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ORIGINAL PAPER

LC-MS analyses of microcystins in fish tissues overestimate toxin levels—critical comparison with LC-MS/MS

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Abstract Microcystins are cyclic peptide toxins with hepatotoxic and tumour-promoting properties which are produced in high quantities in freshwater cyanobacterial water blooms, and several studies have reported microcystin accumulation in fish with possible food transfer to humans. In this study, we provide the first comparison of liquid chromatography with single mass-spectrometric and with tandem mass-spectrometric detection for analyses of microcystins in complex fish tissue samples. Use of traditional single mass spectrometry (i.e. monitoring of ions with *m*/*z* 519.5 for microcystin-RR and *m*/*z* 995.5 for microcystin-LR) was found to provide false-positive responses, thus overestimating the concentrations of microcystins in the tissue samples. More selective tandem mass spectrometry seems to provide more reliable results. The concentrations of microcystins detected by tandem mass spectrometry in fish from controlled-exposure experiments were more than 50% lower in comparison with concentrations obtained by single mass spectrometry. Extensive analyses of edible fish parts—muscles (148 fish specimens from eight different species from five natural reservoirs with dense cyanobacterial water blooms)—showed negligible microcystin concentrations (all analyses below the limit of detection; limit of detection of 1.2–5.4 ng/g fresh weight for microcystin-RR, microcystin-YR and microcystin-LR in multiple reaction monitoring mode). Our findings have practical consequences for critical re-evaluation of the health risks of microcystins accumulated in fish.

Keywords Microcystin · Fish tissue · Liquid chromatography–tandem mass spectrometry

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Introduction

Microcystins are a group of peptide toxins produced by various genera of freshwater cyanobacteria, such as *Anabaena*, *Microcystis* and *Oscillatoria* (*Planktothrix*) [1]. These potent oligopeptides act mainly as inhibitors of serine/threonine protein phosphatases PP1 and 2A, leading to hepatotoxicity and liver tumour promotion. In organisms, microcystins are detoxified mainly by conjugation with cysteine (Cys) and glutathione (GSH) [2]. Microcystins may cause serious health problems, as documented by cases of human and animal intoxications as well as by the results of laboratory studies [3] based on toxicity data. The World Health Organization suggested a tolerable daily intake value (0.04 μg/kg body weight) for one of the microcystin variants microcystin-LR (MC-LR), and the corresponding guideline value of 1.0 μg/L is recommended for drinking waters [4].

Microcystins are produced during massive water blooms of cyanobacteria, which result from worldwide anthropogenic eutrophication of waters [5]. For example, more than

80% of major reservoirs in the Czech Republic (including drinking water supplies and aquaculture ponds) contained microcystins in considerable concentrations [6, 7].

More structural variants of microcystins can be present in one sample and they may represent as much as 1% of the dry cyanobacterial biomass. During massive water blooms, aquatic organisms can be affected by toxins present in water, and microcystin accumulation in biota and its transfer to the food webs—including biomagnification—were previously discussed [8–10]. Some authors reported very high concentrations of microcystins accumulated in fish tissues [10, 11], which might be of toxicological concern for humans. Analyses of microcystins in food and food supplements are thus necessary for critical health risk assessment.

A number of analytical approaches for cyanobacterial toxins (cyanotoxins) in biotic matrices have been described in the literature, including protein phosphatase inhibition assay and immunochemical enzyme-linked immunosorbent assay (ELISA) methods [12, 13]. Further, instrumental analytical methods, namely liquid chromatography (LC), are the most widely used. Microcystins have a characteristic absorption spectrum with a peak at 238 nm, and a number of methods for optical detection in the UV range have been developed. However, biological and traditional instrumental methods may suffer from interferences in the complex matrices, and their application for microcystin analyses in biota is limited. More recent approaches based on LC with mass-spectrometric detection have a number of advantages, including high sensitivity and robustness, which minimizes the above-mentioned complications [14–16].

Although a number of studies used mass-spectrometric detection for analyses of cyanotoxins, no critical comparison of single mass spectrometry (MS) and tandem MS (MS/MS) methods for microcystin analyses in the tissues of biota is available. A number of publications, which relied on a single mass spectrometer for detection and quantification of microcystins, did not consider several drawbacks, such as insufficient selectivity, which might result in overestimated or false-positive results. In this paper, we compare the performance of LC-MS and LC-MS/MS techniques for a series of tissue samples from fish exposed to cyanobacterial microcystins both experimentally (in controlled laboratory conditions) and collected in the field from fish ponds with natural cyanobacterial blooms.

Material and methods

Chemicals and reagents

Standards of microcystins (MC-LR, MC-RR, MC-YR) were obtained from Alexis Biochemicals (Läufelfingen, Switzerland). The solvents (LC-MS grade) were purchased from Sigma-Aldrich (Prague, Czech Republic). Deionized water was prepared with a Simplicity 185 system (Millipore, Bedford, MA, USA).

Experimental design

Fish tissues used for the comparative studies of MS and MS/MS method performance were collected from both laboratory experiments and field samplings.

During the short-term laboratory experiments, fish were injected with high concentrations of microcystins, and the presence of toxin in hepatopancreas and its metabolization were evaluated. For these experiments, common carp (*Cyprinus carpio*; 500±15 g males) were obtained from commercial aquaculture (Pohorelice fishery, Czech Republic). Experimental fish (N=3) were intraperitoneally injected with the mixture of major microcystin variants at a total microcystin dose of 248 μg/kg body weight (injection volume 500 μL). The mixture of microcystins was prepared in phosphate-buffered saline, pH 7.3, and contained 56% MC-RR, 38% MC-LR and 6% MC-YR. Control fish (N=3) received 500 μL of phosphate-buffered saline. After 3 h, the fish were dissected and samples of hepatopancreas and muscle tissues were collected and stored at −80°C until they were analysed.

The second series of experiments studied accumulation of microcystins in fish under natural conditions. Fish specimens of common carp (body weight 32±7 g, n=10) were kept in two experimental ponds for 9 weeks. Pond A contained a massive and dense water bloom dominated by *Microcystis aeruginosa* with microcystin concentrations ranging from 10.1 to 15.4 μg/L during the whole exposure period. Pond B also contained some cyanobacteria but they were present in negligible density and the maximum concentration of microcystins in water reached 2 μg/L. During the experiment, the fish were not externally fed, and no deaths were observed. At the end of the experiment, the fish were dissected and tissue samples were collected for analysis as described above. The concentrations of dissolved microcystins in the ponds were determined by the high performance liquid chromatography (HPLC)–diode-array detector method according to ISO 20179 [17]; the volume of the grab samples was 1 L. Besides this controlled study, fish of different species randomly caught in various aquaculture ponds in the Czech Republic were also analysed for the content of microcystins; details on the environmental conditions and microcystin concentrations are provided in "Results and discussion."

Tissue extractions

Tissue extractions were performed by methods described previously [18]. Briefly, a frozen sample (0.5 g fresh

weight) was homogenized four times with methanol (3 mL), sonicated in an ultrasonic bath for 30 min and centrifuged at 4,000*g* for 10 min. Supernatants were pooled and repeatedly extracted (three times) with 1 mL of hexane to remove lipids (hexane layers were discarded). The methanol extract was evaporated at 50°C, and the residue was dissolved in 300 μL of 50% aqueous methanol (v/v) and analysed. The recovery of the extraction method (approximately 25%; data not shown) was not considered during the calculations to remain consistent with the methods and values previously reported in the literature [8, 10, 11, 18, 19].

Liquid chromatography electrospray ionization mass spectrometry analyses

The present study compared the performance of single MS microcystin analyses, which used the selected ion monitoring (SIM) mode, with the MS/MS approach using the multiple reaction monitoring (MRM) mode.

Analyses were performed with an Agilent Technologies (Waldbronn, Germany) 1200 series HPLC apparatus consisting of a vacuum degasser, a binary pump, an autosampler and a thermostatted column compartment kept at 30°C. The column was a Supelcosil ABZ + Plus RP-18 end-capped (5 μm), 150 mm×4.6-mm inner diameter (Supelco). A SecureGuard C_{18} (Phenomenex, Torrance, CA, USA) guard column was used. The mobile phase consisted of 5 mM ammonium acetate in water, pH4 (solvent A), and acetonitrile (solvent B). The binary pump gradient was as follows: 0–12.00 min, 32–40% solvent B, linear increase; 12.01–20.00 min, 40–42% solvent B, linear increase; 20.01–30.00 min, 90% solvent B. The flow rate was 0.4 mL/min. Twenty microlitres of the individual sample was injected for the analysis.

The mass spectrometer was an Agilent Technologies (Waldbronn, Germany) 6410 triple-quadrupole mass spectrometer with electrospray ionization. Ions were detected in the positive mode. The ionization parameters were as follows: capillary voltage, 5.5 kV; desolvation temperature, 350°C; desolvation gas flow, 11 L/min. In SIM mode the following *m*/*z* were monitored: MC-RR $[M+2H]^{2+}$ 519.8, MC-YR $[M + H]^{+}$ 1,045.5, MC-LR $[M + H]^{+}$ 995.5, MC-RR–GSH $[M+2H]^{2+}$ 673.8, MC-LR–GSH $[M + H]^{+}$ 1,302.5, MC-RR–Cys $[M+2H]^{2+}$ 580.8, MC-LR–Cys $[M + H]^{+}$ 1,116.5. The transitions from the protonated molecular ion to a fragment of the amino acid (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (an unusual amino acid present only in microcystins and related nodularins) at *m*/*z* 135.2 and a fragment at *m*/*z* 127.1 were monitored in MRM mode. The collision energies used for fragmentation were as follows: 50 V for MC-RR and respective conjugates; 40 V for MC-YR and MC-LR and respective conjugates). Quantification of analytes was based on external standards of MC-RR, MC-YR and MC-LR in a matrix (final extract of microcystin-free fish tissue). The mixture of microcystins was added to the extract prior to analysis. The method detection limit (MDL; per gram of tissue, fresh weight) was 1.2 ng/g [standard deviation (SD) of 5%] for MC-RR and 5.4 ng/g (SD=10%) for MC-YR and MC-LR in MRM mode. In SIM mode, the MDL was 3.0 ng/g (SD=5%) for MC-RR and 27.0 ng/g (SD=7%) for MC-YR and MC-LR, respectively. Although other approaches such as the standard addition method could be used, we relied on external calibration as it is considered to have higher precision [20–22].

Results and discussion

A number of field studies which used ELISA and LC-MS for analyses of microcystins in fish tissues reported high

Table 1 Concentrations of microcystins in biological samples reported in the literature with indication of the respective analytical technique

Fish species	Tissue sample	Concentration (MC-LR/g tissue)	Analytical technique	Source
Tilapia sp.	Muscle	3–337 ng/g FW	ELISA	Magalhaes et al. [11]
Tilapia sp.	Muscle	100 ng/g FW	ELISA	Mohamed et al. [9]
Tilapia sp.	Muscle	100 ng/g DW	ELISA	Soares et al. [25]
Hypophtalmichtys sp.	Hepatopancreas	7,000–17,800 ng/g DW	LC/MS	Xie et al. [19]
Hypophtalmichtys sp.	Muscle	500–1,700 ng/g DW	LC/MS	
Hypophtalmichtys sp., *Cyprinus* sp. and others	Muscle, hepatopancreas	1,800–7,700 ng/g DW	LC/MS	Xie et al. [10]
Hypophtalmichtys sp.	Muscle	4.4–29 ng/g FW	ELISA	Adamovsky et al. [18]
Cyprinus sp.	Muscle	5.8–19 ng/g FW	ELISA	
Water chestnut (*Trapa natans*)	–	2–5 ng/g FW	LC/MS	Xiao et al. [26]

MC-LR microcystin-LR, *FW* fresh weight, *DW* dry weight, *ELISA* enzyme-linked immunosorbent assay, *LC* liquid chromatography, *MS* mass spectrometry

concentrations ranging from 5 to 18,000 ng/g (Table 1). However, our previous investigations based on controlled exposures [18] as well as field studies [23, 24] resulted in systematically lower microcystin concentrations in fish tissue. These findings were surprising because the environmental situations and the concentrations of microcystins in the water were similar to those reported in the literature. These differences motivated the present study, which aimed to compare the LC-MS method (commonly reported in the literature) with the more robust and selective LC-MS/MS method.

In the first experiment, we investigated the general ability of the MS and MS/MS approaches to analyse microcystins and their metabolites accumulated in fish after intraperitoneal injection. In Fig. 1, the chromatograms of a tissue extract containing MC-RR obtained in SIM and MRM modes are presented (for MRM with two characteristic transitions; 519.5→135.2, 519.5→127.1). The SIM chromatogram has very high background noise,thus limiting the limit of detection (LOD). On the other hand, the MRM chromatograms seem to be more usable, especially the transition 519.5→135.2.

For correct analysis, also the qualifier ion (transition 519.5→127.1) should be monitored. However, it is present in very low concentrations when working with natural extracts (i.e. at low environmentally relevant levels). Consequently, this fragment may be of limited use because of the higher background noise. MC-YR and MC-LR show very similar behaviour. Table 2 shows the results obtained (MRM mode) for all experimental variants (with/without external standards added before the tissue extraction). The results show the good performance of the MS/MS method to detect microcystins in complex samples with sufficient selectivity and sensitivity. Although no clean-up procedure was applied on the raw extracts, the chromatograms had relatively low background and allowed the detection and quantitation of microcystins in sufficiently low concentrations (the MDL was 1.2 ng/g for MC-RR and 5.4 ng/g for MC-YR and MC-LR). Figure 2 shows the MS/MS chromatogram of a hepatopancreas extract with two peaks of MC-RR and MC-LR, and respective conjugates with GSH. In this experiment, no microcystin–Cys conjugates were detected. As is apparent, the extraction efficiency (variants with external additions of microcystins prior to extraction) was around 25% for total microcystin, which corresponds the finding of to our previous detailed study [18]. The ratio of microcystin variants detected in the hepatopancreas differed from that detected in the original exposure mixtures; no MC-YR was found. This might be affected by a number of factors, including variable uptake/metabolism of microcystin variants as well as differences in MS sensitivity, but detailed investigation is beyond the scope of the present work.

In the follow-up studies, we compared single MS and MS/MS methods by analysing fish caught in natural reservoirs with cyanobacterial blooms. Figure 3 shows the concentrations of microcystins in common carp exposed under controlled conditions for 9 weeks in two ponds with different cyanobacterial water blooms and different microcystin content. In single MS (SIM mode), peaks with retention times of microcystin standards were found in

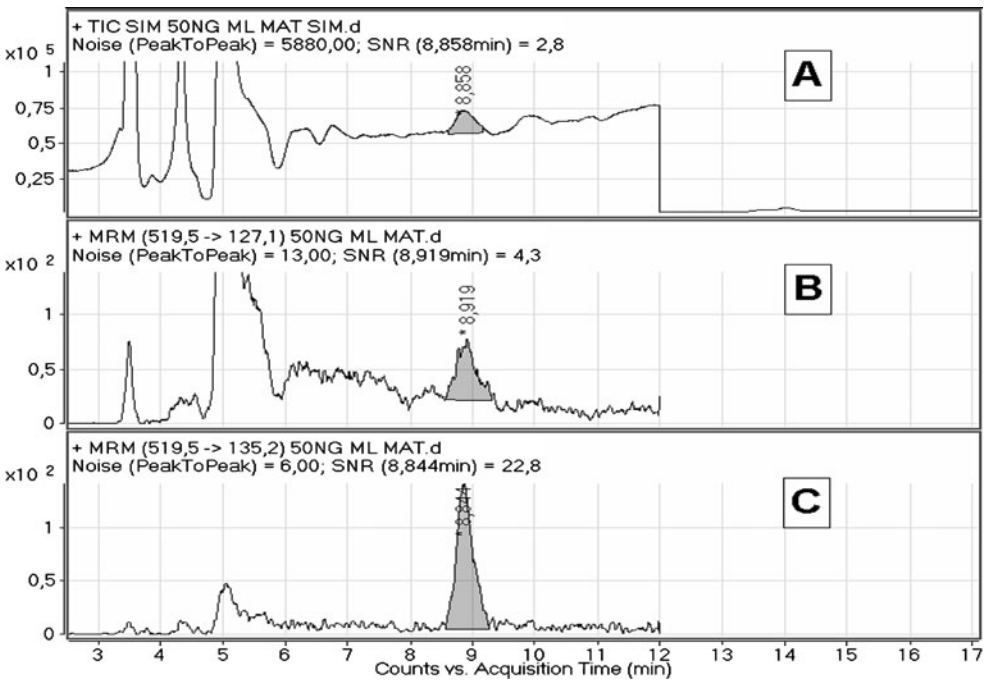

Fig. 1 Chromatograms of microcystin-RR (MC-RR) obtained in selected ion monitoring (SIM; *A*) and multiple reaction monitoring (MRM) (two characteristic transitions; *B* 519.5→127.1, *C* 519.5→135.2) modes. Note the high noise in the SIM chromatogram indicated by the low signal-to-noise ratio (*SNR*)

Table 2 Microcystins determined by tandem MS (MS/MS) in common carp (*Cyprinus carpio*; 500±15 g) hepatopancreas 3 h after intraperitoneal injection of a microcystin mixture into fish (exposed vs. non-exposed). Standards of microcystins were further added to the samples prior to extraction at the doses indicated ("spike" variants)

Experimental variant	Intraperitoneal injection[a] Microcystin (μg/fish)	Pre-extraction addition[b] Microcystin (μg/g FW)	Microcystins detected in hepatopancreas		
			Concentration (total microcystins; μg/g FW) (±SD)	Microcystin congener ratio (MC-RR, MC-YR, MC-LR; %)	Microcystin–GSH conjugates (μg/g FW)
Exposed	124	–	0.909 (±0.375)	5,–, 95	~0.1[c]
Exposed + spike	124	0.424	1.062 (±0.341)	8,–, 92	~0.1[c]
Non-exposed	–	–	0.000 (±0.000)	–	–
Non-exposed + spike	–	0.424	0.124 (±0.019)	32,–, 68	–

SD standard deviation, *GSH* glutathione, *MC-RR* microcystin-RR, *MC-YR* microcystin-YR

[a] Intraperitoneal injection mixture contained 56% MC-RR, 6% MC-YR and 38% MC-LR

[b] Microcystins externally added to the sample before the extraction (mixture with 27% MC-RR, 29% MC-YR and 44% MC-LR)

[c] Low concentration, close to the limit of quantitation (approximate, based on the microcystin standard calibration)

almost all samples, and *m*/*z* values previously reported in the literature were observed (519.5 for MC-RR and 995.5 for MC-LR). Hepatopancreas concentrations based on the SIM mode ranged from 6.0 to 129.0 ng/g tissue fresh weight. In the muscle samples (i.e. the edible part of fish), microcystin concentrations were low, with maxima around 8.0 ng/g fresh weight. However, SIM mode often determined similar "microcystin" peaks also in liver and muscle of control non-exposed fish, and these false positives appeared in all experimental variants. More detailed analyses by MS/MS did not confirm microcystins in all samples. No microcystins were detected in the muscle samples, and the presence of microcystin peaks was confirmed only in hepatopancreas of fish from the pond with high toxin levels. Hepatopancreas concentrations determined by MS/MS ranged from 14.0 to 123.0 ng/g fresh weight, which are more than 50% lower than those calculated from single MS in SIM mode. Fish exposed in the field contained only microcystin–Cys metabolites and in very low concentrations, which were close to the limit of quantification (LOQ; results not shown).

As a final part of the study, muscle samples (edible parts) of eight different fish species collected from five reservoirs during 2008 were analysed by LC-MS/MS. Water blooms of toxic cyanobacteria occurred in all reservoirs during the season and the characteristics of the water blooms and the

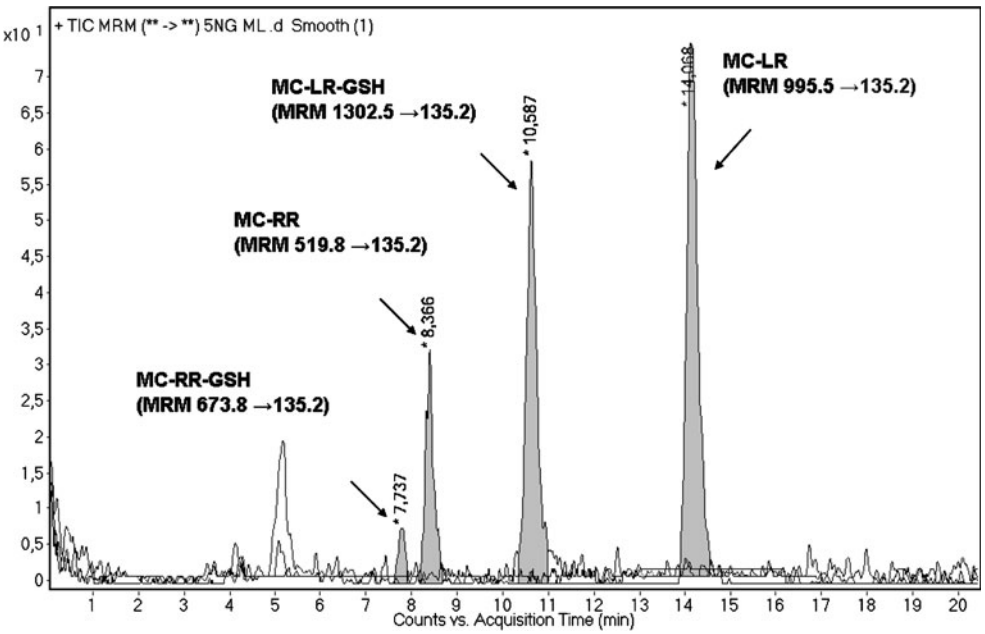

Fig. 2 Chromatogram of hepatopancreas extract from the tandem mass spectrometry (MS/MS) analysis of microcystins (common carp tissues collected 3 h after intraperitoneal injection of microcystins dosed at 248 μg/kg body weight). Peaks of intact molecules as well as their conjugates with glutathione (*GSH*) were detected; retention times and characteristic MRM transitions are shown. *MC-LR* microcystin-LR, *MC-YR* microcystin-YR

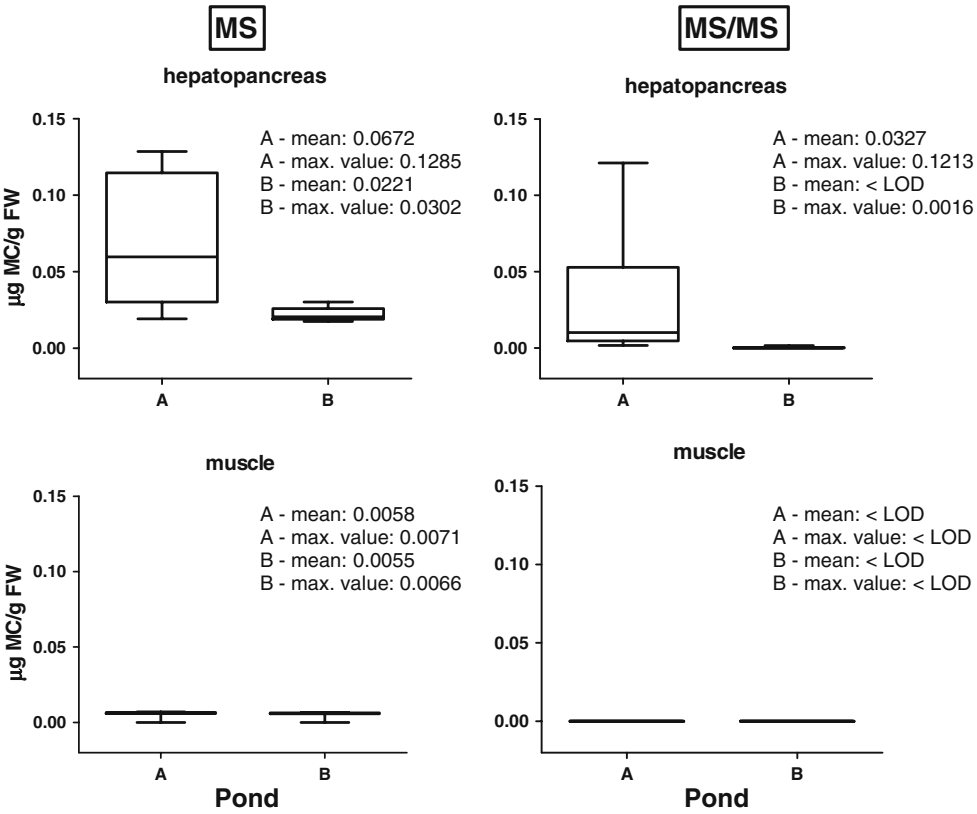

Fig. 3 Concentrations of microcystins (sum of MC-RR and MC-LR) detected by single mass spectrometry (*MS*) and MS/MS (*left graphs* and *right graphs*, respectively) in fish hepatopancreas (*upper graphs*) and muscles (*bottom graphs*). Common carp tissues were analysed after 9-week accumulation under natural conditions in two fish ponds. Pond A contained a massive water bloom during the whole 9-week experiment (dominated by *Microcystis aeruginosa*; concentrations of dissolved microcystins 10–15 µg/L); pond B contained cyanobacteria in low density with maximum microcystin concentration of 2.2 µg/L. Numerical values on each graph show the mean and the maximum for each variant. Limit of detection (*LOD*) of 1.2 ng/g for MC-RR and 5.4 ng/g for MC-YR and MC-LR

microcystin concentrations are given in Table 3. The study covered representatives of all feeding types of fish, including herbivorous (grass carp), omnivorous (bream and common carp), zooplanktivorous (whitefish) and carnivorous (catfish, eel, perch and zander). Of the total 148 fish investigated, none of them were positive for MC-RR, MC-YR or MC-LR. Our results based on tandem mass-spectrometric detection thus indicate lower risk of fish contamination by microcystins, and also lower risks of toxin transfer to humans.

Table 3 Concentrations of microcystins in fish muscles determined by MS/MS in eight fish species collected from five different reservoirs (localities in the Czech Republic) where dense cyanobacterial blooms occurred during the 2008 season. The limit of detection (*LOD*) was 1.2 ng/g for MC-RR and 5.4 ng/g for MC-YR and MC-LR

Locality	Duration of water bloom	Dissolved microcystins in water (µg/L)	Fish species	Number of fish analysed	Microcystins in fish muscle (µg/g FW) (±SD)
Novoveský	May–July	0.638–4.485	Asp	10	<LOD
			Catfish	10	<LOD
			Common carp	10	<LOD
			Eel	1	<LOD
			Grass carp	10	<LOD
			Zander	10	<LOD
Sykovec	August–September	0.125–1.358	Common carp	10	<LOD
			Perch	10	<LOD
			Whitefish	10	<LOD
Medlov	July–September	0.200–0.741	Common carp	10	<LOD
			Perch	10	<LOD
			Whitefish	10	<LOD
Plumlov	June–August	0.212–0.505	Bream	18	<LOD
			Perch	6	<LOD
Vír	June–August	0.000–1.201	Bream	13	<LOD

In summary, our results demonstrate that commonly used methods based on single MS (i.e. monitoring of ions with *m/z* 519.5 for MC-RR and *m/z* 995.5 for MC-LR) may provide false-positive responses, thus overestimating the concentrations of microcystins in the tissue samples. This might have important consequences as some authors have concluded that microcystins accumulated in fish have high human health risks [10, 19]. On the other hand, MS/MS analyses resulted in much cleaner chromatograms with very low background noise, which also lowered the actual LOD/LOQ in comparison with the SIM analyses. It should be pointed out that MS/MS also has its limitations in analyses of extremely complex tissue extracts (e.g. modulations of the MS signal by components of complex matrices, and preferred but not often used quantification using isotopically labelled standards), and these will require further research. Nevertheless, our findings show that selective methods such as MS/MS should be used for analyses of cyanobacterial toxins in tissue samples as they provide more reliable results than single MS methods.

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