

## Development of a wide spectrum method for detection of cyanobacterial toxins by mass spectrometry

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### Abstract

Methods based on liquid chromatography coupled with tandem mass spectrometry (LC/MS-MS) have been developed for detection of cyanobacterial toxins, including specific target compounds of most of the major known toxin groups. Tandem mass spectrometry analyses are commonly conducted in Multiple Reaction Monitoring (MRM) mode and such methods work well, provided that the target variants are known. Application of specific methods depends on knowledge of the prior occurrence of a known toxigenic organism or at least its potential contribution to the toxin content of the sample, as well as some insight into the expected toxin profile. If this information is lacking, toxin analysis can be very tedious. We developed an LC/MS-MS method, with which typical representatives of all known classes of cyanotoxins, including even undescribed derivatives, can be qualitatively monitored. With this method, diagnostic mass fragments of characteristic compounds of the different classes are detected in the Precursor Ion mode, thus allowing for monitoring of a wide spectrum of cyanotoxins in samples for which the toxin composition is undefined.

### Introduction

Cyanobacterial toxins that occur in brackish and fresh-water and occasionally marine ecosystems may be categorized into the following groups: microcystins, nodularins, paralytic shellfish poisoning (PSP) toxins, anatoxins, and cylindrospermopsins. Each class includes a large variety of derivatives, comprising a total of over 100 known toxins.

The development of analytical methods for qualitative and quantitative detection of cyanobacterial toxins has been necessary to monitor these compounds in drinking water and in various other aqueous, plankton, and animal tissue matrices. For example, alternative liquid chromatography (LC)-based methods with mass spectrometric (MS) detection exist for simultaneous determination of various cyanobacterial toxins extracted from phyto-plankton (Dahlmann *et al.* 2003; Dell'Aversano *et al.* 2004; Bogiatti *et al.* 2006). All these methods are based on detection by Multiple Reaction Monitoring (MRM). Yet it is impossible to detect all structural variants in this mode because only common transitions and target variants are considered. In contrast, scanning in Precursor Ion mode using a specific fragment molecule and additionally scanning over a mass:charge ( $m/z$ ) range that includes all spectral masses allows the detection of the entire spectrum of related toxins. This tandem MS/MS mode was first employed in 1993 for detection of mi-

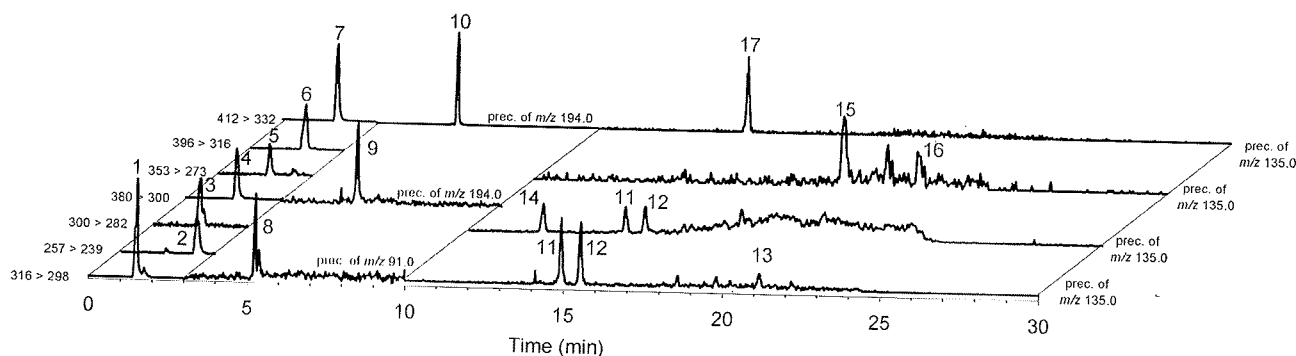
crocystins (MCs), but no further work followed (Edwards *et al.* 1993).

The aim of this method development was to provide a tool for comprehensive toxicity assessment of cyanobacterial samples by qualitative screening for all known classes of toxins. This was achieved in the Precursor Ion mode, which allows the detection of even undescribed toxin variants. The method will be particularly helpful for potentially toxic plankton samples for which the cyanobacterial composition has not been taxonomically characterized.

### Material and Methods

Standard solutions of PSP toxins, microcystins (MCs), nodularin (NOD), anatoxin-a (ANA), and cylindrospermopsin (CYN) were obtained from commercial sources. A sample of *Lyngbya wollei* containing deoxy-cylindrospermopsin (doCYN) was kindly provided by Geoff Eaglesham, Australia.

The analyses were performed on an Agilent Series 1100 HPLC system coupled via Turbo Ion-spray source to a Sciex 4000 Q Trap mass spectrometer (ABI Sciex, Darmstadt, Germany). The chromatographic separation was carried out on a Luna column (3  $\mu$ m, 150 mm x 3.0 mm; Phenomenex, USA) using two eluents containing 50 mM formic acid and 2 mM ammonia formate in water (eluent A) or methanol/water (95/5, eluent B) and gradient elution (0 % B



**Figure 1.** Multi-compound chromatogram for cyanobacterial toxins. First period: MRM chromatogram of PSP toxins – NEO (1), dcSTX (2), STX (3), B1 (4), dcGTX 2/3 (5), GTX 2/3 (6), GTX 1/4 (7); Second period: Precursor Ion spectrum of m/z 91.0 for ANA (8), m/z 194.0 for CYN (9) and doCYN (10); Third period: Precursor Ion spectrum of m/z 135.0 for microcystins (MCs) and nodularins (NODs) – scan range m/z 400-550 with 17 eV CE for MC-YR (11), MC-LR (12), and MC-LW (13), scan range m/z 400-550 with 35 eV CE for MC-RR (14), m/z 900-1100 with 60 eV CE for MC-LA (15) and MC-LF (16), m/z 800-850 with 90 eV CE for NOD (17).

for 1 min, switched to 50 % B, held for 4 min, then further to 90 % B in 10 min, held for 5 min, then re-equilibrated).

Although this MS-MS method was only developed for qualitative screening, the limits of detection (LOD) were estimated for reference standards of cyanobacterial toxins, namely for PSP toxins: neosaxitoxin (NEO), decarbamoyl-saxitoxin (dcSTX), saxitoxin (STX), sulfo-carbamoyl toxin B1 (B1), decarbamoyl-gonyautoxins 2/3 (dcGTX 2/3), gonyautoxins 2/3 (GTX 2/3), gonyautoxins 1/4 (GTX 1/4), as well as for CYN, ANA, MC-LA, -LW, -LF, -LR, -YR, -RR, and NOD. Quantitative determination was carried out in Precursor Ion mode from chromatographic analysis of pure standard solutions of known concentrations. The peak height to averaged background noise ratio (S/N) was measured, whereby the background noise was defined as the baseline near the analytic peak. The LOD was calculated as a S/N of 3:1. For quantification of the epimeric pairs GTX1/GTX4, GTX2/GTX3, dcGTX2/dcGTX3, having the same molecular weight but different fragmentation patterns, an additional analysis yielding complete chromatographic separation of the epimers by hydrophilic interaction liquid ion chromatography (HI-LIC) (Dell' Aversano *et al.* 2004) was performed to determine the toxin response factors for quantification.

## Results and Discussion

The specific chromatographic gradient profile was chosen to keep all cyanotoxins belonging to each group within a limited range of retention times, in order to ensure inclusion of all occurring variants. A baseline separation of all toxin derivatives was there-

fore not achieved, but this is not necessary for such a qualitative method.

Based on the retention times of all cyanobacterial toxins (Table 1), three time periods were defined to optimize detection in different modes and with alternative parameters. For example, during the first three minutes, PSP toxins were detected in MRM mode, but there is no typical daughter ion that could be used in Precursor Ion mode to discriminate these compounds. The PSP toxins were all eluted between 1.4 and 1.7 min on the C18 column, showing no chromatographic separation of NEO, dcSTX, STX, B1, dcGTX 2/3, GTX 2/3, GTX 1/4, but resulting in a positive signal in case of occurrence in sample material (Fig. 1).

In contrast, for ANA, homo-anatoxin (HANA), CYN, doCYN, MCs, and NODs, a characteristic fragment for each toxin group is known and was utilized to analyse the compounds in Precursor Ion mode (Edwards *et al.* 1993; Dell' Aversano *et al.* 2004; James *et al.* 2005). Within the second period (3-10 min), ANA was detected by selecting m/z 91.0 as the precursor ion; CYN and doCYN were analysed based on m/z 194.0 as the daughter ion. Scan ranges for all the anatoxins was m/z 100-300, thereby including ANA, HANA, and degradation products. To include CYN, doCYN, epicylindrospermopsin (EpiCYN), and possible undescribed variants among the cylindrospermopsins, the mass range was set at m/z 300-500.

Microcystin and nodularin variants were analysed within the last period (until 30 min). The MC derivatives containing arginine (Arg) in position 2 and 4 of the peptide ring, such as MC-RR, show doubly charged ions as the base peak in a mass spectrum, in contrast to MCs with only one Arg residue, as for

**Table 1.** Retention times, MRMs, precursor ions and LOD of cyanobacterial toxins.

period 1				period 2				period 3			
Toxin	Retention time (min)	mass transition ( $m/z$ )	LOD (pg) S/N = 3	Toxin	Retention time (min)	Precursor ion ( $m/z$ )	LOD (pg) S/N = 3	Toxin	Retention time (min)	Precursor ion ( $m/z$ )	LOD (pg) S/N = 3
NEO	1.4	316 > 298	1.0	ANA	5.2	91.0	700	MC-RR	12.3	135.0	20
dcSTX	1.4	257 > 239	9.0	CYN	5.4	194.0	9	MC-YR	14.9	135.0	15
STX	1.4	300 > 282	10.0	dcCYN	5.5	194.0	no standard	MC-LR	15.5	135.0	15
B 1	1.5	380 > 300	3.0					MC-LA	19.7	135.0	18
dcGTX 2/3	1.6	353 > 273	25.0					MC-LW	21.0	135.0	100
GTX 2/3	1.6	396 > 316	12.0					MC-LF	22.0	135.0	100
GTX 1/4	1.7	412 > 332	6.0					NOD	14.6	135.0	6

MC-LR or MC-YR. In the mass spectrum of the latter compounds,  $[M+H]^+$  ions are observed as the main peak and the  $[M+2H]^{2+}$  ions give only weak signals. On the other hand, MCs without Arg, such as MC-LA or MC-LF, form only singly charged ions (Yuan *et al.* 1999). Hence, for MCs both mass ranges (i.e.,  $[M+H]^+$  ( $m/z$  900-1100) and  $[M+2H]^{2+}$  ( $m/z$  400-550)) had to be covered using different fragmentation parameters, including the declustering potential (DP) and collision energy (CE). To achieve good sensitivity among the doubly charged ions for the MCs with two Arg residues, milder fragmentation conditions had to be chosen in comparison to conditions for derivatives containing only one Arg. Three different experiments were therefore required for detection of all MCs. In the case of MC-LW, without an Arg residue, a signal of  $[M+2H]^{2+}$  instead of  $[M+H]^+$  was observed. Nodularins form singly charged base ions, within the scan range  $m/z$  800-850. The derivatives MC-RR and MC-LF, as representative MCs that elute early and late, respectively, on a C18 column, were employed to set elution time constraints to cover the entire range of microcystins (Hiller *et al.* 2007).

### Conclusions

We developed an effective analytical tool for the rapid, qualitative detection of cyanobacterial toxins in phytoplankton samples from fresh, marine or brackish waters. This MS-MS method includes detection of all major classes of cyanotoxins, including PSP toxins, ANAs, CYNs, MCs, and NODs, yielding a distinctive positive or negative signal for each toxin or toxin group. If a cyanotoxin peak is detected, a more sensi-

tive and definitive analytical method must be applied to ensure the toxin identity and for quantification.

The application of the Precursor Ion mode instead of the commonly used MRM mode for MS-MS detection of cyanotoxins enables detection of a wide range of structural variants, resulting from the presence of different functional groups and/or amino acid residues in the molecule.

An advantage of this qualitative method is in the ability to detect uncommon and undescribed cyanobacterial toxin variants in a rapid, cursory analysis of putatively toxic samples. Moreover, this capability is maintained even for samples for which both the taxonomic status of the cyanotoxigenic organisms and the toxin profile are unknown.

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