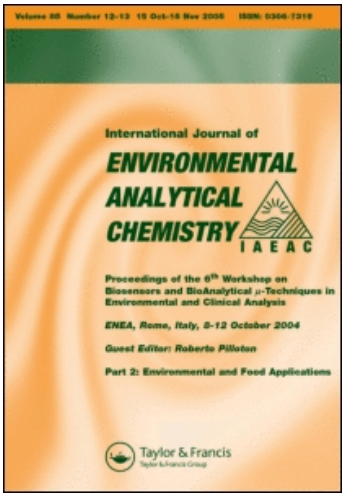

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Determination of microcystins in biological samples from freshwater fish

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A new HPLC–DAD method has been developed to identify and quantify free microcystins (MC) in biological samples from fish (intestine and liver). The toxins were extracted from 500 mg sample with a mixture of methanol–water (85 : 15, v/v) and the extracts obtained were purified employing immunoaffinity columns (IAC). The purification step was optimised by a full factorial 3^2 design. MC were separated using conventional C18 column and an acetonitrile-acidified water (pH 3) gradient. Detection and quantification limits resulted equal for the two toxins assayed (MC-RR and MC-LR) and were 0.15 and 0.5 $\mu g\,g^{-1}$, respectively. The accuracy for each MC in liver samples were 96% (range 80–113%) for MC-RR and 101% (range 93–118%) for MC-LR. The results were slightly lower for intestine samples, with recoveries ranging between 85% (75–93%) for MC-RR and 88% (80–97%) for MC-LR. The proposed method was applied for the determination of free MC in fish intoxicated with these toxins, in order to determine its utility to evaluate the potential risks for human health if MC-contaminated fish are consumed. The results showed the transference of MC-LR from cyanobacterial cells to fish tissues.

Keywords: microcystins; purification; immunoaffinity column; liver; intestine; fish; liquid chromatography–DA

1. Introduction

Water blooms of toxic cyanobacteria in freshwater bodies have been reported all over the world [1] and have gained increased attention due to their undesirable effects on human and animal health [2,3]. Cyanobacteria often produce hepatotoxic cyclic heptapeptides called microcystins (MC), of which over 80 structural variants are currently known. The toxins generally differ in the nature of two L-amino acids and in their degree of methylation, hydroxylation, or epimerisation. Microcystin-LR, microcystin-RR and microcystin-YR are the most frequent types produced by *Microcystis* [4,5]. These molecules represent an increasing environmental and health risk. Their presence in water supplies has caused the death of wild and domestic animals worldwide and has been linked to human fatalities [6]. MC have been shown to be potent and specific inhibitors of protein

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phosphatases (serine/threonine family), especially PP1 and PP2A, resulting in hyper-phosphorylation of proteins, affecting intracellular signalling, cell growth, and differentiation processes, inducing cell disturbance [7]. MC are metabolised primarily by glutathione *S*-transferases (GSTs) and this conjugation appears to be the first step of detoxication of MC in aquatic organisms [8].

No case of human deaths caused by oral consumption of cyanobacteria toxins has been documented yet, whereas chronic toxic effects from exposure through food need to be considered, especially if there is long-term frequent exposure [9]. These toxins have been linked to the high rate of primary liver cancer (PLC) in certain parts of China [10] and were recently identified for the first time in the serum (average 0.228 ng MC-LR eq mL^{-1}) of a chronically exposed human population (fishermen at Lake Chaohu, South China) together with indication of hepatocellular damage [11]. In response to the increase of health-related problems on a global scale, the World Health Organization (WHO) has established safe guidelines for drinking water at 1.0 $\mu g L^{-1}$ [12] and a Tolerable Daily Intake (TDI) value of 0.04 $\mu g Kg^{-1}$ of body weight day^{-1} [2].

In the natural environment, MC are found to accumulate in a wide range of aquatic animals such as shrimps, mussels, gastropods and fish, attracting increasing concern from the public about food safety [9,13–18]. Because these organisms are an important food source not only for birds and fish but also for mammals including humans, MC may be transferred to a higher trophic level through the food chain and could lead to human toxicity. The accumulation and persistence of MC in crustaceans and fish (*Tilapia rendalli*) cultivated in aquaculture ponds and under laboratory conditions have been demonstrated even at low concentrations of toxins, reaching levels above the recommended TDI [13,19,20]. Consequently, it is important to monitor fish and other aquatic animals from water bodies with toxic cyanobacteria species, in order to evaluate the potential human risk by the consumption of them.

There have been extensive studies on the extraction and detection of MC in fish [17,19–25]. The liver followed by the kidney and intestines accumulated most of the MC administered to fish, and small amounts of toxins were detected in the muscles [1,9,14,18,24,26]. Analytical and bioanalytical methods to determine MC included high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), enzyme linked immunosorbent assay (ELISA) and protein phosphatase inhibition assays. Chemical methods are widely used to determine MC in freshwater [27], farm fish [25], and marine food [28,29]. Currently, analysis of MC in animal tissue is mostly carried out using High-Performance Liquid Chromatography–diode array detector (HPLC–DAD) [12,24], and Liquid Chromatography–Mass Spectrometry (HPLC–MS) has also been used widely in animal tissues in recent years [9,17,24]. In previous reports we have employed HPLC–DAD and HPLC–MS to determine MC in natural blooms and cyanobacterial strain cultures and in biological samples from rats injected i.p. with MC-LR or fish orally exposed to toxic cyanobacterial cells [30–32]. The concentrations of MC in foods and biological samples were relatively low, and there were interferences due to matrix effects. Therefore, sample pre-treatment methods capable of providing trace-enrichment and very clean extracts in order to remove the matrix interferences are required. The most common extraction technique for MC is solvent extraction followed by a concentration step and silica gel or octadecyl silica clean-up [25,33]. However, this technique is susceptible to interferences from co-eluting compounds and relies exclusively on the retention times established from authentic standards of which only a few MC congeners are commercially available.

Immunoaffinity columns (IAC) have provided excellent sample clean-up for a variety of toxic chemicals, including MC in waters [34–37] and biological samples, such as green-algae cells [6] and fish samples [22,38], due to the high selectivity towards selected group of compounds. There is still no agreement on the methodology for quantitative analysis of MC in fish tissues. Further investigations of and improvement in routinely applied MC methods for fish tissues are needed for a reliable risk assessment [39].

This paper presents the development of an analytical procedure based on solvent extraction followed by a purification step with IAC and HPLC–DAD technique for the simultaneous determination of MC-LR, and MC-RR from fish liver and intestine. A preliminary study for uncovering the main experimental factors in the purification procedure was carried out using a two-level full factorial design with replications. The method was applied to identify and quantify free MC in liver and intestines excised from Tilapia fish (*Oreochromis* sp.) cultivated under laboratory conditions and exposed to natural blooms containing MC. The present procedure has been intended for routine monitoring of aquatic samples that could be performed by any laboratory worldwide, without the need of expensive detection such as mass spectrometry.

2. Experimental

2.1 *Chemicals and materials*

Microcystin standards (MC-LR, MC-RR) were supplied by Cyanobiotech (Berlin, Germany) with a purity of 99 and 95%, respectively. Stock solutions of each MC at a concentration of 500 $\mu g\,mL^{-1}$ were prepared in methanol and stored in glass-stopper bottles at 4°C. Standard working solutions at the appropriate concentration of each toxin, as well as mixtures of the two toxins, were prepared daily in methanol. All chemicals and reagents were analytical grade materials. HPLC-grade methanol, acetonitrile, and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Phosphate buffered saline solution (PBS) was prepared by dissolving 1.63 g Na_2HPO_4, 0.50 g NaH_2PO_4 and 4.09 g NaCl in 500 mL of water. The pH was adjusted to 7.4 with 1M NaOH. Na_2HPO_4 (99%), NaH_2PO_4 (99%), NaCl (99%) and sodium azide (99%) were purchased from Sigma-Aldrich (Oakville, Ont. Canada). Deionised water (>18 $M\Omega\,cm^{-1}$ resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, USA).

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

2.2 *Exposure study*

Male *Oreochromis* sp. (Nile tilapia, Perciformes: *Cichlidae*) average weight 50 ± 8 g were obtained from a laboratory stock. Two groups of five individuals each (control and MC exposed group) were acclimated for two weeks prior to the experiment in 96-L aquaria containing dechlorinated tap water with a temperature of 21 ± 2°C. During this period they were fed once daily with commercial fish food (Dibaq-Diproteg, Segovia, Spain).

The lyophilised material used in this experiment was obtained from a *Microcystis* bloom which was collected from the Guadiana River in Mértola, Portugal. The concentration of MC-LR, 3300 $\mu g\,g^{-1}$, was determined in a previous study [30]: the cells were extracted three times with 10 mL 0.1 M acetic acid and 20 mL of a mixture of methanol–chloroform (1 : 1 v/v) and determined by HPLC–DAD. A group of fish ($n = 5$) 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, fish were anaesthetised with tricaine methane sulphonate (MS-222) solution for 5–10 min before they were killed by transection of the spinal cord. The liver and intestines were removed, weighed, rinsed with ice-cold saline solution, and kept at –20°C until analysis.

2.3 *Extraction and clean up procedure using immunoaffinity columns (IAC)*

MC extraction from fish tissues was carried out following the method of Moreno *et al*. [32]. *Oreochromis* sp. liver or intestines samples (500 mg) were extracted with 15 mL of methanol 85% by homogenising 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 methanol 85%. The extract was evaporated at 35°C to dryness using a rota-evaporator and redissolved in 0.25 mL methanol. The resulting extract was evaporated to reduce the volume and percolated in PBS buffer to get a methanol concentration under 15% (v/v) until a final volume, depending on assays carried out (200, 250 or 300 μL, see Section 3.1 below).

The immunoaffinity columns on silica-based ImmunoSep™ were purchased from Abkem Iberia (Vigo, Spain) with a reported capacity of 135 ng microcystin-LR/column. The immunoaffinity columns were washed and filled with PBS (pH 7.4) containing sodium azide (0.02% w/v) to prevent mould and bacterial growth. After optimisation of several variables through a full factorial design 3^2, the following clean-up procedure was adopted: the column was conditioned with 10 mL of PBS. After washing with PBS (4 mL), distilled water (4 mL) and 25% methanol (4mL), the MC fraction was passed through the conditioned column and eluted with 80% methanol (15 mL). The eluate was evaporated at 35°C to dryness using a rotary evaporator. The extract was re-dissolved in 250 μL of methanol. An aliquot of 20 μL was injected onto the HPLC–DAD system. Blanks were performed each time after either a standard or a sample was passed through the IAC. Extraction efficiencies were determined by spiking fresh tissue samples with volumes of 250 μL of the working mixtures of MC at appropriate concentrations.

2.4 *Chromatographic conditions*

HPLC–DAD was carried out with a Varian (Palo Alto, CA, USA) system equipped with a tertiary solvent pump (9012), a PDA detector (ProStar 330) set at 238 nm, and a Star analytical work station software (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: 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.

3. Results and discussion

3.1 *Factors screening for purification procedure*

A preliminary study for uncovering experimental factors in the purification procedure was carried out by applying two factors, three-level full factorial design with replications [40].

Table 1. Tested values in the full factorial design for the variables related to the purification step in MC determination in fish.

Variable	Tested range	Coded	Tested range	Coded	Tested range	Coded
Sample volume (μL) (X_1)	200	−1	250	0	300	+1
Clean up volume* (X_2)	3/3/3	−1	4/4/4	0	6/6/6	+1

*mL PBS/mL H_2O/mL MeOH/H_2O (25/75).

The considered factors were sample volume (X_1), and washing solvent volume (X_2). The variable response (Y) was the extraction recovery as a percentage of each MC. Three-level (full) factorial designs require 2f runs with each factor at three levels. First of all, the levels were coded according to the rule: high level = +1, middle level = 0 and low level = −1, as is depicted in Table 1. The design matrix was built considering quintuplicate measurements for the middle of the experiment and only once for the rest of assays. In this way, there are enough degrees of freedom to suitably conduct regression analysis.

The methods available in the literature employing immunoaffinty columns usually have been applied to water samples or blue-green algae [6,34,36,38,41]; only a few authors have used this technique in biological samples from fish [22,38], although none of them have previously optimised this technique. Levels for solvents volume employed to rinse the cartridge were based on this literature. Thus, Lawrence and Menard [22] rinsed the IAC column with 3 mL of PBS, followed by 3 mL of water and then, 3 mL of methanol/water (25/75). Kondo *et al.* [38] only employed for washing the IAC column PBS (5 mL) and distilled water (5 mL). In a previous study [41] we applied these IAC columns in water samples, following the conditions proposed by Aguete *et al.* [37]. In both studies MC analysis in algae and water samples were successfully accomplished by using HPLC techniques. In this work we have optimised the best conditions for fish liver and intestine samples, more complexes matrices than waters, studying the influence of three different sample volumes and washing solvent volumes (Table 1).

The statistical model of a three-level factorial design is linear. These models represent surfaces or hypersurfaces more or less warped owing to the interaction terms but without curvature leading to maxima or minima and are called first order models. The complete model equation for 2 factors (X_1 and X_2) is:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2. \quad (1)$$

The coefficients b_1 and b_2 account for the main effects of the factors X_1 and X_2, respectively. On the other hand, b_{12} represent the second order interaction term. The independent term b_0 represents the response (here the yield) at zero level of every factor, that is, the response at the centre of the design. A significance assay for the regression coefficients is based on the Student t-test once the standard deviation of the regression coefficient b_k, $s(b_k)$, is known: $t_k = b_k/s(b_k)$. So, a coefficient is significant if the t_k value is higher than the critical tabulated value for the degrees of freedom corresponding to the tested regression model (number of runs – number of coefficients to be estimated) at a given confidence level, generally 95% [40].

In our case, the results of the fittings for both MC congeners in intestine homogenates were as follows.

MC-RR

The pure error variance coming from the replication ($n = 5$) at the centre of the design was about 72.9, whereas the residual variance was 71.0. Accordingly, there was no lack-of-fit variance and hence, the model did not contribute to the uncertainty measurement. However, in this case, the correlation coefficient was quite poor (0.69) and the *t*-test applied to the coefficients corresponding to main effects and interactions indicated that the selected two factors were not significant in the proposed model for intestine matrix.

MC-LR

The residual variance was 109.7; the pure error variance obtained from the quintuplicate at the centre of the model was 76.7 and the correlation coefficient was 0.52. We can conclude that in the case of MC-LR neither the model contributed to the uncertainty measurement nor the selected two factors were significant in the proposed model for intestine homogenates.

The results obtained in liver homogenates were similar to those obtained for intestine samples; in this sense the residual variance for MC-RR and MC-LR were 580.3 and 136.1 respectively; the pure error variance coming from the replication at the centre of the design were 482.8 for MC-RR and 120.5 for MC-LR and the correlation coefficient for both toxins were 0.57 (MC-RR) and 0.72 (MC-LR). With these results we can conclude that the model assayed did not contribute to the uncertainty measurement neither for MC-RR nor for MC-LR in liver samples.

Thus, within the range of working levels assayed, there was no influence of the factors on the analytical response and they can be chosen for convenience.

Summarising, with respect to the main factors, in all cases were not significant. This can ensure that any volume of the sample leads to the same results and the solvent volume for the washing step is not always significant. Thus, the purification of the real samples from fish exposed to cyanobacterial cells should be performed at 0 level for the two factors, leading to the best recoveries for both MC. According to these results, the purification step was carried out using a sample volume of 250 μL and 4/4/4 mL of the different solvents employed in the washing step.

3.2 *Features of the methods*

Recovery experiments were performed in quintuplicate spiking 500 mg of fish liver or intestine samples with MC fortification solutions of two commercial standards (MC-LR and MC-RR) at three levels, ranged from 0.5 $\mu g\,g^{-1}$ to 3 $\mu g\,g^{-1}$. Quantitative determination of the recovery rate was performed using the external standard technique; that is, comparing peak areas in sample chromatograms with the corresponding peak areas obtained from standard solutions of the toxins.

The recoveries for each MC in the liver samples ranged from 80 to 113% (mean 96%) for MC-RR and from 93 to 118% (mean 101%) for MC-LR with RSDs between 11 and 12%. The results were slightly lower for intestine samples, with recoveries ranging between 75–93% (mean 85%) for MC-RR and 80–97% (mean 88%) for MC-LR with RSDs of 9%

Table 2. Analytical parameters obtained in the recovery assay for free MC-RR and MC-LR spiked at three levels in fish intestine and liver ($n = 5$).

	Intestine		Liver	
MC	MC-RR	MC-LR	MC-LR	MC-LR
Linearity	0.997	0.999	0.996	0.998
Repetitivity (RSD, %)	14	9	18	12
Reproducibility (RSD, %)	19	12	19	16
LOD ($\mu g\,g^{-1}$)	0.15	0.15	0.15	0.15
LOQ ($\mu g\,g^{-1}$)	0.5	0.5	0.5	0.5
Recovery (%) (mean ± RSD)	85 ± 9	88 ± 9	96 ± 12	101 ± 11

(Table 2). Generally, results obtained for liver samples were better than those obtained for intestine samples. After being used 5 times for extracting MC in fish tissue, the cartridge still had a recovery of over 80%.

The extraction procedure and the HPLC–DAD method proposed to analyse the samples are not suitable to detect MC bound to glutathione or protein phosphatases, so all results in this study refer to free MC in the fish tissues. These IAC columns were also employed by Lawrence and Menard [22] in order to remove MC from spiked fish homogenates (0.1–0.5 $\mu g\,g^{-1}$ MC-RR or MC-LR). In this study, the cartridges were reported to be effective in isolating MC from blue-green algae, fish and water samples, resulting in extracts that were clean enough to enable direct LC–UV detection down to 0.03 $mg\,g^{-1}$ in the blue-green algae and fish samples, and as low as 0.02 $ng\,mL^{-1}$ for water samples. In comparison with our results, lower recoveries were obtained in this work, 73% for MC-RR and 81% for MC-LR. Therefore, the optimisation step together with the extraction technique performed in the present work has led to better recovery rates. Xie *et al*. [18] reported similar recoveries for MC-LR (91%) and MC-RR (89%) from fish liver using an efficient solid phase extraction (SPE) method which employed two types of cartridges (HBL and silica gel/plus silica gel tandem) for the clean-up procedure, but the method is more time-consuming. MC recoveries obtained in this study are in accordance with those reported by other authors; all of them are related as free MC (Table 3).

In order to show the real advantage of the present method to identified and quantified free MC in fish tissues two extracts of liver and two of intestine spiked with 2 $\mu g\,g^{-1}$ MC-LR and MC-RR with and without IAC purification step were analysed by HPLC–DAD. The co-extracted substances in the tissues were effectively eliminated when the IAC column was used and the peaks of the MC analysed were clearly detected (Figure 1A liver and 1A intestine), indicating the usefulness of this method when is applied to tissue samples. In Figure 1B liver and 1B intestine many unknown peaks are observed. Peaks with retention times corresponding to MC-RR and MC-LR standards were obtained as well as several co-eluting peaks making difficult to discern the ones corresponding to MC. Similar results were obtained by Kondo *et al*. [38], who compared two clean-up procedures (C-18 SPE and IAC) in liver extracts added with MC, obtaining the best results with the IAC procedure proposed.

The limit of detection (LOD) is defined as the concentration of the toxin equal to the blank signal plus 3 times the standard deviation (SD) of the blank. According to Miller

Table 3. Comparison of the MC recoveries in aquatic products.

Extraction	Clean-up	Sample	Amount of spiked MC	Average recovery	Reference
SPE (solid phase extraction)	Immunoaffinity	Fish	MC: 0.5–4.0 $\mu g\,g^{-1}$	MC-RR: 73 MC-LR: 81	[22]
LLE (liquid liquid extraction)	–	Zebra mussels	MC-LR: 0.1 and 5 $\mu g\,g^{-1}$	MC-LR: 50 ± 4.2	[45]
LLE	–	Fish	MC: 0.5–3.0 $\mu g\,g^{-1}$	MC > 92	[32]
SPE	C-18	Fish	MC-RR: 5–20 $\mu g\,L^{-1}$	MC-RR: > 80	[46]
SPE	C-18	Fish	MC-LR: 10–100 $\mu g\,g^{-1}$	MC-LR: 58	[39]
LLE	ODS	Fish	MC: 2.5 $\mu g\,g^{-1}$	MC-RR: 78 MC-LR: 81	[9]
SPE	C-18	Fish	MC-RR: 11.4 μg MC-LR: 3.1 μg	MC-RR: 66.4 MC-LR: 68.8	[25]
SPE	ODS and silica	Fish	MC-RR: 11.4 μg MC-LR: 3.1 μg	MC-RR: 89.0 MC-LR: 91.0	[25]
SPE	ODS and silica	Fish	MC-LR: 0.2, 1, 5 $\mu g\,g^{-1}$	MC-LR: 91–103 MC-LR-GSH: 65–76	[43]
LLE	Immunoaffinity	Fish	MC: 0.5–3.0 $\mu g\,g^{-1}$	MC-RR: 85–96 MC-LR: 88–101	Present study

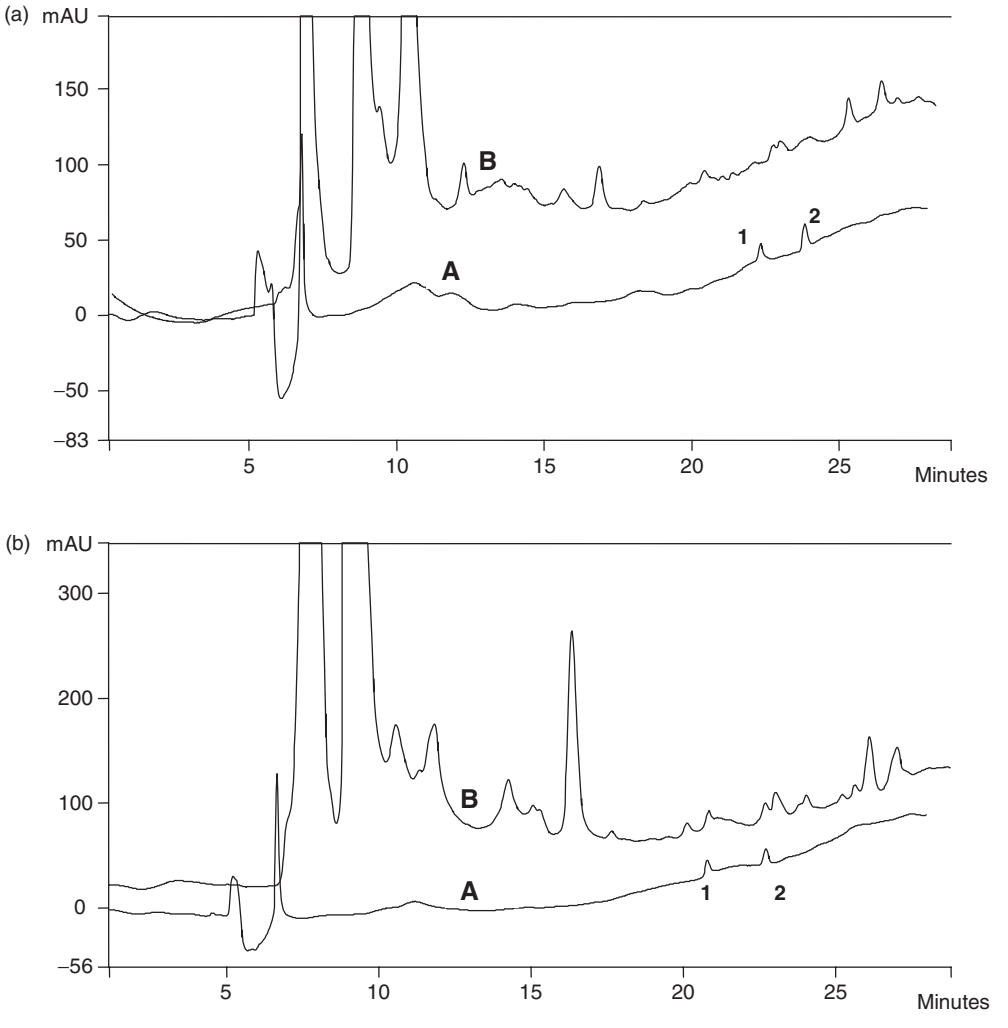

Figure 1. Analysis of (A) liver and intestine extracts of untreated fish spiked with $2\,\mu g\,g^{-1}$ of MC-RR and MC-LR after IAC clean-up and (B) liver and intestine extracts of untreated fish spiked with $2\,\mu g\,g^{-1}$ of MC-RR and MC-LR without purification step. Peak identification: (1) = MC-RR; (2) = MC-LR.

and Miller [42], the SD of regression is equated to the SD of the signal blank, and the intercept is taken as a measure of the signal blank. The limit of quantitation (LOQ) is defined as the lower limit for precise quantitative measurements and is given as the signal blank plus 10 times the SD of the blank. The LOD and LOQ in units of concentration ($\mu g\,g^{-1}$) obtained from calibration curves were $0.15\,\mu g\,g^{-1}$, and $0.50\,\mu g\,g^{-1}$, respectively (Table 2) for each toxin. Sensitivity is, thus, appropriate.

Determination of matrix effects was carried out by analysing a five point calibration curve with a dynamic range that extended over two orders of magnitude ($0.5–50\,\mu g\,mL^{-1}$). Table 4 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

Table 4. Matrix calibration of fish liver and intestine in comparison with standard calibration by IAC–HPLC–DAD.

	MC-RR			MC-LR		
	Slope	*y*-intercept	*r*	Slope	*y*-intercept	*r*
Standard	602699	36862	0.999	732188	21814	0.999
Liver	621393	6831	0.997	723933	7292	0.999
Intestine	633198	10247	0.996	772377	1940	0.998

with standard and spiked extracts were almost equal for the two MC. Both standards and samples showed a good linearity, with correlation coefficients greater than 0.99. Similar responses were noted for MC-RR and MC-LR in liver and intestine samples. Therefore, to avoid biased results, matrix matched standards should always be used for quantifying unknown samples.

3.3 *Evaluation of MC accumulation in Tilapia fish liver and intestine*

The proposed method was applied for the routine monitoring of free MC in fish intoxicated by these toxins, in order to determine its utility to evaluate the potential risks for human health if MC-contaminated Tilapia fish from aquaculture ponds are consumed.

Figures 2 and 3 show typical chromatograms obtained after the MC determination with the proposed method in fish liver and intestine, respectively. The chromatogram in Figure 2A displays the liver extract of untreated fish or control fish. Figure 2B illustrates liver extract obtained from an orally exposed fish to MC (60.0 μg MC-LR $fish^{-1}\,day^{-1}$ during 21 days) and Figure 2C shows the chromatogram of a liver extract of untreated fish spiked at 2 $\mu g\,g^{-1}$of MC-RR and MC-LR.

The results obtained demonstrated that the procedure proposed efficiently extracted free MC-LR from fish exposed approximately to 60.0 μg MC-LR fish day^{-1}, under laboratory conditions. The residues obtained were 1.2 $\mu g\,g^{-1}$ (S.D. 10%) in liver extracts, and 1.5 $\mu g\,g^{-1}$ (S.D. 14%) in intestine extracts; consequently, similar levels of MC-LR were found in both organs, even though the target organ is the liver. These results are in agreement with those obtained in a previous study carried out in our laboratory using a solvent extraction procedure without purification step and HPLC-ESI-MS detection [31], in which 1.04 $\mu g\,g^{-1} \pm 0.14$ MC-LR was detected in the liver of Tilapia orally exposed to MC. This comparison confirms the utility of the IAC purification prior to HPLC–DAD detection as a valuable alternative to determine MC in fish tissues. The less expensive IAC–HPLC–DAD method can be used to do routine monitoring of aquatic samples, as screening method. It is important to emphasise that MC concentration detected in the fish tissues refers to free toxins, once it was not possible to detect MC bound to protein phosphatases or glutathione using the method applied in this work. Consequently, total MC (bound plus unbound) in those tissues were underestimated, as it has been also pointed out by several authors [18,20].

Similarly to our results, most of the experimental and field studies have been focused on the determination of free MC accumulation in fish (Table 5). In Tilapia

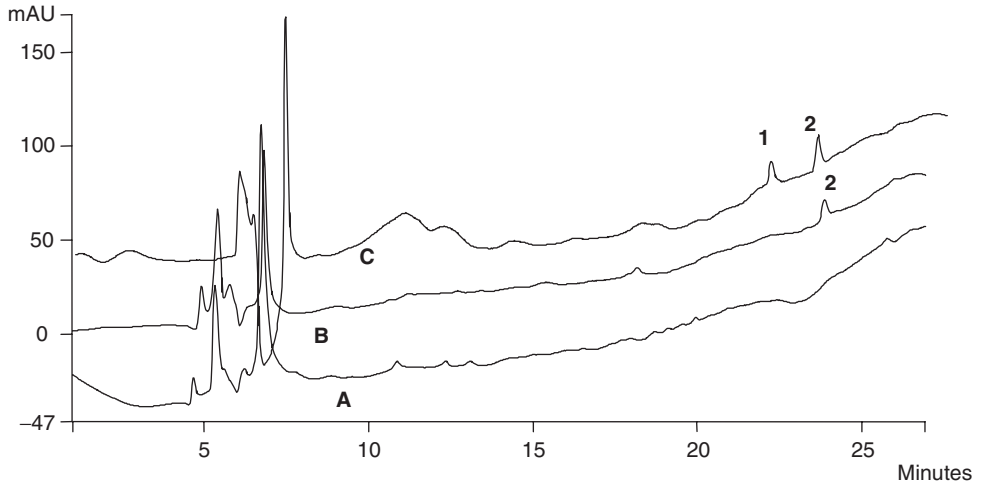

Figure 2. Microcystins determination in fish liver. Figure 2A displays the liver extract of untreated fish. Figure 2B illustrates liver extract obtained from an excised liver from Tilapia exposed to MC-LR from cyanobacterial bloom. Figure 2C shows the chromatogram of a liver extract of untreated fish spiked at $2\,\mu g\,g^{-1}$of MC-RR and MC-LR. Peak identification: (1) = MC-RR; (2) = MC-LR.

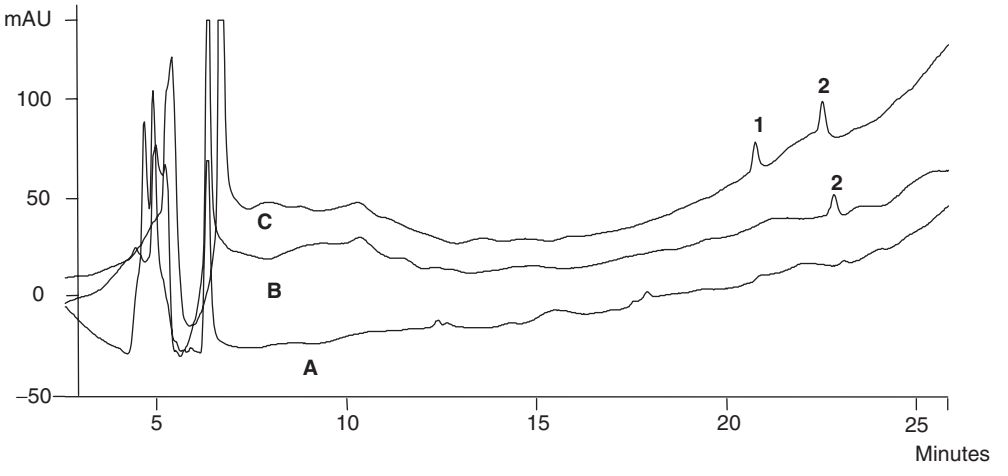

Figure 3. Microcystins determination in fish intestine. Figure 3A displays the intestine extract of untreated fish. Figure 3B illustrates liver extract obtained from an excised intestine from Tilapia exposed to MC-LR from cyanobacterial bloom. Figure 3C shows the chromatogram of an intestine extract of untreated fish spiked at $2\,\mu g\,g^{-1}$of MC-RR and MC-LR. Peak identification: (1) = MC-RR; (2) = MC-LR.

fish (*Oreochromis niloticus*) from natural environments containing heavy blooms of *Microcystis aeruginosa*, $0.53\,\mu g\,g^{-1}$ MC has been quantified in the liver [14]. In general, MC in the organs analysed are within the range of the reported values from the literature. Soares *et al*. [20] reported that, when *Tilapia rendalli* was fed MC at $29.2\,\mu g\,fish^{-1}\,d^{-1}$, on the 42nd day, the accumulation contents of MC were $1.7\,\mu g\,g^{-1}$ in the liver. Previous

Table 5. MC content in various aquatic animals.

MC exposure	Sample	Free MC content	Reference
Jacarepaguá Lagoon (Brazil)	*Tilapia rendalli*	Liver: 6.5 μg g^{-1} Viscera: 14.6 μg g^{-1}	[13]
Egyptian fish farm	*Oreochromis niloticus*	Guts: 821 ng g^{-1} Liver: 531.8 ng g^{-1} Kidneys: 400 ng g^{-1} Muscles: 102 ng g^{-1}	[14]
Laboratory exposure	*Tilapia rendalli*	Liver: 0.6–2.8 μg g^{-1} Muscle: 0.05 μg g^{-1}	[20]
Laboratory exposure	Silver carp	RR: liver: 17.8 μg g^{-1} Muscle: 1.77 μg g^{-1}	[24]
Chinese lake	Freshwater shrimp	Hepatopancreas: 0.53–4.29 μg g^{-1} Gonad: 0.48–1.17 μg g^{-1}	[15]
Chinese lake	Freshwater snail	Hepatopancreas: 4.14 μg g^{-1} Gonad: 0.715 μg g^{-1}	[16]
Laboratory exposure	Bighead carp	Liver: 2.89–5.43 μg g^{-1}	[47]
Laboratory exposure	Tilapia fish	Liver: 1.04 μg g^{-1}	[32]
Chinese lake	Silver carp	Intestine: 24.3 μg g^{-1} Liver: 0.957 μg g^{-1}	[9]
Laboratory exposure	Nile tilapia	Muscle: 0.77–14.62 ng g^{-1} Liver: 423.55–3007.3 ng g^{-1}	[1]
Japanese lake	*Cyprinus carpio*	Liver: 2.06 μg g^{-1}	[18]
Japanese lake	Freshwater snail	Hepatopancreas: 5.38 μg g^{-1} Gonad: 6.90 μg g^{-1}	[18]
Japanese lake	Bighead carp	Liver: 0.374 μg g^{-1} Intestine: 19.3 μg g^{-1}	[17]
Laboratory exposure	Tilapia fish	Liver: 1.2 μg g^{-1} Intestine: 1.5 μg g^{-1}	Present study

experimental studies on silver carp exposed orally to *Microcystis viridis* under laboratory conditions reported 1.77 μg g^{-1} MC-RR in the liver [24], and 2.06 μg g^{-1} in the liver of *Cyprinus carpio* collected from a lake contaminated with *Microcystis* blooms in Japan [18]. Recently, Dai *et al.* [43] have been developing a sensitive and selective liquid chromatography–tandem mass spectrometry method (LC/MS/MS) for the simultaneous quantitative determination of MC-LR and its glutathione conjugate (MC-LR-GSH) in fish tissue.

Although MC are considered mainly hepatotoxins, in fish, field studies demonstrated that after ingestion MC are first detected in gut content and then accumulated mainly in liver [1] but also in muscle and viscera [44]. However, *in situ* studies carried out with silver carp exposed to dense toxic *Microcystis* blooms showed a major accumulation of MC in intestine (24.3 μg g^{-1}) followed by liver (0.957 μg g^{-1}) [9]; similar distribution has been demonstrated in bighead carp (*Aristichthys nobilis*), with MC contents of 19.3 μg g^{-1} in intestine and 0.374 μg g^{-1} in liver [17]. In the present study, the accumulation rate in liver and intestine was similar. Chen *et al.* [17] indicated that there were no significant correlations in MC concentration between the intestine and other organs of bighead carp,

suggesting that MC contents in the intestine may be significantly affected by diverse factors (e.g. digestive degree at sampling or heterogeneity of food resources).

Our results suggest the accumulation of free MC-LR from cyanobacterial cells in fish orally exposed during 21 days. We recommend this analytical procedure for monitoring free MC-producing water blooms in fish farms and as a system of fish tissue monitoring to evaluate the toxic risk associated with MC exposure.

4. Conclusion

This report constitutes a simple, rapid and efficient method for environmental monitoring of free MC in fish tissues, based on solvent extraction, purification with an immunoaffinity columns (IAC) and followed by conventional HPLC–DAD analysis. The work comprises an optimisation of the influents factors in the IAC purification procedure, which improves the recoveries obtained in previous studies. This method provides acceptable quantification limits (MC-RR: $0.15\,\mu g\,g^{-1}$ and MC-LR: $0.5\,\mu g\,g^{-1}$) and high recoveries (>85%) which proves its utility as screening method for routine monitoring of MC in fish potentially contaminated.

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