AUTHOR(S): TURNER, A.D., WAACK, J., LEWIS, A., EDWARDS, C. and LAWTON, L.

TITLE: Development and single-laboratory validation of a UHPLC-MS/MS method for quantitation of microcystins and nodularin in natural water, cyanobacteria, shellfish and algal supplement tablet powders.

YEAR: 2018

Publisher citation: TURNER, A.D., WAACK, J., LEWIS, A., EDWARDS, C. and LAWTON, L. 2018. Development and single-laboratory validation of a UHPLC-MS/MS method for quantitation of microcystins and nodularin in natural water, cyanobacteria, shellfish and algal supplement tablet powders. Journal of chromatography B [online], 1074-1075, pages 111-123. Available from: https://doi.org/10.1016/j.jchromb.2017.12.032

OpenAIR citation: TURNER, A.D., WAACK, J., LEWIS, A., EDWARDS, C. and LAWTON, L. 2018. Development and single-laboratory validation of a UHPLC-MS/MS method for quantitation of microcystins and nodularin in natural water, cyanobacteria, shellfish and algal supplement tablet powders. Journal of chromatography B, 1074-1075, pages 111-123. Held on OpenAIR [online]. Available from: https://openair.rgu.ac.uk

Development and single-laboratory validation of a UHPLC-MS/MS method for quantitation of microcystins and nodularin in natural water, cyanobacteria, shellfish and algal supplement tablet powders

Andrew D. Turner, Julia Waack, Adam Lewis, Christine Edwards, Linda Lawton

PII: S1570-0232(17)31869-X
DOI: https://doi.org/10.1016/j.jchromb.2017.12.032
Reference: CHROMB 20975

To appear in:

Received date: 31 October 2017
Revised date: 22 December 2017
Accepted date: 26 December 2017

Please cite this article as: Andrew D. Turner, Julia Waack, Adam Lewis, Christine Edwards, Linda Lawton , Development and single-laboratory validation of a UHPLC-MS/MS method for quantitation of microcystins and nodularin in natural water, cyanobacteria, shellfish and algal supplement tablet powders. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Chromb(2017), https://doi.org/10.1016/j.jchromb.2017.12.032

Development and single-laboratory validation of a UHPLC-MS/MS method for quantitation of microcystins and nodularin in natural water, cyanobacteria, shellfish and algal supplement tablet powders

Andrew D. Turner[1], Julia Waack[1,2], Adam Lewis[1], Christine Edwards[2], Linda Lawton[2]

[1]Centre for Environment, Fisheries and Aquaculture Science, Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB, United Kingdom.

[2]Robert Gordon University, School of Pharmacy and Life Sciences, Sir Ian Wood Building, Garthdee Road, Aberdeen, AB10 7QB, United Kingdom

Abstract

A simple, rapid UHPLC-MS/MS method has been developed and optimised for the quantitation of microcystins and nodularin in wide variety of sample matrices. Microcystin analogues targeted were MC-LR, MC-RR, MC-LA, MC-LY, MC-LF, LC-LW, MC-YR, MC-WR, [Asp3] MC-LR, [Dha7] MC-LR, MC-HilR and MC-HtyR. Optimisation studies were conducted to develop a simple, quick and efficient extraction protocol without the need for complex pre-analysis concentration procedures, together with a rapid sub 5 min chromatographic separation of toxins in shellfish and algal supplement tablet powders, as well as water and cyanobacterial bloom samples. Validation studies were undertaken on each matrix-analyte combination to the full method performance characteristics following international guidelines. The method was found to be specific and linear over the full calibration range. Method sensitivity in terms of limits of detection, quantitation and reporting were found to be significantly improved in comparison to LC-UV methods and applicable to the analysis of each of the four matrices. Overall, acceptable recoveries were determined for each of the matrices studied, with associated precision and within-laboratory reproducibility well within expected guidance limits. Results from the formalised ruggedness analysis of all available cyanotoxins, showed that the method was robust for all parameters investigated. The results presented here show that the optimised LC-MS/MS method for cyanotoxins is fit for the purpose of detection and quantitation of a range of microcystins and nodularin in shellfish, algal supplement tablet powder, water and cyanobacteria. The method provides a valuable early warning tool for the rapid, routine extraction and analysis of natural waters, cyanobacterial blooms, algal powders, food supplements and shellfish tissues, enabling monitoring labs to supplement traditional microscopy techniques and report toxicity results within a short timeframe of sample receipt. The new method, now accredited to ISO17025 standard, is

simple, quick, applicable to multiple matrices and is highly suitable for use as a routine, high-throughout, fast turnaround regulatory monitoring tool.

Keywords: Microcystins, nodularin, LC-MS/MS, UPLC, shellfish, food safety, natural waters.

1. Introduction

Cyanobacteria, or blue-green algae, are photosynthetic bacteria which are found throughout the world in a variety of aquatic environments including lakes, rivers, ponds and estuaries [1]. Cyanotoxins are natural secondary metabolic products produced by some cyanobacteria from a number of cyanobacterial genera, comprising a wide range of different compounds. Over 35 genera are responsible for the production of cyanotoxins, including *Anabaena*, *Cylindrospermopsin*, *Lyngbya*, *Microcystis*, *Nodularia*, *Nostoc* and *Oscillatoria* (Planktothrix) [2]. Cyanobacteria such as *Microcystis aeruginosa* are known to produce over 100 different analogues of the cyclic peptides known as microcystins (MC) [3,4]. These compounds are cyclic heptapeptides with the generalised structure of cyclo-(D-alanine^{1}-X^{2}-D-MeAsp^{3}-Y^{4}-Adda^{5}-D-glutamate^{6}-Mdha^{7}). The presence of the amino acid Adda [(2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid] is unique to cyanobacteria. The variation in amino acids, primarily X and Y at positions 2 and 4, results in the occurrence of multiple structural variants (**Figure 1**). MC-LR, the most commonly occurring MC congener, is characterised by the presence of leucine (L) and arginine (R) at positions 2 and 4 respectively [5]. In the brackish water *Nodularia spumigena*, a related compound nodularin (Nod) is produced. This cyclic pentapeptide is also a toxin and structurally similar, having the chemical structure cyclo-(D-MeAsp^{1}-L-arginine^{2}-Adda^{3}-D-glutamate^{4}-Mdhb^{5}) (**Figure 1**) where Mdhb is 2-(methylamino)-2-dehydrobutyric acid.

Microcystins (MC) occur globally, most commonly in eutrophic water bodies. The timing and duration of cyanobacterial blooms depends on a number of factors including nutrient inputs as well as the climatic conditions of the region, most notably temperature [6]. Previous studies throughout the world indicate that on average around 60% of cyanobacterial blooms are thought to produce toxins, although this can vary significantly from study to study, ranging from 10% to 95% [7]. Along with Nod, the MC are potent hepatotoxins through

inhibition of protein phosphatases (PP1 and PP2A), which can be responsible for acute and chronic poisonings of humans, as well as wild and domestic animals [7-9]. Acute poisoning from cyanotoxins can lead to gastroenteritis, liver damage, jaundice and neurotoxic effects [10], even leading to fatalities. MC and other cyanotoxins such as anatoxin and cylindrospermopsin can be problematic when present in drinking or recreational waters [2,7,13,14]. MC and Nod have also been reported to accumulate in species such as fish and bivalves, resulting in a potential risk to human health following consumption of contaminated fishery products and animals feeding on toxic filter feeders such as bivalve molluscs [15-20]. The popularity of food supplements prepared from blue-green algae, also raises the possibility of regular human consumption of products containing naturally occurring cyanotoxins [21,22].

Relatively few regulatory limits for cyanotoxins exist. The World Health Organisation (WHO) recommends a safe limit for MC-LR of 1 µg/L in drinking water [10]. For recreational water exposure, the WHO propose a 20 µg MC/L limit as a guideline value for a moderate health alert [7]. Furthermore, for MC concentrations in foodstuffs, a provisional Tolerable Daily Intake (TDI) proposed by the WHO for MC-LR has been used to derive a guideline value equating to a lifetime TDI of 2.4 µg MC-LR per day for a 60 kg human [23]. This is taken in the context of a lack of data on toxicity of other MC congeners, the unknown effects of cyanotoxin mixtures, together with potential synergistic effects between toxins and other unidentified cyanobacterial compounds [16]. A no adverse effect single exposure event dose (acute TI) has also been proposed, relating to 150 µg for a 60 kg human, i.e. ~60 times higher than the level proposed for lifetime intake [24]. These two extremes have subsequently been used to propose a seasonal safety limit of 24 µg for a 60 kg human, relating to exposure on a daily basis for several weeks from shellfish which have naturally accumulated MC in a bloom period [16].

In the UK, monitoring of cyanobacterial blooms is conducted using light microscopic identification of cyanobacterial genera, with cells enumerated and water body closures enforced by local authorities when cell densities exceed a designated threshold of 20,000 cells/mL and/or there is evidence for cyanobacterial scum formation [25]. However, toxic and non-toxic strains from the same cyanobacterial species cannot be differentiated using these criteria. As such, with the assumption of a 60% bloom toxicity, a preferred option would be to conduct analysis of the toxin content of the water or cyanobacterial blooms directly [26]. A range of detection methods have been utilised in recent years for the analysis of MC and Nod [27]. High Performance Liquid Chromatography (HPLC) with ultraviolet (UV) detection has been used most commonly for the analysis of these toxins [8,29], together with the use of enzyme linked immunoassay (ELISA) kits [30,31]. Whilst UV detection is well reported, sensitivity of analysis is low and additional clean-up and concentration steps are required [8]. Mass spectrometric detection methods have been developed more recently [32-34], with ultra-high performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry (MS/MS) becoming the preferred technique for analysis of cyanotoxins [14, 35-38]. Whilst a number of these have been developed for water analysis and animal tissues, and some validated following international guidelines [14,38,39], there are no reports of LC-MS/MS methods which have been developed for application to a wide range of matrices including water as well as tissue and algal supplement tablet powder samples. In addition, there have been reports of LC-MS/MS methods for MCs in tissue samples being subject to performance drift, requiring regular guard column changes, mass spectrometer source cleaning and subsequently great care is needed with quality control [36]. The potential for risks to human health via a number of different routes of exposure is clear, but the overall level of risk is unknown. In order to assess these risks, there is a strong need to establish suitable regulatory monitoring methods for cyanotoxins in a variety of matrices. Such methods

should not only be applicable to a variety of matrices, but also applicable to a high sample throughput and fast turnaround testing environment. Specifically, there is a need for simple, rapid methods which enable testing laboratories to analyse a large number of samples and report results within a short timeframe. Consequently, we have undertaken the testing, development and single-laboratory validation of a simple, rapid LC-MS/MS method for the analysis of multiple cyanotoxins, including MC and Nod in a range of relevant matrices. The sample types assessed were water, cyanobacteria, algal supplement tablet powders and shellfish tissue. The validation followed the requirement of EC regulation 882/2004 that official control methods should be validated and quality assured prior to adoption into EU monitoring programmes [40,41].

The LC-MS/MS method involves the extraction of cyanotoxins from the four matrices, depending on the specific matrix under investigation. Typically, various proportions of aqueous methanol (MeOH) are used by researchers to effectively extract toxins from either cyanobacterial cells or tissue samples, although other solvent mixes and other techniques including the use of immunoaffinity columns and solid phase extraction (SPE) have also been assessed [14,38,42-46]. Extraction of algal cells provides an assessment of toxin concentrations within the cells of the cyanobacteria (intracellular). The analysis of toxins in water, either direct or following a concentration step such as solid phase extraction (SPE) is used to determination the levels of toxins dissolved in the water (extracellular). Aqueous MeOH has also been used previously to extract cyanotoxins from a range of biological tissues, including those from fish, crustacean and shellfish [16]. Such extraction processes will effectively extract toxins which exist freely within the tissue samples. MC congeners are known to form covalent bonds to protein phosphatases in plant and animal cells, and as such are not extracted using standard solvent extraction techniques. However, doubts exist as to whether covalently bound MC would be bioavailable following human consumption [16,47]

and more work is required to fully assess the implications resulting from the presence of conjugated microcystins [48]. Consequently, this study focusses only on free MC, those extracted into methanolic solution. Once extracted into solution, toxins are subjected to UHPLC-MS/MS with selected reaction monitoring (SRM).

The objectives of this study were to assess extraction methods for cyanotoxins in a wide range of sample matrices appropriate to routine regulatory testing, to develop a simple and rapid LC-MS/MS method for sensitive and reproducible quantitation of a range of cyanotoxins from water, cyanobacteria, algal supplement tablet powder and shellfish tissue, and to validate the method, establishing method performance characteristics when applied to each matrix. This work has become increasingly important given the prevalence of cyanobacterial blooms around the world and the potential acceleration in toxic blue-green algal production and subsequent human exposure over recent decades [1,5,6,7,11,16,17,22,24,26,30,49]. Cyanotoxins incorporated into the method were those available at the time of study as commercial reference materials, specifically MC-LR, MC-RR, MC-LA, MC-LY, MC-LF, MC-LW, MC-YR, MC-WR, desmethyl-3-MC LR ([D-Asp3] MC-LR), desmethyl-7-MC LR ([Dha7] MC-LR), homoisoleucine MC IR (MC-HilR) and homotyrosine MC YR (MC-HtyR). Phase 1 assessed the ability of the proposed technique to separate the toxins of concern and give reliable detection. It also focussed on the extraction of the toxins from the four matrices, with a view to developing a quick, simple and readily applicable method for high throughput, fast turnaround analysis. Phase 2 involved the formal assessment of method performance characteristics by conducting an in-house single-laboratory validation (SLV) of the method. This was performed following as closely as possible the guidelines of the International Union of Pure and Applied Chemistry (IUPAC) [41] to obtain information regarding selectivity, linearity, limits of detection, limits of quantitation, accuracy, recovery, ruggedness, instrumental precision, repeatability and reproducibility. Validated method performance

characteristics were also used to generate information expressing the uncertainty of measurement for the methodologies [50,51]. The overall aim was an assessment of the applicability of the method to the analysis of cyanotoxins in each matrix as a potential for use in routine monitoring of water bodies, shellfish and food supplements to guard against human sickness following exposure to these dangerous hepatotoxins.

2. Experimental

2.1 Chemicals and reagents

Instrument solvents used for preparation of mobile phases were of LC-MS-grade (Fisher Optima, ThermoFisher, UK) and all chemicals were LC-MS reagent grade where possible. Sample preparation reagents were HPLC grade. Reference toxin standards (MC-RR, MC-LA, MC-LY, MC-LF, MC-LW, MC-YR, MC-WR, MC-HilR, MC-HtyR, MC-LR, [Asp3] MC-LR and Nod) were all obtained from Enzo Life Sciences, Exeter, UK. A certified standard of [Dha^{7}]-MC-LR and a pre-certified freeze-dried matrix reference material of blue-green algae (RM-BGA, Lot 201301) containing a range of MC was obtained from the Institute of Biotoxin Metrology, National Research Council Canada (NRCC). Reference standards received as solid powders were dissolved in suitable volumes of 50% aqueous methanol, to form stock solutions. A mixed stock solution was subsequently prepared by combining aliquots of each stock, followed by a seven-level suite of working calibration standards resulting in a calibration range between 0.33 ng/mL to 327 ng/m per toxin. RM-BGA (280 mg) was extracted with 28.0 mL 50% aqueous MeOH + 0.1% acetic acid, prior to centrifugation (4,500 g; 10 min) and the supernatant collected prior to analysis. The seven-point calibration standards were used for external calibration of cyanotoxins in all sample matrices, adjusting dilution factors depending on the extraction applied.

2.2 Samples

Blank matrices were obtained as follows.

2.2.1 Water and algae

Natural fresh water used for validation studies was obtained from a municipal boating lake near Bristol, SW England. The lake is subjected to regular cyanobacterial blooms during warmer months, although at the time of water collection, no blooms were in evidence. The sample was selected as it was expected to contain nutrients which are thought to facilitate the rapid blooming of cyanobacteria under suitable conditions. On receipt of the water sample, the water was filtered (0.2 µm) to remove any traces of sediment or other particles. This was kept cool in a fridge (<5 ^{o}C) until required for use. The cyanobacterial algae sample was obtained from a privately-owned lake in Cheshire, NW England. This had previously formed toxic blooms, but at the time of study contained *Microcystis* species and other freshwater algae that were not producing toxins, as confirmed through LC-MS/MS analysis. Mixed water (5 L) and bloom sample was centrifuged, and the cyanobacteria were retained after supernatant removal. The cyanobacterial pellet was aliquoted into 2 mL microcentrifuge tubes where it was kept refrigerated until used for validation experiments.

2.2.2 Algal supplement tablet powders

Algal supplement tablet powders were obtained from commercial suppliers of blue-green algae food supplements. For preparation of a toxin-free blank material, raw materials were sourced from three different suppliers and combined, All three supplements were purchased in tablet or capsule form. Together, the ingredients of the tablets consisted of *Aphanizomenon flos-aquae, arthrospira platensis, spirulina* and *Chlorella.* Tablets consisted either of 100% blue-green algae or contained small proportions of the additive rice

maltodextrin and the anti-adherent magnesium stearate. Blue-green algae from each supplier had been processed through freeze-drying of bulk algae. No other production procedures were made available by commercial providers of the materials. Solid tablets were ground into a fine powder using a pestle and mortar and capsules were opened to decant the internal powders. Ground powder from the three sources of dried algae were stored refrigerated in sealed vials until use.

2.2.3 Mussels

Live mussels utilised for the shellfish validation work were obtained fresh from a commercial seafood supplier. These were shucked to remove all the flesh from their shells, before homogenisation to form a liquid slurry mixture. Aliquots (2.0 g) were weighed into 50 mL centrifuge tubes and used fresh for spiking experiments.

In addition to the blank materials, positive materials were also required for validation and ongoing internal quality control (IQC) work. A toxin-positive mussel (*Mytilus edulis*) tissue reference material was prepared, following the feeding of live mussels in a laboratory tank environment with both *Nodularia spumigena (KAC 66)* and *Microcystis aeruginosa (PCC7813)* cultures. After a week of feeding, mussels were removed from their seawater tanks, shucked and homogenised. After testing the materials to determine the approximate toxin concentrations, homogenised tissues were blended to form a bulk sample, containing appropriate levels of MC and Nod. Once thoroughly homogenised, the tissue was aliquoted into polypropylene tubes, each >4 g, and sealed prior to storing frozen (<-15°C) until required for use. A naturally contaminated algal supplement tablet powder was obtained commercially, following the purchase of a number of blue-green algae food supplements (supplier not disclosed). The positive materials were again either ground into powder or powders combined, and thoroughly homogenised by manual mixing. The bulk powdered material was

transferred into a large plastic screw-top vessel and stored in a fridge (<+4°C) until required for use. Other positive control extracts were obtained from positive algal samples sourced during 2015. These were used only as retention time markers during LC-MS/MS analysis.

2.3 UHPLC-MS/MS of MC and Nod

2.3.1 UHPLC conditions

The UHPLC-MS/MS (abbreviated further to LC-MS/MS) system is as follows. A Waters (Manchester, UK) Xevo TQ tandem quadrupole mass spectrometer (MS/MS) coupled to a Waters Acquity UHPLC system was used for LC-MS/MS analysis. Chromatography was conducted using a 1.7 µm, 2.1x50 mm Waters Acquity UPLC BEH C18 column (P/N 186002350, Lot no. 0249343351) in conjunction with a Waters VanGuard BEH C18 1.7 µm 2.1x5 mm guard cartridge (P/N 186003975, Lot no. 0245343321). The columns were held at +60°C, with samples held in the sample manager at +10°C. The sample injection volume was 5 µL and the mobile phase flow rate was 0.6 mL/min. Mobile phase A consisted of water + 0.025% formic acid, mobile phase B comprised acetonitrile (MeCN) + 0.025% formic acid. The UHPLC gradient was: 2% B initial conditions rising to 25% B1 at 0.5 min holding until 1.5 min, rising to 40% B at 3.0 min, increasing further to 50% B at 4 min, a quick rise to 95% B and 4.1 min and held until 4.5 min until dropping back to 2% B at 5 min. The total run time was 5.5 min. Each instrumental sequence started with a series of instrumental blanks, followed by toxin calibration standards and an extract of RM-BGA to be used as a matrix-based retention time marker and as an IQC. Instrumental sequences finished with a water and MeCN flush, first at 60°C and followed by a second at 30°C. New columns were conditioned as per the manufacturer's instructions. Injections of individual toxin solutions were performed to determine retention times and confirm there was no significant cross-over between determinands. Work was also conducted to optimise the UHPLC conditions of the

cyanotoxins anatoxin-a (ATX), cylindrospermopsin (CYN) and β-N-methylamino-L-alanine (BMAA). However, no acceptable chromatographic retention was found for any of these analytes using the BEH C18 column, with LC peaks observed to elute in the dead volume. As such, work continued without inclusion of these analytes in the cyanotoxin method.

2.3.2 MS/MS conditions

The Waters Xevo TQ tune parameters were as follows: 150°C source temperature, 600°C desolvation temperature, 600 L/hr desolvation gas flow, 0.15 mL/min collision gas flow. Capillary voltage was held at 1.0 kV. Selected Reaction Monitoring (SRM) transitions were built into the MS/MS method using positive mode acquisition for each toxin. Parent and daughter ions, as well as cone and collision voltages were optimized following experiments whereby pure standards were infused into the mass spectrometer in the mobile phase (**Table 1**). The majority of toxins exhibited unique SRM transitions and chromatographic retention times, resulting in good separation of cyanotoxins over the 5 min run time. The exception was [Dha7]-MC-LR and [Asp3] MC-LR, which shared the same transitions and could not be completely resolved. These two analytes are therefore reported together.

The LC-MS/MS MC and Nod method involved the direct quantitation of cyanotoxin toxins against working standards available as certified reference standards. Quantitation was performed using external calibration and results calculated in terms of µg/L of cultures.

2.4 Method optimisation

2.4.1 Extraction optimisation

2.4.1.1 Water and cyanobacteria

Investigations were conducted to develop and optimise suitable extraction methods for each of the four matrices: water, cyanobacteria, algal supplement tablet powder and shellfish

tissue. Water was analysed directly by LC-MS/MS for cyanotoxin content. Upon sample receipt, water samples not containing blooms were mixed and a 1 mL sub-sample pipetted into an autosampler vial for analysis. Samples containing blooms were first centrifuged to remove the algal cells, with the supernatant taken for analysis after filtration through a 0.2 µm syringe filter.

For cyanobacteria, the aim was to provide a rapid, simple and effective protocol for the rapid analysis of algal samples for a range of cyanotoxins. Consequently, centrifugation of algal samples was chosen as an efficient method of isolating cyanobacterial cells. A variety of solvents were assessed for their extraction efficiencies, specifically using different proportions of aqueous MeOH. Algal cultures containing MC were extracted using different proportions of water and MeOH, with results used to determine any differences in extraction efficiency. In case of difficulties with centrifugation, for example if buoyant algal cells were present which did not centrifuge effectively into a solid pellet, an alternative approach was developed to filter the bloom sample and collect the algal cells on the filter paper. This approach was also assessed to determine the optimum parameters for extraction for instances where centrifugation was inappropriate.

2.4.1.2 Mussel

Mussel tissue prepared as a LRM for cyanotoxins was used to optimise the extraction efficiency of the method. Triplicate samples of the LRM were extracted using a variety of solvents, including various proportions of MeOH and water, together with the use of isopropyl alcohol (IPA). In addition to a rapid one-step single dispersive extraction method, the extraction was compared with double, triple and quadruple step extractions performed using 80% MeOH. Extractions were also attempted with a weak acid (1% acetic acid) together with

acidic 80% MeOH. In total 45 LRMs were extracted using different solvent extraction regimes, and the results used to determine the preferred extraction method.

In addition, the single dispersive 80% MeOH extraction was assessed to determine the optimum vortex mixing time for effective toxin recovery. Triplicate 2 g LRMs were extracted by combining with 8 mL 80% MeOH and vortex mixing for a total of 30 s, 60 s, 90 s, 2 min, 3 min, 4 min, 5 min and 10 min. Extracts were analysed by LC-MS/MS and results used to determine the optimum extraction mixing time.

2.4.1.3 Algal supplement tablet powder

The extraction method for algal supplement tablet powders, was assessed using a variety of solvents as per the mussel tissue optimisation. Algal supplement tablets or supplement powders obtained from nutritional food supplement products were spiked with a number of MC toxins (MC-RR, LA, LY, LF, LW, YR, WR, LR) at a concentration of 100 ng/mL per toxin. Results were used to assess extraction recoveries for the different extraction methods tested, using a range of different solvent compositions.

2.5 Validation of the cyanotoxin LC-MS/MS method

In this study, validation was applied to the quantitative analysis of Nod and a range of MC analogues. The aim was to check that the analytical method was fit for purpose over an appropriate range of toxin concentrations in each of the four matrices.

2.5.1 Specificity

Method specificity was assessed with the analysis of toxin-free homogenised samples of each matrix. These were analysed along-side toxin calibration standards to determine

qualitatively whether any of the samples contained any matrix components which may interfere with the detection and quantitation of any of the cyanotoxins.

2.5.2 Linearity

In order to determine the range of selected toxin concentrations over which the quantitation method can be applied, toxins were spiked into matrix extracts as well as solvent (MeOH) to give a range of toxin concentrations between 0.6 and 170 ng per mL of extract, before subsequent LC-MS/MS analysis in triplicate. The linearity of the calibrations was assessed over this standard working range. Linear regression equations were generated and no weighting was placed on the calibration plot. The linearity of the analytical method was evaluated graphically, with visual inspection of calibration plots generated for individual toxins.

2.5.3 Sensitivity

The limit of detection (LOD) was taken as the lowest injected amount of toxin that results in a chromatographic peak height at least three times as high as the baseline noise level surrounding the peak. LODs were determined for each analyte-matrix combination. Method LODs were assessed firstly through the spiking of matrices at the three different concentrations, low, medium and high, used for recovery determination. All matrices were spiked using the following spiking concentrations: mussels (15, 150 and 500 µg/kg), water (3, 30 and 100 ng/mL), cyanobacteria (3, 30 and 100 ng spiked) and algal supplement tablet powder (150 and 500 µg/kg). Only two concentrations were used for algal supplement tablet powder as a 15 µg/kg low spike was found to be too low to facilitate acceptable quantitation. The MC analogues [Asp3] MC-LR and Dha-7 MC-LR cannot be resolved by the UHPLC method, so concentrations were summed and the two analytes reported together.

Limits of quantitation (LOQ) were defined as the concentration of analyte which gives rise to an analytical peak with a signal to noise ratio of 10:1. As such, LOQs were experimentally confirmed with the triplicate analysis of matrices at three concentrations. Using the same approach as above, signal-to-noise ratios for each peak were measured to calculate the predicted concentration which would result in a signal to noise ratio of 10:1.

The Limits of reporting (LOR) are based upon the concentrations of analytes which give rise to peaks for the primary (quantifier) SRM with a S/N ratio of 10 and the secondary (qualifier) SRM with a S/N ratio of 3. Concentrations were rounded up from this amount to include a measure of uncertainty associated with these values, with the uncertainty specifically taken from the variability (standard deviation) of the calculated results. After adding on the uncertainty factor, concentrations were rounded up to the nearest significant figure. Triplicate samples for each spike were used to assess variability of the amount. For the algal matrix where known masses of toxins were spiked, LOD, LOQ and LOR were calculated in terms of mass of toxin. However, an additional calculation was performed in terms of concentration (ng/mL) based upon a standardised extraction of toxins from a 45 mL water sample subjected to centrifugation or filtration to form an algal pellet.

2.5.4 Recovery

In the absence of any appropriate certified matrix reference materials (CRM), traceable to international standards with a known level of uncertainty, estimation of method bias was conducted through the repeat analysis of spiked samples [41]. Each matrix was spiked in triplicate with a mix of cyanotoxins to provide the same expected concentrations as described above for LOD/LOQ assessment. Samples were extracted and analysed, with LC-MS/MS analysis carried out in triplicate. Quantitation was conducted using external calibration, enabling the determination of method recovery.

2.5.5 Precision

The variability in retention time precision was assessed with the repeated analysis over one analytical sequence of extracts containing cyanotoxins spiked at a range of concentrations. Following the assessment of within-batch precision on the same day (n=50), the between batch precision was assessed with the analysis of samples and standards over multiple days within the same week (n=73). Finally, the variability was assessed over the long term, using two different analytical UHPLC columns, with data collected over two months (n=154).

Method precision was assessed with the repeated extraction and analysis of fortified study issues, as conducted for determination of recovery. Spiked recovery samples were assessed in two separate batches enabling the assessment of both short term (intra-batch; n=3) and medium term (inter-batch; n=6; more than 1 week apart) repeatability. Samples were extracted and analysed by LC-MS/MS, with quantitative concentration data used to calculate standard deviations around the calculated means over both single batches and multiple batches. Relative standard deviations were used to assess the overall precision within and between batches.

Long term precision (within-lab reproducibility) was also assessed for each matrix with the repeated extraction and analysis of the mussel and algal supplement tablet powder positive control reference materials. Water and cyanobacteria matrices were spiked with cyanotoxins to generate a suitable material for long term testing. Each set of materials was stored in the freezer until required for use. Over a period of > 3 months, aliquots were removed from frozen storage, thawed and the required amounts processed and quantified by LC-MS/MS. The acceptability of the precision characteristics of the method were examined through the generation of mean, standard deviation and relative standard deviation data and further assessed with the calculation of Horwitz ratio (HorRat) values [52]. RSD values

calculated for each toxin were compared against the Horwitz value derived from the Horwitz equation, with values ≤1.3 and 1.6 inferring satisfactory levels of short term and long term precision respectively [52].

2.5.6 Ruggedness

Ruggedness was assessed to analyse the effects of 7 key method parameters on the stability of the method, comparing the variability of these effects against method precision data. Experimental parameters chosen for the study were based on the judgement of the author, being key method parameters which could practically be assessed during a single Plackett-Burman experiment [53]. These included the accuracy for preparation of the extraction solvent, extraction time, centrifuge speed, centrifuge time, specification and make of extract filter and type of water. It is recognised that whilst many different parameters affect this method, choices were made which relate more to environmental conditions or parameters which may be altered unavoidable or through the use of different analysts. It is noted that due to the experimental design, LC-MS/MS parameters cannot be modified, as all analysis has to take place during one sequence. Eight 2 g homogenates of mussel LRM were extracted according to the method under repeatability conditions to give an assessment of within-batch precision on the same day as the ruggedness experiment. A further eight LRMs were extracted following the ruggedness experimental design. Each centrifuged extract was progressed through the ruggedness experiment as described in **Table 2**. All samples were analysed by LC-MS/MS, with results obtained in the ruggedness experiment (n=8) against the within-batch method precision experiment (n=8) assessed using a significance test (t-test).

2.5.7 Measurement uncertainty

Method performance characteristics from the validation studies were used to calculate an overall value of uncertainty for the measurement of each cyanotoxin in each of the four matrices. Once sources of uncertainty were described, individual component uncertainties were calculated and propagated to calculate an overall measurement uncertainty. Expanded uncertainties were calculated using an appropriate coverage factor (k=2) [50,51].

3. Results

3.1 Method optimisation

Following optimisation of UHPLC gradient parameters, acceptable separation between toxin analytes was achieved in under 4.5 min (**Figure 2**). MC analogue elution order was as expected, depending on the specific molecular substituents. The early eluting MC-RR contains two basic (Arg) substituents, with the most chromatographically retentive analogues (MC-LW and MC-LF) both containing the aromatic Y substituents, tryptophan and phenylalanine respectively, at position 4 (**Figure 1).** No chromatographic separation was achieved between [Asp3] MC-LR and [Dha^{7}]-MC-LR, so these analogues were reported as a summed pair. Full SRM chromatograms, including both quantifier and qualifier transitions are illustrated in supplementary materials (**Figure S1**).

For direct analysis of water, no additional sample concentration step or extraction optimisation was required, so the method validation continued using direct analysis by LC-MS/MS without any clean-up step. Cyanobacterial samples were extracted using varying proportions of water and MeOH, following both centrifugation and filtration to collect cyanobacterial cells. All isolated cell samples were subjected to a rapid freeze-thaw cycle (1 hour in freezer), before solvent extraction, using differing proportions of aqueous MeOH, ranging from 70% to 100% MeOH. For extraction of both centrifugal pellets and filtered cells,

80% aqueous MeOH was found to provide optimum toxin recovery from the algal matrix (**Figure S2**). Consequently, 80% MeOH was chosen as the extraction solvent for both filtered and centrifuged cyanobacterial samples for the remainder of the validation exercises.

For extraction of toxins from mussel tissue, solvent extraction optimisation experiments incorporating a range of different solvents and extraction methods indicated that the 80% MeOH single step dispersive method was as efficient as other solvent compositions (70-100%) for the toxins present in the LRM tissue. Lower recoveries were observed when using lower proportions of MeOH (<70%). No recovery increase was measured when using either double, triple or quadruple exhaustive extraction methods, indicating the faster and simpler single step method was as effective as a full multi-step exhaustive method. Extraction efficiencies were notably worse when using water or weak acetic acid extraction solvents, and the use of IPA was not found to be effective (**Figure S3**). Consequently, 80% MeOH was chosen as the solvent to use for a single-step dispersive extraction method, specifically 2 g homogenate + 8 mL 80% MeOH. Furthermore, no differences were evident in calculated toxin concentrations for the single-step dispersive extraction method using 80% MeOH when employing different vortex mixing times. As such, a 2 min vortex time was standardised for the method (**Figure S4**). Algal supplement tablet powder LRMs were also extracted with a range of solvents including varying proportions of MeOH and water. Results indicated 80% MeOH to again be the optimum solvent composition for extraction of algal supplement powder samples (data not shown). The extraction method for algal supplement tablet powder was consequently defined as a 0.5 g powder + 4.5 mL 80% MeOH, single dispersive extraction.

3.2. Method validation

3.2.1 Specificity

Overall, there were no notable interferences from matrix components affecting the detection and quantitation of cyanotoxins in each of the four matrices. Specifically, no SRM peaks were observed at the same retention times as toxin analyte peaks. **Supplementary figures S5-S8** illustrate the typical SRM chromatograms obtained following the analysis of toxin-free (blank) matrices, alongside the same matrices fortified with high level spike concentrations of cyanotoxins. Overall, there was evidence for good specificity of the method for each of the four matrices validated.

3.2.2 Linearity of the analytical method and matrix effects

The MS/MS detector was calibrated with individual cyanotoxins prepared in either solvent or matrix. In all cases, results showed that a linear-fit model is the preferred model, with separate slopes for each matrix. The summary of all the results following linearity assessment is shown in **Table 3**. The regression slope gradients for each analyte compared well between each of the matrices investigated, indicating the general absence of matrix effects for the majority of toxins. Exceptions included MC-LW, WR, LF and LY in water, although calibration slopes compared well between all other matrices including methanol. Slopes for MC-RR showed the greatest variability in slope gradient between matrices. The linearity, however, for each toxin in each matrix was generally acceptable, as evidenced by all correlation coefficients exceeding 0.98, with the majority > 0.99. Calibrations obtained for each of the matrix-analyte combinations are illustrated in supplementary materials **Figure S9**.

3.2.3. LOD, LOQ and LOR

LOD, LOQ and LOR were calculated for each matrix-analyte combination and are summarised in **Table 4**. LODs were found to show a good level of sensitivity of the cyanotoxins LC-MS/MS method. In algal cells, LODs ranged from 0.03 to 0.15 ng of toxin spiked, which equates to LODs between 0.4 and 3.6 pg/mL for a 45 mL water sample. LORs for cyanobacteria subsequently ranged from 0.1 to 0.9 ng, equating to concentrations of 0.005 to 0.03 ng/mL. For the other matrices, LOD, LOQ and LOR compared well, most notably water and mussel samples. LORs ranged from 0.3 to 1.3 ng/mL for water and 0.3 to 1.5 µg/kg for mussel tissue. LODs for both matrices were found to be acceptably low, dropping to between 0.01 and 0.19 ng/mL for water and 0.01 and 0.21 µg/kg for mussels. Higher LOD, LOQ and LOR were calculated for the analysis of cyanotoxins in algal supplement tablet powders. LODs ranged from 0.12 to 1.18 µg/kg, with LORs between 1.0 and 5.5 µg/kg. Overall the method sensitivities described by these results are acceptable, with a notable increase in sensitivity in comparison to the alternative HPLC-UV method for MC as expected [8].

3.2.4. Recovery

Recoveries were calculated in terms of expected mean recovery for each toxin in each of the spiked, extracted and analysed samples. **Table 5** presents the mean recovery percentages of cyanotoxins from spiked water, cyanobacteria, algal supplement tablet powder and mussels with RSDs calculated from the mean recovery of each triplicate spike. Overall, excellent recoveries were determined for each of the matrices studied, with the majority ranging from 80 to 110%. Some exceptions to this were evident, including a higher recovery of toxins spiked into algal cells at the highest fortification level, a drop in recovery for the later-eluting and more aromatic MC-LF and MC-LW analogues in water at the lowest concentration

and lower recovery for some toxin analogues in mussels at the lowest spiking level. The recovery behaviour was also good for Nod, with no notable differences between Nod and the majority of MC analogues. Overall, the results indicated a good level of recovery for the majority of toxins in each of the matrices studied.

3.2.5 Method precision

Instrumental retention time

Instrumental precision of toxin peak retention times following the repeat analysis (n=10) of spiked samples and standards showed that the level of precision of chromatographic retention times is high (RSD = 0.03% to 0.61% within batch), hence a high degree of confidence can be placed upon the toxin peaks consistently eluting at repeatable retention times during any given sequence or batch. Furthermore, the precision was found to be excellent between sequences run on different days (<1.2% RSD) and even over a period of months, using different columns (RSD 0.3 to 3.5%, with a mean of <1.0% RSD). Overall, therefore, there was evidence for a highly consistent elution pattern for the cyanotoxins assessed in this study, thereby increasing the confidence in the specificity of the method.

Within-batch precision

All toxins spiked at the highest concentration level showed excellent precision in a single analytical batch. For toxins spiked into algal cells, precision varied from 0.6 to 1.7%, for mussels 1.4 to 6.8%, for algal supplement tablet powder, 1.9 to 6% and for water from 2.4 to 13% (**Table 6**). Consequently, the precision of the method for each matrix was well below a target of 15% variability. As expected (Horwitz, 1980), precision decreased at low concentrations, but was found to be <15% for the majority of toxins in each matrix type. The

mean toxin RSDs were all <13% at all concentrations, with the only results >15% found for MC-LA and MC-WR in water (16%) and MC-RR, LA and LY in mussels (20-25%) when spiked at the lowest concentration. Overall this indicated good within-batch precision of the quantitative method for each of the matrices investigated (**Table 6**).

Between-batch precision

For algal cell spikes, mean between batch precision ranged from 2.5 to 4.2% across all concentrations, with similar results returned for algal supplement tablet powder samples. Precision was excellent for water and mussels at high and medium concentrations (mean values <5%). At low concentration spikes, mean precision was higher as expected, but mean values for all toxins were 14% and 16% for water and mussels respectively, with only one toxin-matrix combination exceeding a target precision limit of 25% (MC-LY in mussels; 30%). As such, the results indicate further evidence for acceptable method precision for the analysis of cyanotoxins in each of the studied matrices (**Table 7**).

Within-lab reproducibility

Concentration data from the extraction and analysis of shellfish, water, cyanobacteria and algal supplement tablet powder was generated over a longer period of time (> 3 months), using different batches of reagents and consumables. The data realistically describes the within-laboratory reproducibility of the method, incorporating variable changes such as different working calibration solutions, instruments, and other laboratory conditions experienced over the long term. With the production of a mussel tissue LRM containing levels of Nod, MC-LR, MC-LY, MC-LF, MC-LW, MC-HilR and [Asp3]-MC-LR, this material was used for the assessment of reproducibility in mussels. Further data was generated using the repeat analysis of spikes over the long term to supplement this data, also incorporating the additional

MC toxins (**Table 8**). Results indicate an acceptable level of long-term precision for the materials studied. Long-term repeatability RSDs exhibited values between 5% and 11% for the mussel LRM (mean 7.5%) with the spiked mussels showing slightly higher variability (11-15%, mean 13%). Algal supplement tablet powder materials returned RSD% between 3% and 11% per toxin (mean 6.2%), cyanobacteria 6% to 10% (mean 7.9%) and water 6% to 13% (mean 7.5%). For long term precision assessment these values are excellent. All HorRat values calculated were < 1.0 (**Table 8**), providing further evidence for the acceptability of the within-laboratory reproducibility [51]. Overall, the results therefore indicate a good level of within-laboratory reproducibility and give further evidence for the fitness for purpose of the LC-MS/MS method for cyanotoxins analysis.

3.2.6. Ruggedness

Main effects were calculated as the difference of means for each paired set of parameter levels (parameter differences) and compared against method precision (single batch; n=8) using a t-test (two-tailed, 95% confidence) [53]. All t-test values were lower than t-critical (n=8, 95% confidence) for the ruggedness experiment. As such, none of the ruggedness parameters investigated had a statistically significant effect on the stability of the method, with the assumption that parameters investigated do not interact.

3.2.7. Measurement of uncertainty

The uncertainty of measurement incorporated into the LC-MS/MS method was assessed through the propagation of standard uncertainties inherent in the precision, recovery assessment and within-laboratory reproducibility of the method. The measurement uncertainty inherent in the precision component was evaluated from the statistical distribution of the results of a series of measurements and can be characterised by standard deviations

[51]. Uncertainties were calculated at two concentration levels (medium and high spike levels) for medium term precision and RSDs pooled to give total standardised precision uncertainties. The uncertainties associated with method reproducibility were estimated from the data generated by the repeated extraction and analysis of LRMs and spiked samples. The uncertainties present in the determination of recovery were estimated by calculating the standard deviation for each toxin at each concentration.

Standardised uncertainties for each cyanotoxin were calculated from the square root of the sum of squares of each of the uncertainty contributions (**Table 9**). Results showed a range of combined standardised uncertainties for individual toxins, ranging from 0.06 to 0.21. The mean values of expanded MU were calculated as 0.12 (mussels), 0.09 (algal supplement tablet powder), 0.09 (cyanobacteria) and 0.10 (water). A closer examination of the results shows that the majority of toxins, with the exception of MC-RR, returned very low calculated standardised MU values, ranging from 0.05 to a maximum of 0.16. MC-RR on the other hand was found to be associated with standardised MU values of 0.21 in mussels and 0.16 in water, although values for algal supplement tablet powder and cyanobacteria were significantly lower (0.04 and 0.08 respectively). Expanded uncertainties, calculated using a coverage factor (k) of 2, subsequently result in a range of values from 0.11 to 0.42. The coverage factor, k was taken to be 2 in order to provide a 95% confidence in the distribution of values, assuming a normal distribution.

4. Discussion

There is still no recognised “gold standard” analytical method for the determination of cyanotoxins, with the choice of method depending not just on performance characteristics, but also cost, practicality and reliability [54]. As such, our approach was the development of a simple and rapid method which would facilitate reliable quantitation, with minimal analyst input

and overall cost to the end user. A UHPLC-MS/MS method for detection and quantitation of cyanotoxins was consequently developed to enable the rapid, sensitive and accurate quantitation of MC and Nod in a wide variety of appropriate matrices. The method was optimised to facilitate the extraction and analysis of cyanotoxins from a wide variety of matrices including shellfish tissues and algal supplement tablet powders, as well as water and cyanobacterial bloom cells. Optimisation experiments demonstrated that excellent recoveries for all analytes in all four matrices could be achieved through use of 80% aqueous MeOH as the extraction solvent, thereby showing similar results to those determined previously for extraction of cell material using 70% aqueous methanolic extraction [8], 75% aqueous MeOH [42] and other solvent extraction approaches [44] without the need to use more complex extraction techniques such as SPE and immunoaffinity columns [39,43-46,55-57]. For extraction of algal supplement tablet powder and shellfish tissue, a wide range of solvents and extraction techniques were tested, but again the single step dispersive extraction using 80% aqueous MeOH was found to provide optimum extraction of toxins from both matrix types prior to LC-MS/MS analysis, eliminating the need for more complex and time-consuming extraction and clean-up protocols including prolonged ultrasonication, solvent evaporation steps and SPE [21,28]. The use of 80% aqueous MeOH as the optimum extraction solvent was therefore in direct agreement with the solvent used for extraction of other tissue samples, including carp larvae [57] as well as the 75% MeOH solvent methods used for extraction of mussels and fish liver [36] and other fish tissues [38] and 90% MeOH for fish tissues, mussels and oysters [34]. In addition to a simple and rapid extraction method, the chromatographic method was designed to carry out separation of MC analogues and Nod within an extremely short time-frame. Whilst the overall cycle time of 5.5 min is not quite as quick as reported previously by [58], this equates in our laboratory to the ability to run more than 140 samples per day, not including full calibrations at the start and end of the sequence,

regular calibration control checks and other quality control materials such as positive controls and procedural blanks. This high throughput capability compares extremely favourably with the more time-consuming analytical methods reported to date [34,39,46,55].

Consequently, the optimised method for each matrix facilitated the rapid and simple extraction of high numbers of samples in a short time-frame, thereby being ideally suited to the routine, high-throughput regulatory monitoring environment. In addition, there was no evidence for drift issues, either in retention time or peak area response following the analysis of large numbers of samples, as reported previously by [36]. Following our validation, long sequences of samples have been analysed, with some extending to five days without break. In these scenarios there has been no evidence of performance drift and no requirement for guard cartridge changes or MS cone cleaning. The optimised method was validated for each matrix and for each commercially-available MC and Nod to assess the specificity, linearity, limits of detection and quantitation, recovery, precision, repeatability/reproducibility and ruggedness of the method. The method was found to be specific with no interferences observed in any of the four matrices from naturally occurring matrix coextractives. The method linearity was acceptable for all analytes in the four matrices over the full calibration range, 0.6 to 170 ng/mL extract, similar to the linear range reported by other authors [14,34-35,37,46]. In addition, calibration slopes were, for the majority of analytes, similar between matrix types including methanol solvent, indicating a lack of suppression or enhancement in the mass spectrometer source as generally reported in raw water matrices by [14].

Method sensitivity was assessed through the determination of LOD, LOQ and LOR, with results showing the sensitivity was fit for purpose. With LODs equating to less than 1 pg on column, sensitivity was similar to or improved in comparison to LC-UV [8], direct injection LC-MS/MS methods [14,34-35,45] and even some MS/MS methods employing pre-analysis concentration and/or clean up steps [36,55]. As such, method sensitivity is fit for the purpose

of quantifying cyanotoxins in natural waters, noting both the 1 µg/L safe limit for MC-LR in drinking water and the 20 µg/L WHO moderate health alert guideline for recreational exposure to microcystins in water [7]. The LOQs for quantitation of MC variants in this method range from 0.04 to 0.64 µg/L per analogue, so fall well under these limits. For the analysis of algal supplement tablet powders, the likely potential ingestion of up to 10 g powder daily by supplement users, and the WHO proposed TDI of 2.4 µg MC-LR per day, resulted in a requirement to establish as a minimum the quantitation of MC-LR at a concentration of 240 µgkg. As such, this method is capable of quantitation at concentrations more than 200 times lower than this guidance threshold for this toxin. Similarly, even with the daily consumption of 500 g shellfish flesh, resulting in a required safety limit of 4.8 µg MC-LR per kg shellfish tissue, this method is capable of quantitation of MC analogues well below this level, with the majority of toxin analogues showing an LOQ of < 0.5 µg/kg. There are also significant practical and performance advantages to be gained with elimination of pre-analysis concentration steps [35]. Method recoveries, assessed with the repeat analysis of spiked matrix samples were shown to be acceptable, with the majority ranging from 80 to 110%. Results were therefore significantly improved in comparison to the recoveries of seven MCs reported from mussels by [36], almost identical on average to those reported by [34] and [38] for the analysis of six MC variants in shellfish and fish tissues by LC-MS/MS. For recovery in waters, results were similar to or improved in comparison to those reported following immunoaffinity clean-up [43] and SPE clean-up of water samples prior to LC-UV [8] and LC-MS/MS [37,46,55] agreeing with [35] and [14] that the direct analysis approach without clean-up is appropriate for rapid analysis of natural water samples. The variability of the analyte concentrations determined during recovery assessment was used to confirm the acceptable level of within-batch and between-batch precision of the method, with all within-laboratory reproducibility data resulting in HorRat values <1.0. Again, repeatability compared favourably

with values reported elsewhere [35-36,43,45-46,55]. An assessment of method ruggedness using a Placket-Burman experimental design, showed that the method was robust for all parameters investigated assuming parameters do not interact. Standardised uncertainties associated with method performance characteristics were used to calculate and pool overall measurement uncertainties for each matrix-analyte combination. Uncertainties calculated appeared acceptable, and will be reported with any future analytical results.

Overall, the results presented show that the optimised LC-MS/MS method for cyanotoxins is fit for the purpose of detection and quantitation of a range of MC and Nod in shellfish, algal supplement tablet powder, water and cyanobacteria. It is now available for use as a routine monitoring tool for each of these matrices and following assessment is now accredited at Cefas to ISO17025 standard. With excellent sensitivity and recovery, the method provides an early warning tool for the presence of harmful cyanotoxins in water/cyanobacteria bloom samples, as well as providing the potential for analysis of shellfish during periods where freshwater cyanobacterial blooms may impact upon estuarine shellfishery beds. As such, the method will provide excellent support to the current traditional microscopic analysis, providing toxin concentration data to supplement bacterial cell detection and cell density enumeration. Further work will be conducted in the future to extend the method to other cyanotoxins, potentially including cylindrospermopsin and anatoxins. The method will be considered for further assessment by collaborative study.

Acknowledgements

Cefas internal funding (contract DP345A) is acknowledged for supporting this development.

References

[1] Codd, G.A., Morrison, L.F. and Metcalf, J.S. (2005) Cyanobacterial toxins: risk management for health protection. *Toxicol. Appl. Pharmacol*. 203, 264-272

[2] van Apeldoorn, M.E., van Egmond, H.P., Speijers, G.J.A., Bakker, G.J.I., 2007. Toxins of cyanobacteria. *Mol. Nutr. Food Res*. 51, 7–60.

[3] Chia, A.M., Oniye, S.J., Ladan, Z., Lado, Z., Pila, A.E., Inekwe, V.U., Mmerole, J.U., 2009. A survey for the presence of microcystins in aquaculture ponds in Zaria, Northern-Nigeria: possible public health implication. *Afr. J. Biotechnol*. 8, 6282–6289.

[4] Vesterkvist, P.S.M., Misiorek, J.O., Spoof, L.E.M., Tiovola, D.M., Meriluoto, J.A.O. (2012). Comparative cellular toxicity of hydrophilic and hydrophobic microcystins on caco-2 cells. *Toxins* 4, 1008–1023.

[5] Carmichael W.W, (1998). Toxins of freshwater algae. In: Handbook of Natural Toxins, Vol. 3: Marine Toxins and Venoms, A.T. Tu, Ed., pp. 121–147, Marcel Dekker, New York.

[6] Chorus, I. and Bartram, J. (1999). Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. WHO Guide. E & FN Spon, St Edmundsbury Press, Suffolk, UK

[7] WHO (2003) World Health Organisation report, 2003. Guidelines for safe recreational water environments. Volume 1: coastal and fresh waters. Chapter 8. Algae and cyanobacteria in fresh water. 136-158

[8] Lawton, L.A., Edwards, C. and Codd, G.A (1994) Extraction and high-performance liquid chromatographic method for the determination of microcystins in raw and treated waters. *Analyst.* 19, 1525-1530

[9] Roegner, A.F., Brena, B., Gonzalez-Sapienza, G., Puschner, B., 2014. Microcystins in potable surface waters: toxic effects and removal strategies. *J. Appl. Toxicol*. 34, 441–457.

[10] WHO (2008). World Health Organisation report, 2008. The World Health Report – Guideline for Drinking Water Quality, third ed., incorporation the first and second addenda. World Health Organisation, Geneva, Switzerland.

[11] Pilotto, L.S., Douglas, R.M., Burch, M.D., Cameron, S., Beers, M., Rouch, G.J., Robinson, P., Kirk, M., Cowie, C.T., Hardiman, S., Moore, C., Attewell, R.G., (1997). Health effects of exposure to cyanobacteria (blue-green algae) during recreational water-related activities. *Aust. N.Z. J. Public Health* 21, 562–566.

[12] Backer, L.C., McNeel, S.V., Barber, T., Kirkpatrick, B., Williams, C., Irvin, M., Zhou, Y., Johnson, T.B., Nierenberg, K., Aubel, M., LePrell, R., Chapman, A., Foss, A., Corum, S., Hill, V.R., Kieszak, S.M. and Cheng, Y-S. (2009) Recreational exposure to microcystins during algal blooms in two California lakes. *Toxicon.* 55, 909-921

[13] Merel, S., Clement, M., Thomas, O., (2010). State of the art on cyanotoxins in water and their behaviour towards chlorine. *Toxicon* 55, 677–691.

[14] Pekar, H., Westerberg, E., Bruno, O., Laane, A., Persson, K.M., Sundstrom, L.F. and Thim, A-M. (2016) Fast, rugged and sensitive ultra high pressure liquid chromaotgrpahy tandem mass spectrometry method for analysis of cyanotoxins in raw water and drinking water – first findings of anatoxins, cylindrospermopsins and microcystin variants in Swedish source waters and infiltration ponds. *J. Chrom. A.* 1429, 265-276

[15] Vasconcelos, V. (1995) Uptake and depuration of the heptapeptide toxin microcystin-LR in *Mytilus galloprovincialis. Aquatic toxicology.* 32, 227-237

[16] Ibelings, B.W., Chorus, I., 2007. Accumulation of cyanobacterial toxins in freshwater ‘‘seafood’’ and its consequences for public health: a review. *Environ. Pollut*. 150, 177–192.

[17] Miller, M.A., Kudela, R.M., Mekebri, A., Crane, D., Oates, S.C., Tinker, M.T., Staedlier, M., Miller, W.A., Toy-Choutka, S., Dominik, C., Hardin, D., Langlois, G., Murray, M., Ward, K.

and Jessup, D.A. (2010) Evidence for a novel marine harmful algal bloom: cyanotoxins (microcystin) transfer from land to sea otters. *Plos One.* 5(9) e12576 doi: 10.1371

[18] Preece, E.P., Moore, B.C., Hardy, F.J., Deobald, L.A., 2015. First detection of microcystin in Puget Sound, Washington, mussels (*Mytilus trossulus*). *Lake Reserv. Manag*. 31, 50–54.

[19] Gibble, C.M., Peacock, M.B. and Kudela, R.W. (2016) Evidence for freshwater algal toxins in marine shellfish: implications for human and aquatic health. *Harmful algae.* 59, 59-66

[20] Preece, E.P., Hardy, F.J., Moore, B.C. and Bryan, M. (2017) A review of miscrocystin detections in estuarine and marine waters: environmental implications and human health risk. *Harmful Algae.* 61, 31-45

[21] Heussner, A.H., Mazija, L., Fastner, J. and Dietrich, D.R. (2012) Toxin content and cytotoxicity of algal dietary supplements. *Toxicology and Applied Pharmacology.* 265, 263-271

[22] Vichi, S., Lavorini, P., Funari, E., Scardala, S. and Testai. (2012) Contamination by *Microcystis* and microcystins of blue-green algae food supplements (BGAS) on the Italian market and possible risk for the exposed population. *Food and Chem. Tox.* 50, 4493-4499

[23] Dietrich, D., Hoeger, S., 2005. Guidance values for microcystins in water and cyanobacterial supplement products (blue-green algal supplements): a reasonable or misguided approach? *Toxicol. Appl. Pharmacol*. 203, 273-289.

[24] Fromme, H., Kohler, A., Krause, R., Fuhrling, D., (1999). Occurrence of cyanobacterial toxins Microcystins and anatoxin-a in Berlin water bodies with implications to human health and regulations. *Environmental Toxicology*. 15, 120-130

[25] EA (2016) Monitoring freshwater and marine algal incidents, version 8. Unpublished operational instruction 906_08. Environment Agency Report, issued 08/03/2016.

[26] Chorus, I., Falconer, I.R., Sala, H.J. and Bartram, J. (2000). Health risks caused by freshwater cyanobacteria in recreational waters. *J. Toxicol. Environ. Health* 3, 323–347.

[27] Kaushik, R. and Balasubramanian, R. (2013) Methods and approaches used for detection of cyanotoxins in environmental samples: a review. *Crit. Rev. Env. Sci. Tech.* 43, 1349-1383

[28] Harada, K-I., Kondo, F. and Tsuji, K. (2001) Trace analysis of microcystins in environmental samples. *J. AOAC International.* 84(5) 1636-1642

[29] Gurbuz, F., Metcalf, J.S., Karahan, A.G. and Codd, G.A. (2009) Analysis of dissolved microcystins in surface water samples from Kovada Lake, Turkey. *Sci. Tot. Env.* 407, 4038-4046

[30] Merel, S., Walker, D., Chicana, R., Snyder, S., Baures, E., Thomas, O., 2013. State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. *Environ. Int*. 59, 303–327.

[31] McElhiney, J., Lawton, L.A., (2005). Detection of the cyanobacterial hepatotoxins microcystins. *Toxicol. Appl. Pharmacol*. 203, 219–230.

[32] Edwards, C., Lawton, L.A., Beattie, K.A., Codd, G.A., Pleasance, S. and Dear, G.J. (1993) Analysis of microcystins from cyanobacteria by liquid chromatography with mass spectrometry using atmospheric-pressure ionization. *Rap. Comm. Mass Spec.* 7, 714-721

[33] Dahlmann, J., Budakowski, W.R. and Luckas, B. (2003) Liquid chromatography-electrospray ionization-mass spectrometry based method for the simultaneous determination of algal and cyanocaterial toxins in phytoplankton from marine waters and lakes followed by tentative structural elucidation of microcystins. *J. Chrom. A.* 994, 45-57

[34] Mekebri, A., Blondina, G.J. and Crane, D.B. (2009) Method validation of microcystins in water and tissue by enhanced liquid chromatography mass spectrometry. *J. Chrom. A.* 1216, 3147-3155

[35] Allis, O., Dauphard, J., Hamilton, B., Shuilleabhain, A.N., Lehane, M., James, K.J. and Furey, A. (2007) Liquid chromatography-tandem mass spectrometry application, for the

determination of extracellular hepatotoxins in Irish lake and drinking waters. *Anal. Chem.* 79(9), 3436-47

[36] Karlsson, K.M., Spoof, L.E.M. and Meriluoto, J.A.O. (2005) Quantitative LC-ESI-MS analyses of microcystins and Nodularin-R in animal tissue – matrix effects and method validation. *Env. Toxicol..* 20(3), 381-389

[37] Kaloudis, T., Zervou, S-K., Tsimeli, K., Triantis, T.M., Fotiou, T. and Hiskia, A. (2013) Determination of microcystins and nodularin (cyanobacterial toxins) in water by LC-MS/MS. Monitoring of Lake Marathonas, a water reservoir of Athens, Greece. *J. Haz. Mat.* 263(1), 105-115

[38] Greer, B., McNamee, S.E., Boots, B., Cimarelli, L., Guillebault, D., Helmi, K., Marcheggiani, S., Panaiotov, S., Breitenbach, U., Akcaalan, R., Medlin, L.K., Kittler, K., Elliott, C.T. and Campbell, K. (2016) A validated UPLC-MS/MS method for the surveillance of ten aquatic biotoxins in European brackish and freshwater systems. *Harmful Algae*. 55, 31-40

[39] Beltran, E., Ibanez, M., Sancho, J.V. and Hernandez, F. (2012) Determination of six microcystins and nodularin in surface and drinking waters by on-line solid phase extraction-ultra high pressure liquid chromatography tandem mass spectrometry. *J. Chrom. A.* 1266, 61-68

[40] Anon. (2006). Commission Regulation (EC) No 1664/2006 of 6th Nov. 2006 amending Regulation (EC) No 2074/2005 as regards implementing measures for certain products of animal origin intended for human consumption and repealing certain implementing measures. *Official Journal of the European Union*. L320, 13-45.

[41] Thompson, M., Ellison, S.L.R., Wood, R. (2002). Harmonized guidelines for single laboratory validation of methods of analysis (IUPAC technical report). *Pure Appl. Chem*. 74 (5), 835-855.

[42] Fastner, J., Flieger, I. and Neumann, U. (1998) Optimised extraction of microcystins from field samples – a comparison of different solvents and procedures. *Wat. Res.* 32(10) 3177-3181

[43] Aranda-Rodriguez, R., Kubwabo, C. and Benoit, F.M. (2003) Extraction of 15 microcystins and nodularin using immunoaffinity columns. *Toxicon.* 42, 587-599

[44] Amé, M.V., Galanti, L.C., Menone, M.L., Gerpe, M.S., Moreno, V.J. and Wunderlin, D.A. (2010) Microcystin-LR, -RR, -YR ad -LA in water samples and fishes from a shallow lake in Argentina. *Harmful Algae.* 9, 66-73

[45] Chen, J., Yan, T., Xu, J., He, S., Zhao, P. and Yan, X. (2012) Simultaneous determination of toxins in algae and water samples by high-performance liquid chromatography with triple quadrupole mass spectrometry. *J. Sep. Sci.* 35, 1094-1101

[46] Zervou S-K., Christophoridis, C., Kaloudis, T., Triantis, T.M. and Hiskia, A. (2017) New SPE-LC-MS/MS method for simultaneous determination of multi-class cyanobacterial and algal toxins. *J. Haz. Mat.* 323, 56-66

[47] Vasconcelos, V., Oliveira, S. and Teles, F.O. (2001) Impact of a toxic and a non-toxic straing of *Microcystis aeruginoasa* on the crayfish *Procambarus clarkia. Toxicon.* 39, 1461-1470

[48] Schmidt, J.R., Wilhelm, S.W. and Boyer, G.L. (2014) The fate of microcystins in the environment and challenges for monitoring. *Toxins.* 6, 3354-3387

[49] Taranu, Z.E., Gregory-Eaves, I. (2015) Acceleration of cyanobacterial dominance in north temperate subartic lakes during the Anthropocene. *Ecology Letters.* Doi: 10.1111/ele.12420

[50] ISO (1993) Guide to the expression of uncertainty in measurement, ISO, Geneva (1993).

[51] Anon (2012). Quantifying uncertainty in analytical measurement. Eurachem/Citac guide, 3rd edition. Eds: Ellison, S.L.R., Rosslein M. and Williams, A.

[52] Horwitz, W., Kamps, L.R. and Boyer, K.W. (1980). Quality assurance in the analysis of foods and trace constituents. *J. AOAC.* 63(6) 1344-43

[53] Plackett, R.L. and Burman, J.P. (1946). The design of optimum multifactorial experiments. *Biometrika*, 23(4), 305-325

[54] Gaget, V., Lau, M., Sendall, and Froscio, S. (2017) Cyanotoxins: which detection technique for an optimum risk assessment? *Wat. Res.* 118, 227-238

[55] Shan, Y., Shi, X., Dou, A., Zou, C., He, H., Yang, Q., Zhao, S., Lu, X. and Xu, G. (2011) A fully automated system with on-line micro solid-phase extraction combined with capillary liquid chromatography-tandem mass spectrometry for high throughput analysis of microcystins and nodularin-R in tap water and lake water. *J. Chrom. A.* 1218, 1743-1748

[56] Munoz, G., Duy, S.V., Roy-Lachapelle, A., Husk, B. and Sauvé, S. (2017) Analysis of individual and total microcystins in surface water by on-line preconcentration and desalting coupled to liquid chromatography tandem mass spectrometry. *J. Chrom. A.* 1516, 9-20

[57] El Ghazali, I., Saqrane, S., Carvalho, A.P., Ouahid, Y., Del Campo, F.F., Oudra, B. and Vasconcelos, V. (2010) Effect of different microcystin profiles on toxin bioaccumulation in common carp (*Cyprinus carpio*) larvae via *Artemia* nauplii. *Ecotox. Env. Safety.* 73, 762-770

[58] Neffling, M-R., Spoof, L. and Meriluoto, J. (2009) Rapid LC-MS detection of cyanobacterial hepatotoxins microcystins and nodularins – comparison of columns. *Anal. Chem. Acta.* 653, 234-241

Table 1. Positive ion mode SRM transitions used for MC detection and quantitation

Analyte	SRM transitions	Cone, V	CE, eV
MC-RR*	519.9 > 134.9; 126.9; 102.8	30	30; 50; 70
Nod	825.5 > 135.1; 103.1	55	60; 100
MC-LA	910.1 > 135.1; 106.9	35	70: 80
[Dha^{7}]-MC-LR	981.5 > 135.0; 106.8	75	75: 80
[Asp3] MC-LR	981.5 > 134.9; 106.9	75	70; 80
MC-LF	986.5 > 213.0; 135.0	35	60; 65
MC-LR	995.6 > 135.0; 127.0	60	70; 90
MC-LY	1002.5 > 135.0; 106.9	40	70; 90
MC-HilR*	1009.7 > 134.9; 126.9; 106.9	75	75; 90; 80
MC-LW	1025.5 > 134.9; 126.8	35	65; 90
MC-YR	1045.6 > 135.0; 126.9	75	75; 90
MC-HtyR	1059.6 > 134.9; 106.9	75	70; 90
MC-WR	1068.6 > 134.9; 106.9	80	75; 100

CE = Collision energy. *3 SRM transitions used throughout validation to assess which would ultimately be most applicable

Table 2. Experimental design for ruggedness testing of microcystins and nodularin in mussel tissue

	Experiment number							
Parameter	1	2	3	4	5	6	7	8
Extraction solvent (%MeOH)	78%	78%	78%	78%	82%	82%	82%	82%
Extraction time (s)	100	100	140	140	100	100	140	140
Centrifuge speed (rpm)	4,500	3,500	4,500	3,500	4,500	3,500	4,500	3,500
Centrifuge time (min)	10	10	9	9	9	9	10	10
Filter spec (µm)	0.2	0.45	0.2	0.45	0.45	0.2	0.45	0.2
Filter make	Type 1	Type 2	Type 2	Type 1	Type 1	Type 2	Type 2	Type 1
Water grade	HPLC	MilliQ	MilliQ	HPLC	MilliQ	HPLC	HPLC	MilliQ

Table 3. Summary of calibration parameters for cyanotoxins in methanol, cyanobacteria, mussels, powder and water (n=3)

Matrix	Parameter	NOD	MC-LR	MC-RR	MC-LA	MC-LY	MC-LF	MC-LW	MC-YR	MC-WR	[Asp3] MC-LR	MC-HilR	MC-HtyR
MeOH	Gradient	342	218	306	101	98	179	94	170	163	149	202	110
	r2	0.995	0.996	0.989	0.995	0.994	0.995	0.995	0.996	0.997	0.996	0.996	0.996
Cyanobacteria	Gradient	332	215	270	97	95	159	102	161	152	124	193	104
	r2	0.996	0.997	0.991	0.996	0.996	0.997	0.997	0.997	0.996	0.990	0.996	0.996
Mussels	Gradient	369	236	516	99	96	162	103	177	169	149	210	117
	r2	0.995	0.996	0.990	0.998	0.997	0.998	0.998	0.995	0.994	0.996	0.997	0.994
Powder	Gradient	354	233	371	94	93	159	103	173	167	149	208	117
	r2	0.996	0.997	0.993	0.994	0.994	0.994	0.995	0.996	0.997	0.996	0.996	0.997
Water	Gradient	328	206	254	89	55	74	49	153	114	133	185	99
	r2	0.996	0.991	0.982	0.990	0.998	0.997	0.999	0.984	0.987	0.989	0.986	0.985

Table 4. Summary of LOD, LOQ and LOR for each toxin in the four validated matrices

		NOD	MC-LR	MC-RR	MC-LA	MC-LY	MC-LF	MC-LW	MC-YR	MC-WR	[Asp3] MC-LR & [Dha7]-MC-LR	MC-HilR	MC-HtyR
Algal cells **ng spiked**	LOD	0.02 ± 0.01	0.06 ± 0.05	0.02 ± 0.01	0.04 ± 0.03	0.16 ± 0.11	0.15 ± 0.05	0.15 ± 0.05	0.06 ± 0.06	0.04 ± 0.03	0.09 ± 0.06	0.09 ± 0.08	0.03 ± 0.04
	LOQ	0.05 ± 0.04	0.19 ± 0.16	0.07 ± 0.04	0.12 ± 0.12	0.54 ± 0.36	0.50 ± 0.15	0.49 ± 0.15	0.21 ± 0.18	0.13 ± 0.10	0.30 ± 0.21	0.31 ± 0.28	0.10 ± 0.12
	LOR	0.1	0.35	0.15	0.25	0.9	0.7	0.7	0.4	0.25	0.6	0.6	0.25
Algal cells **ng/mL**	LOD	0.0004 ± 0.0002	0.0013 ± 0.0011	0.0005 ± 0.0003	0.0008 ± 0.0008	0.0036 ± 0.0024	0.0034 ± 0.0010	0.0033 ± 0.0010	0.0014 ± 0.0012	0.0009 ± 0.0007	0.0020 ± 0.0014	0.0020 ± 0.0019	0.0007 ± 0.0008
	LOQ	0.0012 ± 0.0008	0.0043 ± 0.0036	0.0016 ± 0.0010	0.0028 ± 0.0026	0.0119 ± 0.0079	0.0112 ± 0.0034	0.0110 ± 0.0034	0.0048 ± 0.0041	0.0030 ± 0.0022	0.0066 ± 0.0048	0.0068 ± 0.0063	0.0022 ± 0.0027
	LOR	0.005	0.01	0.03	0.006	0.02	0.02	0.02	0.01	0.01	0.02	0.02	0.01
Water **ng/mL**	LOD	0.04 ± 0.02	0.05 ± 0.03	0.01 ± 0.01	0.10 ± 0.06	0.08 ± 0.04	0.14 ± 0.06	0.13 ± 0.04	0.05 ± 0.03	0.06 ± 0.03	0.19 ± 0.20	0.14 ± 0.14	0.06 ± 0.05
	LOQ	0.13 ± 0.06	0.18 ± 0.10	0.04 ± 0.04	0.33 ± 0.21	0.26 ± 0.14	0.46 ± 0.22	0.45 ± 0.13	0.18 ± 0.10	0.20 ± 0.10	0.64 ± 0.65	0.46 ± 0.46	0.21 ± 0.15

	LOR	0.30	0.30	0.10	0.60	0.50	0.90	0.90	0.50	0.40	1.30	1.00	0.40
Mussels	LOD	0.05 ± 0.01	0.09 ± 0.08	0.01 ± 0.01	0.12 ± 0.05	0.11 ± 0.03	0.10 ± 0.03	0.15 ± 0.08	0.05 ± 0.04	0.06 ± 0.04	0.21 ± 0.17	0.14 ± 0.08	0.05 ± 0.04
µg/kg	LOQ	0.16 ± 0.03	0.31 ± 0.27	0.03 ± 0.03	0.39 ± 0.17	0.37 ± 0.08	0.35 ± 0.12	0.51 ± 0.26	0.18 ± 0.13	0.20 ± 0.14	0.69 ± 0.57	0.45 ± 0.26	0.18 ± 0.14
	LOR	0.30	1.30	0.20	0.60	0.50	0.90	1.30	1.50	0.40	1.30	1.20	0.40
Powder	LOD	0.12 ± 0.06	0.40 ± 0.18	0.21 ± 0.12	1.08 ± 0.25	1.12 ± 0.52	0.91 ± 0.34	1.18 ± 0.30	0.29 ± 0.18	0.39 ± 0.10	0.64 ± 0.11	0.57 ± 0.24	0.27 ± 0.17
µg/kg	LOQ	0.40 ± 0.20	1.35 ± 0.62	0.70 ± 0.41	3.59 ± 0.83	3.74 ± 1.73	3.05 ± 1.15	3.94 ± 1.01	0.95 ± 0.61	1.29 ± 0.32	2.15 ± 0.36	1.89 ± 0.82	0.90 ± 0.57
	LOR	1.00	2.00	1.50	4.50	5.50	4.50	5.00	2.00	2.00	3.00	3.00	2.00

Table 5. Mean percentage recoveries (and RSDs of triplicate spikes) of cyanotoxins from water, powder, cyanobacteria and mussel samples spiked at three different concentrations

		NOD	MC-LR	MC-RR	MC-LA	MC-LY	MC-LF	MC-LW	MC-YR	MC-WR	[Asp3] MC-LR & [Dha7]-MC-LR	MC-HilR	MC-HtyR
Cyanobacteria cells	High	83 ± 3.4	126 ± 8.6	124 ± 4.0	123 ± 6.4	130 ± 6.0	132 ± 7.3	131 ± 3.6	123 ± 4.7	118 ± 3.8	127 ± 3.2	84 ± 4.5	122 ± 3.7
	Medium	80 ± 1.6	86 ± 1.3	83 ± 3.3	85 ± 4.2	88 ± 3.3	88 ± 1.4	89 ± 3.4	84 ± 3.3	80 ± 2.9	87 ± 2.4	87 ± 2.7	83 ± 1.7
	Low	84 ± 5.2	93 ± 10.1	108 ± 11.4	106 ± 13.0	110 ± 14.8	111 ± 10.5	116 ± 12.8	108 ± 12.8	106 ± 13.2	114 ± 10.6	94 ± 5.5	105 ± 10.5
Water	High	92 ± 3.0	94 ± 2.9	96 ± 11.0	92 ± 2.5	90 ± 3.9	81 ± 5.8	77 ± 5.3	90 ± 4.0	82 ± 2.1	92 ± 3.5	93 ± 3.5	91 ± 4.9
	Medium	99 ± 1.9	92 ± 4.2	90 ± 10.0	87 ± 5.4	89 ± 1.2	72 ± 3.9	73 ± 3.4	91 ± 2.2	75 ± 3.2	88 ± 3.1	83 ± 2.5	92 ± 2.6
	Low	93 ± 4.0	79 ± 6.7	114 ± 32.8	74 ± 8.5	75 ± 6.0	48 ± 10.0	49 ± 6.7	83 ± 3.2	75 ± 5.4	76 ± 5.5	84 ± 7.5	82 ± 7.7
Mussels	High	87 ± 14.0	92 ± 14.4	91 ± 7.2	83 ± 12.1	89 ± 15.5	86 ± 13.3	84 ± 13.4	96 ± 8.6	95 ± 16.7	86 ± 7.7	97 ± 14.2	89 ± 8.0
	Medium	71 ± 11.2	84 ± 10.5	88 ± 7.4	81 ± 8.6	85 ± 13.7	82 ± 10.3	82 ± 10.5	94 ± 5.7	94 ± 13.9	83 ± 7.0	92 ± 12.0	83 ± 6.1
	Low	70 ± 7.6	61 ± 7.9	111 ± 28.8	61 ± 5.6	68 ± 12.2	67 ± 6.6	72 ± 10.9	88 ± 7.3	91 ± 8.8	73 ± 10.8	79 ± 5.9	72 ± 3.0
Powder	High	94 ± 1.8	97 ± 3.0	99 ± 3.4	96 ± 11.0	101 ± 6.5	125 ± 16.3	98 ± 4.7	101 ± 2.9	91 ± 2.8	107 ± 6.7	97 ± 4.7	98 ± 4.7
	Medium	96 ± 2.7	96 ± 4.5	98 ± 4.3	96 ± 12.1	103 ± 4.8	124 ± 20.9	99 ± 3.7	97 ± 1.3	91 ± 4.6	103 ± 6.9	88 ± 5.8	97 ± 3.0

Table 6. Summary of within-batch precision (RSD%) for cyanotoxins in cyanobacteria, water, mussels and powder

		NOD	MC-LR	MC-RR	MC-LA	MC-LY	MC-LF	MC-LW	MC-YR	MC-WR	[Asp3] MC-LR & [Dha7]-MC-LR	MC-HilR	MC-HtyR
Cyanobacteria													
cells	High	0.87	0.88	1.00	1.42	1.45	1.65	0.67	0.90	1.77	1.62	1.47	1.10
	Medium	3.65	2.79	1.63	1.99	5.19	2.47	1.59	1.20	3.48	1.27	2.82	2.78
	Low	2.99	8.09	1.75	6.62	6.90	2.19	1.96	3.46	4.01	4.28	3.98	6.27
Water	High	3.27	3.30	13.04	3.76	4.19	4.86	4.06	2.41	3.92	4.09	4.70	3.34
	Medium	2.84	4.31	9.07	2.89	5.07	9.40	1.81	2.41	2.80	1.56	3.62	5.21
	Low	8.15	8.53	10.44	16.16	10.19	19.87	12.36	11.20	16.19	14.84	14.01	12.64
Mussels	High	1.40	3.47	6.76	3.14	2.54	3.83	2.64	3.89	3.22	2.62	2.60	5.60
	Medium	1.47	5.70	18.40	3.66	2.81	4.08	3.07	2.45	3.56	4.67	3.24	5.78
	Low	6.94	7.01	20.01	24.58	20.92	10.43	12.23	5.70	18.15	11.30	8.47	8.02
Powder	High	1.91	3.79	1.57	5.90	3.76	2.37	4.62	2.75	2.80	4.34	2.71	3.54
	Medium	3.67	6.26	2.02	7.23	6.19	5.07	5.98	2.87	4.09	7.19	5.66	5.39

Table 7. Summary of between-batch precision (RSD%) for cyanotoxins in cyanobacteria, water, mussels and powder

		NOD	MC-LR	MC-RR	MC-LA	MC-LY	MC-LF	MC-LW	MC-YR	MC-WR	[Asp3] MC-LR & [Dha7] MC-LR	MC-HilR	MC-HtyR
Cyanobacteria													
cells	High	2.45	1.98	1.85	3.52	2.77	2.75	2.39	2.24	2.49	1.92	3.34	2.07
	Medium	2.93	2.23	1.32	2.28	4.61	2.23	1.81	2.09	2.95	1.92	3.08	3.66
	Low	3.03	6.34	1.75	8.23	5.00	2.03	4.00	2.81	3.86	4.03	3.77	5.80
Water	High	2.57	2.38	10.11	2.73	3.32	3.28	3.46	3.15	4.29	4.14	5.14	2.88
	Medium	4.08	3.90	10.08	6.33	5.54	6.61	3.19	4.40	3.19	2.29	4.82	4.56
	Low	6.40	7.12	36.49	15.10	10.68	17.61	14.33	12.38	16.43	13.35	11.63	9.76
Mussels	High	3.00	2.81	9.31	3.26	2.94	2.94	2.27	2.80	2.43	2.15	2.31	3.97
	Medium	2.04	4.44	12.02	5.50	4.75	3.01	3.64	4.17	5.32	4.53	3.53	4.24
	Low	8.44	11.22	47.87	22.01	30.28	11.50	9.51	9.25	14.32	10.10	9.69	10.53
Powder	High	1.69	3.49	1.56	5.08	3.16	2.43	3.80	2.13	2.63	3.55	2.92	2.41
	Medium	2.87	5.26	1.48	6.90	5.18	5.11	4.03	3.56	4.35	5.77	5.63	4.70

Table 8. Summary of within-laboratory reproducibility (RSD%) plus associated HorRat value for cyanotoxins in cyanobacteria, water, mussels and powder

		NOD	MC-LR	MC-RR	MC-LA	MC-LY	MC-LF	MC-LW	MC-YR	MC-WR	[Asp3] MC-LR & [Dha7]-MC-LR	MC-HilR	MC-HtyR
Cyanobacteria													
cells	Mean	65.64	58.99	56.30	44.91	38.76	38.96	31.00	49.42	40.03	87.72	52.77	49.21
	sd	4.80	5.05	4.19	2.92	2.51	3.98	2.57	3.99	3.01	7.28	4.49	3.75
	RSD%	7%	9%	7%	7%	6%	10%	8%	8%	8%	8%	9%	8%
	HorRat	0.30	0.35	0.30	0.25	0.25	0.39	0.31	0.32	0.29	0.36	0.34	0.30
Water	Mean	89.61	282.44	134.15	132.08	140.51	155.78	136.71	133.89	132.71	251.66	92.58	133.15
	sd	5.05	25.83	8.36	8.15	8.49	19.74	9.92	9.85	11.21	15.96	7.19	9.70
	RSD%	6%	9%	6%	6%	6%	13%	7%	7%	8%	6%	8%	7%
	HorRat	0.25	0.47	0.29	0.28	0.28	0.60	0.34	0.34	0.39	0.32	0.34	0.34
Mussels	Mean	74.42	81.17	75.24	69.87	74.50	64.65	72.30	86.10	84.24	159.19	88.92	81.95
(spike)	sd	8.83	10.72	8.68	9.04	8.54	8.60	8.57	11.52	12.92	20.49	12.05	11.02
	RSD%	12%	13%	12%	13%	11%	13%	12%	13%	15%	13%	14%	13%

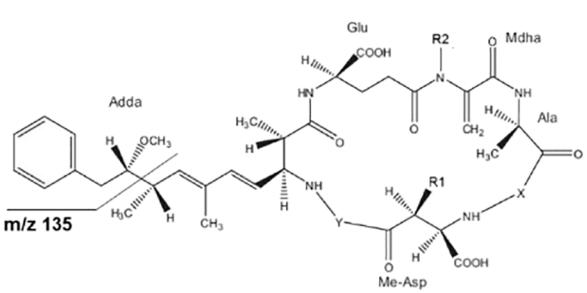
	HorRat	0.50	0.57	0.49	0.54	0.48	0.55	0.50	0.58	0.66	0.61	0.59	0.58
Mussels	Mean	115.84	73.58	-	-	12.44	27.82	26.58	-	-	14.24	1.53	-
(LRM)	sd	5.47	6.06	-	-	0.78	3.05	1.38	-	-	0.84	0.17	-
	RSD%	5%	8%	-	-	6%	11%	5%	-	-	6%	11%	-
	HorRat	0.21	0.35	-	-	0.20	0.40	0.19	-	-	0.20	0.26	-
Powder	Mean	43.37	51.30	41.58	44.33	46.30	40.69	44.59	47.88	49.91	96.42	48.25	49.58
	sd	1.66	4.60	1.23	1.51	1.64	3.92	3.15	2.42	5.54	5.64	2.96	3.32
	RSD%	4%	9%	3%	3%	4%	10%	7%	5%	11%	6%	6%	7%
	HorRat	0.15	0.36	0.11	0.13	0.14	0.37	0.28	0.20	0.44	0.26	0.24	0.27

Table 9. Combined uncertainties calculated from validation data for mussels, powder, cyanobacteria and water showing uncertainties as (a) standardised uncertainty and (b) expanded uncertainty (k=2)

	Mussels		Powder		Cyanobacteria		Water	
	Std MU	Exp MU	Std MU	Exp MU	Std MU	Exp MU	Std MU	Exp MU
NOD	0.06	0.11	0.05	0.11	0.08	0.16	0.07	0.15
MC-LR	0.10	0.20	0.11	0.23	0.09	0.18	0.10	0.21
MC-RR	0.21	0.42	0.04	0.08	0.08	0.15	0.16	0.33
MC-LA	0.14	0.28	0.10	0.19	0.07	0.15	0.09	0.17
MC-LY	0.08	0.16	0.08	0.15	0.08	0.17	0.09	0.18
MC-LF	0.12	0.24	0.11	0.22	0.11	0.21	0.16	0.31
MC-LW	0.07	0.13	0.10	0.19	0.09	0.17	0.09	0.17
MC-YR	0.14	0.28	0.06	0.13	0.08	0.17	0.09	0.17
MC-WR	0.16	0.32	0.12	0.24	0.08	0.17	0.10	0.20
[Asp3] MC-LR & [Dha7]-MC-LR	0.08	0.16	0.10	0.19	0.09	0.17	0.08	0.16
MC-HilR	0.12	0.24	0.09	0.18	0.09	0.19	0.10	0.20
MC-HtyR	0.15	0.30	0.09	0.18	0.08	0.17	0.09	0.19

Highlights

- Novel UHPLC-MS/MS cyanotoxin method
- Developed for shellfish, powder and cyanobacteria
- Fully validated following international guidelines
- Rapid, simple, accurate, fast turnaround method
- Highly suited to high throughput regulatory testing



a) **Microcystins**

	X	Y	R1	R2
MC-LR	Leu	Arg	Me	Me
MC-RR	Arg	Arg	Me	Me
MC-LA	Leu	Ala	Me	Me
MC-LY	Leu	Tyr	Me	Me
MC-LF	Leu	Phe	Me	Me
MC-LW	Leu	Try	Me	Me
MC-YR	Tyr	Arg	Me	Me
MC-WR	Try	Arg	Me	Me
[Asp3] MC-LR	Leu	Arg	H	Me
[Dha7] MC-LR	Leu	Arg	Me	H
MC-HilR	HomoisoLeu	Arg	Me	Me
MC-HtyR	Homotyr	Arg	Me	Me

b) **Nodularin**

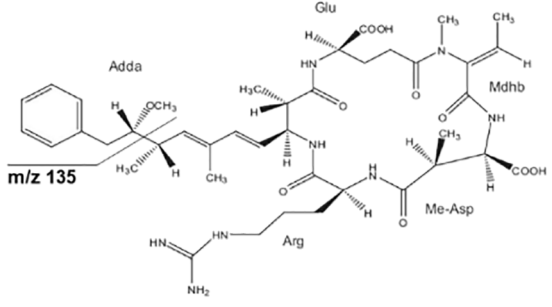


Figure 1

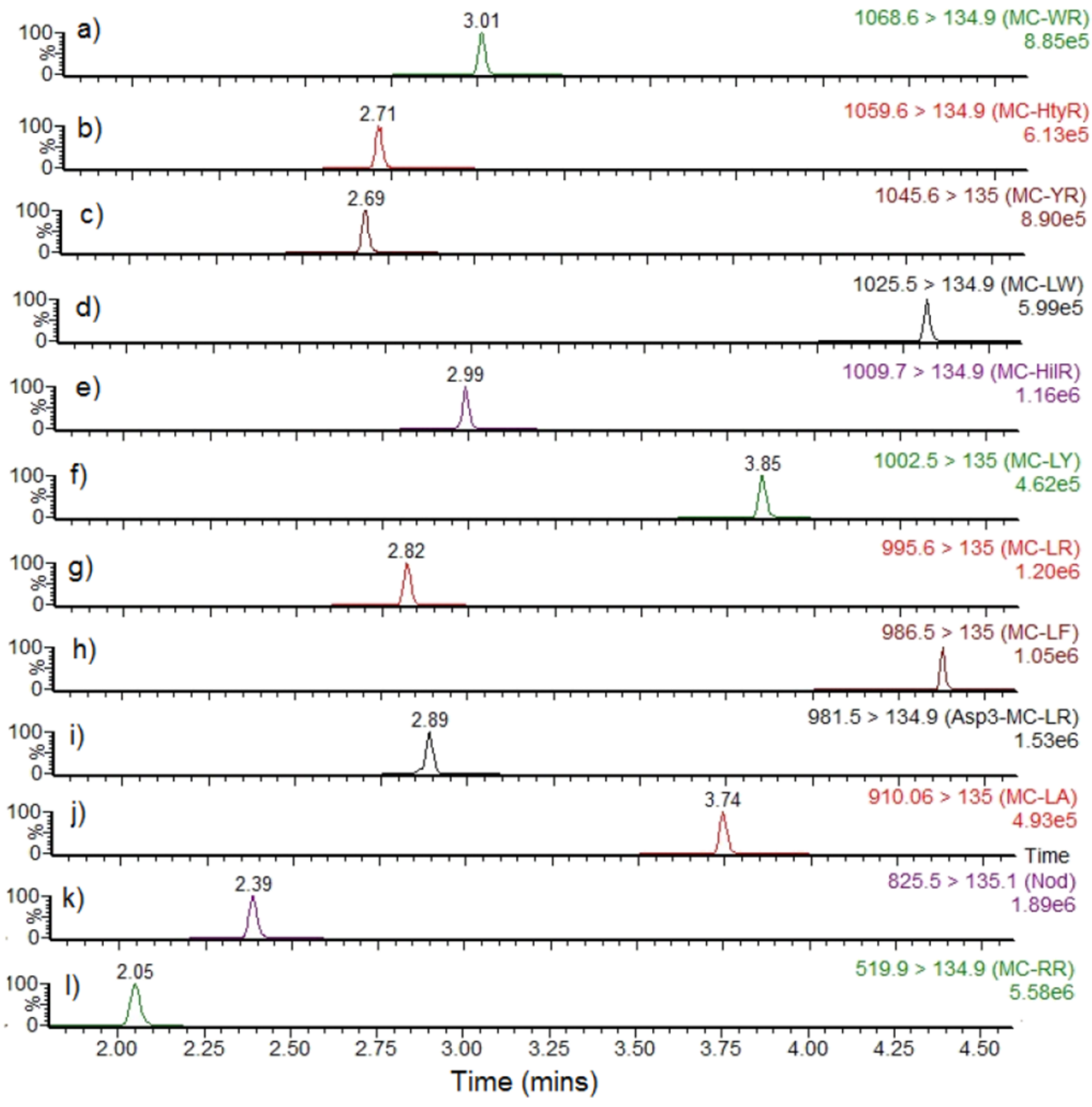

a)
3.01
1068.6 > 134.9 (MC-WR)
8.85e5
b)
2.71
1059.6 > 134.9 (MC-HtyR)
6.13e5
c)
2.69
1045.6 > 135 (MC-YR)
8.90e5
d)
1025.5 > 134.9 (MC-LW)
5.99e5
e)
2.99
1009.7 > 134.9 (MC-HilR)
1.16e6
f)
3.85
1002.5 > 135 (MC-LY)
4.62e5
g)
2.82
995.6 > 135 (MC-LR)
1.20e6
h)
986.5 > 135 (MC-LF)
1.05e6
i)
2.89
981.5 > 134.9 (Asp3-MC-LR)
1.53e6
j)
3.74
910.06 > 135 (MC-LA)
4.93e5
Time
k)
2.39
825.5 > 135.1 (Nod)
1.89e6
l)
2.05
519.9 > 134.9 (MC-RR)
5.58e6
100
%
0
2.00
2.25
2.50
2.75
3.00
3.25
3.50
3.75
4.00
4.25
4.50
Time (mins)

Figure 2