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Analysis of cyanobacterial toxins by hydrophilic interaction liquid chromatography–mass spectrometry

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Abstract

The combination of hydrophilic interaction liquid chromatography with electrospray mass spectrometry (HILIC–MS) has been investigated as a tool for the analysis of assorted toxins produced by cyanobacteria. Toxins examined included saxitoxin and its various analogues (**1**–**18**), anatoxin-a (ATX-a, **19**), cylindrospermopsin (CYN, **20**), deoxycylindrospermopsin (doCYN, **21**), and microcystins-LR (**22**) and -RR (**23**). The saxitoxins could be unequivocally detected in one isocratic analysis using a TSK gel Amide-80 column eluted with 65% B, where eluent A is water and B is a 95% acetonitrile/water solution, both containing 2.0 mM ammonium formate and 3.6 mM formic acid. The analysis of ATX-a, CYN and doCYN required 75% B isocratic. Simultaneous determination of **1**–**21** was also possible by using gradient elution. HILIC proved to be suitable for the analysis of microcystins, but peak shape was not symmetric and it was concluded that these compounds are best analysed using existing reversed-phase methods. The HILIC–MS method was applied to the analysis of field and cultured samples of *Anabaena circinalis* and *Cylindrospermopsis raciborskii*. In general, the method proved quite robust with similar results obtained in two different laboratories using different instrumentation.

Keywords: Hydrophilic interaction chromatography; Bacteria; Toxins; Cyanotoxins; Saxitoxins; Cylindrospermopsin; Anatoxins; Microcystins

1. Introduction

Cyanobacteria are found in fresh and brackish water throughout the world and can present a public safety hazard through contamination of drinking water supplies. In addition, numerous deaths of wildlife and domestic animals occur each year when water becomes contaminated with cyanobacterial blooms [1]. Such organisms are known to produce a number of toxins including neurotoxins, such as the saxitoxins and anatoxin-a (ATX-a), and hepatotoxins, such as cylindrospermopsin (CYN) and microcystins (Fig. 1) [2]. The saxitoxins (**1**–**18**), also known as paralytic shellfish poisoning (PSP) toxins, are fast acting neurotoxins which inhibit nerve conduction by blocking sodium channels [1,3] and, among cyanobacteria, are produced primarily by *Anabaena circinalis* [4,5], *Lyngbya wollei* [6] and *Aphanizomenon flos-aquae* [7,8]. Taxonomic re-evaluation of *A. flos-aquae*, however, has been reported [9], indicating that other species are emerging as having been identified as *A. flos-aquae*. The isolation of saxitoxin (**1**) and its analogues **3** and **4** from a Brazilian strain of *Cylindrospermopsis raciborskii* has also been reported recently [10]. Anatoxin-a (**19**) is a potent depolarising neuromuscular blocking agent [11] and is produced by different strains of *Anabaena* [2,12], *Planktothrix* and *Aphanizomenon*. Cylindrospermopsin (**20**) and its deoxy-derivative (doCYN, **21**) are alkaloids produced by *C. raciborskii* [13], *Umezakia natans* [14], *Aphanizomenon ovalisporum* [15], and *Raphidiopsis curvata* [16]. Microcystins (**22** and **23**), produced mainly by *Microcystis* sp., are heptapeptides [2,17,18] and show tumour promoting activity on rat liver with inhibition of protein phosphatases 1 and 2A [19].

The monitoring of drinking water supplies for the presence of these toxins is of critical importance for the assessment of environmental and health risks. Therefore, the development of an analytical method that could provide simultaneous detection and unambiguous identification of

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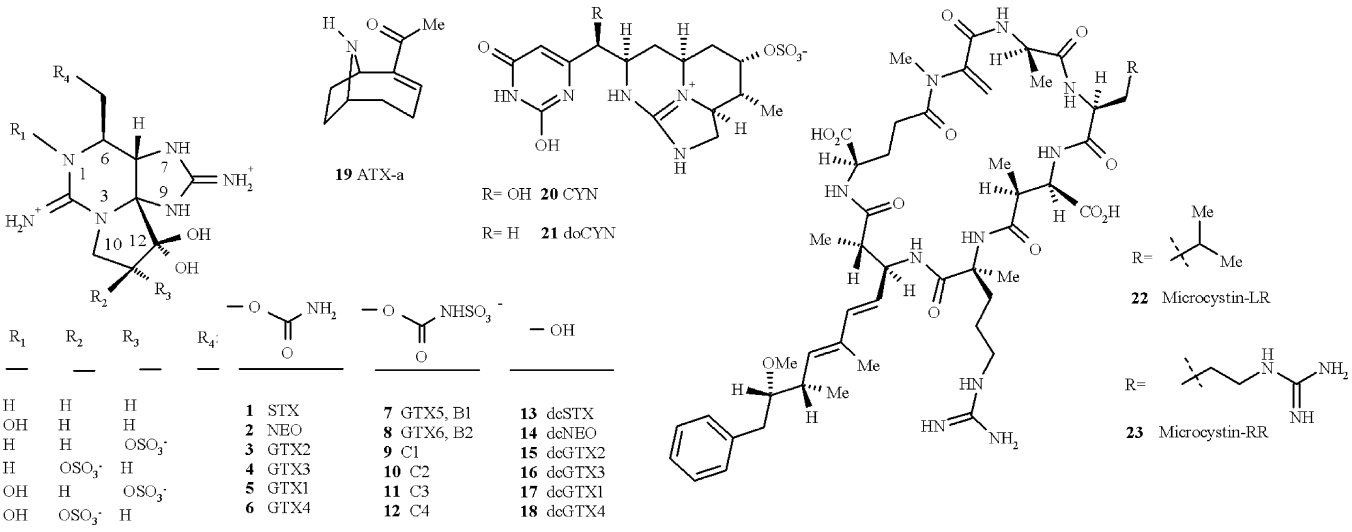

Fig. 1. Structures of a number of toxins produced by cyanobacteria: the saxitoxin group, which includes saxitoxin (**1**), neosaxitoxin (**2**), gonyautoxin-2, -3, -1, -4, -5, -6 (**3**–**8**), *N*-sulfogonyautoxin-1, -2, -3, -4 (**9**–**12**), decarbamoylsaxitoxin (**13**), decarbamoylneosaxitoxin (**14**), decarbamoylgonyautoxin-2, -3, -1, -4 (**15**–**18**), anatoxin-a (**19**), cylindrospermopsin (**20**), deoxycylindrospermopsin (**21**), microcystin-LR (**22**) and -RR (**23**).

different cyanobacterial toxins would be highly desirable. Liquid chromatography–mass spectrometry (LC–MS) with electrospray ionisation (ESI) has proven to be a powerful tool for the analysis of toxins at trace levels, since the first reports of its use in this field 11 years ago [20–22]. Recent developments, involving both improved design and lower costs of LC–MS instruments, are making this technique a viable tool in many laboratories involved in both monitoring and toxin research [23].

The LC–MS methods that have been reported for cyanotoxins are usually based on reversed-phase liquid chromatography (RPLC) separations [24–27]. However, the wide range of structures and charge states of most cyanobacterial toxins make it difficult to resolve all of the toxins in one analysis. In addition, due to the high water solubility of some of the toxins, ion-pairing agents are required in most cases to achieve sufficient chromatographic retention on reversed-phase columns. Unfortunately, such ion-pair agents can increase background noise and decrease electrospray ionisation efficiency resulting in poor detection limits.

In this work, we have examined the suitability of hydrophilic interaction liquid chromatography coupled with electrospray mass spectrometry (HILIC–MS) [28] for the analysis of assorted toxins produced by cyanobacteria known to contaminate freshwater supplies. For this purpose, standard solutions of various saxitoxins, CYN, doCYN, ATX-a and microcystins, as well as field and cultured samples of *A. circinalis* and *C. raciborskii*, were tested.

2. Experimental

2.1. Chemicals

All organic solvents were of distilled-in-glass grade (Caledon Labs., Georgetown, Canada). Water was distilled and passed through a Milli-Q water purification system (Millipore, Bedford, MA, USA) to 18 MΩ quality or better. Formic acid (90%, laboratory grade) and ammonium formate (analytical-reagent grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Standard solutions of saxitoxins (**1**–**18**) were provided by the Marine Analytical Chemistry Standard Program (NRC, Halifax, Canada). Serial dilutions of certified standards of STX, NEO, GTX2, and GTX3 were used to obtain calibration plots. ATX-a (**19**) was purchased from Calbiochem (La Jolla, CA, USA) and microcystins-LR (**22**) and -RR (**23**) were purchased from Sigma–Aldrich (St. Louis, MO, USA). CYN (**20**) and doCYN (**21**) standards were prepared by methanol extraction of lyophilised *C. raciborskii* grown in culture in Queensland Health Scientific Services laboratory (Australia) followed by LC purification. The purity of this material was confirmed by NMR and mass spectrometry and was shown to be 93.5% by comparing the absorbance at 262 nm to literature values [29].

2.2. Samples

Field samples of *A. circinalis* were collected in October 1997 from a large scum forming bloom from Coolmunda Dam, in south-east Queensland (Australia). Approximately 20 l of material was collected, the cells were lyophilised and stored at −20 °C until used. Other samples of *A. circinalis* were obtained from a bloom in an ornamental pond at Sandgate (Queensland, Australia) which occurred in May 2001 and from a laboratory culture grown in Jaworskis media [30] at Queensland Health Scientific Services.

C. raciborskii (culture strain AWT 205/1) was provided by Australian Water Technologies (Peter Hawkins, P.O. Box 73, West Ryde 211, NSW, Australia). This strain was grown in a batch culture at Queensland Health Scientific Services

using Jaworskis media [30]. The cells were lyophilised and stored at −20 °C until used.

2.3. Extraction procedure

Lyophilised cells (20 mg, dry mass) were suspended in 80 μl of water (neutral pH) at room temperature (about 15 °C) stirring at medium speed in the dark for 2 h. Then 125 μl of acetonitrile–water–formic acid (80:19.9:0.1) was added, mixed well and allowed to sit for 10 min. The mixture was filtered using an Ultrafree-MC 0.45 μm membrane (Millipore) at 628.3 rad. s^{-1} for 10 min. The filter was washed twice with 125 μl of the same extracting solvent. The filtrates were combined, the volume was adjusted to 500 μl with extracting solvent, and analysed directly by RPLC–fluorescence detection (FLD) and/or HILIC–MS. For quantitation of toxins present in the samples of strains of *A. circinalis* tested, the above extraction procedure was modified slightly: accurately weighed samples of material (20–40 mg) after suspension in water were extracted with 4× 800 μl aliquots of acetonitrile–water–formic acid (80:19.9:0.1) and the combined extracts made to a final volume of 4 ml.

2.4. Instrumentation

2.4.1. LC–MS and MS–MS

LC–MS and LC–MS–MS experiments were performed using either a Perkin-Elmer Sciex API-III+ triple quadrupole mass spectrometer (Thornhill, Canada) equipped with a pneumatically assisted electrospray (ionspray) ionisation source coupled to an HP 1090 liquid chromatograph (Hewlett-Packard, CA, USA), or a PE Sciex API-300 mass spectrometer equipped with a turbo-ionspray source (Thornhill, Canada) coupled to a Perkin-Elmer 200 series HPLC system (Perkin-Elmer, Norwalk, CT, USA). HILIC–MS analyses were carried out using a 5 μm TSK gel Amide-80, 2 mm × 250 mm column (Tosohaas, PA, USA). Eluent A was water and B was acetonitrile–water (95:5), both eluents containing 2.0 mM ammonium formate and 3.6 mM formic acid (pH 3.5). The following eluting systems were used: (a) 65% B isocratic for **1**–**18**; (b) 75% B isocratic for **19**–**21**; (c) gradient (90–65% B over $T = 13$ min and hold 5 min) for **22** and **23**; and (d) gradient (75% B for 5 min, 75–65% B over 1 min, hold 13 min, 65–45% B over 4 min, hold 10 min) for multiple toxins (**1**–**21**). The flow rate was 0.2 ml/min and a post-column split was employed to deliver approximately 20 μl/min to the ionspray interface for the API-III+ system. The API-300 instrument with turbo-ionspray interface was used at 0.2 ml/min without splitting. A sample injection volume of 5 μl was used in most cases. MS detection was carried out in the selected ion monitoring (SIM) and selected reaction monitoring (SRM) modes for the positive ions reported in Table 1. Ion dwell times were adjusted to give a total scan time of 1 s. MS–MS experiments were carried out using argon (nitrogen in API-300) as the collision gas in the second radio-frequency only quadrupole and a collision energy of 30 V (26 V in API-300).

2.4.2. RPLC–FLD

Extracts of *A. circinalis* were analysed for saxitoxins by RPLC with post-column oxidation and fluorescence detection according to the method detailed by Oshima [31]. The analyses were carried out on a Waters 600MS HPLC system equipped with a Waters post-column reaction system (Waters, Milford, MA, USA). Data were acquired using a Shimadzu C-R6A integrator (Shimadzu Australia, Rydalmere, Australia).

3. Results and discussion

3.1. General aspects

ESI-MS has proven to be a good technique for detection of all of the toxins of concern [20,32], which being quite basic all give strong $[M + H]^+$ ions. The LC–MS challenge lies in the chromatographic separation method. In fact, many of the very polar cyanotoxins require ion-pairing agents [33] which are either incompatible with ESI or lead to reduced ionisation efficiency. HILIC overcomes these drawbacks, the basis of the separation being the combination of an amide bonded stationary phase and a mobile phase containing high percentage of organic solvent and low concentration of volatile buffer. The mechanism appears to involve the hydrophilic interaction of polar compounds with a stagnant aqueous phase at the stationary phase surface, as well as some additional ion exchange interactions [34]. The elimination of ion-pairing agents and the use of such a volatile mobile phase facilitate high sensitivity for MS detection.

3.2. Development of HILIC–MS method

Previously, we have investigated the HILIC–MS and MS–MS of saxitoxins [28]. It was shown that all the saxitoxins could be separated on the Amide-80 column using isocratic elution with 65% B. Sensitive and selective detection was afforded by SIM of molecular and/or main fragment ions or SRM based on specific collision-induced dissociations (Table 1).

In order to develop a HILIC–MS method for the detection of cylindrospermopsins and anatoxin-a, a standard mixture of CYN (**20**) and doCYN (**21**) as well as a commercially available standard of ATX-a (**19**) were used to acquire mass spectra and adjust mobile phase strength.

Electrospray spectra were acquired for CYN and doCYN. The full scan mass spectrum of CYN (Fig. 2a) showed an abundant $[M + H]^+$ ion at *m/z* 416, as well as the ammonium, sodium and potassium adduct ions: $[M + NH_4]^+$ at *m/z* 433, $[M + Na]^+$ at *m/z* 438, and $[M + K]^+$ at *m/z* 454, respectively. No significant fragment ions were observed with the conditions used. The MS–MS product ion spectrum

Table 1
Retention times for cyanobacterial toxins and detection ions used for LC–MS analyses in the selected ion monitoring (SIM) and selected reaction monitoring (SRM) modes

No.	Toxin	Retention time (min)[a]	SIM ions[b] *m/z* (%RI)	SRM ions *m/z* → *m/z* (%RI)
1	STX	20.3	300 (100)	300 → 282 (100), 300 → 204 (70)
2	NEO	21.0	316 (100)	316 → 298 (100), 316 → 220 (65)
3	GTX2	9.6	316 (100), 396 (25)	396 → 316 (100), 316 → 298 (30), 396 → 298 (5)
4	GTX3	10.7	396 (100), 316 (30)	396 → 298 (100), 396 → 316 (20), 316 → 298 (10)
5	GTX1	9.8	332 (100), 412 (30)	412 → 332 (100), 412 → 314 (1)
6	GTX4	10.9	412 (100), 332 (30)	412 → 314 (100), 412 → 332 (40)
7	GTX5 (B1)	13.1	380 (100), 300 (40)	380 → 300 (100), 380 → 282 (15), 300 → 282 (5), 300 → 204 (5)
8	GTX6 (B2)	14.6	396 (100), 316 (30)	396 → 316 (100), 396 → 298 (40), 316 → 298 (5)
9	C1	7.2	396 (100), 476 (20)	396 → 316 (100), 396 → 298 (40),316 → 298 (5)
10	C2	8.0	396 (100), 476 (20)	396 → 298 (100), 396 → 316 (20), 316 → 298 (5)
11	C3	7.9	492 (100), 412 (30)	412 → 332 (100), 412 → 314 (20)
12	C4	8.8	492 (100), 412 (30)	412 → 314 (100), 412 → 332 (10)
13	dcSTX	21.1	257 (100)	257 → 239 (100)
14	dcNEO	20.8	273 (100)	273 → 255 (100)
15	dcGTX2	10.2	273 (100), 353 (70)	353 → 273 (100), 273 → 255 (30)
16	dcGTX3	11.3	353 (100), 273 (20)	353 → 273 (100), 273 → 255 (30)
17	dcGTX1	10.1	289 (100), 369 (50)	369 → 289 (100)
18	dcGTX4	11.4	369 (100), 289 (1)	369 → 289 (100)
19	ATX-a[c]	5.8	166 (100), 149 (30)	166 → 91 (100), 166 → 131 (30), 166 → 149 (10)
20	CYN[d]	7.1	416 (100), 433 (30)	416 → 194 (100), 416 → 176 (50), 416 → 336 (40), 416 → 274 (30)
21	Deoxy-CYN	6.2	400 (100), 417 (30)	400 → 194 (100), 400 → 320 (50), 400 → 274 (40)

[a] Retention times are referred to the following chromatographic conditions: 5 μm TSK gel Amide-80, 2 mm × 250 mm column; mobile phase 65% B isocratic for **1**–**18** and 75% B isocratic for **19**–**21** with eluent A being water and B acetonitrile–water (95:5), both containing 2.0 mM ammonium formate and 3.6 mM formic acid (pH 3.5); flow rate 0.2 ml/min.

[b] $[M + H]^+$ ions are underlined.

[c] Estimated limit of detection (S/N = 3) in picomoles injected on column is 1.4 pmol (225 pmol, lowest amount of toxin actually injected) in SIM. The injection volume was 3 μl.

[d] Estimated limit of detection (S/N = 3) in picomoles injected on column is 3.2 pmol (315 pmol, lowest amount of toxin actually injected) in SIM and 0.3 pmol (240 pmol, lowest amount of toxin actually injected) in SRM. The injection volume was 5 μl.

of the $[M + H]^+$ ion of CYN (Fig. 2c), acquired using the triple quadrupole MS API-III+, showed fragment ions corresponding to the loss of SO_3 and H_2O from $[M + H]^+$, at *m/z* 336 and 318, respectively. Another fragment ion, $[M + H - 142]^+$ at *m/z* 274, resulted from loss of the [6-(2-hydroxy-4-oxo-3-hydropyrimidyl)]hydroxymethinyl moiety of the molecule and this ion subsequently underwent loss of SO_3 and H_2O to afford ions at *m/z* 194 and 176, respectively. The full scan and tandem mass spectra of doCYN Fig. 2b and d, respectively) were very similar, the only difference being the shift of ions at *m/z* 336 and 318 in CYN down 16 mass units in doCYN. The proposed fragmentation of the two compounds is also summarized in Fig. 2e.

The full scan mass spectrum of ATX-a (Fig. 3a) showed an abundant $[M + H]^+$ ion at *m/z* 166 and fragment ions at *m/z* 149 and 131 due to sequential elimination of NH_3 and H_2O. The MS–MS product ion spectrum (Fig. 3b) showed the same ions, as well as a prominent ion at *m/z* 43 corresponding to CH_3CO^+ from the acetyl function. The assignment of the other ions has not yet been made.

The protonated and ammonium-adduct ions of CYN and doCYN as well as the $[M + H]^+$ and the $[M + H - NH_3]^+$ ions of ATX-a were selected as ions to monitor in SIM experiments. The most abundant fragment ions contained in the MS–MS spectra of each toxin were selected for SRM experiments. Selected ions and transitions for compounds **1**–**21** are contained in Table 1.

Under the same conditions used previously for saxitoxins (65% B), ATX-a, CYN and doCYN eluted too quickly. A higher percentage of solvent B was required for effective retention of these toxins. In particular, with 75% B isocratic, **19**–**21** eluted at 5.8, 7.1 and 6.2 min, respectively. A gradient was developed for the analysis of all the above toxins (**1**–**21**) in one run and the results are shown in Fig. 4 for a mixture of standard compounds (**1**–**7**, **9**–**16**, **19**, **20**). The gradient used (see Section 2) includes three different steps to elute in a single 32 min analysis: (i) anatoxin-a (**19**) and cylindrospermopsins (**20** and **21**); (ii) the neutral and singly-charged saxitoxins (**3**–**12**, **15**–**18**); and (iii) the double-charged saxitoxins (**1**, **2**, **13**, **14**). This result holds great promise for multi-toxin screening.

A gradient elution was also required for analysis of standard solutions of microcystin-LR (**22**) and -RR (**23**), which eluted at 7.9 and 13.5 min, respectively. Mono- and bicharged protonated molecules (*m/z* 995 and 498 for **22** and *m/z* 1038 and 520 for **23**, respectively) were selected as ions to monitor in selected ion monitoring experiments. HILIC–MS technique proved to be capable of selectively detecting the microcystins. However, the peak shapes were

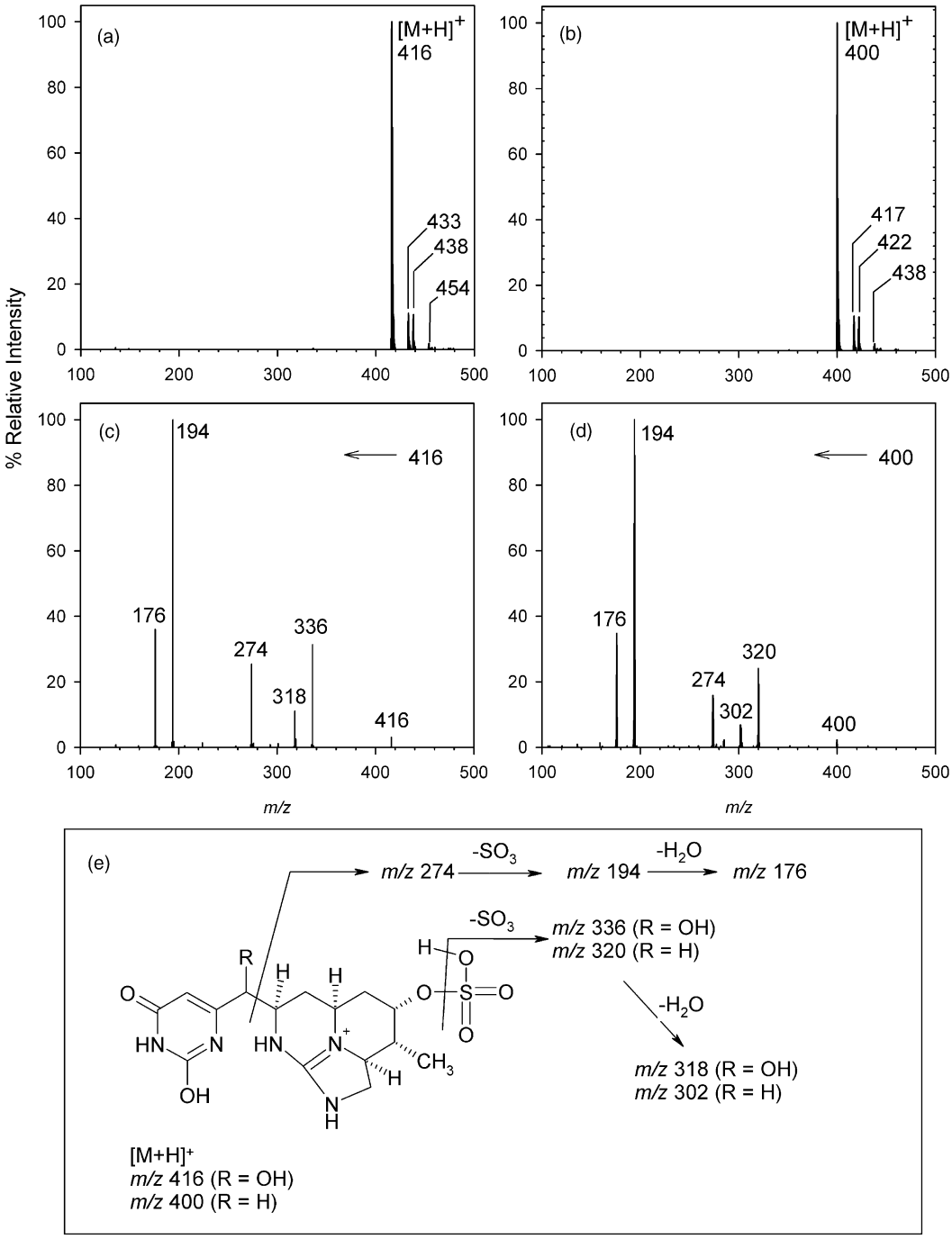

Fig. 2. Electrospray mass spectra in positive ion mode of cylindrospermopsin (CYN) (a), and its deoxy-derivative (doCYN) (b). MS–MS product ion spectra of the $[M+H]^+$ ions of CYN and doCYN are shown in (c) and (d), respectively. MS–MS spectra were carried out on API-III+ system using a collision energy of 30 V. Assignments of labelled fragment ions are shown (e).

not satisfactory, showing increased broadening and tailing compared to the other toxins. It is possible that an adjustment of mobile phase, pH or buffer might help the peak shape but many RPLC methods for the analysis of this class of cyanotoxins have been reported in literature [26,27,35–37]. These methods, using a variety of reversed-phase columns and aqueous mobile phases commonly added of volatile buffers, appear to offer advantages of robustness and better chromatographic performance for the large range of microcystins.

3.3. Application to samples

The developed method was tested by analysing field and cultured cyanobacterial samples at our disposal, namely *A. circinalis* and *C. raciborskii*. For HILIC–MS analysis,

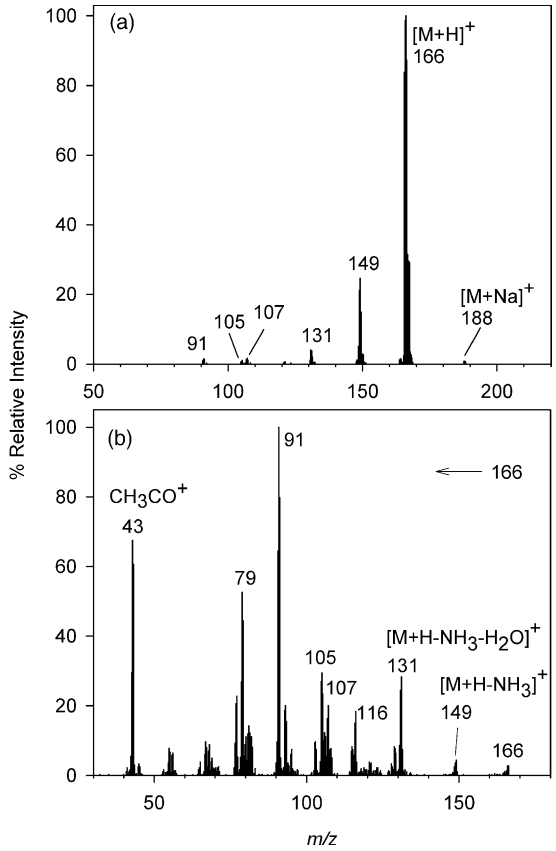

Fig. 3. Electrospray mass spectrum in positive ion mode of anatoxin-a (ATX-a) (a) and MS–MS product ion spectrum of the $[M + H]^+$ ion (b). MS–MS spectra were carried out on the API-III+ system using a collision energy of 30 V.

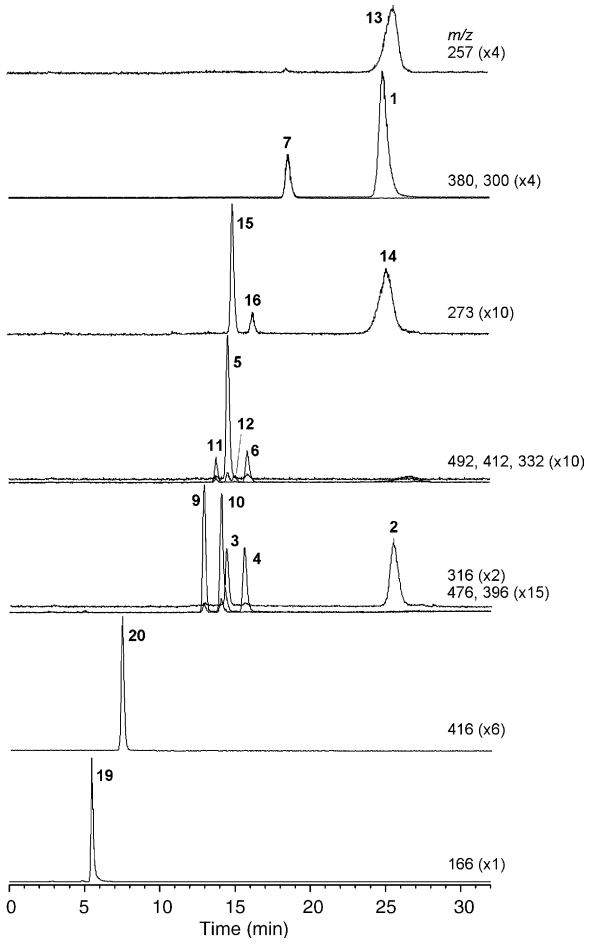

Fig. 4. HILIC–MS analyses of a standard mixture containing most saxitoxins (**1**–**7**, **9**–**16)**, ATX-a (**19**) and CYN (**20**). Experiments were carried out in the SIM mode on the API-III+ system. Some traces are plotted with expanded scales as indicated. Chromatographic conditions were as in Section 2.4.1 using gradient elution system "d".

a simple extraction method was used and no cleanup was performed on the crude extracts in order to demonstrate rapid analysis.

In the selection of the extraction solvent, consideration was given to compatibility with the mobile phase used in the HILIC–MS analysis. Hydrophilic interaction liquid chromatography of cyanotoxins utilises a high percentage of acetonitrile in the mobile phase (65–75% B). Therefore, in order to minimise interferences of extraction solvent on the chromatographic performance, extraction of the cyanobacterial sample was implemented using an acidified aqueous acetonitrile solution. In particular, lyophilised cells (20 mg) were first re-hydrated to 80% water content and then extracted with 80% acetonitrile plus 0.1% formic acid in a centrifugal filter unit. To the best of our knowledge no toxin degradation should occur in the above extraction conditions [38–40]. Although the main purpose of this study was to explore qualitative aspects, some quantitative work was carried out using a more exhaustive extraction procedure.

3.3.1. A. circinalis

A sample of the cyanobacterium, *A. circinalis*, collected in October 1997 from the Coolmunda Dam, south-east Queensland (Australia), was acquired for testing the suitability of our existing method first developed for PSP toxins in marine algae and shellfish [28].

A preliminary RPLC–FLD analysis had shown several saxitoxin-related toxins to be present in the sample at concentrations in the range of 75–1000 μg/g: STX (**1**), GTX2 (**3**), GTX3 (**4**), C1 (**9**), C2 (**10**), and dcSTX (**13**). HILIC–MS analysis of the crude extract was carried out using the API-III+ system in SIM mode (Fig. 5). The protonated molecules $[M + H]^+$ and the main fragmentation ions caused by loss of SO_3 $[M + H - 80]^+$ of each toxin were selected as ions to monitor in this experiment (Table 1). SIM allowed the detection of all toxins previously revealed by RPLC–FLD and also indicated the presence of dcGTX2 (**15**) and dcGTX3 (**16**). Unfortunately, the reconstructed ion chromatograms were quite confusing. In particular, three factors made unambiguous interpretation of the results difficult: (i) extra peaks from other components in the crude extract; (ii) the high background signal in some ion traces, which is always a problem when the ESI is performed over low mass ranges; and (iii) a hump in the chromatograms

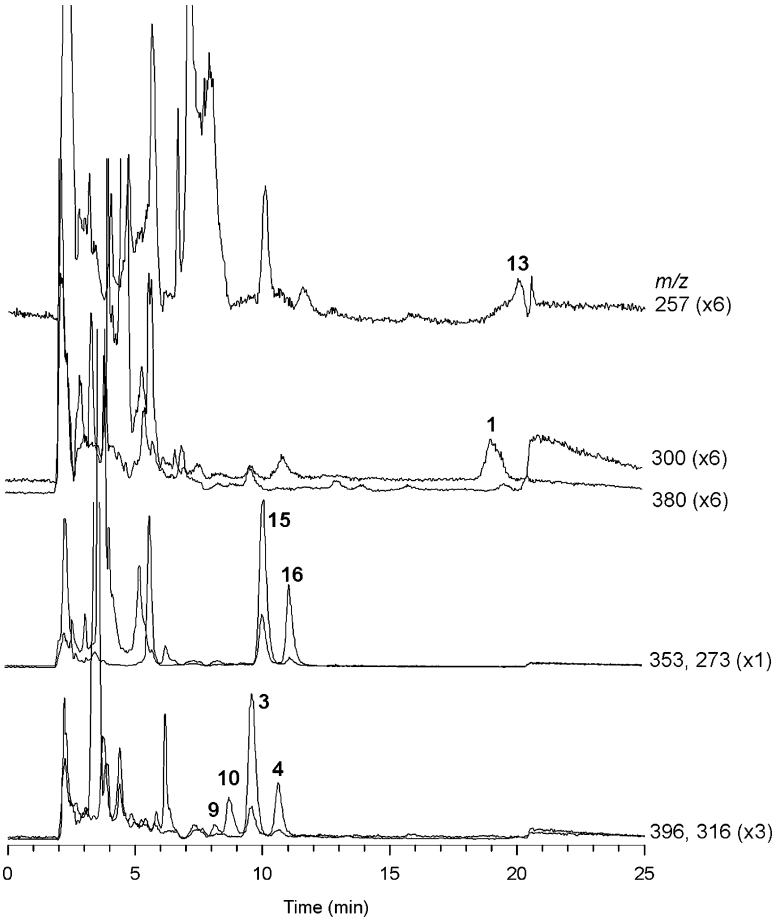

Fig. 5. HILIC–MS analyses of an *A. circinalis* extract containing saxitoxins using the SIM mode on the API-III+ system. See Fig. 1 for structures associated with various codes. Protonated and/or fragment ions were selected for monitoring (Table 1). Some traces are plotted with expanded scales as indicated. Chromatographic conditions were as in Section 2.4.1 using isocratic elution system "a".

at about 20 min, which appeared to be associated with the sample matrix. A clean-up step by solid-phase extraction (SPE) could help to solve such problems, but to the best of our knowledge, there is no SPE method that works for all cyanobacterial toxins. Thus, instrumental solutions were investigated.

LC–MS in the SRM mode of the same *A. circinalis* sample (Fig. 6) proved to be useful in overcoming the drawbacks of SIM. All of the toxins detected by RPLC–FLD, as well as GTX5 (**7**), dcGTX2 (**15**) and dcGTX3 (**16**), were easily confirmed by matching ion transition signals and retention times with those of standards. Furthermore, an additional significant peak was present in the chromatogram for the *m/z* 380 → 300 transition at 9.7 min. The identity of this compound could not be assigned just on the basis of MS data and further investigation is required for its identification. The SRM method gave better results because it is highly selective, very sensitive and presents almost zero background signal in the chromatograms. For the above reasons, it is strongly recommended as the method of choice for the analysis of the saxitoxin class of toxins.

Different field and cultured samples of *A. circinalis* were analysed in the Australian laboratory using the API-300 system in the SRM mode. All contained varying concentrations of the same toxins with none of the *N*-hydroxylated toxins being detected (Table 2). Sensitivity varied with the different toxins but was generally excellent, ranging from 60 to 600 fmol injected on column (80–800 fmol, lowest amount of toxin actually injected) and more than adequate to determine toxins present in the samples. Calibration plots using serial dilutions of certified standards of STX (**1**), NEO (**2**), GTX2 (**3**), and GTX3 (**4**) were obtained. Good reproducibility and linearity were observed between the amounts of injected toxin and the peak areas within the tested concentration ranges, r^2 values being 0.9991, 0.9971, 0.9963 and 0.9958 for STX, NEO, GTX2 and GTX3, respectively.

The Coolmunda Dam *A. circinalis* extract was also analysed for ATX-a (**19**). The detection limit for the ATX-a standard on the *m/z* 166 ion was estimated (S/N = 3) to be 1.4 pmol injected on column (225 pmol, lowest amount of toxin actually injected) in SIM mode and the sample was found to contain no detectable ATX-a. It is interesting

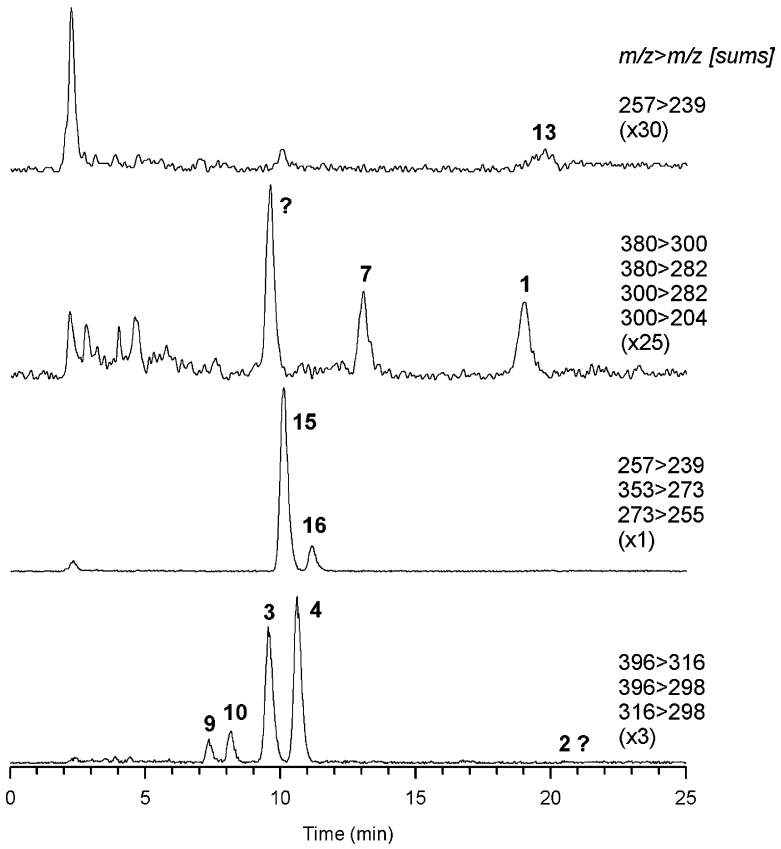

Fig. 6. HILIC–MS analyses of an *A. circinalis* extract containing saxitoxins using the SRM mode on the API-III+ system. SRM was performed on a series of ion transitions consistent with the fragmentation pattern of each toxin (Table 1) and signals common to groups of toxins were summed. Some traces are plotted with expanded scales as indicated. Chromatographic conditions were as in Section 2.4.1 using isocratic elution system "a".

that European strains of *A. circinalis* have been observed to produce ATX-a while Australian strains have never been observed to do so [41]. Unfortunately, no sample containing ATX-a was available for testing.

3.3.2. *C. raciborskii*

A cultured sample of *C. raciborskii*, known to contain CYN (**20**) and doCYN (**21**) at levels as high as 2 mg/g, was acquired for testing. A HILIC–MS analysis of the crude extract based on SIM of the $[M+H]^+$ ions (Fig. 7a) provided sufficient selectivity and sensitivity. Detection limit for the CYN standard was estimated (S/N = 3) to be 3.2 pmol injected on column (315 pmol, lowest amount of toxin actually injected). However, both CYN and doCYN could be detected easily in this sample even in full scan mode. A SRM experiment was also performed on the sample for added confirmation. The results (Fig. 7b) confirmed the SIM results and also provided excellent sensitivity, the detection limit being

Table 2
Concentrations (μg/g, dry mass) of PSP toxins measured in various specimens of *A. circinalis* from south-east Queensland in API 300

No.	Toxin	Coolmunda Dam	Laboratory culture	Pond at Sandgate	LOD (fmol)[a]
1	STX	20 ± 2	37 ± 3	39 ± 3	300 (560)
3	GTX2	200 ± 10	190 ± 10	123 ± 7	600 (800)
4	GTX3	66 ± 3	86 ± 3	151 ± 6	60 (80)
7	B1(GTX5)	13.8 ± 0.6	23.1 ± 0.9	63 ± 3	130 (300)
9	C1	(+)[b]	(+)[b]	(+)[b]	
10	C2	(+)[b]	(+)[b]	(+)[b]	
13	dcSTX	7.1 ±. 0.7	76 ± 8	10 ± 1	390 (500)
15	dcGTX2[c]	430	110	14	600
16	dcGTX3[c]	18	2.5	1.0	60

[a] LOD is the estimated limit of detection (S/N = 3) in femtomoles injected on column. The lowest amount of toxin actually injected is reported in parentheses. The injection volume was 5 μl.

[b] (+) indicates compound is present in the sample but concentration was not determined as no standard was available at the time of analysis.

[c] Concentrations estimated from GTX2 and GTX3 standards, respectively, by assuming similar molar response in the mass spectrometer.

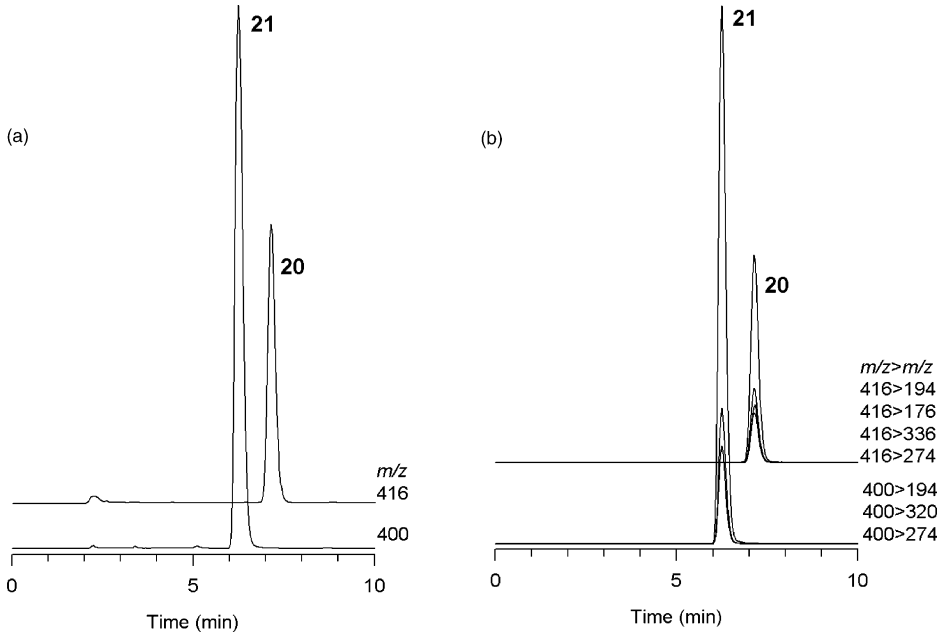

Fig. 7. HILIC–MS analyses of a *C. raciborskii* extract containing cylindrospermopsin (**20**) and its deoxy-derivative (**21**). Experiments were carried out on the API-III+ system using (a) SIM on the $[M+H]^+$ ions, and (b) SRM on a series of ion transitions. Chromatographic conditions were as in Section 2.4.1 using isocratic elution system "b".

(S/N = 3) 0.3 pmol injected on column (240 pmol, lowest amount of toxin actually injected). The *C. raciborskii* extract was also analysed for saxitoxin-related toxins (**1**–**18)** but none was detected in this strain.

4. Conclusion

HILIC–MS is a technique suitable for a number of cyanotoxins, including the saxitoxins, CYN, doCYN, and ATX-a. Microcystins could also be chromatographed but they are best analysed by existing RPLC–MS methods. For saxitoxin and its analogues, SRM is the preferred method due to its higher selectivity. Indeed, a clean-up step is needed if SIM is the only acquisition mode available. For cylindrospermopsin and anatoxin-a, the SIM method is adequate, but SRM can provide additional selectivity for confirmatory analyses. Multi-toxin determination is possible, thus allowing the rapid, simultaneous screening of an entire range of toxins (**1**–**21**). The developed HILIC–MS method has been shown to be sensitive, straightforward and readily automated. Analysis of algal samples required no sample clean-up or pre-concentration step, and proved to be quite robust with similar results obtained in two different laboratories using different instrumentation.

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References

[1] W.W. Carmichael, Sci. Am. 270 (1994) 78.
[2] W.W. Carmichael, N.A. Mahmood, E.G. Hyde, in: S. Hall, G. Strichartz (Eds.), Marine Toxins, ACS Symposium Series No. 418, American Chemical Society, Washington, DC, 1990, p. 87.
[3] T. Narahashi, Fed. Proc. 31 (1972) 1124.
[4] H. Onodera, Y. Oshima, M.F. Watanabe, M. Watanabe, C.J. Bolch, S. Blackburn, T. Yasumoto, in: T. Yasumoto, Y. Oshima, Y. Fukuyo (Eds.), Proceedings of the Seventh International Conference on Toxic Phytoplankton, UNESCO, Sendai, Japan, 1995, p. 563.
[5] A.R. Humpage, J. Rositano, A.H. Bretag, R. Brown, P.D. Baker, B.C. Nicholson, D.A. Steffensen, Aust. J. Mar. Freshwater Res. 45 (1994) 761.
[6] H. Onodera, M. Satake, Y. Oshima, T. Yasumoto, W.W. Carmichael, Nat. Toxins 5 (1997) 146.
[7] E. Jackim, J. Gentile, Science 162 (1968) 915.
[8] F.M.B. Ferriera, J.M.F. Soler, M.L. Fidalgo, P. Fernandez-Vila, Toxicon 39 (2001) 757.
[9] R. Li, W.W. Carmichael, Y. Liu, M.M. Watanabe, Hydrobiologia 438 (2000) 99.
[10] N. Lagos, H. Onodera, P.A. Zagatto, D. Andrinolo, S.M.F.Q. Azevedo, Y. Oshima, Toxicon 37 (1999) 1359.
[11] W.W. Carmichael, D.F. Biggs, M.A. Peterson, Toxicon 17 (1979) 229.
[12] K. Sivonen, in: M. Miraglia (Ed.), Mycotoxins and Phycotoxins—Developments in Chemistry, Toxicology and Food Safety, Proceedings of the Ninth International IUPAC Symposium on Mycotoxins and Phycotoxins, Rome, 1998, p. 547.
[13] I. Ohtani, R.E. Moore, M.T.C. Runnegar, J. Am. Chem. Soc. 114 (1992) 7941.
[14] K.-I. Harada, I. Ohtani, K. Iwamoto, M. Suzuki, M.F. Watanabe, M. Watanabe, K. Terao, Toxicon 32 (1994) 73.

[15] R. Banker, S. Carmeli, O. Hadas, B. Teltsch, R. Porat, A. Sukenik, J. Phycol. 33 (1997) 613.
[16] R.H. Li, W.W. Carmichael, S. Brittain, G.K. Eaglesham, G.R. Shaw, Y.D. Liu, M.W. Watanabe, J. Phycol. 37 (2001) 1.
[17] W.W. Carmichael, V.R. Beasley, D.L. Bunner, J.N. Eloff, I. Falconer, P. Gorham, K.-I. Harada, Y. Krishnamurthy, M.-J. Yu, R.E. Moore, K.L. Rinehart, M. Runnegar, O.M. Skulberg, M. Watanabe, Toxicon 26 (1988) 971.
[18] K.L. Rinehart, K.-I. Harada, M. Namikoshi, C. Chen, C.A. Harvis, M.H.G. Munro, J.W. Blunt, P.E. Mulligan, V.R. Beasley, A.M. Dahlem, W.W. Carmichael, J. Am. Chem. Soc. 110 (1988) 8557.
[19] R. Matsushima, S. Yoshizawa, M.F. Watanabe, K.-I. Harada, M. Furusawa, W.W. Carmichael, H. Fujiki, Biochem. Biophys. Res. Commun. 171 (1990) 867.
[20] M.A. Quilliam, B.A. Thomson, G.J. Scott, K.W.M. Siu, Rapid Commun. Mass Spectrom. 3 (1989) 145.
[21] S. Pleasance, M.A. Quilliam, A.S.W. De Freitas, J.C. Marr, A.D. Cembella, Rapid Commun. Mass Spectrom. 4 (1990) 206.
[22] S. Pleasance, M.A. Quilliam, J.C. Marr, Rapid Commun. Mass Spectrom. 6 (1992) 121.
[23] W.M.A. Niessen, J. Chromatogr. A 794 (1998) 407.
[24] G.K. Eaglesham, R.L. Norris, G.R. Shaw, M.J. Smith, R.K. Chiswell, B.C. Davis, G.R. Neville, A.A. Seawright, M.R. Moore, Environ. Toxicol. 14 (1999) 151.
[25] K.–I. Harada, H. Nagai, Y. Kimura, M. Suzuki, H.-D. Park, M.F. Watanabe, R. Luukkainen, K. Sivonen, W.W. Carmichael, Tetrahedron 49 (1993) 9251.
[26] J. Pietsch, S. Fichtner, L. Imhof, W. Schmidt, H.-J. Brauch, Chromatographia 54 (2001) 339.
[27] G.K. Poon, L.J. Griggs, C. Edwards, K.A. Beattie, G.A. Codd, J. Chromatogr. 628 (1993) 215.
[28] M.A. Quilliam, P. Hess, C. Dell'Aversano, in: W.J. deKoe, R.A. Samson, H.P. Van Egmond, J. Gilbert, M. Sabino (Eds.), Mycotoxins and Phycotoxins in Perspective at the Turn of the Millennium, Wageningen, The Netherlands, 2001, p. 383.
[29] I. Ohtani, R. Moore, M. Runnegar, J. Am. Chem. Soc. 114 (1992) 7941.
[30] A.S. Thompson, J.C. Rhodes, I. Pettman, in: Natural Environmental Research Council Culture Collection of Algae and Protozoa-Catalogue of Strains, Freshwater Biology Association, Ambleside, UK, 1988, p. 22.
[31] Y. Oshima, J. AOAC Int. 78 (1995) 528.
[32] F. Kondo, K.-I. Harada, J. Mass Spectrom. Soc. Jpn. 44 (1996) 355.
[33] M.A. Quilliam, in: B. Reguera, J. Blanco, M.L. Fernandez, T. Wyatt (Eds.), Harmful Algae, Proceedings of the Eighth International Conference on Harmful Algae, IOC, UNESCO, Vigo, Spain, 1997, p. 509.
[34] M.A. Strege, Anal. Chem. 70 (1998) 2439.
[35] J. Meriluoto, Anal. Chim. Acta 352 (1997) 298.
[36] L. Lawton, C. Edwards, G.A. Codd, Analyst 119 (1994) 1525.
[37] K.-I. Harada, F. Kondo, K. Tsuji, J. AOAC Int. 84 (2001) 1636.
[38] W.M. Indrasena, T.A. Gill, Food Chem. 71 (2000) 71.
[39] R.K. Chiswell, G.R. Shaw, G. Eaglesham, M.J. Smith, R.L. Norris, A.A. Seawright, M.R. Moore, Environ. Toxicol. 14 (1999) 155.
[40] D.K. Stevens, R.I. Krieger, Toxicon 29 (1991) 167.
[41] D.J. Fitzgerald, D.A. Cunliffe, M.D. Burch, Environ. Toxicol. 14 (1999) 203.