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Liquid chromatography–electrospray ionisation-mass spectrometry based method for the simultaneous determination of algal and cyanobacterial toxins in phytoplankton from marine waters and lakes followed by tentative structural elucidation of microcystins

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Abstract

A liquid chromatography (LC)-based method with mass spectrometric (MS) detection was developed for simultaneous determination of various algal and cyanobacterial toxins extracted from phytoplankton occurring world-wide in marine waters and lakes. The method enables quantification of saxitoxin, anatoxin-A, domoic acid, nodularin, microcystins, okadaic acid and dinophysistoxin-1 with a single chromatographic run. In addition, the applied chromatographic conditions allow isolation and identification of substances suspected to be ''new'' microcystins (cyclic peptides) by fraction collection, hydrolysis, derivatisation of resulting free amino acids with the modified chiral Marfey's reagent *N*-α-(2,4-dinitro-5-fluorophenyl)-L-valinamide (L-FDVA) and enantioselective analysis of the amino acid derivatives by LC–ESI-MS.
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1. Introduction

During the past two decades a variety of structurally different algae and cyanobacteria borne toxins have been identified. Such toxins present in marine waters are: okadaic acid (OA), dinophysistoxin-1 (DTX-1) [1], saxitoxin (STX) [2], domoic acid (DA) [3], and nodularin (NOD) [4]. The cyanobacterial heptapeptides—called microcystins (MCs)—and the neurotoxic anatoxin-A mainly occur in freshwater lakes [5–8]. The molecular structures of these toxins (Fig. 1) reveal chemical differences that are important with respect to their simultaneous determination.

The threat to human consumers from algal and cyanobacterial toxins in marine organisms and the related economic problems for the fishery industry have been described extensively [9–14]. Preventive measures for consumer protection, as well as basic scientific research on harmful algal blooms (HABs), require powerful analytical methods. Such methods have been developed for most of the toxin groups [15]. Due to the structural differences among the toxins, analytical methods are typically based on

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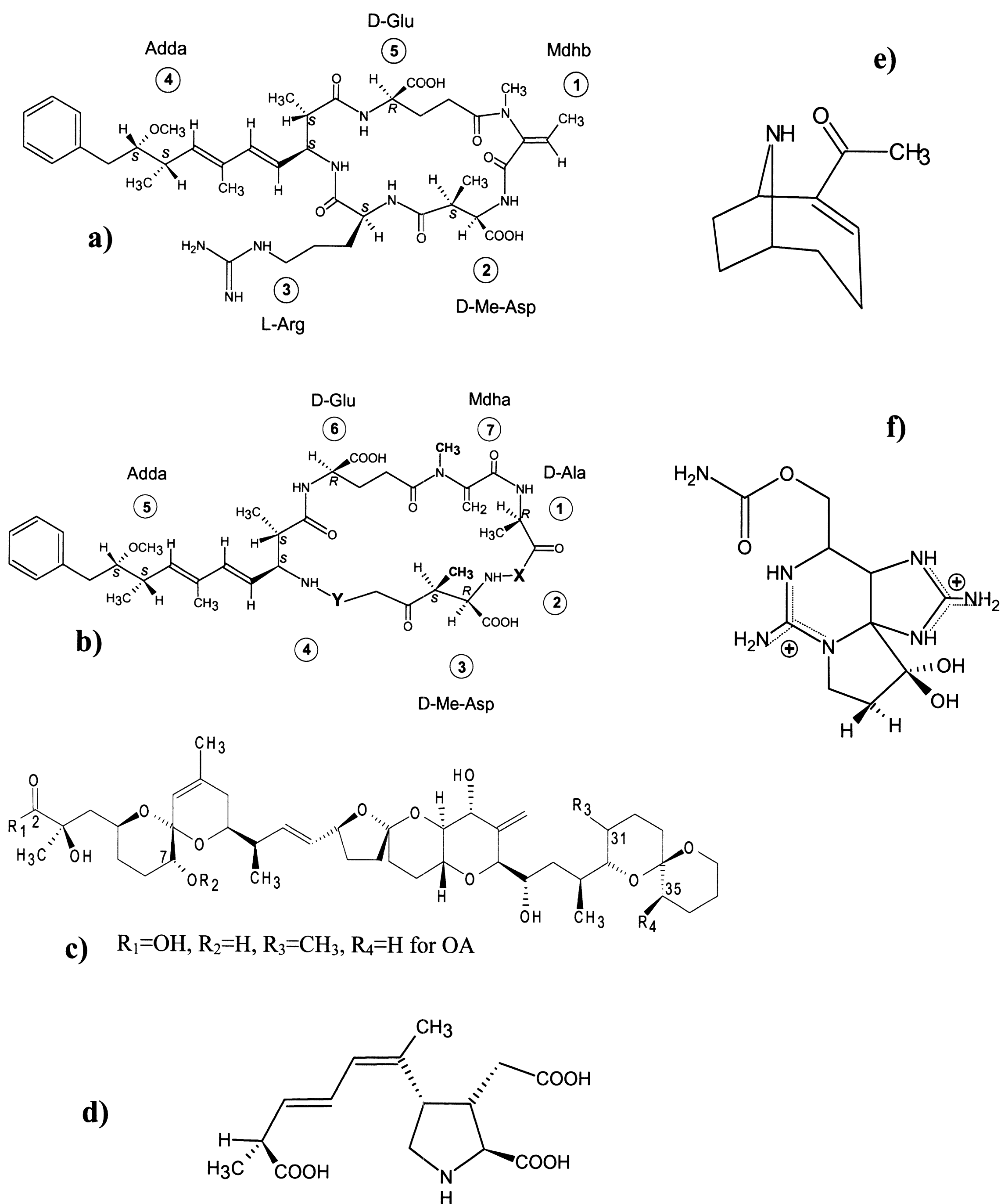

Fig. 1. Chemical structure of (a) nodularin, (b) microcystins (general structure), (c) okadaic acid, (d) domoic acid, (e) anatoxin-A and (f) saxitoxin.

ion-pair chromatography [16], ion-exchange chromatography [17] and reversed-phase chromatography coupled to UV [18] or fluorescence detection [19]. When using optical detection methods, pre- or post-column derivatisation procedures are necessary for toxins without chromophoric groups [20,21], and cumbersome clean-up procedures, prior to the chromatographic separation, are typically necessary to exclude interferences from matrix compounds [22–26]. These toxin-specific methods have their benefits and are in use at numerous analytical laboratories. However, there is a need for simultaneous detection methods, especially if large batches of samples have to be screened for HAB toxins. Recently, liquid chromatography–electrospray ionisation-mass spectrometry (LC–ESI-MS) methods became available, which combine efficient separation power with generic detection for the different toxin groups [27–30]. By application of the highly selective mass detector, reliable results are obtained and sample preparation protocols are usually simpler and less laborious.

Ideally, risk assessments should give information regarding the aforementioned toxins present in the phytoplankton under investigation. This information should become available immediately during, for example, research cruises or when complex phytoplankton communities from aquacultures have to be analysed. Therefore, a universal solvent is needed for extraction of the different algal and cyanobacterial toxins and highly selective and sensitive mass spectrometry is required for measurement of these toxins with one analytical method. Consequently, efforts have been made to develop LC–ESI-MS methods to analyse most of the HAB toxins in contaminated biological materials.

Within the group of paralytic shellfish poisoning (PSP) toxins, besides STX, further PSP toxins such as neosaxitoxin, gonyautoxins, and their decarbamoyl variants can be found in sample material obtained from cyanobacteria. The presented method allows extension for quantitation of further PSP toxins. However, since the naturally available material in our laboratory contained mainly STX, this paper concentrates on STX and we refer to a recently published LC–MS method designed for various PSP toxins [17].

In the field of HAB toxin analyses problems arose due to the lack of standards for numerous structural variants of the hepatotoxic MCs. In the early 1980s, the structure of these cyanobacterial hepatotoxins was clarified and found to consist of seven amino acids forming a cyclic peptide [31,32]. Two amino acids (at positions 2 and 4 of the ring) are variable L-amino acids (X and Y, Fig. 1b). The structural variations are indicated by suffix letters; e.g., MC-LR contains leucine (L) at position 2 and arginine (R) at position 4. Further research identified other MCs. Here structural variations originating from side chain modifications other than at positions 2 or 4. (Dha)MCs result when *N*-methyldehydroalanine (Mdha) is demethylated at position 7, while the presence of 2-amino-2-butenoic acid (Dhb) at position 7 leads to (Dhb)MCs. Demethylation of D-*erythro*-β-methyl aspartic acid (D-MeAsp) at position 3 leads to (D-Asp)MCs. In addition, variations of the Adda (3-amino-9-methoxy-2,6,8,-trimethyl-10-phenyl-deca-4(*E*),6(*E*)-dienoic acid) at position 5 have also been described. To date more than 70 MCs have been isolated and characterized [33]. Therefore, determination of such MC variants, in addition to the commercially available MC-RR, MC-YR, MC-LR, MC-LA, MC-LW and MC-LF, is of importance.

Structure elucidation of unknown MCs was achieved by fraction collection of the separated compounds subsequent to their LC separation. The LC–ESI-MS device allows one to split the eluate, for partial collection by an automated fraction collector and synchronical MS or MS–MS detection. The fractions were subjected to microwave-assisted hydrolysis [34] followed by derivatisation of the resulting amino acids with the chiral reagent *N*-α-(2,4-dinitro-5-fluorophenyl)-L-valinamide (L-FDVA). This allows separation of D- and L-amino acids on a non-chiral C_{18} column and determination as diastereomeres with both UV and mass spectrometric detectors [35]. The principle of this derivatisation method for amino acids was already reported in 1984 [36].

The LC–ESI-MS method presented here is based on the use of a single quadrupole MS. Liquid chromatography–mass spectrometry with triple–quadrupole mass spectrometers (MS–MS) may enhance sensitivity and selectivity of toxin determination by measuring characteristic fragmentation patterns of the molecules [37]. In this study, MS–MS

techniques were applied only to obtain the fragmentation pattern of a compound suspected to be a ''new'' MC and for verification of our results regarding the structure of MCs in cyanobacteria after determination of the MC amino acid profile.

Recovery and repeatability of the proposed LC–ESI-MS method were tested with solutions containing six MCs (MC-RR, MC-YR, MC-LR, MC-LA, MC-LW, MC-LF), NOD, anatoxin-A, DA, OA, DTX-1 and STX. In addition, the method was applied to different phytoplankton cultures of algae and cyanobacteria grown under laboratory conditions and to sample material obtained during research cruises.

2. Materials and methods

2.1. Chemicals

DA, OA, DTX-1 and STX standard solutions were obtained from the National Research Council (NRC) Canada (Halifax, NS, Canada). Non-certified standards of, anatoxin-A, MC-RR, MC-YR, MC-LR, MC-LA, MC-LW, MC-LF, and NOD were purchased from Calbiochem (La Jolla, CA, USA). Pure D- and L-amino acids were obtained from Sigma (St. Louis, MO, USA). The amino acids were dissolved in 0.1 N HCl. The algal and cyanobacterial toxin standards were diluted in methanol–water (50:50, v/v).

Acetonitrile and methanol obtained from Baker (Deventer, The Netherlands) were HPLC grade. Hydrochloric acid, trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA) were obtained from Sigma. Water was purified to HPLC-grade quality with a Millipore-Q RG ultra pure water system from Millipore (Milford, CT, USA). Nitrogen for the turbo ion-spray (TIS) interface of the LC–ESI-MS system was generated using a Nitrox UHPLCMS nitrogen generator from Domnick Hunter (Durham, UK). All chemicals were at least analytical grade.

2.2. Sample material

Samples of phytoplankton and aqueous phytoplankton were collected from the Baltic (West-German coast) and from freshwater lake ''Behlendorf'' (Rendsburg, Schleswig-Holstein, Germany) under a monitoring program of the ''Landesamt für Natur und Umwelt'' (LANU), Schleswig-Holstein, Germany. Isolates from cultured *Pyrodinium bahamense* were obtained from the Institute of Biology, University of the Philippines, Quezon City, Philippines. The *Microcystis aeruginosa* bloom material was collected by the Center of Biotechnology, Vietnam National University, Hanoi, Vietnam. The phytoplankton mix consisting of *Alexandrium tamarense*, *Nodularia spumigena*, and *Pseudonitschia* sp. originated from the Kalmar Algae Collection (KAC) Kalmar, Sweden. All field samples were taken from 1–2 m water depth.

2.3. Extraction of HAB toxins

Aqueous phytoplankton samples were filtered over glass fiber filters (retention size 2 μm). Filters with planktonic material or lyophilized biomass were placed in 2-ml Eppendorf cups and extracted twice with a mixture of water–methanol (50:50, v/v) by 10 min sonication in an ultrasonic bath and subsequent treatment for 2 min with an ultrasonic homogeniser Sonopuls GM 70 (Bandelin, Berlin, Germany). The extracts were centrifuged (14 000 rpm) and the supernatant was filtered using 0.22-μm PTFE syringe filters (Roth, Karlsruhe, Germany). The extracts were directly subjected to LC–ESI-MS analysis.

2.4. Hydrolysis of the MCs

The hydrolysis of the MCs was done according to Reichelt et al. [34].

2.5. LC–ESI-MS determination of the individual toxins

LC of algal and cyanobacterial toxins was performed with a PE Series 200 Quaternary Pump and a PE Series 200 autosampler (Perkin-Elmer, Shelton, CT, USA). The chromatographic separation was carried out on a reversed-phase column (Aqua, 5 μm C_{18}, 250×4.60-mm I.D., Phenomenex, Torrance, CA, USA) using gradient elution (Table 1). The flow-rate was 0.7 ml min^{-1}, throughout.

MS measurements were performed using an API

Table 1
Gradient for LC elution

Time (min)	Eluent A: acetonitrile (%)	Eluent B: aqueous 0.01 *M* TFA containing 0.01% HFBA (%)
0	5	95
0–12	40	60
12–17	70	30
17–27	70	30
27–28	5	95
28–36	5	95

165 mass spectrometer with an atmospheric pressure ionization (API) source operating in turbo ion-spray (TIS) mode (Applied Biosystems, Foster City, CA, USA). The eluate from the LC column was transferred to the MS device using a split ratio of 5:1 (volume to waste/volume transferred), and nitrogen (heated to 450 °C, 7.5 l min^{-1}) was applied to dry the ion-spray aerosol. Nitrogen was also used as nebuliser gas with a flow of 0.6 l min^{-1}. The ionization voltage of the TIS interface was set to 5.2 kV. The MS system was operated in positive multiple ion detection (MID) mode to give highest sensitivity and selectivity. $[M+H]^+$ ions centered at: m/z 166.5 (anatoxin-A), 300.2 (STX), 312.2 (DA), 520.2 (MC-RR), 825.8 (NOD), 1045.8 (MC-YR), 995.7 (MC-LR), 910.8 (MC-LA), 1026.8 (MC-LW), 987.8 (MC-LF), 805.7 (OA), and 819.7 (DTX-1) were monitored. For determination of unknown MCs the following mass ranges were scanned in additional runs: 500–600 Da (doubly charged MCs) and 800–1200 Da (singly charged MCs). Fractions were collected in 10-s intervals using a Abimed 202 automated fraction collector (Abimed, Langenfeld, Germany).

2.6. ESI-MS–MS measurements

ESI-MS–MS experiments for confirmation of ''new'' MCs in the collected fractions were performed on an API QSTAR Pulsar System (Applied Biosystems, Foster City, CA, USA) by flow injection. The ion source parameters applied were the same as those used for LC–ESI-MS. Specific MS–MS parameters were: collisional activated dissociation (CAD; collision gas pressure 3 au), collision energy (CE; 45 eV), and declustering potential (DP; 25 eV).

2.7. Amino acid analysis

Derivatisation and chromatographic separation of free amino acids formed by hydrolysis of MCs was carried out according to Fujii et al. [35], but L-FDVA was used for derivatisation and the chromatographic separation was carried out on a reversed-phase column Aqua, 5 μm C_{18}, 250×4.60-mm I.D. (Phenomenex).

For detection of the amino acid derivatives the ion source parameters for the API 165 were the following: ionisation voltage of the TIS interface was set to 4.0 kV, nitrogen (heated to 280 °C, 7.5 l min^{-1}) was applied to dry the ion-spray aerosol. Nitrogen was also used as nebuliser gas with a flow of 0.6 l min^{-1}. The MS system was operated in positive MID mode. $[M+H]^+$ ions m/z 312.2 (MeAmin), 370.2 (Ala), 386.4 (Ser), 412.5 (Leu, iso-Leu), 414.3 (Asp), 428.4 (Glu and MeAsp), 447.5 (Phe), 455.4 (Arg), 462.3 (Tyr), and 612.4 (Adda) were analysed. For the determination of other amino acid derivatives scan-mode (300–650 Da, step size: 0.2 Da) was applied.

2.8. Linearity, repeatability, precision and limit of detection

Since most HAB toxins are not commercially available as certified reference standards, the repeatability and linearity of the LC–ESI-MS method were examined by injecting a series of solutions containing different concentrations of STX, OA, DTX-1, DA, MCs, NOD, anatoxin-A, and the amino acid derivatives. Each analyte solution was injected in triplicate. The limits of detection for the different toxins were calculated from the chromatograms, at a signal-to-noise ratio of 5:1.

Quantitative determination of the toxins was carried out by comparing peak areas in sample chromatograms with the corresponding peak areas obtained from the pure toxins. The recovery of the individual HAB toxins was tested by spiking a non-toxic extract from the cyanobacterium *Spirulina* sp.

3. Results and discussion

3.1. LC–ESI-MS analyses of algal and cyanobacterial toxins

Observations made during research cruises in the Baltic Sea confirmed that naturally occurring phytoplankton communities often consist of numerous algal and cyanobacterial species.

A broad spectrum of HAB toxins, related to various toxic syndromes, were measured. The proposed LC–ESI-MS multitoxin method allows rapid determination of the relevant toxins within one chromatographic run; this method is ideally suited for on board analyses of phytoplankton and for monitoring phytoplankton in aquacultures.

The toxins of interest were well separated under the applied LC–ESI-MS conditions (Figs. 2 and 3). However, matrix interferences may hamper quantification while operating the instrument in MID or scan mode. In these cases extraction of the mass from the target compound will give baseline resolved peaks and enable quantification (for example, $[M+H]^+$ for STX, inset in Fig. 4).

Comparison of the accuracy of quantitation of phytoplankton toxins in showed values around 10%, similar to those values obtained with other methods (Table 3). This is remarkable, since the multitoxin method is based on a universal extraction solvent (water–methanol, 50:50, v/v).

The multitoxin method was applied in the determination of HAB toxins in various environmental samples and in the analysis of laboratory-grown phytoplankton. Since cell numbers were not determined, the toxin content was calculated as ng toxin per 1 ml culture medium (or per 1 ml sea water), or expressed as toxin content per mg lyophilized phytoplankton (Table 2). In one case, laboratory-grown phytoplankton species were pooled in order to prepare an artificial multitoxin mixture.

3.2. Sample preparation

The methanol–water (50:50, v/v), extraction sol-

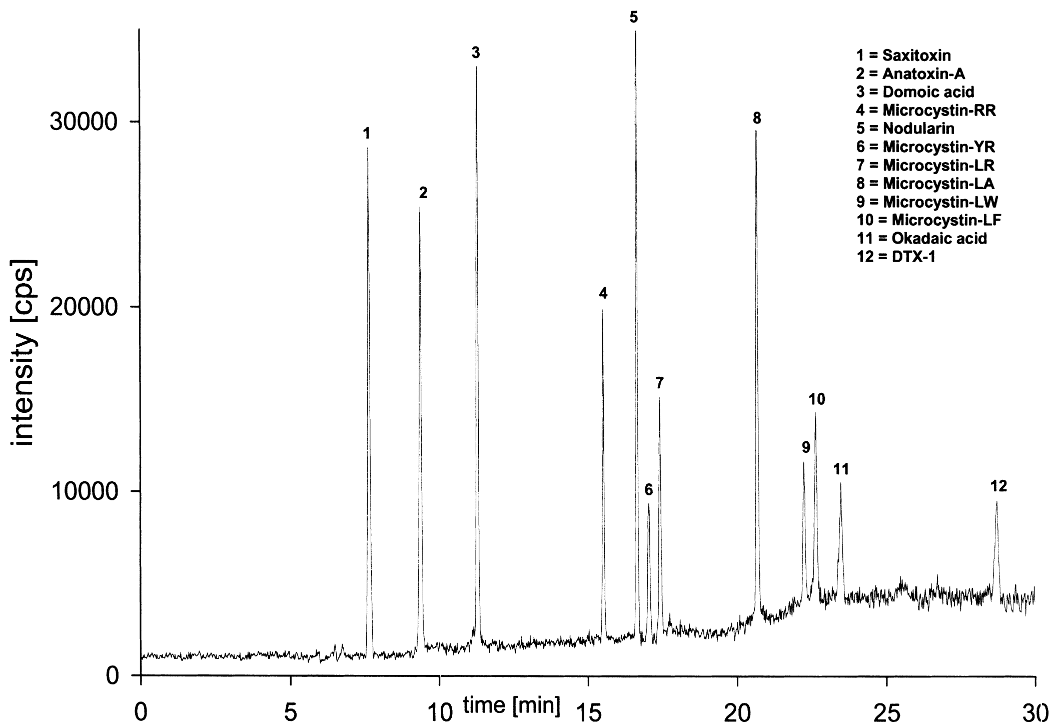

Fig. 2. LC–ESI-MS: MID chromatogram of standards of HAB toxins.

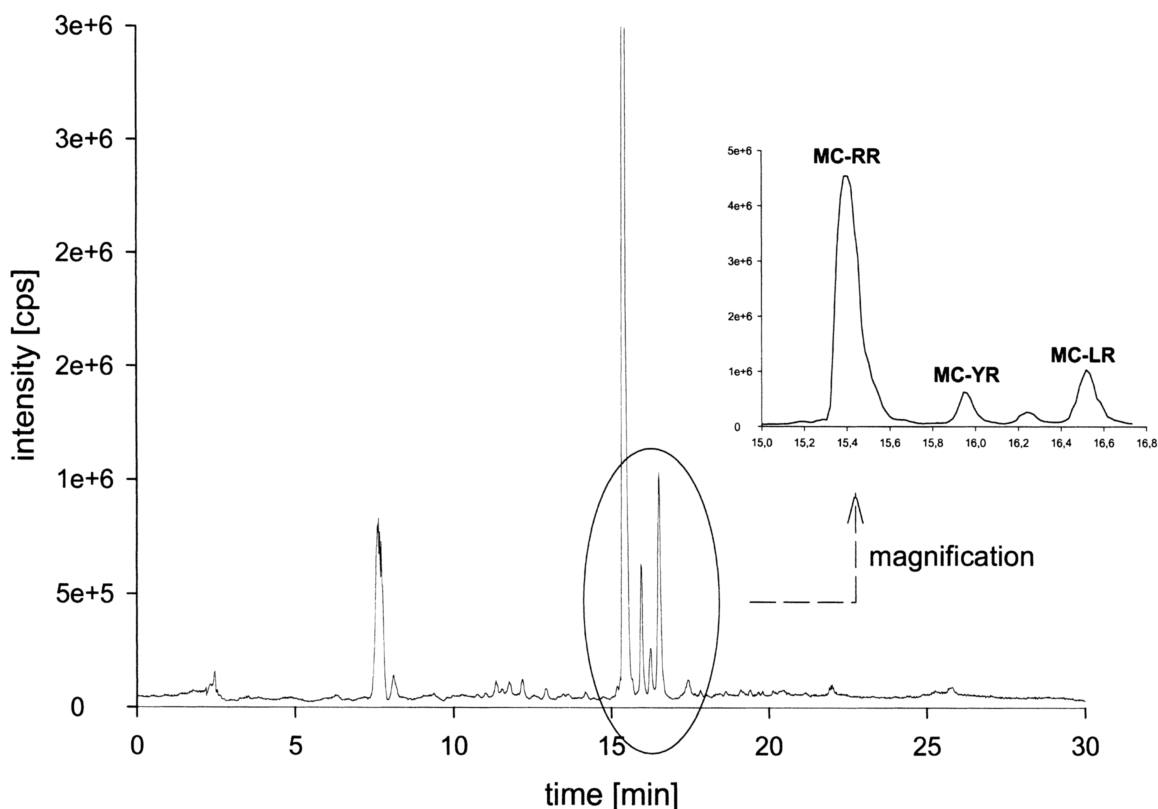

Fig. 3. LC–ESI-MS: MID chromatogram of a *Microcystis aeruginosa* extract (Hai Phong, Vietnam).

vent proved to be a powerful tool for extraction of all HAB toxins under investigation. The recovery of the multitoxin method was about 90%, which compared well with established methods for toxin determination using selective extraction solvents. The extraction efficiency was determined by comparing samples extracted according to established protocols with those extracted with the universal solvent (Table 3).

3.3. Structure elucidation of MCs

The advantage of MC structure elucidation by the presented LC–ESI-MS method was demonstrated by analysing a phytoplankton sample from ‘‘Lake Behlendorf’’, Rendsburg, Schleswig-Holstein, Germany. The dominant species in this lake during the summer 2001 bloom was identified as *Planktothrix rubescence*, a cyanobacterium also found in other German water-bodies [38]. None of the six standard MCs were detected in this sample. Therefore, the determination of a suspected ‘‘new’’ MC was carried out by setting the mass spectrometer to scan mode (500–600 and 800–1200 Da). The base peak was found at a retention time (RT) of 16.2 min, having a mass of 513.2 Da and corresponding to a $[M+2H]^{2+}$ ion. Additionally, a low intensity signal at 1024.5 Da was observed, corresponding to a $[M+H]^{+}$ ion (Fig. 5). For confirmation, the amino acid composition was analysed after fraction collection, hydrolysis and derivatisation with L-FDVA. The amino acids D-Ala, D-Asp, D-Glu, L-Arg and Adda (not available as a standard, but identified from comparison of retention time, and m/z 612.2, of Adda obtained from a hydrolysed MC-RR standard) were detected. Methylamine (MeAmine) and D-MeAsp were not detected (Fig. 6).

Moreover, the fact that the dominant ion in this sample is doubly charged (a $[M+2H]^{2+}$ peak of 513.2 Da), which is typical for a MC containing two

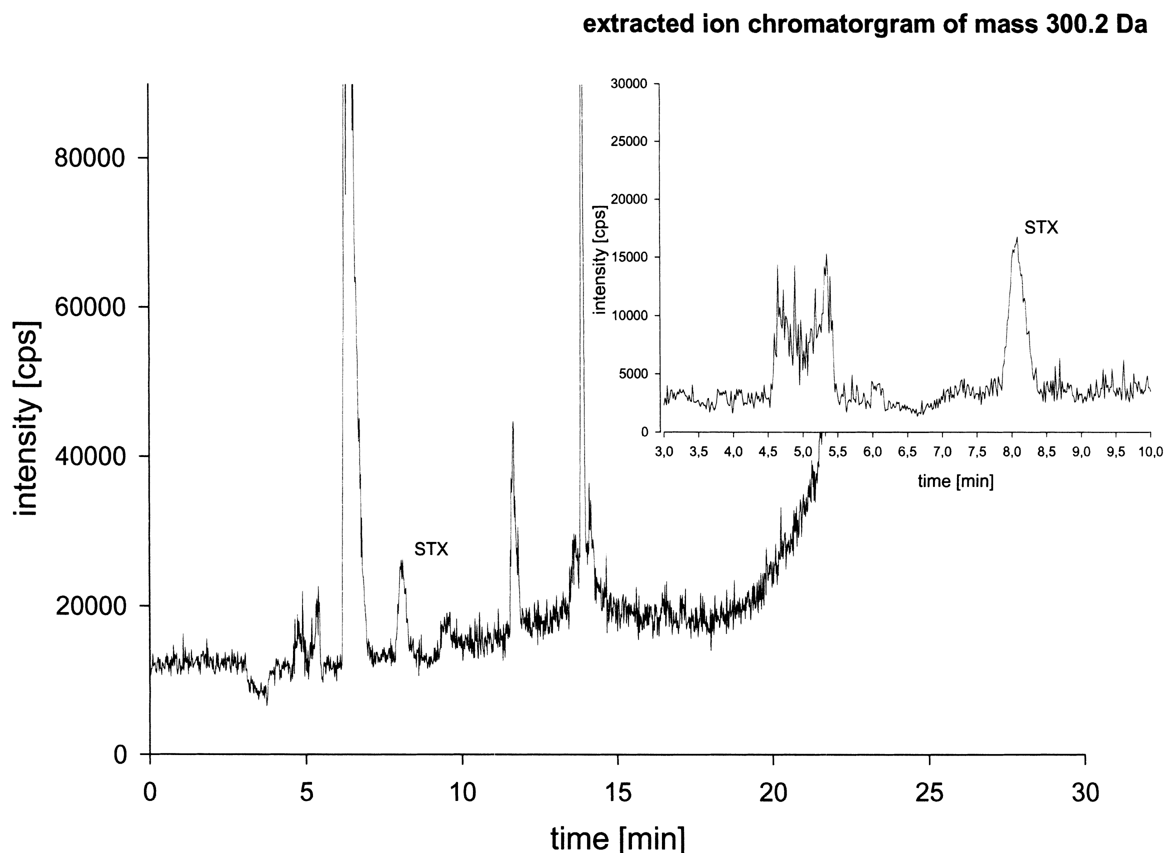

Fig. 4. LC–ESI-MS: MID chromatogram of extracted cultured *Pyrodinium bahamense*. Inset showing extracted mass for $[M+H]^+$ of m/z 300.2 for STX.

Arg residues [39], and that the RT is close to MC-RR led to the conclusion that an MC-RR variant, [D-Asp^3,(E)-Dhb^7]MC-RR, was present. This MC produced by *Planktotrix rubescence* is composed of 2-amino-2-butenoic acid (Dhb) instead of *N*-methyldehydroalanine (Mdha), and D-Asp instead of D-MeAsp. Dhb is not stable as a free amino acid; therefore, it cannot be derivatised with L-FDVA and remains undetected in the amino acid analysis (Fig. 6). [D-Asp^3,(E)-Dhb^7]MC-RR has been previously reported to be produced by *Planktothrix* spp. (formerly the *Oscillatoria*) [40–42]. The structure of this MC and the ESI-MS–MS spectrum with the fragment ion list are shown in Fig. 7.

The *Microcystis aeruginosa* sample from Vietnam contained MC-RR, MC-YR, MC-LR, in addition to several MCs not available as standards (Table 2 and Fig. 3). Of these MCs we focused on the unambiguous detection and exact quantification of MC-WR (W=tryptophan) by application of the previously described method for amino acid analysis. In addition, the LC–ESI-MS method allowed confirmation of the presence of MC-WR by comparing retention time and molecular mass with a self prepared standard. Demethylated MC variants (probably [D-Asp^3]MC-LR and [Dha^7]MC-LR) in this *Microcystis aeruginosa* sample from Vietnam were identified by matching retention times and molecular masses [43].

Although the multitoxin LC–ESI-MS method allows successful separation and general identification, with regards to structure elucidation, co-elution of some demethylated MC variants with other MCs (during the fraction collection for amino acid analysis) may hamper structure assignment.

Table 2
HAB toxins found in phytoplankton samples (determined by LC-ESI-MS)

HAB toxin	Mixture of cultured *Alexandrium*, *tamarense*, *Nodularia spumigena* pseudo-*nitschia* sp. (ng/ml)	Sample of *Planktotrix rubescence* Lake Behlendorf, Germany, May 2001 (ng/ml)	Sample of *Microcystis aeruginosa*, Bay of Hai Phong, Vietnam, April 2001 (ng/mg dry mass)	Culture of *Pyrodinium bahamense*, isolated in the Philippines (ng/ml)	Phytoplankton sample from the South-Western Baltic, German coast, July 2001 (ng/ml)
STX	0.05	n.d.	n.d.	0.15	n.d.
DA	17	n.d.	n.d.	n.d.	n.d.
MC-RR	n.d.	n.d.	1013	n.d.	n.d.
NOD	354	n.d.	n.d.	n.d.	90
MC-YR	n.d.	n.d.	84	n.d.	n.d.
MC-LR	n.d.	n.d.	420	n.d.	n.d.
[D-Asp^3, Dhb^7] MC-RR[a]	n.d.	352	n.d.	n.d.	n.d.
MC-WR**	n.d.	n.d.	60	n.d.	n.d.
Demethylated MC-LR variant[b]	n.d.	n.d.	54	n.d.	n.d.

n.d., not detected.
[a] Quantified using MC-RR standard.
[b] Quantified using MC-LR standard.

Table 3
Statistical parameters for calibration and recovery data

Toxin	RSD (%)	No. of datapoints	R^2	Recovery (%)[a]	LOD (ng) abs.	Extraction efficiency (%)[b]	Reference for single toxin methods
STX	2.7	7	0.996	96	0.5	86	[44]
Anatoxin-A	3.2	4	0.959	103	0.5	102	[46]
DA	3.7	5	0.987	96	0.5	92	[47]
MC-RR	2.4	5	0.997	99	1.0	95	[48]
NOD	2.5	5	0.997	100	0.5	102	[48]
MC-YR	2.8	4	0.998	103	1.5	91	[48]
MC-LR	3.4	5	0.983	97	1.0	99	[48]
MC-LA	2.4	5	0.995	102	0.5	89	[48]
MC-LW	1.8	5	0.991	113	1.0	91	[48]
MC-LF	3.2	5	0.989	101	1.0	91	[48]
OA	0.6	5	0.994	98	1.0	88	[45]
DTX-1	7.1	6	0.992	93	1.0	89	[45]

RSD, relative standard deviation (conc. range tested: 2–100 ng toxin on column); LOD, limit of detection.
[a] Based on comparison of peak areas obtained by single toxin LC methods with peak areas obtained by the multitoxin method injecting the same amount of toxin at a concentration of 10 ng/μl (STX: 5 ng/μl).
[b] Extraction efficiency (mean values of triplicate analyses) is based on comparing the extraction yield using methanol–water (50:50, v/v) with the extraction yield using established single toxin methods (see Refs.).

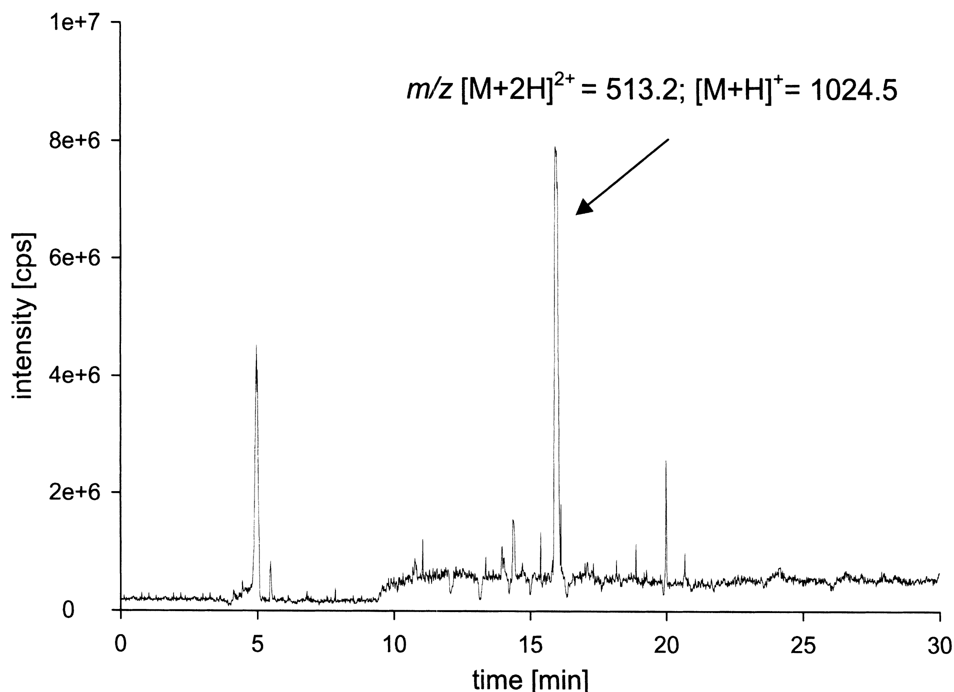

Fig. 5. LC–ESI-MS: scan mode chromatogram (500–600, 800–1200 Da) of phytoplankton extract from lake Behlendorf, Germany, containing *Planktothrix rubescence*.

3.4. Method calibration and validation

Table 3 lists the statistical parameters for the simultaneous LC–ESI-MS determination of the investigated HAB toxins. For all toxins, correlation coefficients are provided to illustrate the linearity of the calibration curves regarding the calibration range from 1 to 50 ng toxin on column. The mean values of the peak areas of the toxins, and their relative standard deviations (RSD) demonstrate the good precision and repeatability of the method. Most RSDs were less than 3.9%, the late eluting DTX-1 showed a RSD of 7.1% due to slight variances in the peak shape which resulted in a slightly different integration. The absolute limit of detection (LOD) of the different toxins were determined to be 0.5 ng for STX, DA, anatoxin-A, MC-LA, NOD, and 1.0 ng for MC-RR, MC-YR, MC-LR, MC-LW, MC-LF, OA and DTX-1. In addition, a toxin-free *Spirulina* sp. extract was spiked with the HAB toxins to determine the recovery under the influence of the matrix. The recoveries ranged between 96 and 114%, and the RSD of the peak areas were comparable to those determined for the standard solutions. Thus, both recovery and accuracy were satisfactory, especially taking into consideration that 11 HAB toxins could be quantified rapidly and unambiguously.

4. Conclusion

The proposed LC–ESI-MS method for simultaneous determination of several classes of algal and cyanobacterial toxins proved to be a powerful tool for monitoring phytoplankton blooms. The biomass was extracted with methanol–water (50:50, v/v). By application of this solvent mixture, the HAB toxins that arise during a phytoplankton bloom are extracted in high yields. The extracts are injected directly into the LC–ESI-MS device, and are well separated in a single 30-min chromatographic run, and unambiguously detected by ESI-MS. In addition, the presented LC–ESI-MS multitoxin method provides reliable MC analyses. Structure elucidation for those MC

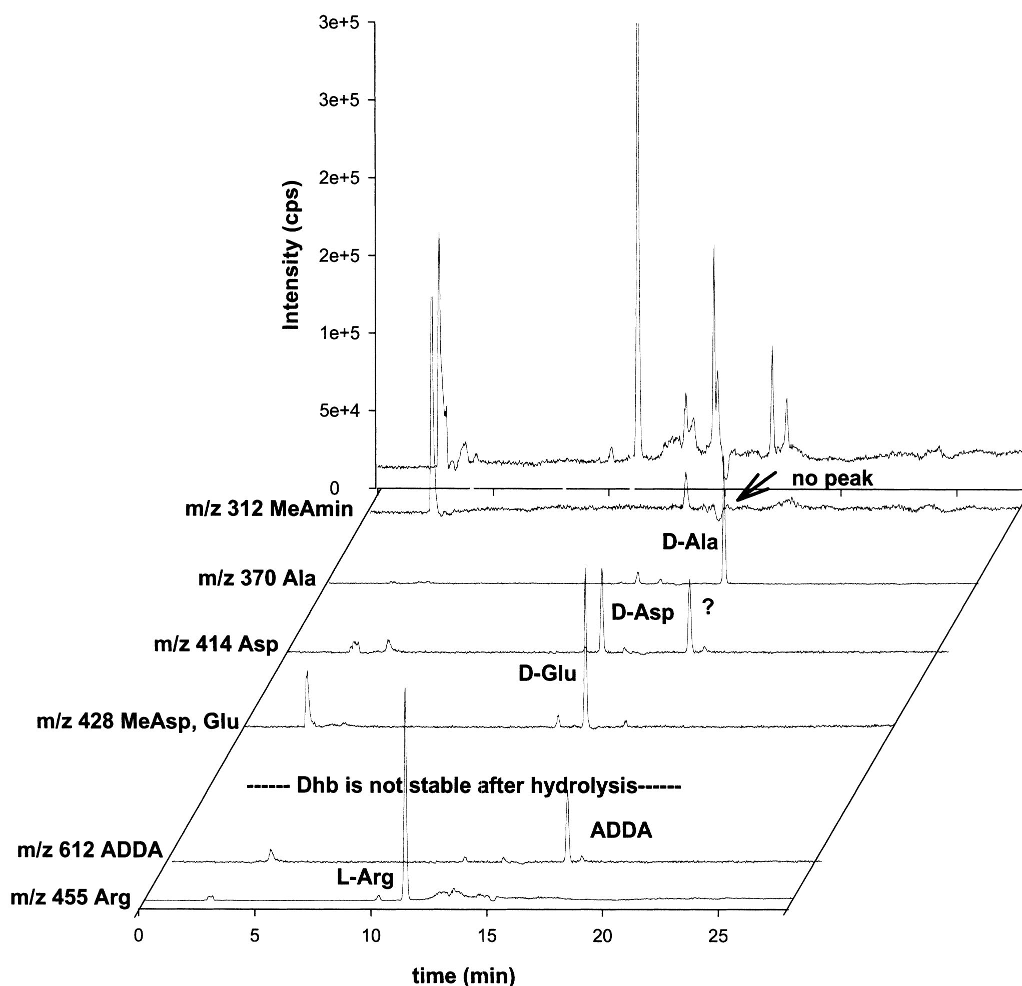

Fig. 6. LC–ESI-MS: MID chromatogram of amino acids derivatised with L-FDVA obtained after hydrolysis of compound with m/z 513.2 for $[M+2H]^{2+}$ produced by *Planktothrix rubescence* (Lake Behlendorf, Germany).

variants that are not available as standards is possible by fraction collection of the hydrolysed cyclic peptides followed by L-FDVA derivatisation to determine the amino acid composition.

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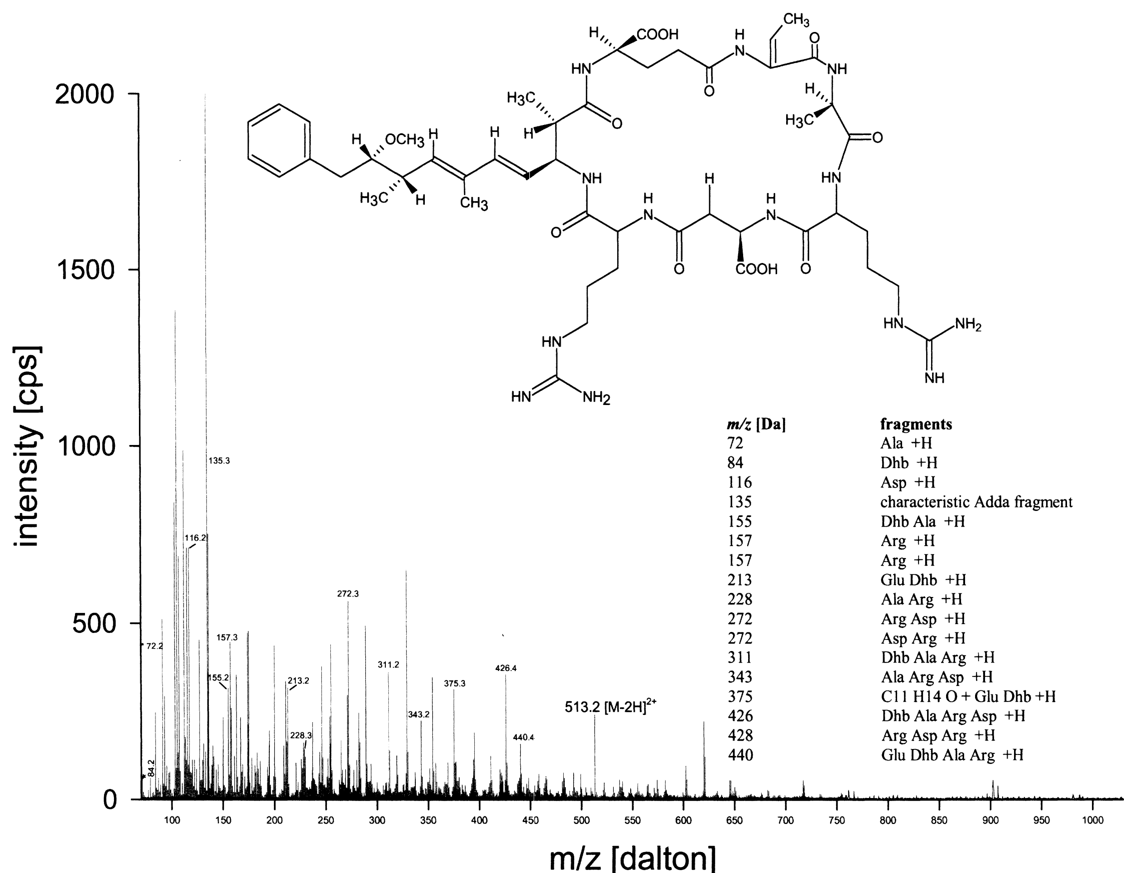

Fig. 7. ESI-MS–MS spectrum with fragment ion list (calculated with Cyanotox, Version 1.5.3, hyphen MassSpec Consultancy) and structure of [D-Asp[3],Dhb[7]]-MC-RR with *m/z* 513.2 for $[M+2H]^{2+}$ produced by *Planktothrix rubescence* (Lake Behlendorf, Germany).

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