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Analysis of cyanobacterial hepatotoxins in water samples by microbore reversed-phase liquid chromatography–electrospray ionisation mass spectrometry

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Abstract

A method based on liquid chromatography coupled to mass spectrometry with positive electrospray ionisation was developed for the analysis of cyanobacterial hepatotoxins in environmental samples. The chromatographic separation was performed using two microbore columns, 2 mm and 1 mm I.D. columns, which allowed the coupling of liquid chromatography to mass spectrometry with no flow splitting. Analytes were eluted using two different water–acetonitrile, both acidified with formic acid gradients. Mass spectrometric parameters were optimised in order to maximise sensitivity. Detection limits for the 2 mm I.D. column ranged from 0.077 to 2.057 ng in full scan and from 0.021 to 1.153 ng in SIM mode. However, limits of detection as low as 60–340 pg in full scan and 6–72 pg in SIM mode were achieved for the 1 mm I.D. column. Finally, the proposed method was applied to the analysis of microcystins and nodularins in real samples.

Keywords: Water analysis; Hepatotoxins; Cyanobacterial toxins; Microcystins; Nodularins

1. Introduction

Microcystins are a family of hepatotoxic peptides produced by cyanobacteria of *Microcystis*, *Anabaena*, *Planktothrix* (*Oscillatoria*) and *Nostoc* genera. These toxins have been responsible for the death of farm, domestic and wild animals [1]. Moreover, they have been related to the illness [2], and more recently, to the death of human beings [3].

Microcystins have a common structure (Fig. 1a) containing three D-amino acids (alanine, β-linked *erythro*-β-methylaspartic acid, and α-linked glutamic acid), two variable L-amino acids, R_1 and R_2, and two unusual amino acids, *N*-methyldehydroalanine (Mdha) and 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid (Adda) [4]. To date, more than 60 microcystins have been identified [5]. Nodularins, produced by the cyanobacterium *Nodularia spumigena*, are hepatotoxic cyclic pentapeptides of similar structure to microcystins (Fig. 1b): α-linked D-glutamic acid, β-linked D-*erythro*-β-methylaspartic acid, L-arginine, 2-(methylamino)-2-dehydrobutyric acid (mdhb) and Adda [6].

Since human-health problems related to the presence of microcystin in water supplies have been reported, there is a need for a reliable, rapid and

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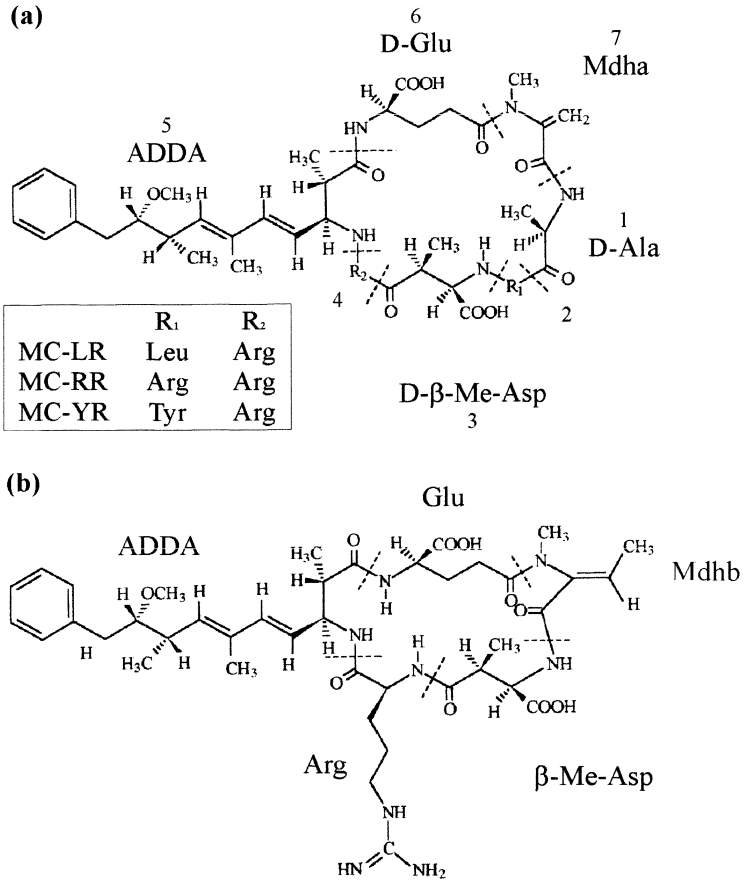

Fig. 1. General chemical structure of (a) microcystins and (b) nodularins.

sensitive method for the identification of microcystins in water samples. The methods for the detection and analysis of hepatotoxins can be classified into biological, including both bioassay and biochemical assay methods, and instrumental methods.

Some biological methods developed include mouse bioassay [7], protein phosphatase assay [8] and enzyme-linked immunosorbent assay (ELISA) [9]. These methods, which provide total microcystin concentration, are suitable for rapid screening analyses. However, if further information about the microcystin composition of the sample is required, instrumental methods have to be used.

The most commonly used analytical method for the detection and quantification of microcystins is reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) [10] and photodiode array (PDA) detection [11]. Nevertheless, the lack of specificity of UV detection results in some problems in identifying microcystins in the presence of matrix interferences.

As mass spectrometry provides some structural information, some methods have been reported for the characterisation of microcystins and nodularins. High resolution and tandem (MS–MS) mass spectrometry with fast atom bombardment (FAB) [12], electrospray [12,13] and more recently MALDI [14] ionisation have been used to characterise microcystins. Since atmospheric pressure ionisation interfaces (API) were developed, LC–MS is considered one of the most powerful hyphenated techniques for the analysis of a wide range of analytes. Ionspray [15] and electrospray [13,16] ionisation LC–MS with HPLC effluent splitting has been described for the identification of microcystins present in a bloom

material with reported detection limits of 400 [15] and 250 pg [13], respectively.

Typical HPLC separations of microcystins are carried out using C_{18} silica columns with gradients of water and acetonitrile both containing trifluoroacetic acid (TFA) as both pH modifier and ion-pairing reagent [11]. However, in LC–ESI-MS both non-volatile buffers and ion-pairing reagents must be avoided, as they suppress ionisation. Then, formic acid can be used instead of TFA.

In this study, a method based on LC–ESI-MS with two microbore C_{18} columns was developed. The optimum flow rates of the 2 and 1 mm I.D. columns were suitable for electrospray ionisation, but the use of the 1 mm I.D. column allowed enhanced sensitivity. Mass spectrometric parameters were evaluated in order to optimise the analyte ionisation. Finally, both columns were applied to the determination of microcystins and nodularins in water samples. The mass spectrometric detection allowed the identification of microcystins, even though no standards were available.

2. Experimental

2.1. Chemicals and materials

All reagents used were of analytical grade or high-performance liquid chromatographic (HPLC) grade. Acetonitrile, methanol and trifluoroacetic acid were obtained from Merck (Darmstadt, Germany) and formic acid from Panreac (Montcada i Reixac, Spain). High-purity water produced with a Milli-Q Organex-Q system (Millipore, Bedford, MA, USA) was used.

Microcystin-LR, -RR, -YR and nodularin standards were purchased from Calbiochem (La Jolla, CA, USA). Standard solutions of each analyte were prepared in methanol and stored at −20 °C.

End-capped Isolute C_{18} cartridges, 1 g in 3 ml, were purchased from IST (Mid Glamorgan, UK) and the SPE vacuum manifold from J&W (Folsom, CA, USA).

2.2. Sample preparation

Water samples were collected in brown glass bottles and stored at 4 °C until analysed. The water samples were then filtered through GF/C discs (Whatman, Maidstone, UK), and the discs, containing the cell material, were frozen and stored at −20 °C.

Two 500-ml subsamples were taken and a standard solution containing a mixture of nodularin and microcystin-LR, -RR and -YR was added to one of them. The samples were then concentrated according to the method reported by Lawton et al. [11].

2.3. High-performance liquid chromatography

The HPLC system consisted of a P 580 A LPG liquid chromatograph (Gynkotek, Munich, Germany) and a Rheodyne 7725 switching valve (Cotati, CA, USA). Two columns with different internal diameter were used to separate the target compounds: Kromasil C_{18} column, 3.5 μm×10 cm, with an internal diameter of 2.1 mm and 1 mm (Tracer, Teknokroma, Sant Cugat del Vallès, Spain). The mobile phases were Milli-Q water and acetonitrile, both containing 0.08% (v/v) formic acid. The injection volume was 5 μl.

2.4. Mass spectrometry equipment

Mass spectrometry experiments were conducted on a Navigator quadrupolar mass spectrometer (Finnigan, MassLab Group, Manchester, UK) with a coaxial electrospray source. Toxins were analysed in positive mode. Nitrogen was used as the drying gas. Spectra and chromatograms were processed with Masslab software version 2.0.

In LC–ESI-MS analyses, full-scan mass spectra from 500 to 1200 m/z were acquired in continuum mode at 3.00 s/scan. Eight ions (519.8, 1038.6, 825.5, 826.5, 1045.5, 1046.5, 995.6, 996.6) were monitored in continuum mode at 0.8 s/cycle with a dwell time of 100 ms when acquiring in selected ion monitoring (SIM) mode. Samples were introduced directly to the mass spectrometer via the HPLC column.

2.5. Identification

Microcystin-LR, -RR, -YR and nodularin were identified on the basis of both their retention time

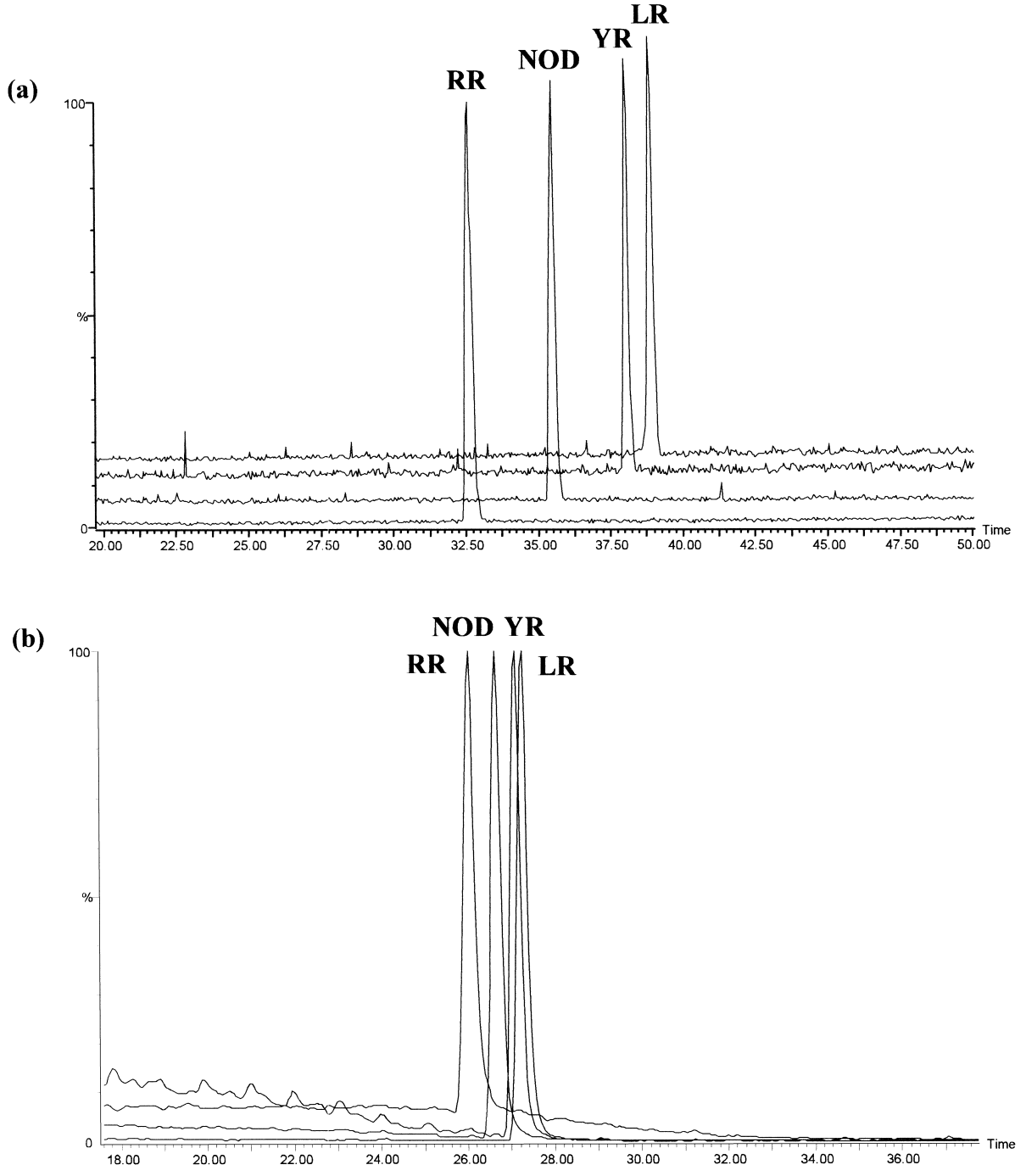

Fig. 2. Reconstructed ion chromatograms of a standard solution containing microcystin-LR, -RR, -YR and nodularin using (a) the 2 mm I.D. column and (b) the 1 mm I.D. column.

and mass spectra. Toxins different from the standards available were tentatively identified by comparing the mass spectrum provided by this technique to those available in the literature.

2.6. Quantitation

Quantitative analyses were carried out by external standard. Toxins different from microcystin-LR, -RR, -YR and nodularin were quantified related to microcystin-LR. Calibration curves were calculated daily.

3. Results

3.1. Chromatographic conditions

In the present work, two C_{18} columns with different internal diameter were used for the analysis of hepatotoxins by LC–ESI-MS. The mobile phases consisted of water (A) and acetonitrile (B), both containing 0.08% formic acid. Different gradients and flow rates were studied in order to optimise different parameters, such as the sensitivity, the chromatographic separation and the time of analysis.

The best separation with the 2 mm I.D. column was obtained at a flow rate of 200 μl min^{-1} with the following gradient: 10–30% B 20 min, 30–40% B 30 min, 40–70% B 5 min, 70% B 5 min. As can be observed in the reconstructed ion chromatogram obtained for a standard mixture of microcystin-LR, -RR, -YR and nodularin (Fig. 2a), an acceptable separation between chromatographic peaks was achieved. On the other hand, a second column with an internal diameter of 1 mm was used to improve the sensitivity of the method. A linear gradient of 0–100% acetonitrile in 20 min at a flow rate of 45 μl min^{-1} was chosen in order to reduce the time of analysis. Although separation observed between the chromatographic peaks when using the 1 mm I.D. column (Fig. 2b) is not very good, MS detection allows microcystins and nodularins to be identified and quantified correctly.

3.2. Optimisation of ESI parameters

Different ESI parameters, including the mobile phase and the drying gas flow rates, the source temperature and the capillary voltage, were optimised for each column in order to get the best sensitivity. The cone voltage was chosen in order to obtain only the protonated molecules with single or double charge, $[M+H]^+$ and $[M+2H]^{2+}$, respectively. The optimum ESI+ parameters for each column are summarised in Table 1.

3.3. Quality parameters

Some quality parameters for each analyte are shown in Table 2. As can be observed, detection limits, calculated by using a signal-to-noise ratio of 3, as low as 60–340 pg in full scan and 6–72 pg in SIM mode were achieved when using the 1 mm I.D. column. Limits of quantification were calculated by using a signal-to-noise ratio of 5. Repeatability and reproducibility ranged from 2.8 to 6.7 and from 6.6 to 11.7%, respectively, for the 2 mm I.D. column. Nevertheless, they varied from 6.4 to 9.7 and from 9.1 to 13.3%, respectively, for the 1 mm I.D. column.

3.4. Environmental samples

The method based on LC–ESI-MS using the 2 mm I.D. column was applied to the routine analysis of hepatotoxins in different water samples, such as water blooms and surface waters. On the other hand, the 1 mm I.D. column was used for the analysis of microcystins and nodularins in samples from treat-

Table 1
Optimum ESI+ parameters for the analysis of microcystins and nodularins using both the 2 mm and 1 mm I.D. HPLC columns

Parameter	Value	
Capillary voltage	3.25 kV	
Cone voltage	50.0 V	
Skimmer	1.6 V	
Skimmer lens offset	5.0 V	
RF lens	0.2 V	
Ion energy	1.1 V	
	2 mm I.D. column	1 mm I.D. column
Mobile phase flow rate	200 μl min^{-1}	45 μl min^{-1}
Gas flow rate	250 l h^{-1}	225 l h^{-1}
Source temperature	150 °C	100 °C

Table 2
Quality parameters for each analyte with both the 2 and 1 mm I.D. columns

Compound	LOD (ng)				LOQ (ng)				CV (%)			
	2 mm I.D. column		1 mm I.D. column		2 mm I.D. column		1 mm I.D. column		2 mm I.D. column		1 mm I.D. column	
	Full scan	SIR	Full scan	SIR	Full scan	SIR	Full scan	SIR	Repeat.	Reprod.	Repeat.	Reprod.
Mcyst-LR	0.18	0.06	0.08	0.01	0.30	0.09	0.14	0.02	6.7	12	7.8	9.2
Mcyst-RR	0.69	0.33	0.34	0.07	1.1	0.54	0.57	0.12	3.6	6.6	7.9	9.1
Mcyst-YR	2.1	1.2	0.14	0.02	3.4	1.9	0.24	0.04	2.8	6.8	6.4	13
Nod	0.08	0.02	0.06	0.01	0.13	0.03	0.10	0.01	6.0	7.2	9.7	12

Mcyst, microcystin; Nod, nodularin; Repeat., repeatability; Reprod., reproducibility.

ment water plants supplied by the reservoirs where water blooms were observed. However, no microcystins were detected in any treated water. Table 3 shows extracellular microcystin levels in some water bloom samples collected in Catalonia, northeast Spain, in 1998 and 2000. Microcystin-LR was identified in all the samples, while microcystin-RR was only identified in two samples (Sau and Susqueda reservoirs, 1998). The highest level of extracellular microcystin-LR, 270 $\mu g\ l^{-1}$, was detected in the sample collected from Sau reservoir in 2000.

As an example, extracellular microcystins iden-

Table 3
Levels of extracellular microcystins in samples from Sau and Susqueda reservoirs in Catalonia, northeast Spain, during a water bloom in 1998 and 2000

Sample	Concentration ($\mu g\ l^{-1}$)		
	Mcyst-LR	Mcyst-RR	Mcyst-YR
Sau reservoir, 1998	0.447	0.189	–
Susqueda reservoir, 1998	4.384	0.340	–
Caròs, Susqueda reservoir, 1998	35	–	–
Sau reservoir, 2000	270	–	–
Pasteral reservoir, 2000	0.240	–	–

A 5-μl sample was injected into the 2 mm I.D. column and mass spectra were acquired in full scan mode.

Table 4
Levels of extracellular microcystins identified in a water bloom sample extract from the river Tajo, central Spain, September 1999

Base peak (m/z)	Ion	Concentration ($\mu g\ l^{-1}$)[a]	Microcystin identity
520.0	$[M+2H]^{2+}$	3.578	Mcyst-RR
1049.6	$[M+H]^{+}$	1.157	Mcyst-(1,2,3,4-tetrahydrotyrosine)YR
1045.5	$[M+H]^{+}$	1.521	Mcyst-YR
995.6	$[M+H]^{+}$	1.623	Mcyst-LR
1029.7	$[M+H]^{+}$	2.537	Mcyst-FR, mcyst-M(O)R or [Dha^{7}]mcyst-HphR
1059.7	$[M+H]^{+}$	0.800	Mcyst-HtyR
1068.6	$[M+H]^{+}$	1.079	Mcyst-WR
1009.5	$[M+H]^{+}$	0.889	[D-Asp^{3}, $ADMAdda^{5}$]mcyst-LR, mcyst-HilR or [D-$Glu(OCH_3)^{6}$]mcyst-LR
949.7	$[M+H]^{+}$	3.064	Not identified

A 5-μl sample was injected into the 2 mm I.D. column and mass spectra were acquired in full scan mode. Mcyst, microcystin.
[a] Microcystins different from microcystin-RR and -YR were quantified related to microcystin-LR due to the lack of standards available.

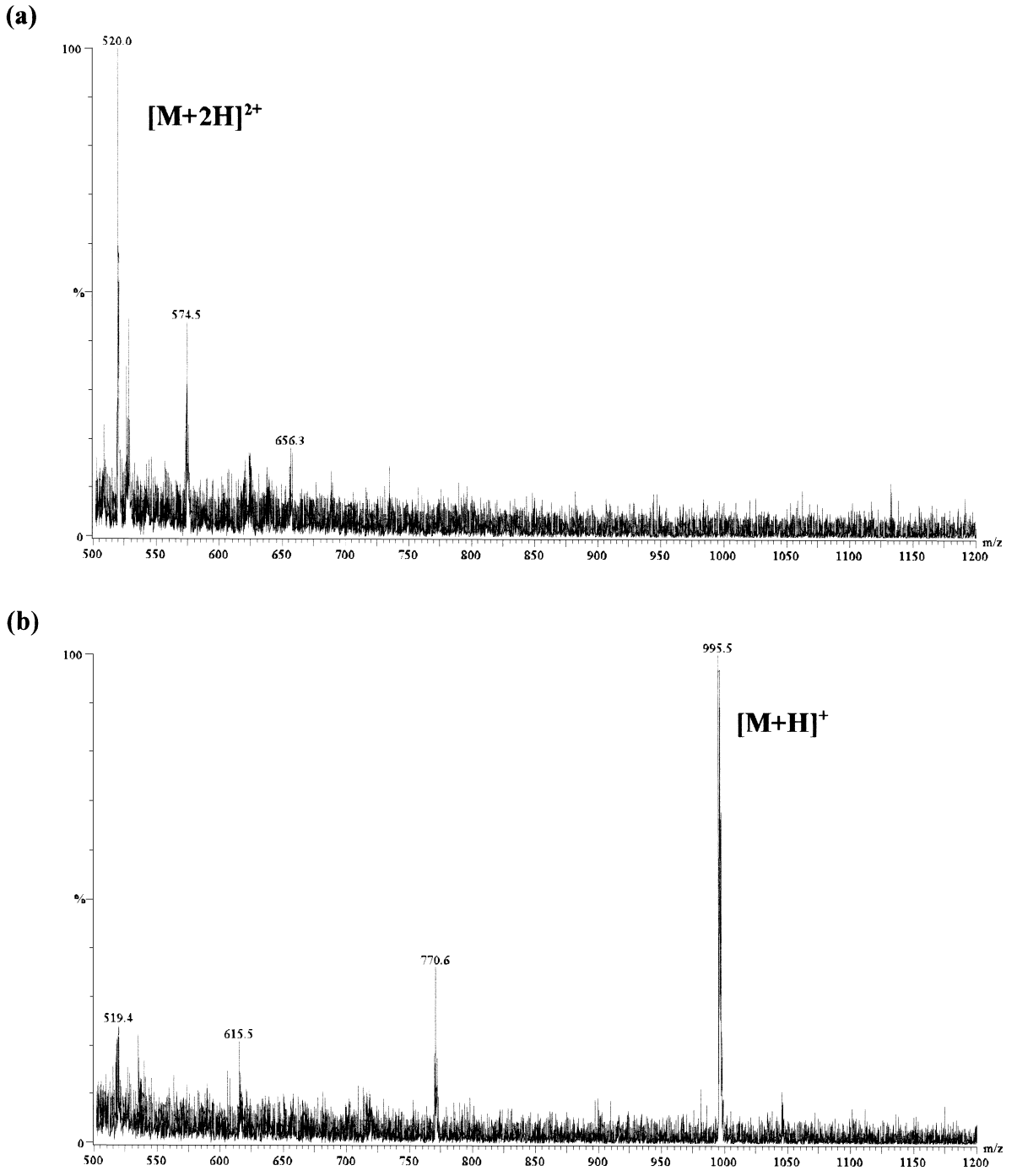

Fig. 3. Full scan electrospray mass spectra of (a) microcystin-RR, (b) microcystin-LR and (c) microcystin with the base peak at 1029.7 m/z detected in the water sample collected from the river Tajo in 1999.

tified and quantified in a water bloom sample extract from the river Tajo, central Spain, are summarised in Table 4. Microcystin-RR was found to be the main microcystin in this sample. Microcystin-YR and -LR could also be identified. Three different peaks with intense ions at 1049.6, 1059.7 and 1068.6 m/z were tentatively identified as microcystin-(1,2,3,4-tetrahydrotyrosine)YR, -HtyR and -WR, respectively. Two microcystins with 1029.7 and 1009.5 m/z ions as the base peaks were also observed. Three different analogs for each peak have been reported in the literature. Unfortunately, it was not possible to further determine the identity of these compounds. Finally, a compound with the base peak at 949.7 m/z was detected. However, it could not be matched with any of the known microcystins [5] and further experiments by tandem mass spectrometry must be performed in order to characterise this compound. Mass spectra of some microcystins detected in this sample (Fig. 3a–c) show unfragmented protonated molecules as the base peaks. Depending on the basic amino acids contained in the structure, mono $[M+H]^+$ or double charged $[M+2H]^{2+}$ ions were obtained. The mass spectra of microcystin-LR and microcystin with 1029.7 m/z ion as the base peak mainly presented the single charged ions $[M+H]^+$ at 995.5 and 1029.7, respectively (Fig. 3b,c). However, the main peak observed in the mass spectrum of microcystin-RR was the double charged ion $[M+2H]^{2+}$ at 520.0 m/z, probably due to the presence of two arginine residues (Fig. 3a). All ESI+ mass spectra obtained were similar to those reported in the literature [16].

4. Discussion

An analytical method for the analysis of microcystins and nodularins by LC–ESI-MS using microbore columns was developed. As is known, the low optimum flow rates, 200 and 45 μl min^{-1} for the 2 mm and 1 mm I.D. columns, respectively, were suitable for electrospray ionisation mass spectrometry. Therefore, no split was required when coupling LC to MS with the subsequent enhancement of sensitivity. With the optimum electrospray ionisation conditions obtained, limits of detection of 11, 72, 21 and 6 pg were achieved for microcystin-

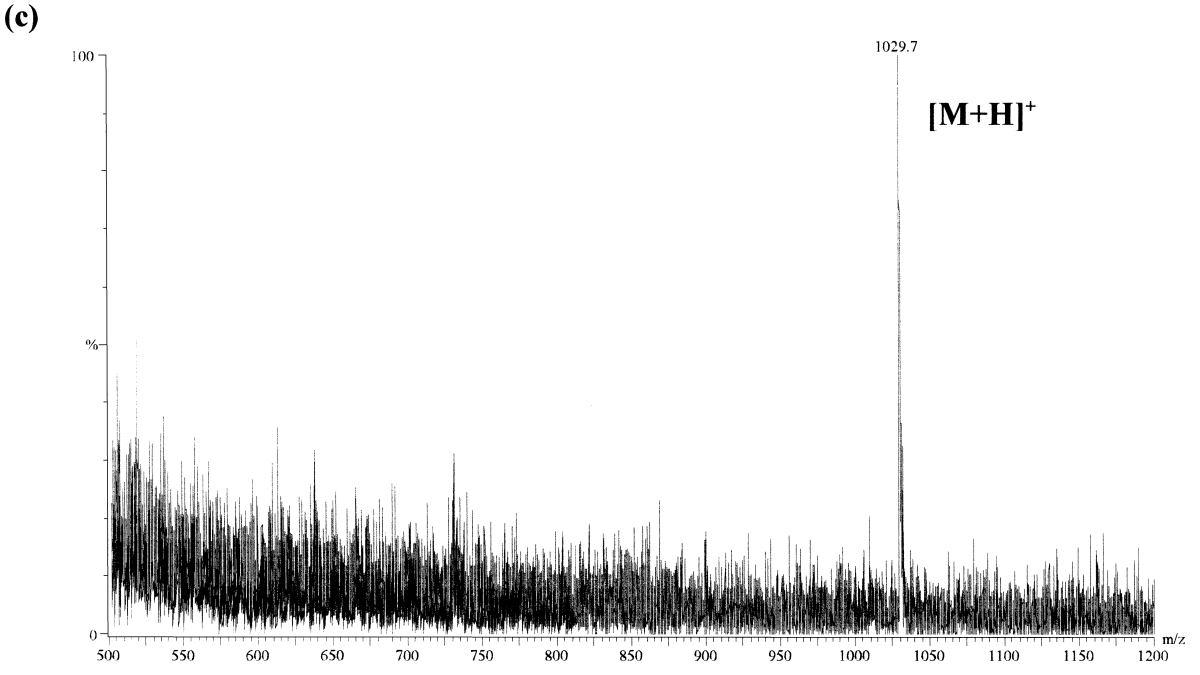

Fig. 3. (*continued*)

LR, -RR, YR and nodularin, respectively. These values, obtained when working with the 1 mm I.D. column and SIM acquisition mode, have been found to be the lowest detection limits ever reported in the analysis of hepatotoxins by LC–MS. This report shows that LC–ESI-MS is a simple, sensitive and suitable technique for the monitoring and identification of a wide range of cyanobacterial hepatotoxins in environmental samples. Nevertheless, tandem mass spectrometry must be considered when more accurate identification of unknown compounds is required.

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