

Determination of microcystins in fish tissues using HPLC with a rapid and efficient solid phase extraction

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Abstract

The presence of toxic cyanobacterial blooms have caused severe problems for aquatic animals, wildlife and humans. Monitoring of microcystins (MCs) in fish and other aquatic animals is important for evaluating the potential risk for human consumption. Existing methods for extracting and purifying microcystins in fish tissue are both time- and solvent-consuming. The present study used HLB (0.5 g) and silica gel (2 g)/plus silica gel (0.69 g) tandem cartridges to isolate microcystins from fish tissue samples for subsequent analysis by high-performance liquid chromatography with photodiode array detection (HPLC-PDA). The microcystins (MC-RR, MC-LR) were isolated from a *Microcystis* bloom in Chikato Pond, Japan, and the purity was confirmed by HPLC. This report has presented a simple and rapid solid phase extraction procedure for determining microcystins in fish tissue, since it yielded high recovery (>85%) and consistency (SD<5%) in isolating microcystins from fish tissue.

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1. Introduction

Toxic cyanobacterial blooms occur frequently in eutrophic fresh waters worldwide (Paerl et al., 2001, Xie and Liu, 2001). Many cyanobacterial species are able to produce toxins, and microcystins are usually the predominant cyanotoxins present in drinking, fisheries and recreational waters during cyanobacterial blooms (Carmichael, 1994). Microcystins contamination in the field has been widely reported for various groups of aquatic animals, such as fish (Magalhães et al., 2001; Xie et al., 2004, 2005), mussels (Yokoyama and Park, 2002), snails (Zurawell et al., 1999) and shrimps (Chen and Xie, 2005), attracting increasing concern from the public about

food safety. The most widely studied microcystins are microcystin-RR (MC-RR) and microcystin-LR (MC-LR). Mass occurrences of toxic cyanobacteria have been associated with feral fish kills (Toranzo et al., 1990; Jewel et al., 2003). Cyanotoxins from freshwater cyanobacterial bloom have been reported to cause substantial damage in estuarine and marine aquaculture facilities (Shumway, 1990; Hallegraef, 1993).

It is important to establish how microcystins are transferred in the food web when toxic cyanobacterial blooms occur. It is also important to monitor microcystins in tissues of fish and other aquatic animals in order to evaluate the potential risk for human health from contaminated food. A reliable and sensitive method for extracting microcystins from a sample is therefore needed. Extensive studies on the extraction and detection of microcystins have led to a number of methods, such as

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protein phosphatase inhibition assays (PPIA, Li et al., 2004), enzyme-linked immunosorbent assay (ELISA, Rapala et al., 2002), liquid chromatography (LC, Moreno et al., 2004) and capillary electrophoresis (CE, Gago-Martínez et al., 2003). Currently, routine analysis of microcystins in animal tissue is mostly carried out using HPLC-PDA.

Therefore, the development of an easy-to-use, rapid, robust and inexpensive method for the measurement of low concentrations of these toxins has been recognised as a high priority. Nevertheless, there is still no agreement on the methodology for quantitative analysis of microcystins in fish tissue. Further investigation of and improvement in routinely applied MC methods for fish tissue are needed for a reliable risk assessment (Ernst et al., 2005).

The aim of this study was to develop an efficient analytical procedure, including solvent extraction and HPLC, for the simultaneous determination of MC-RR and MC-LR in fish tissue.

2. Materials and methods

2.1. Cartridges

HLB (Oasis[®], 0.5 g), which is an acronym for hydrophilic–lipophilic balance, describes the two major features of this sorbent: (1) it remains wetted with water, and (2) it retains a wide spectrum of both polar and non-polar compounds. The sorbent is manufactured by Waters corporation (Milford, Massachusetts, USA, Oasis[®], Part No: 189000116).

Silica gel (2 g)/plus silica gel (0.69 g) cartridges are polar-sorbent, and used primarily to adsorb analytes from non-polar solvents such as hydrocarbons, chloro- or fluoro-substituted hydrocarbons or less polar esters and ethers; elution can be done with more polar solvents such as polar esters, ethers, alcohols, acetonitrile or water. The binding mechanism can be hydrogen bonding or dipole–dipole interaction. This product is manufactured by Waters corporation (Milford, MA, USA).

2.2. Reagents and materials

The microcystins (MC-RR, MC-LR) were isolated from a *Microcystis* bloom which was collected from Chikato Pond, Japan (July 14, 2002), using a plankton net (40 μ m mesh size). The samples were stored immediately in a portable refrigerator (around 0 °C) and then transported to the laboratory. They were then immediately frozen at –40 °C, and freeze-dried. To determine the concentrations of microcystins in the cells, the cells were homogenized in a mortar and extracted three times with

5% acetic acid and the supernatant was applied to an HLB cartridge (0.5 g, Oasis[®], Waters, Milford, Massachusetts, USA). The purity was confirmed by HPLC (Park et al., 1998). The concentration of the MC-RR and MC-LR was 826 and 520 μ g/g, respectively.

The method used to extract the microcystins from fish tissues is shown in Fig. 1. Lyophilized samples (muscle, liver, blood and kidney of *Hypophthalmichthys molitrix*, two fishes were utilized, which were collected from Lake Chaohu, China (August 21, 2003)) were homogenized in a mortar and extracted three times with 10 mL of BuOH:MeOH:H₂O (1:4:15), sonicated for 3 min (30% amplitude, 60 W, 20 kHz), then centrifuged at 4000 rpm (3200 \times g) for 20 min at room temperature. The dry weight of the extracted tissue ranged from 30 to 50 mg.

Microcystins (MC-RR: 11.4 μ g; MC-LR: 3.1 μ g) were added to the supernatant, transferred to a pear-shaped glass flask (250 mL) and evaporated at 35 °C to about 20 mL using a roto-evaporator, then applied directly to an HLB cartridge, which had been preconditioned by 100% MeOH and distilled water. The column was first washed

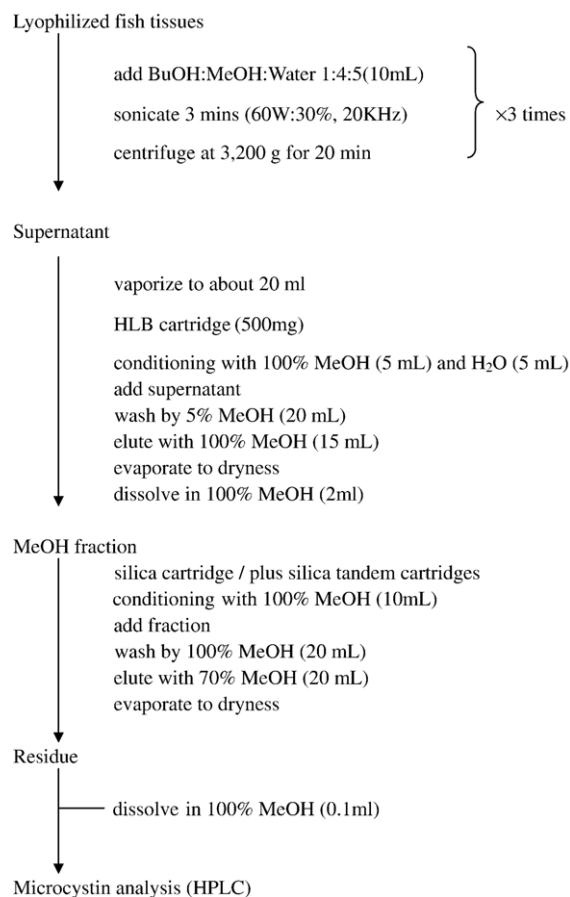


Fig. 1. Procedure for analysis of microcystins in fish.

Table 1
The recoveries of the different kinds of cartridges

Cartridges	Added MCs (μg)	Recovery (100%)	
		MC-RR	MC-LR
Alumina A	MC-LR: 12.6	–	0.70
Silica gel (2 g)	MC-LR: 7.10	–	69.3
Plus silica gel (0.69 g) tandem	MC-LR: 7.10	–	96.5
Plus silica gel (0.69 g)	MC-LR: 6.21	–	56.3 \pm 4.9
MCX (0.5 g)	MC-RR: 11.4 MC-LR: 3.10	57.5 \pm 2.5	66.0 \pm 0.3
ODS (5 g)	MC-RR: 11.4 MC-LR: 3.10	91.5 \pm 4.9	75.0 \pm 3.2
HLB (0.5 g)	MC-RR: 11.4 MC-LR: 3.10	94.1 \pm 3.0	97.2 \pm 0.7
Silica gel (2 g)/ plus silica gel (0.69 g) tandem	MC-RR: 11.4 MC-LR: 3.10	100.2 \pm 0.6	98.9 \pm 0.2

Three replicates were used in the present study.

– Means only used microcystin-LR.

with 5% MeOH and the toxin then eluted with the 100% MeOH. The microcystin-containing fraction was evaporated to dryness and the residue then re-dissolved in 100% MeOH. This solution was applied to a silica gel cartridge (2 g)/plus silica gel (0.69 g) tandem cartridge, which had been preconditioned by 100% MeOH. The column containing the toxins was washed with 100% MeOH and then eluted with 70% MeOH. This elution fraction was also evaporated to dryness and the residue then dissolved in 100% MeOH. This solution was injected into

the high-performance liquid chromatograph (HPLC) for analysis. Pre-accumulated MCs in the fish was very low ($<0.1 \mu\text{g}$) as compared to the MC-LR and MC-RR spikes and was not expected to exaggerate recovery of microcystins from tissue.

The treatment of the ODS, MCX and Alumina cartridges was as follows: Supernatant diluted 1:1 with water was directly applied to a 5 g reverse-phase ODS cartridge (Chromatocores ODS, 100–200 mesh, packed into a polypropylene cartridge), which had been preconditioned by washing with 50 mL of 100% MeOH and 50 mL of H₂O. The column was washed with water (50 mL), then with water–MeOH (4:1, 100 mL). Elution from the column with 90% MeOH (100 mL) yielded the fraction. Supernatant was acidified to 0.05 N HCL and applied to a 0.5 g MCX cartridge (Waters corporation, Milford, MA, USA), which had been preconditioned by 6 mL 100% MeOH and 6 mL distilled water, then load sample. The column was washed with 0.1 N HCL 10 mL and 100% MeOH 10 mL. Elution from the column with 95% MeOH/5% NH₄OH (20 mL) yielded the fraction of interest. Supernatant was applied to Alumina cartridge, which had been preconditioned by 100% MeOH. The column containing the toxins was washed with 100% MeOH and then eluted with 70% MeOH.

2.3. Chromatographic conditions

The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-9A pump coupled to an SPD-10A UV–visible detector (238 nm), an SPD-M10A photodiode

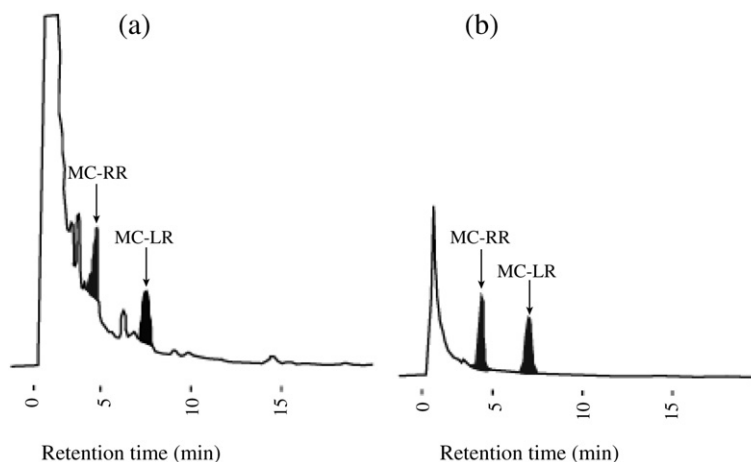


Fig. 2. High-performance liquid chromatograms monitored at 238 nm and MC-RR and MC-LR measured for the spiked tissue (blood) of *Hypophthalmichthys molitrix* after the first clean-up with HLB (a) and the second clean-up with silica gel/plus silica gel tandem cartridges (b).

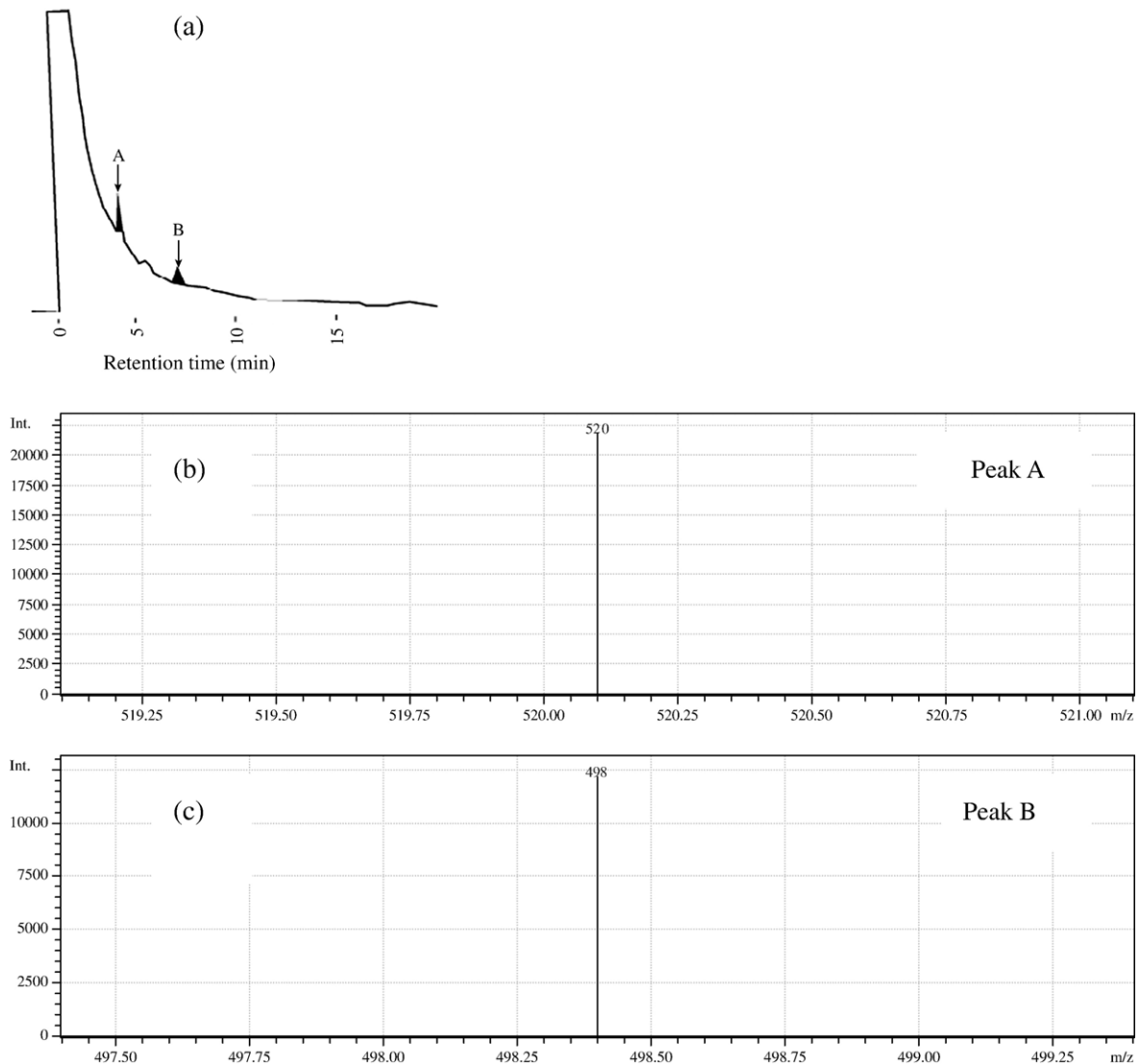


Fig. 3. Chromatograms measured for microcystin in the intestine of *Hypophthalmichthys molitrix*: (a) High-performance liquid chromatograms monitored at 238 nm; (b) LC/ESI–MS mass spectra of microcystins-RR; (b) LC/ESI–MS mass spectra of microcystins-LR; mass chromatograms monitored at m/z 498, 520 ($M+2H$)²⁺, respectively.

array detector, a C-R6A integrator, and an ODS column (Cosmosil 5C18-AR; 4.6 mm × 150 mm, Nakarai, Japan). The sample was separated with a mobile phase consisting of methanol:0.05 M phosphate buffer (pH 3.0; 58:42) at a flow rate of 1 mL min⁻¹. The MC concentration was quantified against standard MC-RR and MC-LR (Wako Ltd., Japan). Liquid chromatography electrospray ionization/mass spectrometry (LC/ESI–MS) analysis of microcystins was conducted under the following conditions: curved desolvation line (CDL) temperature, 200 °C; nebulizing gas (N₂) flow, injected volume 5 μL, detector gain, 1.5 kV.

3. Results and discussion

3.1. Recovery of microcystins using different kinds of cartridges

Table 1 shows the recovery of microcystins using different kinds of cartridge. Recovery was highest in the HLB (MC-RR: 94.1 ± 3.00, MC-LR: 97.2 ± 0.66) and silica gel/plus silica gel tandem cartridge (MC-RR: 100.2 ± 0.62, MC-LR: 98.9 ± 0.17). These two methods had an excellent ability to retain the microcystins and they were highly reproducible. Therefore, we selected

Table 2

The recoveries of different tissues of fish in spike experiment (replication=3) using HLB and silica gel/plus silica gel tandem cartridges

Organs	Dry weight of extracted tissue (mg)	Recovery (100%)	
		MC-RR	MC-LR
Muscle	50	88.5±0.8	89.3±0.1
Liver	40	88.5±2.3	91.5±1.4
Blood	40	83.5±1.9	85.8±1.6
Kidney	30	95.5±1.4	97.5±1.2

the HLB (0.5 g) cartridge to concentrate toxins followed by use of the silica gel/plus silica gel tandem cartridge to remove the co-extracted materials. Chromatograms monitored for microcystins in spiked blood of *H. molitrix* were compared after the first clean-up with those after the second clean-up (Fig. 2). Following the first clean-up step (HLB cartridge) there were additional peaks which were removed by the second step (silica gel/plus silica gel tandem cartridge). The peaks then became clear enough for identification. The chromatograms showed that the treatment using these two types of silica cartridges was effective for eliminating co-existing substances other than microcystins. A chromatogram used for measurement of microcystins in the intestine tissue of *H. molitrix* (Lake Chaohu, China) is shown in Fig. 3. The peaks are clearly evident. LC/ESI–MS mass spectra indicated that microcystins-RR and -LR were present in the sample.

3.2. Confirmation of the extraction procedure

Recovery experiments were performed by spiking fish tissue with microcystins (MC-RR, MC-LR) at MC concentrations between 3.10 and 11.4 µg/g (Table 2). After being used three times for extracting microcystins in fish tissue, both types of cartridge still had a recovery of over 80%.

Cyanobacteria water blooms phenomena will often last year-round occurring in many eutrophic water-bodies when the climate permits, especially in some fishery ponds. Fish can be exposed to MC via the consumption of toxic cyanobacteria (Xie et al., 2004) or aquatic organisms that had previously accumulated MC in their tissues. The professional freshwater fishery biologist has been faced with growing problems due to metalimnic blooms and their toxins. Monitoring of microcystins in fish and other aquatic animals is important for evaluating the potential risk for human consumption. This study presented a reliable and sensitive method for extracting microcystins from tissues of fish.

Solid phase extraction (SPE) is typically used to enrich environmental concentrations of microcystins, or to eliminate contaminants from complex samples such as animal tissues (McElhiney and Lawon, 2005). In spite of considerable time and effort being spent on choosing an appropriated SPE sorbent and extraction protocol, there are still serious limitations for using these sorbents (Oasis® Applications Notebook). For instance, those involved with the analysis need to take great care to maintain control in the extraction procedure. Even with

Table 3

A comparison of the determination of microcystins with different kinds of methods

Methods	Cartridge	Sample	The amount of spiked MCs	Average recovery (%)	Extraction solvent	Reference
LLE (Liquid liquid extraction)	–	Zebra mussels	MC-LR: 0.1 and 5 µg/g	LR: 50±4.2	75% MeOH (v/v)	Pires et al. (2004)
LLE	–	Fish	MCs: 0.5–30 µg/g	MCs>92 RSDs<16	85% MeOH (v/v)	Moreno et al. (2005)
MSPD (Matrixsolid-phase dispersion)	C ₁₈ bonded porous silica	Rat	MCs: 1–10 µg/g	RR: 56.2±5.9 LR: 61.2±4.5	MeOH or 70% MeOH (v/v)	Ruiz et al. (2005)
SPE (Solid phase extraction)	Immunoaffinity	Fish	MCs: 0.5–4.0 µg/g	RR: 73 LR: 81	75% MeOH (v/v)	Lawrence and Menard (2001)
SPE	C-18	Fish	MC-RR: 5–20 µg/L	RR:>80%	70%MeOH:1%TFA (v/v)	Cazenave et al. (2005)
SPE	C-18	Fish	MC-LR: 10–100 µg/g	LR: 58±9.0	BuOH:MeOH:water (1:4:15) (v/v/v)	Ernst et al. (2005)
SPE	ODS and silica	Fish	MC-RR: 11.4 µg MC-LR: 3.1 µg	RR: 66.4±2.2 LR: 68.8±0.7	BuOH:MeOH:water (1:4:15) (v/v/v)	Present study
SPE	HLB silica gel/plus silica gel tandem	Fish	MC-RR: 11.4 µg MC-LR: 3.1 µg	RR: 89.0±1.6 LR: 91.0±1.1	BuOH:MeOH:water (1:4:15) (v/v/v)	Present study

– Means only used solvent.

all precautions, it is still difficult and time-consuming to achieve high, reproducible recoveries during analysis of important toxicant and metabolites.

The clean-up procedure reported previously by Harada et al. (1988) was developed for algal samples, but is not efficient for animal samples due to the presence of many more impurities. A modified method developed by Watanabe et al. (1997) enabled the detection of microcystins in freshwater mussels by using two types of cartridges (ODS and silica gel cartridges) for the clean-up procedure, but the method is time-consuming. Moreover, it did not give satisfactory results when applied to fish, as the recovery of microcystins in the spiked tissues was only about 60% (present study). Lawrence and Menard (2001) reported that anti-microcystin-LR immunoaffinity cartridges are effective for isolating microcystins from fish, but this cartridge has a finite capacity to retain microcystins (<0.03 µg/g). It is important that the sample extract passing through the cartridges contains less toxin than the cartridge capacity, otherwise losses will occur. Moreno et al. (2005) reported a simple and rapid solvent extraction procedure that did not require laborious pretreatment or clean-up. However, their chromatograms showed high variation in baseline noise, indicative of low reliability. Also they spiked the samples with too much MC (3300 µg/g) in their experiment. To date, the highest accumulated MC concentration in the aquatic animals is 1387 µg/g DW (Watanabe et al., 1992). Ernst et al. (2005) described the recovery of MC from fish liver using the colorimetric protein phosphatase inhibition (cPPA) method and yielded high recovery (97%). However, they concluded that this unspecific PP inhibition causes an overestimation of the MC contamination in tissues in general and of MC-LR contamination in fish liver homogenates specifically, and therefore is inappropriate for routine MC detection in tissue samples. The present study showed that the HLB and silica gel/plus silica gel tandem cartridge provides excellent cleaning-up efficiency for samples of microcystins from fish tissue, involving much lower amounts of organic solvent in the clean-up procedure.

Table 3 compares the recovery rates of the different methods in extracting microcystins present in animal tissue. The method presented in this paper ranks the first in the SPE methods and is therefore recommended as the method of choice for extracting microcystins from fish tissue.

4. Conclusion

In conclusion, this report has presented a simple and rapid solid phase extraction procedure, followed by

conventional HPLC, for determining microcystins in fish tissue. Our method proved to be of value for monitoring microcystins in fish tissue, since it yielded high recovery (>85%) and consistency (SD<5%).

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