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Short communication

Determination of microcystins in fish by solvent extraction and liquid chromatography

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Abstract

A liquid chromatography electrospray mass spectrometry (LC/ESI/MS) method has been developed to identify and quantify microcystins in fish liver and intestine. Microcystins (MCs) were extracted from 500 mg sample with methanol–water (85:25, v/v) and the extracts concentrated to 250 μl. The parameters were optimized by a full factorial 2^3 design. Neither laborious pre-treatment nor clean up were necessary. MCs were separated using conventional C_{18} column and an acetonitrile–acidified water (pH 3) gradient. Negative samples (without MCs) were discriminated by liquid chromatography diode array detection (LC/DAD). The limits of detection (LOD) and the limits of quantification (LOQ) resulted equal for MC-RR, MC-YR, and MC-LR and were 0.1 and 0.5 μg g^{-1}, respectively. MCs recoveries at three levels in spiked samples (0.5–3.0 μg g^{-1}) were >92%, with relative standards deviations (RSDs) <16% for liver samples and >68% with RSDs <18% for intestine samples. The proposed method was applied to determine MC-LR in exposed fish to evaluate the bioaccumulation risk. The results showed the transference of MC-LR from cyanobacterial cells to fish tissues.

Keywords: Microcystins; Solvent extraction; Liver; Intestine; Fish; LC/MS; LC/DAD

1. Introduction

In addition to the implication of being widespread and often bloom forming, many cyanobacterial species share the ability to produce toxins [1,2]. Microcystins (MCs), the most abundant of those cyanotoxins, are cyclic heptapeptides with severe hepatotoxic action. There are over 70 MCs, which structurally differ in the nature of the two L-amino acids and in the degree of methylation, hydroxylation, and/or epimerization [1,3]. Their bioactivity is mainly based on an inhibition of eukaryotic protein phosphatases, being potentially harmful for higher organisms and humans [4].

MCs are usually associated to freshwater environments, and their bioaccumulation by aquatic animals, including zooplankton, fish, and molluscs, has been reported by several authors [5–8]. Liver, followed by kidney and intestine accumulate most of the MCs administered in fish; small amounts of toxins were also detected in the muscles [6,7,9]. Because these organisms are an important food source, not only for birds and fish but also for mammals, including humans, MCs can be transferred to the higher trophic level through the food chain leading to human toxicity. Consequently, it is important to monitor MCs in fish and other aquatic animals in order to evaluate the potential risk for human health derived from their consumption.

There is a great diversity of analytical methods to detect and identify cyanotoxins that comprise protein phosphatase inhibition assays [10], enzyme linked immunosorbent assay

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(ELISA) [11], liquid chromatography (LC) [12–14], and capillary electrophoresis (CE) [15,16]. LC combined with different detectors, such as diode array detector (DAD) or mass spectrometry (MS), can identify and quantify MCs in freshwater [12], natural blooms [13], fish and shellfish [17], and biological samples [14].

The aim of this study was to develop an analytical procedure, based on solvent extraction and liquid chromatography electrospray mass spectrometry (LC/ESI/MS), for the simultaneous determination of MC-LR, MC-RR and MC-YR in fish liver and intestine. The extraction procedure was optimized using a two-level full factorial design with replications. Samples were firstly analyzed by liquid chromatography with diode array detection (LC/DAD) for discriminating negative samples. The method was applied to identify and quantify MC-LR in liver and intestine excised from Tilapia fish (*Oreochromis* sp.) cultivated under laboratory conditions, and exposed to natural blooms containing MC-LR. There are more than 70 MCs and this study covers only three. However, the selected ones are the most ubiquitous, produced in largest amounts by the cyanobacteria, and the only for which there are commercially available standards.

2. Experimental

2.1. Reagents and materials

Microcystins (MC-LR, MC-YR, MC-RR) were obtained as analytical standards from Calbiochem-Novabiochem (Nottingham, UK). Stock solutions of each MC, at a concentration of 500 $\mu g\,ml^{-1}$, and the appropriate standard working solutions were prepared in methanol and stored in glass-stopper bottles at 4 °C. HPLC-grade methanol and acetonitrile, and trifluoroacetic acid (TFA) for UV spectroscopy were purchased for Merck (Darmstadt, Germany). Deionized water (>18 $M\Omega\,cm^{-1}$ resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, USA).

Statistical analysis of all results by full factorial design 2^3 was performed with the statistical package, Statistica 99, from Statsoft (Alges, Portugal).

2.2. Exposition study

Oreochromis sp. (Nile tilapia, Perciformes: Cichlidae) males with mean body mass (BM) of 49.92 ± 9.1 g weight were obtained from a laboratory stock. Two groups of five individuals each (experimental and control) were kept in separate test aquariums (96 l) at constant pH of 7.6–7.7 in a temperature-controlled room at 21 ± 2 °C.

The test substance MC-LR was from disrupted cyanobacterial cells (lyophilized blooms of cyanobacteria from the Guadiana River in Mértola, Portugal). The concentration of MC-LR, 3300 $\mu g\,g^{-1}$, was determined in a previous study [13]. After a fortnight of acclimatization, the experimental group ($n = 5$) was exposed to cyanobacterial cells (ca. 60.0 μg MC-LR $fish^{-1}\,day^{-1}$) through the diet (0.3 g day^{-1} fish food and toxic cells), for 21 days. Control group ($n = 5$) was treated only with the commercial fish food for the same period. After the exposure time, the fishes were anaesthetized with tricaine and killed by transection of the spinal cord. Liver and intestine were removed, weighed, rinsed with ice-cold saline solution, and kept at −20 °C until analysis.

2.3. Solvent extraction procedure

After optimisation of several variables through a full factorial design 2^3, the following extraction procedure was adopted: *Oreochromis* sp. liver or intestines samples (500 mg) extracted with 15 ml of methanol 85% by homogenizing for 1 min using a Polytron (Glen Mills, Clifton, NJ) and centrifuging at 4500 rpm for 10 min at 18 °C. The supernatant was transferred to a clean glass flask. The residue was re-extracted with 10 ml of the methanol 85%. The extract was evaporated at 35 °C to dryness using a rota-evaporator, and redissolved in 0.25 ml of methanol. An aliquot of 20 μl was injected onto the LC-system. Extraction efficiencies were determined by spiking fresh tissue samples with volumes of 250 μl of the working mixtures at appropriate concentrations.

2.4. Chromatographic conditions

LC/DAD was carried out with a Varian (Palo Alto, CA, USA) system equipped with a tertiary solvent pump (9012), and a PDA detector (ProStar 330) set at 238 nm, and a Galaxy analytical work station (Varian). The analytes were separated at a flow rate of 1 ml min^{-1} through a LiChrospher C_{18} (250 mm × 4.6 mm I.D., 5 μm) stainless steel column with a guard column LiChropher RP-18 (4 mm × 4.6 mm, 5 μm), both from Merck (Darmstadt, Germany), with the following gradient: from 90/10 acetonitrile/water with 0.05% TFA to 60/40 in 20 min and held for 5 min. Then, the system returned to the initial conditions in 5 min.

LC/MS analysis was achieved with a Hewlett Packard (Palo Alto, CA, USA) HP-1100 Series LC/MSD system equipped with a binary solvent pump, an autosampler, an electrospray ionization (ESI) interface, a single quadrupole MS, and a computer with HP Chem Station. Analytical column and mobile phase were the same but the gradient selected was acetonitrile 35%, linearly increased to 65% in 15 min and held at 65% for 5 min at flow rate of 0.4 ml min^{-1}. The ESI/MS interface in positive mode operated at 350 °C gas temperature, 13.0 l min^{-1} drying gas flow, 60 psi nebulizer gas pressure and 4000 V capillary voltage. MS detection was performed in selected ion monitoring (SIM), recording from 0 to 13 min, *m*/*z* 519.9 at 120 V fragmentor voltage for MC-RR; and from 13 to 20 min, *m*/*z* 991.5 and 1045.5 for MC-YR, and *m*/*z* 861.5 and 995.6 for MC-LR at 180 V fragmentor voltage. SIM acquisition was carried out at gain EMV of 2 and using the high resolution setting.

3. Results and discussion

3.1. Optimization of the extraction procedure

The extraction procedure was optimized to achieve a reduction of the analysis time and analyte losses, common in sample manipulation, by applying three factors, two-level full factorial design with replications [18]. The considered factors were methanol concentration (X_1), solvent volume (methanol) (X_2) and homogenization time (X_3). The variable response (Y) was the extraction recovery in % of each MC. The levels are coded according to the rule: high level = + 1 (X_1 = 100%; X_2 = 25 + 15 ml; X_3 = 3 min), low level = −1 (X_1 = 85%; X_2 = 10 + 15 ml; X_3 = 1 min). Results showed that the factor X_1 was never significant, accordingly methanol concentration from 85 to 100% leads to the same results. The alcohol volume was significant because the coefficient b_2 was always negative. The best results were obtained extracting twice consecutive with 15 and 10 ml of methanol. The homogenization time was only significant for the extraction of MC-YR, and its coefficient was negative after 1 min of homogenization. Thus, the experiment should be performed at −1 level for the three factors, leading to the best recoveries for the three MCs.

3.2. Validation of the extraction procedure

Recovery experiments were performed in quintuplicate spiking 500 mg of fish liver or intestine with MCs fortification solution of the three commercial standards available (MC-RR, MC-YR and MC-LR) at three levels, between 0.5 and 3 μg g^{-1}. For liver, recoveries ranged from 95 to 105% for MC-RR, from 72 to 104% for MC-YR, and from 87 to 99% for MC-LR with RSDs between 5 and 16%. For intes-

Table 1
Matrix calibration of fish liver and intestine in comparison with standard calibration by LC/DAD and LC/ESI/MS

	MC-RR			MC-RR			MC-RR		
	Slope	*y*-Intercept	*r*	Slope	*y*-Intercept	*r*	Slope	*y*-Intercept	*r*
LC/DAD									
Standard	524432	167282	0.996	545139	75448	0.998	674162	61263	0.996
Liver	533198	180861	0.996	471885	102111	0.991	772377	77446	0.998
Intestine	556212	71463	0.997	563413	107477	0.996	713978	83909	0.999
LC/ESI/MS									
Standard	14028	2914	0.9991	19002	−1898	0.9996	12768	1241	0.9987
Liver	12345	2578	0.9972	17102	−1430	0.9990	12881	1354	0.9972
Intestine	14193	3218	0.9983	18947	−2002	0.9982	12507	1008	0.9968

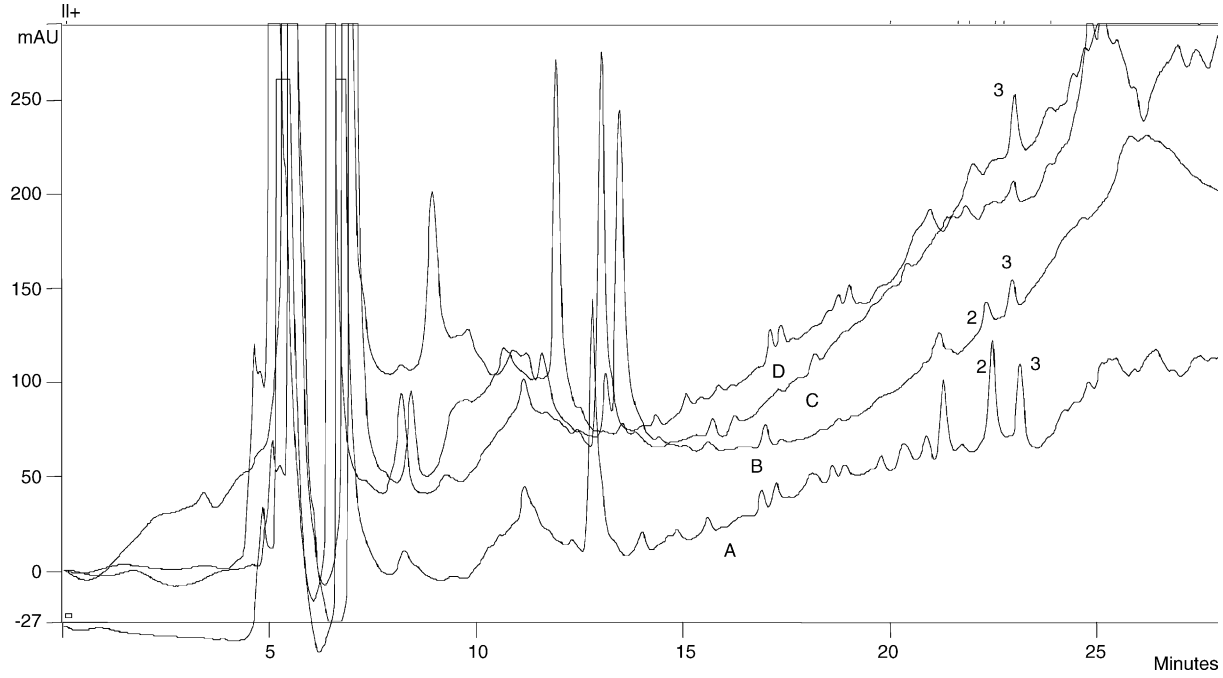

Fig. 1. LC/DAD chromatograms obtained from liver extract of (A) untreated fish spiked at 2 μg g^{-1} of each MC, (B) untreated fish spiked at 0.5 μg g^{-1} of each MC (LOQ level), (C) an exposed fish (concentration 1.16 μg g^{-1} ± 0.08), and (D) untreated fish. Peak identification: (1) = MC-RR; (2) = MC-YR; (3) = MC-LR.

tine, recoveries ranged from 71 to 85% for MC-RR, from 69 to 76% MC-YR, and from 68 to 73% MC-LR with RSDs between 11 and 18%. Results obtained for liver were better than those obtained for intestine.

The limit of detections (LODs), calculated from $3S_b$/slope of calibration curve, in which S_b is the standard deviation of a blank measurement, were 0.15 $\mu g\,g^{-1}$ for each MC using LC/DAD and 0.1.$\mu g\,g^{-1}$ using LC/MS. The limits of quantification (LOQs), determined as the lowest concentration of compound that gave a response that could be quantified with an inter-assay RSDs of less that 20%, was 0.50 $\mu g\,g^{-1}$ for each MC on either detector. Sensitivity is, thus, appropriate since it is almost the same by both determination techniques.

The response, as function of concentration, was measured by a five-point calibration curve with a dynamic range that extended over two orders of magnitude (0.5–50 $\mu g\,ml^{-1}$). Table 1 shows the regression equation calculated from standards prepared in methanol as well as from standards prepared in liver and intestine blank extracts. The slopes obtained with standard and spiked extracts were almost equal for the three MCs using LC/DAD whereas, using LC/MS, a slight matrix effect was noted. The signal for MC-RR and MC-YR decreased in liver (with maximum value of 12%). This reduction in the response is almost negligible.

Figs. 1 and 2 show typical chromatograms obtained from unspiked and spiked fish liver obtained by LC/DAD and LC/MS, respectively. Chromatograms obtained by LC/DAD showed too much variable baseline noise, which could indicate method's lack of rugedness and reliability. On the contrary, chromatograms obtained with LC/ESI/MS turned out to be robust with biological samples: no inestability was found. Although, quantitative results obtained by both methods analyzing the treated fish samples in triplicate (see Figs. 1 and 2) present sufficient agreement and acceptable standard deviation (SD), the LC/MS method is more appropriate. The LC/DAD method can be useful to discriminate the negative samples.

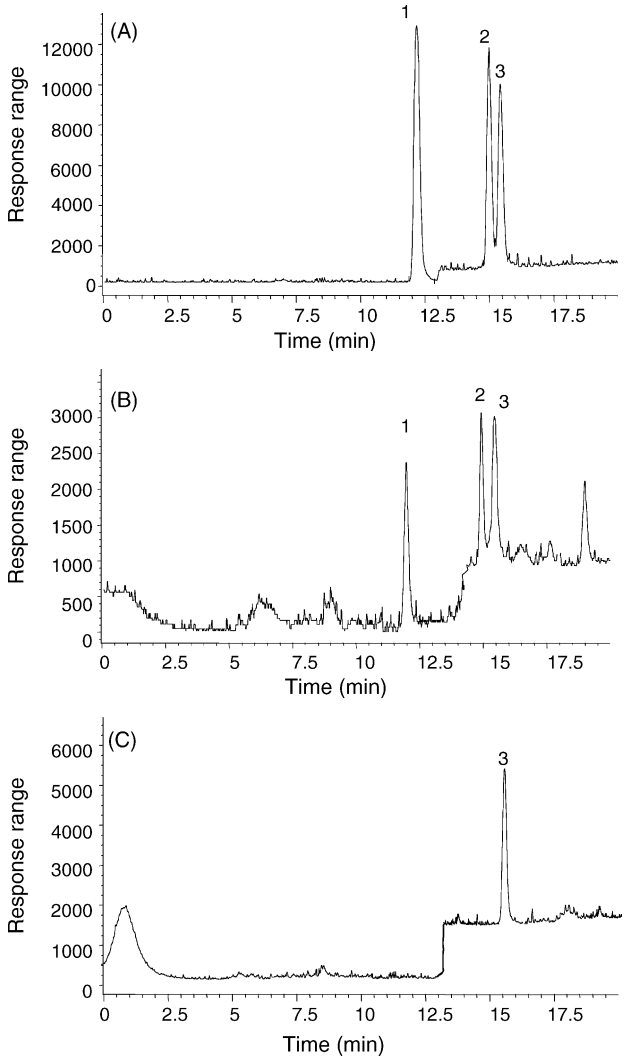

Fig. 2. LC/ESI/MS chromatograms obtained from liver extract of (A) untreated fish spiked at 2 $\mu g\,g^{-1}$ of each MC, (B) untreated fish spiked at 0.5 $\mu g\,g^{-1}$ of each MC, (C) an exposed fish (concentration 1.04 $\mu g\,g^{-1} \pm 0.14$), Peak identification as in Fig. 1.

4. Conclusion

This report presents a simple and rapid solvent extraction procedure, followed by conventional LC/MS, for determining MCs in fish tissues. This method provides detection limits acceptable for environmental studies and proves its utility for monitoring MCs in fish tissues.

The application of the proposed method to study the effect of toxins produced by cyanobacterial bloom demonstrates the transference of MCs from cyanobacterial cells to different fish tissues.

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