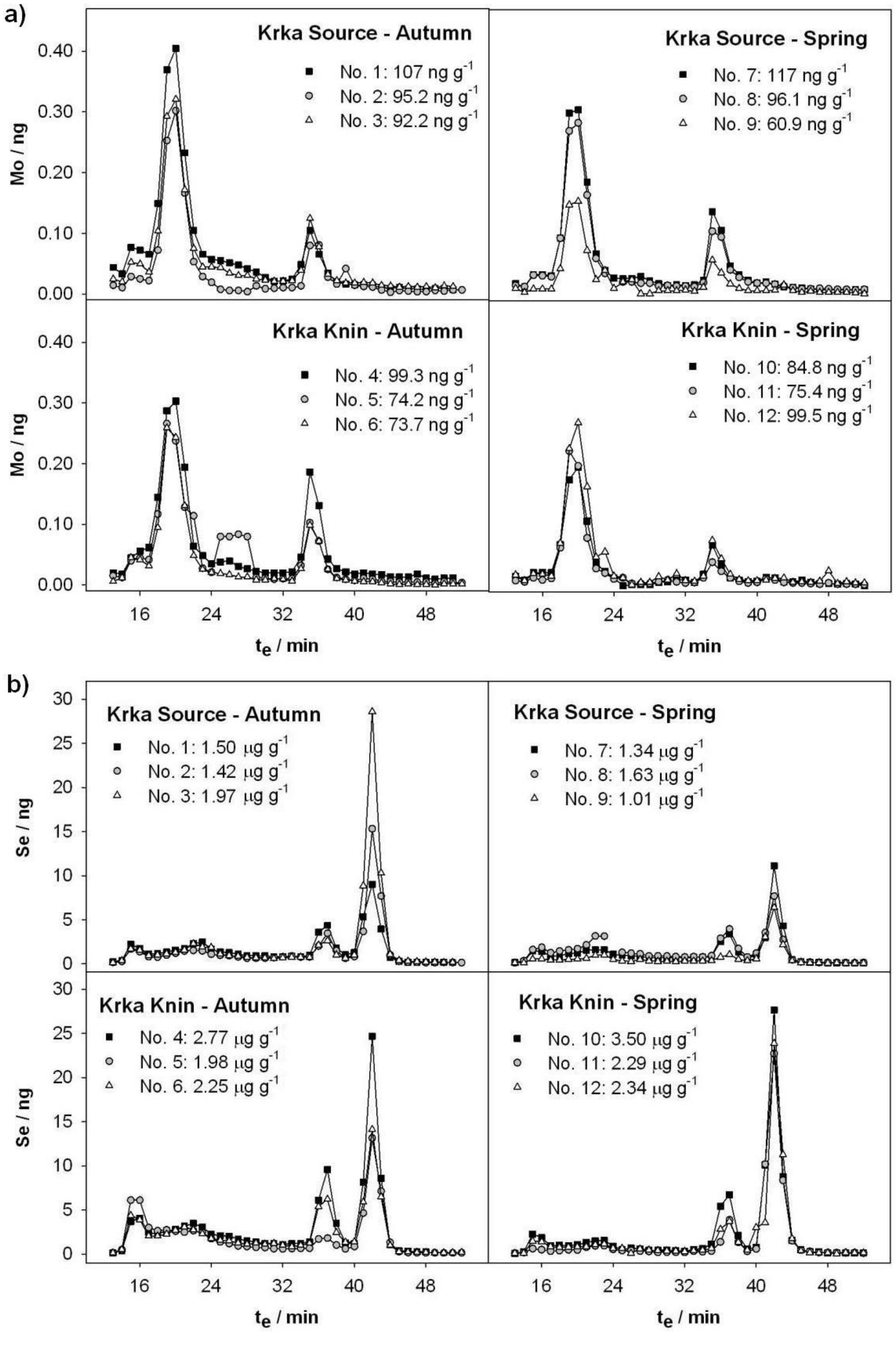
a)
Krka Source - Autumn
No. 1: 107 ng g-1
No. 2: 95.2 ng g-1
No. 3: 92.2 ng g-1
Krka Source - Spring
No. 7: 117 ng g-1
No. 8: 96.1 ng g-1
No. 9: 60.9 ng g-1
Krka Knin - Autumn
No. 4: 99.3 ng g-1
No. 5: 74.2 ng g-1
No. 6: 73.7 ng g-1
Krka Knin - Spring
No. 10: 84.8 ng g-1
No. 11: 75.4 ng g-1
No. 12: 99.5 ng g-1
Mo / ng
te / min
b)
Krka Source - Autumn
No. 1: 1.50 µg g-1
No. 2: 1.42 µg g-1
No. 3: 1.97 µg g-1
Krka Source - Spring
No. 7: 1.34 µg g-1
No. 8: 1.63 µg g-1
No. 9: 1.01 µg g-1
Krka Knin - Autumn
No. 4: 2.77 µg g-1
No. 5: 1.98 µg g-1
No. 6. 2.25 µg g-1
Krka Knin - Spring
No. 10: 3.50 µg g-1
No. 11: 2.29 µg g-1
No. 12: 2.34 µg g-1
Se / ng
te / min

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Figure 5. Distribution profiles of essential metal Zn among cytosolic biomolecules of different molecular masses in the liver of brown trout (*Salmo trutta*) from two sites at the Krka River (Krka River source and Krka River downstream of Knin town) in two sampling campaigns (autumn: October 2015; spring: May 2016). The results are presented in the same way as in Figure 1. Unlike the other analyzed elements (Fig. 1-4), the results for only 10 fish are presented for Zn. Two samples from spring season were excluded from interpretation due to evident Zn contamination during the sample processing.