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Recovery of MC-LR in Fish Liver Tissue

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ABSTRACT: Cyanotoxins, particularly microcystins (MCs), have been shown to be a hazard to human health. MCs accumulate in aquatic organisms probably as a result of irreversible binding to liver protein phosphatases. The aim of this study was to describe the recovery of MC from fish liver using various detection methods, with MC-LR as the representative congener. These findings are discussed in conjunction with the current procedures and limit values used for human risk assessment. Following incubation of liver homogenates with various MC-LR concentrations, the homogenates were extracted by a water/methanol/butanol mixture via different treatments and subsequently analyzed via the colorimetric protein phosphatase inhibition assay (cPPA), HPLC, and anti-Adda ELISA. Detection via cPPA appeared to yield the highest recovery of MC-LR, although the presence of unspecific background may have resulted in overestimation of the true recovery. The recoveries determined via HPLC and anti-Adda ELISA were comparable to each other. The limits of detection were 0.01–2.4 μg MC-LR/g liver tissue, depending on the method used. Maximum MC-LR recovery from samples incubated with 10 and 100 μg MC-LR/g ranged between 44% and 101%. Recovery from samples incubated with 1 μg MC-LR/g liver tissue was below 3%. Lower recovery is assumed to result from irreversible, covalent MC protein binding, as confirmed by Western blotting of liver homogenates with anti-Adda immunoprobing. The results demonstrate that further investigation of and improvement in routinely applied MC methods for fish tissue and/or food analyses are needed for a reliable risk assessment.

Keywords: microcystin; recovery; fish; tissue; risk assessment; cyanobacteria; liver

INTRODUCTION

Cyanobacteria occur worldwide in coastal and surface waters. To date, at least 46 cyanobacterial species have been shown to produce potent hepato- and/or neurotoxins. Approximately 75% of water samples containing cyanobacteria also contain toxic cyanobacterial metabolites (Sivonen and Jones, 1999).

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The most widespread cyanobacterial toxins are the microcystins (MC) and the related nodularins. These cyclic peptides are produced mainly by the cyanobacterial genera *Anabaena*, *Anabaenopsis*, *Microcystis, Oscillatoria,* and *Nostoc*. So far, nearly 80 variants of microcystins have been identified (Dietrich and Hoeger, 2005), which are responsible for the deaths of terrestrial wildlife, livestock (Briand et al., 2003), and fish (Landsberg, 2002) all over the world.

Human injury, that is, liver necrosis and acute diarrhea/gastroenteritis after acute exposure to microcystins (Byth, 1980; Turner et al., 1990; Teixeira et al., 1993; Pouria et al., 1998; Annadotter et al., 2001) and an increased incidence of primary liver or colorectal cancer after chronic exposure (Yu, 1995; Zhou et al., 2002) have been reported. This highlights the need to consider the acute and chronic effects of microcystin exposure via nutritional intake, including water, especially as human deaths have been

associated with, but not proven to result from, the consumption of drinking water or food contaminated with cyanobacterial toxins (Chorus et al., 2000; Falconer, 2001; Dietrich and Hoeger, 2005). Indeed, the World Health Organization (WHO) has recommended a provisional guideline of a maximum microcystin concentration of 1.0 μg MC-LR/L final drinking water. In this regard, it was assumed that most of the microcystin ingested daily came from contaminated drinking water (80%). However, other sources of oral microcystin exposure exist, for example, contaminated food, uptake during recreational activity, and self-inflicted exposure via cyanobacterial food supplements (Dietrich and Hoeger, 2005).

Microcystins have been shown to accumulate in various aquatic organisms including mussels (Williams et al., 1997c; Karlsson et al., 2003a), crustaceans (Liras et al., 1998; Kankaanpää et al., 2005), and fish (Williams et al., 1997a; Sipiä et al., 2001a; Kankaanpää et al., 2002a; Karlsson et al., 2003b; Soares et al., 2004). The microcystin concentrations detected in field samples of aquatic organisms varied between 0.01 and 100 μg/g tissue (Williams et al., 1997b, 1997c; Magalhaes et al., 2001, 2003; Mohamed et al., 2003). Most MCs accumulate in the liver because of the first-pass effect; however, sufficient MCs can pass via the liver to other organs including muscle, kidney, and brain (Williams et al., 1997a; Fischer and Dietrich, 2000).

For detection, microcystins are routinely extracted from animal tissue by freezing/thawing or sonication using methanol or a mixture of water/methanol/butanol (water/MeOH/BuOH) as extraction solvent (Eriksson et al., 1989; Prepas et al., 1997; Amorim and Vasconcelos, 1999; Sipiä et al., 2001b; Kankaanpää et al., 2002b; Mohamed et al., 2003). Microcystins are routinely detected in the resulting tissue extracts via HPLC-UV (Eriksson et al., 1989; Andersen et al., 1993; Lawrence and Menard, 2001; Magalhaes et al., 2001), colorimetric (cPPA), or radioactive protein phosphatase inhibition assay (Andersen et al., 1993; Williams et al., 1995, 1997c; Prepas et al., 1997; Tencalla and Dietrich, 1997; Malbrouck et al., 2003, 2004), or ELISA (Amorim and Vasconcelos, 1999; Sipiä et al., 2001b; Magalhaes et al., 2001, 2003). However, discussion on the applicability and quality of these analytical methods is ongoing, as microcystins are at least partly covalently bound to PPs, and therefore the microcystin concentrations reported in tissue samples may reflect only freely available microcystins (Meriluoto, 1997; Dietrich and Hoeger, 2005).

The aim of this study was to compare different microcystin detection methods, sample pretreatments, and extraction steps in order to determine the most reliable method or methods for routine determination of microcystin concentrations in fish tissue. These findings are discussed in conjunction with the currently employed human risk assessment procedures and limit values for contaminated foodstuffs (e.g., fish).

MATERIALS AND METHODS

Sample Preparation and MC-LR Incubation

Double-distilled water was purified to 18.2 MΩcm using a Milli-Q system (Millipore, Germany). All other chemicals were of the highest analytical grade commercially available. Microcystin-LR was obtained from Alexis (Switzerland).

Rainbow trout (*Oncorhynchus mykiss*) were obtained from a local fish hatchery (250–300 g/fish). The fish were killed with a blow to the head, and the livers (2–4 g/fish) were removed, weighed, pooled, and placed in a sample buffer (10 mL of buffer/g tissue) containing 10 mM Tris HCl, 140 mM NaCl, 5 mM EDTA, Triton X-100 (1%), 1 mM PMSF, and 1 mM DTT. Tissue was minced using an Ultra Turrax T25 (Janke & Kunkel, Germany) and homogenized using a Dounce Homogenizer 3431-E20 (Thomas Technological Service, USA).

Each homogenate was divided into aliquots, one of which served as a control. Homogenates were incubated with 1, 10, and 100 μg MC-LR/g tissue (Table I). MC-LR incubation took place in continuously rotating glass vials at 30°C for 20 h in order to achieve a representative amount of covalently bound microcystin complexes (Craig et al., 1996). Four or five different homogenates of pooled liver samples were used for each MC-LR concentration (Table I). Controls and MC-LR samples were handled identically.

Sample Splitting and Extraction

All liver homogenate aliquots (samples), incubated for 20 h with or without MC-LR, were divided into 3 subsamples after incubation. The first subsample was stored at −20°C until use for SDS-PAGE/Western blotting. The second subsample was centrifuged (15,000 × g) for 20 min at 4°C. The supernatant (treatment S) obtained was stored at −20°C until further cleanup steps prior to MC analyses. The remaining pellet (treatment P) and subsample 3 (treatment E) were subsequently subjected individually to a single MC-LR extraction (Fig. 1). Extraction was performed using a 75:20:5 (v/v/v) mixture of water/methanol/butanol, which has been demonstrated to yield the best possible extraction (Kankaanpää et al., 2002b). The extraction involved alternate shaking and ultrasonication at 35 kHz at hourly intervals over an 8-h period. Extracts were centrifuged (20 min at 15,000 × g), and the resulting supernatants were stored at −20°C for further cleanup steps prior to microcystin analysis (Fig. 1).

Analytical Subsample Pretreatment and Microcystin Analysis

Prior to microcystin analysis, all three subsample pretreatment types (S, P, and E) were purified and concentrated

TABLE I. Experimental setup with incubated fish liver weights and MC-LR quantities applied

Incubation [μg MC-LR/g tissue]	Quantity of Incubated Liver [g]	MC-LR Added [μg]	Volume of Resuspension [mL]	Theoretical Concentration on Column/Assay [μg MC-LR/mL]	Number of Incubations/Controls
Control	0.2–1	0	1	0	5
1	1, 2.5	1, 2.5	1, 0.5	1, 5	4
10	0.5	5	1	5	4
100	0.2	20	1	20	5

using C18 end-capped solid-phase extraction (SPE) cartridges (Chromabond C18ec, 500 mg; Macherey-Nagel, Germany). For SPE, samples were diluted with water to give methanol concentrations of <5%. The cartridges were preconditioned using 9 mL of 100% methanol, followed by 9 mL of water. Samples were applied to the cartridges slowly, followed by two washing steps using 9 mL of MQ and 9 mL of 10% methanol. Samples were then eluted from the solid phase in the cartridge using 12 mL of 100% methanol, and the eluents were dried under a nitrogen stream and finally resuspended in 20% methanol (Table I) to give the final treatment type–specific microcystin analytes (MC analytes; Fig. 1), which were stored at −20°C until microcystin analysis.

The colorimetric protein phosphatase assay (cPPA) with 4-nitrophenylphosphate (Acros Organics, Belgium) as substrate was performed as described by Heresztyn and Nicholson (2001), using recombinant protein phosphatase 1 from *E. coli* (New England BioLabs Inc., UK) at an end concentration of 0.375 units/mL with MC-LR as a standard. The PP-inhibiting capacities of the respective MC analytes were compared to an MC-LR standard curve in the same assay. The detection range (20%–80% inhibition of PP1) of the colorimetric PPA used was 1.5–15 μg MC-LR/L, with a derived IC_{50} of 4.8 μg MC-LR/L. Enzyme solution (20 μL/well) was added to 20 μL of sample in 96-well plates and incubated at 37°C for 5 min. Substrate solution (200 μL/well) was added and incubated at 37°C for 2 h, and the absorption was measured at 405 nm using an SLT Reader. The absorption was measured before and after incubation, and substrate conversion was determined as the difference between the first and second measurements. MC concentrations were calculated via comparison with substrate conversion of the MC-LR standards. Each MC analyte was analyzed 3 times in duplicate.

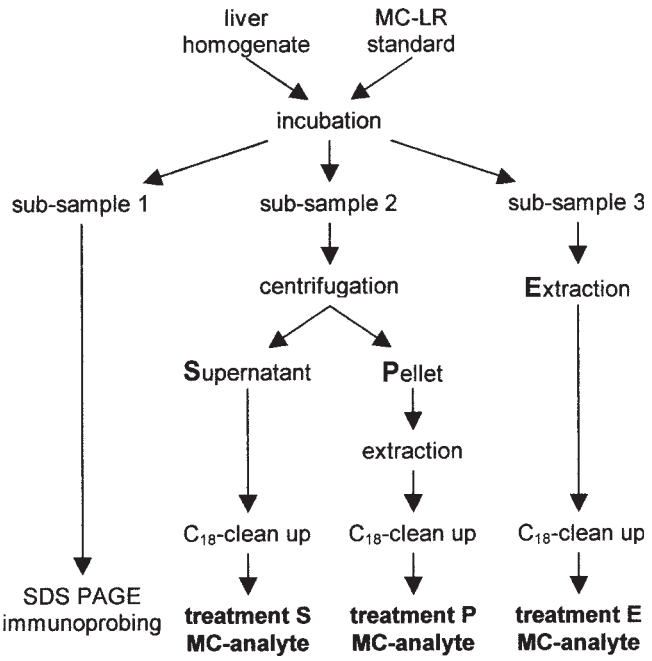

Fig. 1. Experimental setup: scheme of sample aliquots, subsample extraction, and pretreatment.

The anti-Adda ELISA Kit (Abraxis LLC, USA) employed in the tests is based on an antiserum raised against the unique C_{20} amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadieonic acid (Adda; Fischer et al., 2001). The ELISA was performed according to the manufacturer's instructions. Each MC analyte was analyzed 3 times in duplicate.

HPLC was performed using Beckman (Germany) HPLC equipment (Autosampler 507e, Solvent Module 125) with an analytical C_{18} column (Grom-Sil 120 ODS-4 HE, 5 μm, 250 × 4 mm). A gradient with water (0.05% TFA) and acetonitrile (0.05% TFA) as the mobile phase was used according to the method described by Lawton et al. (1994). MC-LR was detected using a photodiode array SPD-M10A VP (Shimadzu, Germany) and identified via retention time and typical spectrum in comparison with internal MC-LR standards. MC-LR concentrations were calculated using peak area and peak height. HPLC MC-LR analysis was carried out once for each MC-LR analyte.

For qualitative detection of covalently bound microcystin adducts, subsamples 1 (Fig. 1) were separated via 10% SDS PAGE in accordance with Laemmli (1970). The protein content of each treatment was determined according to the method of Bradford (1976) and adjusted to give a protein load of 60 μg protein/lane. Separated proteins were transferred onto a nitrocellulose membrane via Western

blot. The membranes were blocked using TTBS + 1% BSA for 30 min, and MC-LR adducts were detected via incubation with polyclonal sheep anti-Adda serum (diluted 1:1000 in blocking buffer) at room temperature for 1 h according to Fischer and Dietrich (2000). Membranes were washed using TTBS (3 × 5 min) and incubated with secondary antibody (antisheep IgG-AP, diluted 1:5000 in TTBS; Sigma-Aldrich, Germany) at room temperature for 1 h. After washing with TTBS (3 × 5 min) and TBS (1 × 15 min), specific bands were finally stained using Sigma Fast Red® (Sigma-Aldrich, Germany) according to the manufacturer's instructions. The molecular weights of detected adducts were estimated by comparison with full-range rainbow marker proteins RPN 800 (Amersham, UK).

Statistics

Data analyses were carried out using JMP® (USA) software. Values represent the mean ± SD of at least three separate experiments. Results of the HPLC, ELISA, and PPA analyses, as well as results of different treatments, were analyzed for statistical differences using analysis of variance (ANOVA) and the Tukey–Kramer multiple comparisons test ($p \geq 0.05$). The sum of MC-LR concentrations analyzed in analyte samples of treatments S and P (S+P) were tested for statistical differences ($p \geq 0.05$) to concentrations analyzed via treatment E using the Student's *t* test.

RESULTS

All MC analytes were analyzed by cPPA, HPLC, and anti-Adda ELISA. Detected MC-LR concentrations in the respective analytes and their corresponding deduced tissue concentrations and recoveries are shown in Tables II–IV. Analyte samples of treatment S were analyzed for quantification of soluble MC-LR. Samples of treatment P were analyzed to recover the remaining, extractable, and not readily soluble MC-LR. Analyte samples of treatment E were analyzed to quantify the overall extractable MC-LR in the incubated tissue homogenates.

In all treatment approaches, the sum of MC-LR concentrations analyzed in the S+P analyte samples were not significantly different from recovery from whole tissue homogenate extraction (treatment E), regardless of the MC-LR concentration and detection methods used. The highest MC-LR recovery generally was achieved using whole-tissue homogenate extraction (treatment E) without previous centrifugation and separation (Tables II–IV). However, the overall extractable MC-LR analyzed by treatment E did not always differ significantly from the quantities of soluble MC-LR in treatment S.

When comparing the different detection methods, determinations by HPLC and ELISA yielded comparable MC-LR recoveries in all MC-LR concentrations and sample treatments applied. Analyses with cPPA generally resulted in higher recovery than detection via either HPLC or ELISA (Tables II–IV), although this was not significant at all MC-LR concentration levels (1, 10, and 100 μg/g) used.

Analyses of controls via cPPA resulted in an average background noise equivalent to 0.3–0.4 μg MC-LR/mL analyte (Table II). Nonspecific positive signals were also observed with the anti-Adda ELISA and control sample analytes (Table IV). This background noise, however, corresponded to $\leq$0.01 μg MC-LR/mL analyte and therefore

TABLE II. MC-LR concentrations detected, corresponding tissue concentrations, and calculated recovery after a 20-h incubation of fish liver tissue with various MC-LR concentrations using different sample treatments (Fig. 1) with the colorimetric protein phosphatase inhibition assay (cPPA) as the analytical method

		PPA			
Incubation (μg MC-LR/g)		Detected Concentration (μg/mL)	Detected Tissue Concentration (μg/g)	Recovery (%)	Number of Incubations Analyzed
S	Control	0.27 ± 0.212			4
	1	i.d.	i.d.	i.d.	3
	10	3.51 ± 0.511	7.02 ± 1.022	70 ± 10.2	3
	100	14.19 ± 2.793	70.95 ± 13.96	71 ± 13.9	4
P	Control	0.38 ± 0.415			4
	1	i.d.	i.d.	i.d.	3
	10	1.21 ± 0.298	2.42 ± 0.596	24 ± 05.9	3
	100	1.89 ± 0.964	9.45 ± 4.820	9 ± 04.8	3
E	Control	0.39 ± 0.219			4
	1	i.d.	i.d.	i.d.	3
	10	5.05 ± 1.288	10.10 ± 2.576	101 ± 25.8	4
	100	18.59 ± 5.593	92.95 ± 27.96	93 ± 28.0	3

i.d. = indistinguishable from controls.

TABLE III. MC-LR concentrations detected, corresponding tissue concentrations, and calculated recovery after a 20-h incubation of fish liver tissue with various MC-LR concentrations using different sample treatments (Fig. 1) with high-performance liquid chromatography as the analytical method

		HPLC			
Incubation (μg MC-LR/g)		Detected Concentration (μg/mL)	Detected Tissue Concentration (μg/g)	Recovery (%)	Number of Incubations Analyzed
S	Control	nd			3
	1	nd	nd	nd	3
	10	2.25 ± 0.31	4.5 ± 0.60	45 ± 06.2	3
	100	12.63 ± 1.66	63.2 ± 8.30	63 ± 08.3	4
P	Control	nd			3
	1	nd	nd	nd	3
	10	0.21 ± 0.11	0.4 ± 0.22	4 ± 02.2	3
	100	1.03 ± 0.50	5.2 ± 2.50	5 ± 02.5	4
E	Control	nd			3
	1	nd	nd	nd	3
	10	2.19 ± 0.50	4.4 ± 1.00	44 ± 10.0	4
	100	16.10 ± 1.37	80.5 ± 6.85	81 ± 06.8	4

nd, not detectable.

was at least 30 times lower than the background observed in the cPPA analyses. Conversely, no background noise relevant to the MC-LR peak retention time was observed in the HPLC analyses. To compare the results obtained with the three methods of MC analysis, concentrations detected via cPPA and ELISA were corrected for nonspecific background noise (subtraction of the background noise from the raw value in the analysis).

Limits of detection for MC-LR in the liver homogenate samples used in this study were 1.2 μg MC-LR/mL (cPPA, Table II), 0.2 μg MC-LR/mL (HPLC, Table III), and 0.01 μg MC-LR/mL (anti-Adda ELISA, Table IV). These detection limits translate to minimum MC-LR tissue concentrations of 2.4 μg MC-LR/g for cPPA (Table II), 0.4 μg MC-LR/g for HPLC (Table III), and 0.01 μg MC-LR/g for anti-Adda ELISA (Table IV).

Maximum MC-LR recovery from liver homogenate samples incubated with 10 and 100 μg MC-LR/g, for example, for treatment E (whole homogenate extract), ranged between 44% and 101%, depending on the initial MC-LR concentration and detection method used (Tables II–IV). The reliability of the recovery data (the variance) largely

TABLE IV. MC-LR concentrations detected, corresponding tissue concentrations, and calculated recovery after a 20-h incubation of fish liver tissue with various MC-LR concentrations using different sample treatments (Fig. 1) with anti-Adda-ELISA as the analytical method

		ELISA			
Incubation (μg MC-LR/g)		Detected Concentration (μg/mL)	Detected Tissue Concentration (μg/g)	Recovery (%)	Number of Incubations Analyzed
S	Control	< 0.01			3
	1	0.01 ± 0.003	0.01 ± 0.003	1 ± 00.3	3
	10	2.33 ± 0.116	4.66 ± 0.232	47 ± 02.3	3
	100	10.42 ± 2.009	52.10 ± 10.05	52 ± 10.1	3
P	Control	< 0.01			3
	1	0.02 ± 0.006	0.02 ± 0.006	2 ± 00.6	3
	10	0.37 ± 0.135	0.74 ± 0.270	7 ± 02.7	3
	100	0.99 ± 0.247	4.95 ± 1.235	5 ± 01.2	3
E	Control	0.01 ± 0.005			3
	1	0.03 ± 0.013	0.03 ± 0.013	3 ± 01.3	3
	10	2.89 ± 0.536	5.78 ± 1.072	58 ± 10.7	3
	100	13.49 ± 0.352	67.45 ± 1.760	68 ± 01.8	3

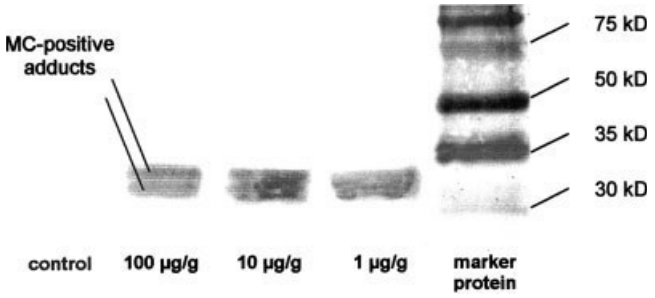

Fig. 2. Immunostaining of MC-LR adducts in fish liver homogenates following a 20-h incubation with 1, 10, and 100 μg MC-LR/g tissue. Molecular weights were estimated via comparison with marker proteins.

depended, however, on the analytical method used. The analytical methods ranked in order from worst to best are: cPPA < HPLC < anti-Adda ELISA.

In contrast, MC-LR recovery from samples incubated with 1 μg MC-LR/g liver tissue was extremely low (1%–3%), even after water/MeOH/BuOH extraction. Indeed, anti-Adda ELISA analyses provided a detectable MC-LR concentration of only 0.01–0.03 μg MC-LR/g liver tissue, depending on the sample pretreatment used (Table IV). As a result of having a higher limit of detection, neither the cPPA (Table II) nor the HPLC-PDA (Table III) analysis was able to detect MC-LR in the 1 μg/g liver homogenate samples.

Western blot analysis showed that anti-Adda immunostaining of positive protein adducts (30–35 kD) could be detected in all liver homogenate samples incubated with MC-LR (1, 10, and 100 μg/g liver; Fig. 2).

DISCUSSION

The results demonstrated varying levels of MC-LR recovery from fish liver homogenates, depending on the treatment of the homogenate subsamples and the detection method used. Comparing recovery resulting from different subsample treatments, whole-tissue homogenate extraction provided comparable MC-LR recovery to the sum of recoveries of treatments S and P (E = S + P). This suggests that water/MeOH/BuOH extraction increases MC-LR recovery from incubated liver tissue. However, as the overall extractable MC-LR analyzed in treatment E samples did not always differ significantly from those of soluble MC-LR in treatment S, the additional MC-LR recovery achieved by homogenate water/MeOH/BuOH extraction appears to be limited. For example, water/MeOH/BuOH extraction is not useful for extracting covalent bond MC.

Indeed, repetition of the extraction procedure resulted in no additional MC-LR recovery (data not shown). These findings are in agreement with those of Kankaanpää et al. (2002b), who demonstrated that extraction of nodularin from animal tissue yielded the best extraction results (highest recovery) using water/MeOH/BuOH 75:20:5 (*v*/*v*/*v*) with an 8-h extraction time and that extraction repetition did not improve recovery. On the contrary, repetitious extraction increased the amount of matrix compounds interfering with HPLC analysis of nodularin.

MC-LR recovery detected by cPPA was generally higher than that determined by anti-Adda ELISA or HPLC, independent of subsample treatment and the amount of MC-LR used for incubation of the homogenate. This is most likely a result of the high background of non-MC-LR-related PP inhibition, as strongly suggested by the PP inhibition observed in the controls. It is assumed that this high background of non-MC-LR-related PP inhibition may be the result of (i) specific endogenous PP1 inhibitors (Oliver and Shenolikar, 1998) in the liver homogenates liberated during the liver homogenization process and/or (ii) unspecific influences arising from matrix effects. These observations were corroborated by the findings of Sipiä et al. (2001a), who demonstrated that nodularin could not be detected in the muscle of Atlantic salmon via MC-LR ELISA, whereas analysis by cPPA resulted in 55–65 ng NOD/g. Sipiä et al. (2001a) also concluded that the disparate findings between the analyses by MC-LR ELISA and by cPPA were the result of turbidity and color as well as of matrix-interfering compounds in the tissue homogenates and the resulting extracts.

Consequently, this unspecific PP inhibition causes an overestimation of the MC and nodularin contamination in tissues in general and of MC-LR contamination in fish liver homogenates specifically, as presented in this study. Therefore, cPPA with PP1 appears inappropriate for routine MC and nodularin detection in tissue samples.

In contrast to cPPA detection, overall MC-LR recovery from liver homogenates determined by HPLC was comparable to that determined by anti-Adda ELISA. However, no MC-LR was detectable by HPLC in homogenates incubated with 1 μg MC-LR/g, whereas the analysis with anti-Adda ELISA resulted in detectable MC-LR concentrations of up to 0.03 μg/mL analyte.

Similar observations were made by Kankaanpää et al. (2002a), who demonstrated that no nodularin was detectable via HPLC analysis in liver tissue from sea-trout, orally dosed with nodularin; in contrast MC-LR ELISA analysis resulted in nodularin concentrations of up to 1.2 μg/g tissue. These concentrations exceeded the detection limit for nodularin quantification in liver tissue (0.15 μg NOD/g) of the chromatographic system employed and thus should actually have been detectable via HPLC. Kankaanpää and co-authors concluded, in accord with Metcalf et al. (2000), that the discrepancy between HPLC and ELISA analyses is most likely a result of the detection of additional nodularin conjugates via the ELISA assay, whereas these conjugates would not be readily detectable via HPLC.

The detection limit of the HPLC system employed in this study was approximately 10 ng per injection. Thus, the

injection volume of 50 μL of analyte resulted in an absolute detection limit of 0.2 μg MC-LR/mL analyte. As the MC-LR concentrations (anti-Adda ELISA) that could be determined in the homogenates incubated with 1 μg MC-LR/g were far below the HPLC detection limit, it is not surprising that no MC-LR was detectable in these samples using HPLC.

Karlsson et al. (2003b) reported that matrix effects hindered HPLC-UV detection of nodularin in liver tissue samples of flounder, and Sipiä et al. (2001a) described HPLC as an inappropriate method for nodularin analysis in liver tissue samples using mobile and stationary phases with UV detection, as small concentrations ($\leq$0.3 μg NOD/ 10 μL injection) of nodularin easily escaped detection. Contrary to these findings, the present results suggest that HPLC-UV is an accurate analytical method for quantification of MC in tissue samples, as long as MC tissue contamination is greater than 0.2 μg MC-LR/mL analyte, as specified by the sample treatment and extraction method used in this study.

Unspecific competitive background binding also was observed in the anti-Adda ELISA with control samples, representing less than 0.01 μg MC-LR/mL approximately. Sipiä et al. (2001a) described nodularin concentrations of less than 0.01 μg/g liver tissue to be below the level of quantification in MC-LR ELISA because of matrix effects. Kankaanpää et al. (2002a) described a level of nonspecific binding to antibodies of approximately 0.02 μg MC/g liver tissue of sea trout analyzed for nodularin with MC-LR ELISA. Kankaanpää et al. (2005) also suggested that the theoretical detection limits of MC-LR ELISA for nodularin analysis in hepatopancreas and muscle tissue of prawns are affected by low-level matrix effects because of unspecific binding to and/or denaturing of the antibodies. Matrix effects in the analyses of nodularin in liver tissue samples of flounder also have been reported by Karlsson et al. (2003b), suggesting that analysis via MC-LR ELISA is not optimal if it is the only means of toxin analysis in tissue. In contrast, the results of the study reported here demonstrated that background/matrix-associated effects and thus the limit of detection in the anti-Adda ELISA employed were at least 30 times lower than in the cPPA and HPLC methods used. Moreover, in conjunction with the MC-LR amounts recovered from incubated homogenates in this study, overestimation of MC-LR contamination of tissue because of unspecific measurement by the anti-Adda ELISA appeared negligible.

In summary, when comparing the MC detection methods employed in this study and the results obtained with those previously reported, anti-Adda ELISA appears to be the most appropriate method for the detection of MC in tissue samples. In tissue samples contaminated with relatively high MC concentrations (>0.4 μg MC-LR/g), both anti-Adda ELISA and HPLC-UV appear to be suitable methods for reliable MC detection in tissue samples. However, generally, a prerequisite for acceptable analyses is triplicate analyses and relevant standards and controls. The complementary use of different detection methods, that is, simultaneously using two or more analytical methods for the same analytes, is highly recommended, in agreement with other authors (Metcalf et al., 2000; Meriluoto, 2004), in order to achieve reliable detection of MC contamination in tissue samples.

Kankaanpää et al. (2002b) specified three possible reasons for incomplete recovery of nodularin and MC, as also observed in the study reported here: (i) loss during the analytical procedure, (ii) metabolism (conjugation) in tissue and, (iii) covalent binding of MC to macromolecules (proteins and peptides). One possible way to verify the abundance of MC-protein adducts is through immunoprobing using antibodies raised against MC or MC fragments, for example, the Adda moiety (Hitzfeld et al., 1999; Fischer and Dietrich, 2000; Mikhailov et al., 2003). As noncovalently bound MC-LR is expected to elute from a SDS-PAGE denaturing gel, Adda-positive bands observed in the Western blots most likely represent MC-LR protein adducts in the liver homogenates analyzed. That cyanobacterial peptides such as nodularin, which do not appear to covalently bind to proteins, could not be detected in Western blots using the appropriate antibodies corroborates this (Mikhailov et al., 2003; Schmid et al., 2004). The presence of bands visible by anti-Adda immunostaining is therefore a distinct indication of the presence of covalently bound MC-LR adducts in incubated homogenates. This is in agreement with former studies, showing adducts in the 28- to 38-kD range that most likely represent PP-MC adducts to the liver endogenous protein phosphatases (Hitzfeld et al., 1999; Fischer and Dietrich, 2000; Ernst et al., 2001; Mikhailov et al., 2003). In agreement with the findings of previous investigations (Meriluoto, 1997; Williams et al., 1997a, 1997b; Amorim and Vasconcelos, 1999; Kankaanpää et al., 2002b), we observed loss in recovery that was probably attributable to irreversible covalent MC binding.

According to MacKintosh et al. (1990), the MC-binding capacity in mice liver is expected to be approximately 1 μg/g. Similarly, Yoshida et al. (1998) estimated the amount of irreversibly bound MC in mice liver as 0.7 μg MC/g liver. Assuming a similar MC-binding capacity for fish, binding capacities may be expected also to be saturated for homogenates incubated with 1 μg MC/g. Consequently, MC recovery was only 1%–3% in homogenates incubated with 1 μg MC/g.

To extrapolate the above findings and conclusions to the routine situation of laboratory analyses of food samples and their safety assessment according to regulatory recommendations, the WHO recommendations and guidance values were employed in the analysis of the tissue levels used in this study in order to provide a reasonable example of risk calculation and extrapolation.

The WHO suggests a tolerable daily intake (TDI) of 0.04 μg MC-$LR_{equivalents}$/kg food a day, where MC-$LR_{equivalents}$ is the sum of all MC congener concentrations

TABLE V. Estimation of an interim maximum acceptable contamination (IMAC) for fish based on a tolerable daily intake of 0.04 μg MC/kg bw, calculated based on average body weight of a 60 kg, diverse average fish consumption (AFC) and various percentages of uptake of toxin (POT) via fish consumption

AFC (g/d)/POT (%)	0.25	0.5	0.75
	IMAC [μg/g]		
10	0.060[E,H]	0.120[E,H]	0.180[E,H]
50	0.012[E]	0.024[E]	0.036[E]
300	0.002	0.004	0.006

[E] Detectable using anti-Adda ELISA.
[H] Detectable using HPLC.

likely to be in the food as contaminants. To determine an interim maximum acceptable concentration (IMAC) in fish used for consumption, the following equation was applied in accordance with the publication by Falconer (2001): IMAC = TDI × BW × POT/AFC where BW is body weight, POT is the proportion of toxin consumed in the form of contaminated fish, and AFC is the average fish consumption.

Average human body weight is assumed to be 60 kg. Based on Egyptian and Brazilian eating habits, the AFC ranges from 100 to 300 g per day per person (Magalhaes et al., 2001; Mohamed et al., 2003). According to the European Commission (2004), worldwide fish consumption is calculated as 43 g per person per day (including fish from marine water, brackish water, and freshwater). People in Europe on average consume 67 g per person per day, ranging from 31 g in Austria to 167 g in Portugal. Average German fish consumption is 35 g/day. However, only 8 g of that is freshwater fish or fish from mildly saline waters, which are more likely to be affected by cyanobacterial blooms (http://europa.eu.int/comm/fisheries). Performing a universal risk assessment is difficult because of large differences in consumption and exposure conditions (high variation in AFC and POT). Consequently, guideline values for MC contamination of fish must be based on local customs, conditions, and circumstances.

Using various assumed AFC and POT levels, the interim maximum acceptable contamination for fish was calculated and demonstrated to vary between 0.002 and 0.18 μg/g tissue (Table V). Assuming detection limits in liver tissue (as demonstrated here) to be similar to that in muscle tissue, these IMACs were then compared to the three analytical methods used for MC-LR analysis in this study, from which it was determined that cPPA is generally inappropriate for MC tissue contamination analysis. Considering the relevant limits of detection, anti-Adda ELISA and HPLC may be used for certain IMACs (Table V). However, it also was clearly demonstrated that for providing safe and healthy food for consumers neither anti-Adda ELISA nor HPLC, depending on the AFC, is sufficiently sensitive to allow reliable detection and thus regulation of fish contaminated with MC (Table V). In addition, on the basis of the findings of MacKintosh et al. (1990), Yoshida et al. (1998), and the results reported here, it can be assumed that in tissue contaminated with concentrations of ≤1 μg MC/g, most MC is bound covalently.

In this respect, and given the unsatisfactory detection limits as mentioned above, further investigation and improvement of routinely applicable MC methods for fish tissue and/or food analyses are essential requirements for an effective risk assessment. Current investigations have demonstrated that such recent developments and improvements might include immunoaffinity chromatography, LC-MS, and MALDI-TOF analyses (Hormazabal et al., 2000; Lawrence and Menard, 2001; Karlsson et al., 2003a, 2003b).

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