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Simple Assay for Analyzing Five Microcystins and Nodularin in Fish Muscle Tissue: Hot Water Extraction Followed by Liquid Chromatography−Tandem Mass Spectrometry

SARA BOGIALLI,† MILENA BRUNO,§ ROBERTA CURINI,† ANTONIO DI CORCIA,*,† ALDO LAGANÁ,† AND BARBARA MARI†

Dipartimento di Chimica, Università "La Sapienza", Piazza Aldo Moro 5, 00185 Roma, Italy, and Dipartimento d'Igiene Ambientale, Istituto Nazionale di Sanità, Viale Regina Elena 299, 00161 Roma, Italy

A simple, specific, and sensitive procedure for determining six cyanotoxins, that is, microcystins RR, LR, YR, LA, and LW and nodularin, in fish muscle tissue is presented. This method is based on the matrix solid-phase dispersion technique with heated water as extractant followed by liquid chromatography (LC)−tandem mass spectrometry (MS) equipped with an electrospray ion source. Target compounds were extracted from tissue by 4 mL of water acidified to pH 2 and heated at 80 °C. After acidification and filtration, 0.2 mL of the aqueous extract was injected in the LC column. MS data acquisition was performed in the multireaction monitoring mode, with at least two precursor ion > product ion transitions selected for each target compound. Analyte recovery ranged between 61 and 82% and was not substantially affected by either the analyte concentrations or the type of fish. The nonexcellent recovery of some of the microcystins was traced to binding of these compounds to protein phosphatases in fish tissue occurring during sample treatment. The existence of covalently bound microcystins in fish has been evidenced by several studies. Compared to an older sample preparation procedure, this one extracted larger amounts of the analytes in a simpler and much more rapid way. On the basis of a signal-to-noise ratio of 10, limits of quantification were estimated to range between 1.6 and 4.0 ng/g. The effects of temperature and volume of the extractant on the analyte recovery were studied.

KEYWORDS: Microcystins; nodularin; cyanotoxins; fish; muscle tissue; LC-MS/MS

INTRODUCTION

Heavy blooms of cyanobacteria (blue-green algae) are one of the consequences of the worldwide trend of increasing eutrophication in many waters. This phenomenon is thought to result from increased exogenous nutrient loadings. In addition to the ecological problems related to cyanobacteria blooms, these algae produce a wide range of secondary metabolites, some of which are known to be toxic to human and animal health (*1*). Among cyanobacteria, the microcystins (MCs) are the most investigated, but nodularin is also partly characterized (*2*, *3*−*6*). The toxicity of nodularin and MCs is due to severe inhibition of protein phosphatases 1 (PP-1) and 2A (PP-2A), causing mainly functional and structural disturbances of the liver (*7*, *8*). In addition to the hepatotoxicity, nodularin has also been reported as a direct carcinogen in rat liver and not only as a tumor promoter like the MCs (*9*).

Bioaccumulation of cyanotoxins by aquatic animals, including fishes and mollusks, is well documented (*10*−*14*). In these studies, the authors outlined the risk for human health associated with the consumption of cyanotoxin-contaminated fishes. Therefore, in addition to direct ingestion of water contaminated by cyanotoxins, another route of exposure of humans to these toxins is the consumption of aquatic animals that have ingested cyanobacteria and accumulated their toxins. Cyanotoxins are rarely ingested by man in amounts large enough to reach a lethal acute dose, but damages provoked by chronic effects are rather probable if exposure is frequent and prolonged for long periods. On the basis of various considerations, the World Health Organization (WHO) established 40 ng/kg of body weight as a tolerable daily intake of MC LR (*15*). Therefore, it is of importance to monitor fishes and other aquatic animals living in water bodies where toxic cyanobacteria species are present.

Several nonchromatographic analytical techniques for cyanotoxins have been proposed. These include mouse and invertebrate bioassays (*15*), protein phosphatase inhibition assays (*15*, *16*), and immunoassays (*15*, *16*−*18*). All of the reported methods have deficiencies that limit their application to large-

* Author to whom correspondence should be addressed (fax +39-06-49913680; e-mail antonio.dicorcia@uniroma1.it.
† Università "La Sapienza".
§ Istituto Nazionale di Sanità.

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scale occurrence surveys and human exposure studies. Briefly, the mouse bioassay is time-consuming and cannot distinguish among a large number of potential toxins. Like immunoassays, the phosphatase inhibition assay cannot distinguish among the various toxicants. Moreover, it was recently reported (*19*) that MC LR concentrations measured by ELISA in livers of treated animals were >1000 times higher than those measured by liquid chromatography (LC) and gas chromatography (GC)−mass spectrometry (MS), indicating that ELISA results were almost certainly due to cross-reactivity with something other than intact MC LR.

In recent years, LC coupled to MS has become the technique of choice for analyzing cyanotoxins in a variety of matrices. With LC-MS/MS, the multiple-reaction monitoring (MRM) data acquisition technique provides great signal/noise enhancement, selectivity, and quantitation capabilities. Although the potential of LC-MS/MS has been well exploited for analyzing cyanotoxins in water bodies (*20*−*22*), only one method based on LC-MS/MS has been elaborated for measuring nodularin and demethylnodularin in mussels and flounders (*23*).

Various sample preparation methods have been employed for analyzing cyanotoxins in tissue samples. Erikssson et al. (*24*), Vasconcelos (*13*), Xie et al. (*25*), Kankaanpää et al. (*26*), and Karlson et al. (*23*) made use of multiple extraction with butanol/methanol/water mixtures of various compositions for isolating cyanotoxins from aquatic animal tissues. Extraction was assisted by ultrasonication with times varying from 0.5 (*13*, *24*) to 8 h (*23*, *26*) or by stirring for 24 h (*25*). Orr et al. (*19*) used overnight extraction with a phosphate buffer (pH 6.8) to isolate MC LR from beef liver tissue. Pure methanol was used by Magalhães et al. (*27*) for extracting MCs from fish liver and muscle tissues. To make extracts amenable to LC analysis, the authors cited above made use of a single cleanup step by using a C-18 cartridge with the exception of Xie et al. (*25*), who adopted an additional purification step by using a silica gel cartridge. All of the above sample preparation methods are rather laborious and time-consuming because they were not thought to be methods of routine use but rather as a means for occasionally studying the effects on the animal life of the ingestion of cyanotoxins.

After the pioneering work of Barker and his colleagues (*28*), many researchers have successfully adopted the so-called matrix solid-phase dispersion (MSPD) technique for extracting xenobiotics from solid biological matrices (*29*). A fine dispersion of the biological matrix onto a solid support such as silica, alumina, diatomaceous earth, C-18-bonded silica, and other sorbents is easily obtained by blending the sample and the sorbent with a mortar and pestle. After blending, this material is packed into a minicolumn, and analytes are eluted by a suitable extractant. The abrasive action of the sorbent during blending has been demonstrated to disrupt the gross architecture of the matrix (*29*), so that a tight and quasi-homogeneous layer of the matrix components is formed on the sorbent surface. Over classical sample treatment procedures, MSPD offers distinct advantages in that (1) the analytical protocol is drastically simplified and shortened, (2) the possibility of emulsion formation is eliminated, (3) consumption of toxic, flammable, and expensive solvents is substantially reduced, and (4) last but not least, the extraction efficiency of the analytes is enhanced as the entire sample is exposed to the extractant.

Recently, we have proposed a rapid method for determining 12 sulfonamide antibacterials in fish tissue (*30*). This method is based on analyte extraction from the matrix dispersed on sand by hot water followed by extract filtration and injection of a large aliquot of the final extract on a LC column. Detection of the analytes was performed by a MS system equipped with an ESI ion source and a single quadrupole.

The aim of this work has been that of adopting the above analytical strategy for determining microcystins RR, LR, YR, LA, and LW and nodularin in fish muscle tissue. In this study, selective and sensitive detection was performed by tandem MS in the MRM mode.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals. MC RR, MC YR, MC LR, MC LA, and MC LW were from Calbiochem (La Jolla, CA). Nodularin was from ICN Biomedicals Inc. (Aurora, OH). Trimethacarb, an obsolete carbamate insecticide, was obtained from Riedel-de-Haen and used as internal standard (IS). Structures of the analytes are shown in **Figure 1**.

For each analyte, individual standard stock solutions of each analyte were prepared by dissolving them in methanol to obtain final concentrations of 25 μg/mL. After preparation, MCs and nodularin solutions were stored at −18 °C to minimize their decompositions. A 1 mg/mL IS solution was prepared by dissolving 100 mg of trimethacarb in 100 mL of methanol and stored at 4 °C. For recovery studies, a single working composite standard solution was prepared by combining aliquots of each of the six individual stock solutions and diluting with methanol to obtain a final concentration of 1.0 μg/mL.

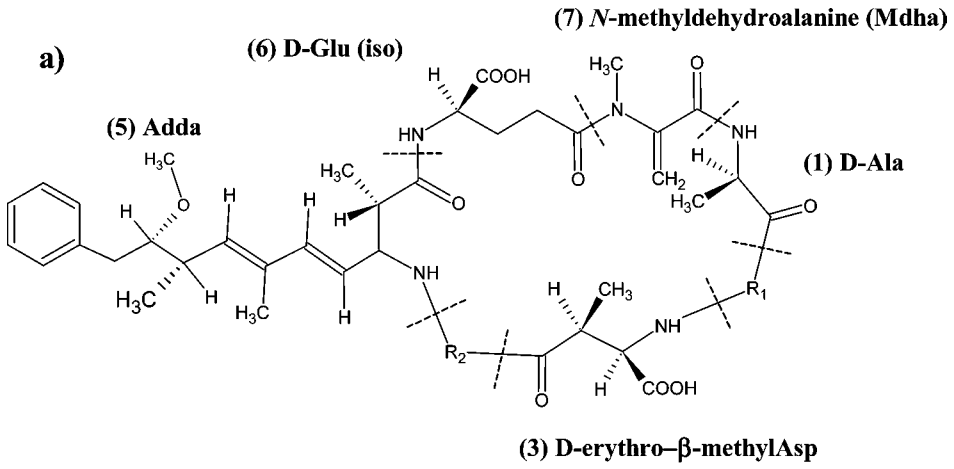
Sand (Crystobalite, 40−200 mesh size) was provided by Fluka AG, Buchs, Switzerland. Acetonitrile "Plus" of gradient grade and RS grade methanol were obtained from Carlo Erba, Milano, Italy.

Fish Samples. Trout, mullet, perch, and goldfish samples were from retail markets. Preliminary analyses showed they were analyte-free. These fish species were chosen because they live in sites, such as lakes or basins designed for aquaculture, which, being prone to eutrophication, may contain algal toxins.

Extraction Apparatus. The design of the homemade extraction apparatus used in this work was very similar to that shown in a previous paper (*31*), with the exception that the analyte-containing water leaving the extraction cell was collected in a calibrated glass tube instead of a sorbent cartridge. An 8.1 cm × 8.3 mm i.d. stainless steel column was used as extraction cell.

Prior to blending with sand, fish fillet samples were finely diced with scissors. For recovery studies, 1 g of tissue was put in a porcelain mortar and spiked with variable volumes of the partially aqueous working standard solution, taking care to uniformly spread it on the sample. An intimate contact between the analytes and the sample was obtained by pounding with the pestle for some minutes. Then, 1 h was allowed for equilibration, storing the mortar at 4 °C. Thereafter, 5 g of sand was added to the mortar, and the mixture was blended with the pestle for ~10 min, until an apparently dry material was obtained. This material was then packed into the extraction cell, taking care to tap the tube to avoid loose packing of the particles. Any void space remaining after the solid material had been packed was filled with sand. A stainless steel frit (2 μm pore size) and a polyethylene (20 μm pore size) frit were located, respectively, above and below the mixture. The tube was then put into the oven and heated at 80 °C for 5 min. Water acidified to pH 2 (HCl) was then passed through the cell at a 1 mL/min flow rate to extract the analytes, and 4 mL of it was collected in a tube. After extraction, 15 ng of the IS was added, and then the extract was acidified to pH 3.1 with 10 mol/L formic acid and filtered through a regenerated cellulose filter (0.2 μm pore size, 25 mm diameter, Alltech, Sedriano, Italy) to prolong the life of the guard column. By doing so, the guard column was replaced with a new one after >150 injections of fish extracts. Two hundred microliters of the final extract was injected into the LC column.

LC-MS/MS Analysis. The liquid chromatograph consisted of a Waters pump (model 1525μ, Milford, MA), a 200 μL injection loop, and Alltima HP 5 μm C-18 guard (7.5 × 4.6 mm i.d.) and analytical (250 mm × 4.6 mm i.d.) columns (Alltech) thermostated at 35 °C and was interfaced to a benchtop triple-quadrupole mass spectrometer (model Micromass 4 MICRO API, Waters). Mobile phase component



Microcystin	R_1	R_2	MW
MC-RR	Arg	Arg	1037
MC-YR	Tyr	Arg	1044
MC-LR	Leu	Arg	994
MC-LA	Leu	Ala	909
MC-LW	Leu	Trp	1024

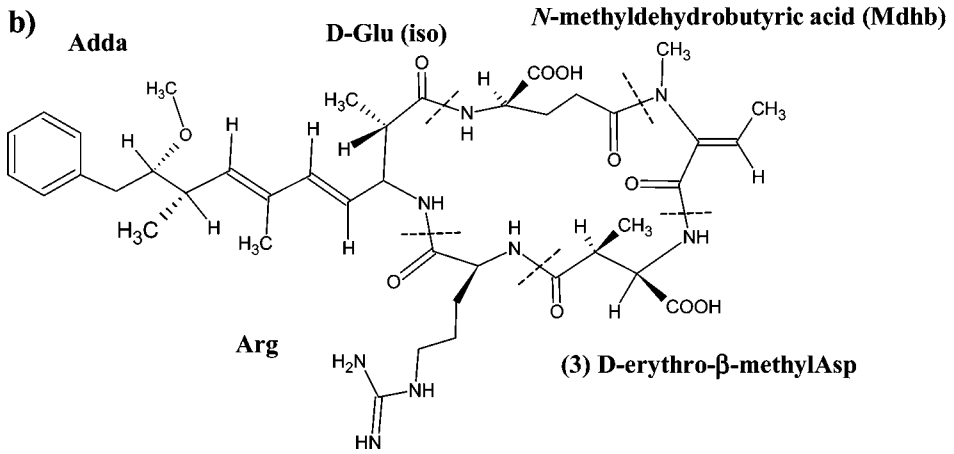


Figure 1. Chemical structures of selected cyanotoxins: (**a**) microcystins; (**b**) nodularin.

A was 10 mM formic acid in acetonitrile, and component B was aqueous 10 mM formic acid. At 1.0 mL/min, the mobile phase gradient profile was as follows (t in minutes): t_0, A = 0%; t_5, A = 0%; t_6, A = 35%; t_{11}, A = 45%; t_{12}, A = 57%; t_{17}, A = 67%; t_{18}, A = 100%; t_{20}, A = 100%; t_{21}, 0%; t_{30}, A = 0%. Analyte retention times varied by ≤0.5% over 2 weeks. A diverter valve led 400 μL/min of the LC column effluent into the ion source that was operated in the PI mode only between 10 and 22 min of the chromatographic run. High-purity nitrogen was used as drying and curtain gases; high-purity argon was used as collision gas. Nebulizer gas was set at 650 L/h, whereas the cone gas was set at 50 L/h; the probe and desolvatation temperatures were maintained, respectively, at 100 and 350 °C. The setting for the gas pressure in the collision cell was set at 3 mTorr. Capillary voltage was 3000 V, and extractor voltage was 0 V. Declustering potential, collision energy, and other transmission parameters were optimized for each analyte (data are reported in **Table 1**). Mass axis calibration of each mass-resolving quadrupole Q_1 and Q_3 was performed by infusion of a sodium and cesium iodide solution at 10 μL/min. Unit mass resolution was established and maintained in each mass-resolving quadrupole by keeping a full width at half-maximum of ∼0.7 amu. All of the source and instrument parameters for monitoring cyanotoxins were optimized by standard solutions of 5 μg/mL infused at 10 μL/min by a syringe pump. The multiple reaction-monitoring (MRM) mode was used for quantitation by selecting at least two fragmentation reactions for each analyte (**Table 1**).

Quantitation. Recovery of each analyte added to fish tissue samples at any given concentration was assessed by summing the ion current profiles relative to the transitions considered, normalizing them to the peak area of the IS, and comparing these ratios to those obtained by injecting a blank sample extract to which the analytes were added postextraction. We followed this procedure to obviate matrix effects that weakened the analyte ion signal intensities, especially those of MC RR and MC YR. The mass spectrometry data handling system used was the Mass Lab software from Waters.

RESULTS AND DISCUSSION

Recovery Studies. When not explicitly mentioned, recovery studies were conducted by spiking trout fillets samples with analytes at 50 ng/g and analyzing. Following conditions reported elsewhere (*30*), initial extraction experiments were performed by using pure water heated at 80 °C as extractant (**Table 2**). Under this condition, poor recovery of the analyte was observed.

Table 1. Time-Scheduled Selected Reaction Monitoring Conditions for Detecting Selected Cyanotoxins

compound	MRM transition,[a] *m/z*	cone voltage, V	collision energy, eV	retention window, min	dwell time, s
microcystin RR	**520** → 135	35	35	0–15.5	0.2
	520 → 887		24		0.2
nodularin	*825* → 135	45	60	15.5–19.0	0.1
	825 → 781		40		0.1
microcystin YR	**523** → 135	18	15		0.1
	523 → 910	18	10		0.1
	1045 → 135	70	70		0.1
microcystin LR	**498** → 135	25	15		0.1
	995 → 135	70	60		0.1
	995 → 599	70	40		0.1
internal standard	*194* → 137	25	10	19–23.5	0.2
microcystin LA	*910* → 402	35	25		0.2
	910 → 776				0.2
microcystin LW	*1025* → 1007	30	30		0.2
	1025 → 890				0.2

[a] Single charged precursor ions are reported in italic, whereas double-charged precursor ions are reported in boldface.

Table 2. Extraction Efficiency of Selected Cyanotoxins in a Trout Fillet Sample at the 50 ng/g Level and Various pH Values of the Extractant (Water at 80 °C)

compound	recovery[a] (RSD)		
	H_2O	H_2O (pH 2)	H_2O (pH 1.5)
microcystin RR	41 (3)	62 (6)	47 (6)
nodularin	45 (8)	80 (5)	48 (6)
microcystin YR	51 (8)	76 (9)	39 (9)
microcystin LR	49 (7)	71 (9)	37 (8)
microcystin LA	50 (8)	72 (9)	35 (9)
microcystin LW	51 (4)	63 (4)	40 (6)

[a] Mean values from four experiments.

This unsatisfying result was traced to the presence on the siliceous material (sand) supporting the biological matrix of silanols able to strongly bind compounds bearing amino groups, such as cyanotoxins. Protonation of the amino groups by extraction with heated water acidified to pH 2 increased remarkably the recovery of the analytes. Attempts to extract larger amounts of cyanotoxins, in particular of MC RR and MC LW, by further decreasing the pH of the water failed, presumably because of analyte decomposition.

By progressively increasing the temperature, water becomes more and more effective in extracting organic compounds from solid matrices as a result of its decrease in polarity (*32*). On the other hand, a risk inherent to the use of hot water as extractant is that it could decompose those compounds that are thermolabile and/or prone to hydrolytic attack. With the aim of enhancing the recovery of cyanotoxins in fish tissue obtained by extracting them with acidified water at 80 °C, we performed two recovery experiments in which the extraction temperature was in one case lower and in the second case higher than 80 °C. At each temperature, four extractions were carried out, and results are reported in **Figure 2**. As can be seen, the best results were those obtained by extraction at 80 °C. Presumably, the low extraction yield observed at 100 °C was due to decomposition of cyanotoxins by pH 2 acidified water.

According to our previous work (*30*), initial recovery experiments were performed by using 4 mL of extractant. Additional recovery studies of cyanotoxins in fish tissue were conducted

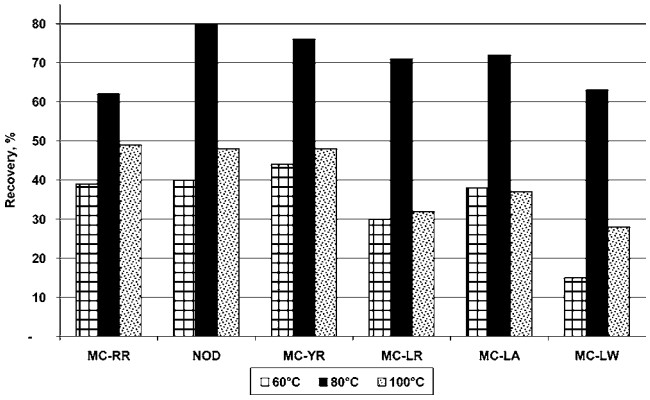


Figure 2. Recovery of trout fillet spiked with 50 ng/g of cyanotoxins by varying the extraction temperature. MC, microcystin; NOD, nodularin. Extractant volume was 4 mL.

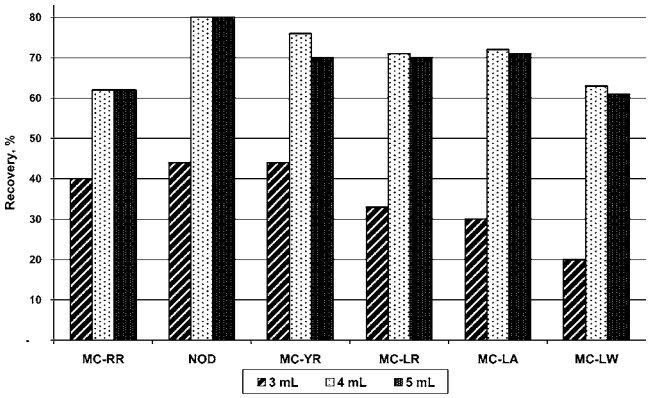


Figure 3. Recovery of trout fillet spiked with 50 ng/g of cyanotoxins by varying the extractant volume (water heated at 80 °C). MC, microcystin; NOD, nodularin.

by both increasing and decreasing the extractant volume mentioned above. The experiment performed by using a larger water volume had the obvious purpose of ascertaining if a larger water volume successfully extracted larger amounts of cyanotoxins, whereas the rationale behind the use of a lower extractant volume was that of extracting the maximum amount of analytes with a minimum extractant volume. Because this method does not include any concentration step of the extract, the extractant volume influences directly the sensitivity of the method. At any extractant volume considered, four experiments were performed, and the results are visualized in **Figure 3**. As can be seen, the use of only 3 mL of extractant gave unacceptably low recovery of all of the analytes, whereas no significant increase of the extraction yield was observed by extraction with 5 mL of the extractant.

To determine if recovery rates were affected by the concentrations of cyanotoxins in fish tissue, we spiked four samples of trout fillets with various amounts of cyanotoxins. Data reported in **Table 3** show that recovery of the analytes was not substantially affected by their concentrations.

We evaluated if the type of fish could affect recovery of target compounds. For this purpose, we spiked trout, perch, mullet, and goldfish fillet samples with 50 ng/g of the target compounds. Results from quadruplicate experiments for each type of fish considered are reported in **Table 4**. It can be seen that recovery of the analytes was not substantially dependent on the type of matrix.

Method Comparison. Williams et al. (*14*, *33*, *34*) had direct evidence for the fact that remarkable portions of microcystins in fish tissues exist as covalent complexes with the PP-1 and

Table 3. Recovery of Cyanotoxins in a Trout Fillet Sample at Three Different Analyte Concentrations

	recovery [a] (RSD) at		
compound	25 ng/g	50 ng/g	100 ng/g
microcystin RR	63 (5)	62 (6)	65 (5)
nodularin	76 (7)	80 (5)	79 (6)
microcystin YR	76 (10)	76 (9)	72 (7)
microcystin LR	75 (6)	71 (9)	70 (5)
microcystin LA	70 (8)	72 (9)	73 (8)
microcystin LW	66 (6)	63 (4)	62 (6)

[a] Mean values from four measurements.

Table 4. Recovery of Cyanotoxins Added to Muscle Tissues of Four Different Types of Freshwater Fishes at the 50 ng/g Level

	recovery[a] (RSD)			
compound	trout	goldfish	mullet	perch
microcystin RR	62 (6)	61 (9)	66 (7)	65 (6)
nodularin	80 (5)	76 (7)	75 (8)	82 (8)
microcystin YR	76 (9)	72 (8)	76 (10)	75 (8)
microcystin LR	71 (9)	67 (8)	73 (5)	69 (5)
microcystin LA	72 (9)	70 (7)	66 (8)	73 (8)
microcystin LW	63 (4)	66 (5)	62 (7)	70 (8)

[a] Mean values from four measurements.

Table 5. Mean Recovery of Cyanotoxins Added to a Trout Muscle Sample at the 50 ng/g Level Following Two Different Sample Treatment Protocols

	recovery[a] (RSD)	
compound	method A[b]	this method
microcystin RR	53 (5)	62 (6)
nodularin	57 (5)	80 (5)
microcystin YR	54 (8)	76 (9)
microcystin LR	52 (4)	71 (9)
microcystin LA	59 (7)	72 (9)
microcystin LW	51 (8)	63 (4)

[a] Mean values from four measurements. [b] From ref *27*.

PP-2A enzymes. We recently observed that sulfaquinoxaline, a sulfonamide antibacterial, added to bovine liver was rapidly converted by enzymatic oxidation to a more polar compound during the operation of deposition of the matrix on the siliceous support (*35*). On the basis of these two pieces of evidence, we took into consideration that the nonexcellent recovery data of MCs could be traced to a rapid partial binding of MCs to PP-1 and PP-2A enzymes occurring during deposition of the fish tissue on sand, rather than to a failure of our extraction method. For this reason, we compared our sample treatment method with one quoted in the literature and developed by Magalhães et al. (*27*). It has to be noted that the latter sample treatment is similar to other ones used by several researchers (*13*, *23*−*26*). Briefly, the method followed by Magalhães et al. is based on double extraction with pure methanol followed by defatting with hexane. After methanol removal, the residue was reconstituted with water, and this solution was passed through a C-18 cartridge. Microcystins were eluted with 30 mL of 20% methanol and 50 mL of pure methanol. With both of the two extraction protocols, four measurements were performed, and results in terms of analyte recovery are shown in **Table 5**. Compared to our extraction procedure, it appears that the conventional procedure gave even lower recovery of microcystins. Thus, we assumed that a fraction of microcystins added to fish tissue is rapidly and irreversibly bound to PP-1 and PP-2A enzymes and that only unreacted microcystins can be recovered by any extraction procedure. From an analytical point of view, it has to be pointed out that our method, in addition to being much simpler and more rapid, is able to extract larger amounts of cyanotoxins than a conventional sample extraction procedure.

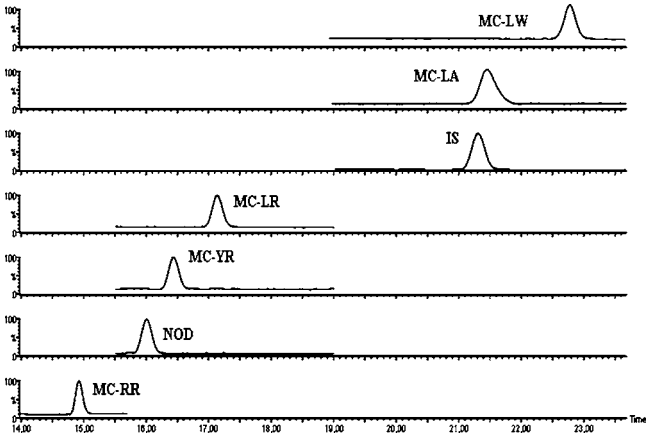


Figure 4. Time-scheduled LC-ESI/MS/MS MRM chromatogram resulting from the analysis of a trout fillet spiked with 25 ng/g of cyanotoxins. MC, microcystin; NOD, nodularin.

Table 6. Limits of Detection (LODs) and Quantification (LOQs) of the Method for Determining Cyanotoxins in Fish Fillet

compound	LOD, ng/g	LOQ, ng/g
microcystin RR	1.6 (520 → 887)	1.6
nodularin	1.5 (825 → 807)	1.6
microcystin YR	2.1 (523 → 135)	3.9
microcystin LR	2.3 (995 → 599)	4.0
microcystin LA	1.9 (910 → 402)	3.1
microcystin LW	1.7 (1025 → 890)	3.8

Limits of Detection (LODs) and Quantification (LOQs). LOQs of the method were estimated from a MRM LC-MS-MS chromatogram shown in **Figure 4** and resulting from analysis of a trout muscle tissue extract spiked with cyanotoxins at the 25 ng/g level. After extraction of the sum of the ion currents of the transitions selected for each analyte, the resulting trace was smoothed twice by applying the mean smoothing method (Mass Lab software, Waters). Thereafter, the peak height-to-averaged background noise ratio was measured. The background noise estimate was based on the peak-to-peak baseline near the analyte peak. LOQs were then calculated on the basis of a minimal accepted value of the signal-to-noise ratio (S/N) of 10. These data are listed in **Table 6**. In the same table, LODs of the method are also presented. When detection with a MS/MS arrangement is performed, the most important condition to be satisfied for ascertaining the presence of a targeted compound is that at least two precursor ion → product ion transitions give signals distinguishable from the background ion current. Accordingly, a definition of LOD (S/N 3) of each analyte was adopted, considering in each case the transition giving the worst S/N. When more than two transitions were selected for analyte identification, the LODs were estimated by selecting the two signals giving the best S/N ratios. It can be read that cyanotoxins can be detected in fish tissue at a few nanograms per gram levels.

Linear Dynamic Range. Under the instrumental conditions reported under Experimental Procedures, the linear dynamic range of the ESI/MS/MS detector was estimated for all of the analytes. Amounts of each analyte varying from 0.2 to 600 ng

and a constant amount of 0.75 ng of the internal standard were injected from suitably prepared standard solutions into the LC column. At each analyte amount, three replicate measurements were made. Signal against amount-injected curves were then constructed by averaging the peak area resulting from the sum of the signals for the two parent ion → daughter ion transitions of each analyte and relating this area to that of the internal standard. Results showed that ion signals of the six cyanotoxins were linearly correlated with injected amounts up to 400 ng, with R^2 ranging between 0.9957 and 0.9987.

Conclusions. This work has again shown that environmentally friendly and inexpensive water, besides being an effective extractant for free cyanotoxins in fish tissue, produces sufficiently clean extracts requiring little manipulation before final analysis by LC-MS. Besides being highly specific, the ESI/MS/MS detector provides sensitivity for analyzing cyanotoxins in fish fillets at levels of a few nanograms per gram.

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