

Extraction and High-performance Liquid Chromatographic Method for the Determination of Microcystins in Raw and Treated Waters

Linda A. Lawton,* Christine Edwards and Geoffrey A. Codd

Department of Biological Sciences, University of Dundee, Dundee, UK
DD1 4HN

Increasing concern over the presence of microcystins (cyanobacterial/blue-green algal hepatotoxins) in water supplies has emphasized the need for a suitable analytical method. As many microcystins are known to exist, a method was developed that permits the determination of numerous variants by a single procedure. The method involves filtration to separate cyanobacterial cells from water, allowing intracellular and extracellular toxin levels to be assessed. The cellular components of the samples are extracted repeatedly in methanol, which was found to be the most versatile solvent tested for the extraction of microcystins. The efficiency of this extraction procedure was found to be independent of cell biomass. The filtered water was subjected to trace enrichment using a C₁₈ solid-phase extraction cartridge, followed by identification and determination by photodiode-array high-performance liquid chromatography. The procedure was assessed using four water samples (two raw and two treated) spiked with a mixture of five microcystins and the cyanobacterial hepatotoxin nodularin. Recoveries of all but one microcystin were found to be good when spiked with concentrations as low as 250 ng l⁻¹. The linearity and precision of the experimental procedure were assessed for five microcystins and nodularin. The proposed method permits rapid sample processing and determination of several microcystins.

Keywords: Microcystin; cyanobacterial toxin; blue-green algae; water analysis; high-performance liquid chromatography

Introduction

Bloom-forming cyanobacteria (blue-green algae) are commonly observed in eutrophic water bodies and pose a serious water quality problem because of the potent toxins that they often produce.^{1,2} The most commonly reported toxins are the hepatotoxins, the largest group being heptapeptides, known as microcystins. These are monocyclic peptides with a common structure containing three D-amino acids (alanine, *erythro*-β-methylaspartic acid and glutamic acid), two L-amino acids, which are variable, plus two unusual amino acids, *N*-methyldehydroalanine and a hydrophobic, 20-carbon chain, unique to these toxins, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (ADDA).³ The toxins are named using the one-letter abbreviations to indicate the two variable amino acids present;⁴ for example, microcystin-LR contains leucine (L) and arginine (R). Many other minor variations in the chemical structure are known, especially the lack of the methyl group on either methylaspartic acid or

methyldehydroalanine, or both.⁵ Other minor variations include modifications in the ADDA part of the molecule. There are now reports of more than 40 microcystins isolated from cyanobacterial species.⁶ Nodularin, isolated from the brackish-water cyanobacterium *Nodularia spumigena*, is a hepatotoxic cyclic pentapeptide of similar structure to microcystins containing glutamic acid, ADDA, arginine, methylaspartic acid and methyldehydrobutyryne.²

Microcystins and nodularin are potent inhibitors of protein phosphatases 1 (PP1) and 2A (PP2A) from animals and higher plants.⁷⁻⁹ PP1 and PP2A are two of the major protein phosphatases in eukaryotic cells which have been shown to be important enzymes in tumour suppression.¹⁰ The action of microcystins and nodularin in inhibiting such enzymes might suggest that they may in turn act as tumour promoters, which has been indicated by the work of Nishiwaki-Matsushima *et al.*¹¹ Concern over established acute^{1,2} and the possible chronic effects has increased the pressure to monitor levels of these toxins in drinking water.

It has been reported that at least 50% of recorded cyanobacterial blooms have been found to contain toxins¹² and the occurrence of toxicity can vary both spatially and temporally.¹³ It is known that microcystins are produced within the cyanobacterial cell and become free in the water column after cell lysis.¹² It is therefore important not only to develop a rapid method of analysis but also to provide a means of assessing intracellular levels of toxins in addition to those free in water. This is particularly important where affected waters are destined for water treatment, allowing remedial action to be taken, where necessary, at water treatment works. Knowledge of the levels of intracellular toxins also permits advance warning of potentially high levels of free toxin.

Although an analytical method for microcystin has been proposed,¹⁴ it was developed for only one of the many naturally occurring variants of these toxins (microcystin-LR). The other main shortfall with the method is that it fails to take account of intracellular toxins, only providing a method for free microcystin-LR analysis.¹⁴ The method reported here allows the determination of both intra- and extracellular microcystins in a wide range of water types, including raw and treated reservoir water.

Experimental

Reagents and Standards

All reagents were of analytical-reagent or high-performance liquid chromatographic (HPLC) grade; acetonitrile and methanol were purchased from Rathburn (Walkerburn, Peebles-shire, UK), trifluoroacetic acid from Fisons (Loughborough, Leicestershire, UK) and sodium sulfite from BDH (Poole,

* Present address: School of Applied Sciences, Robert Gordon University, St. Andrew Street, Aberdeen, UK AB1 1HG.

Dorset, UK). High-purity water produced with a Milli-Q system (Millipore, Milford, MA, USA) was used throughout.

Microcystins and nodularin standards were purified from cyanobacterial sources, including laboratory isolates, and from bloom samples. Constituent amino acids were determined using the Waters Pico Tag HPLC system (Millipore) for amino acid analysis. All standards were stored dry at -20°C and dissolved in methanol when required.

Apparatus

HPLC analysis was carried out using Waters instrumentation consisting of a Model 600E solvent-delivery system, a Model 717 WISP autosampler with detection using a Model 991 photodiode-array (PDA) detector at 200–300 nm with 3 nm resolution. A Waters μ Bondapak C_{18} column (300×3.9 mm i.d.) (Millipore) was used. The eluents were water–0.05% v/v trifluoroacetic acid (TFA) (A) and acetonitrile–0.05% v/v

Table 1 Linear gradient conditions at 1 ml min^{-1} used in HPLC of microcystins and nodularin. Solvent A = water–0.05% TFA and B = acetonitrile–0.05% TFA

	Time/min						
	0	10	40	42	44	46	50
Solvent A (%)	70	65	30	0	0	70	70
Solvent B (%)	30	35	70	100	100	30	30

Table 2 Correlation coefficients as an indication of linearity for the method calibration graph obtained over the concentration range indicated (zero to maximum) for five microcystins (MC) and nodularin (NOD)

Microcystin/ nodularin	Water type	Range (min.–max.)/ $\mu\text{g l}^{-1}$	Correlation coefficient (r)
MC-RR	Treated	0.082–8.177	1.000
	Raw	0.409–8.177	0.998
MC-LR	Treated	0.089–8.880	0.996
	Raw	0.444–8.880	0.998
MC-LY	Treated	0.034–3.400	0.994
	Raw	0.170–3.400	0.997
MC-LW	Treated	0.069–6.860	0.963
	Raw	0.343–6.860	0.979
MC-LF	Treated	0.087–8.701	0.998
	Raw	0.435–8.701	0.998
NOD	Treated	0.073–7.273	0.995
	Raw	0.364–7.273	0.965

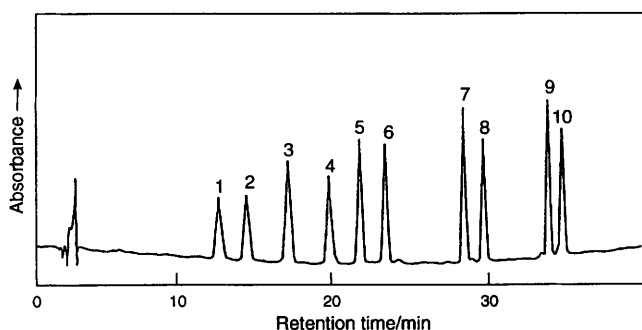


Fig. 1 HPLC separation of a solution of standards containing nine microcystins and nodularin: 1, [D-Asp³]microcystin-RR; 2, microcystin-RR; 3, nodularin; 4, microcystin-YR; 5, microcystin-LR; 6, microcystin-FR; 7, microcystin-LA; 8, microcystin-LY; 9, microcystin-LW; and 10, microcystin-LF.

TFA (B). Separation was achieved using a linear gradient as described in Table 1. The column temperature was maintained at 40°C using a Waters temperature control module.

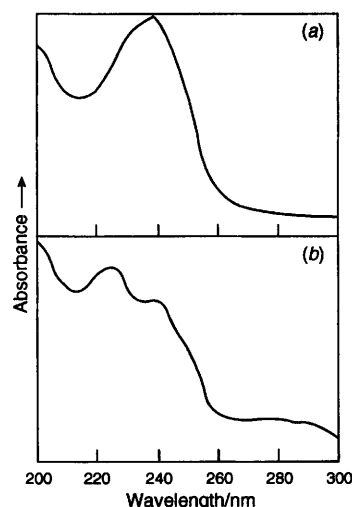


Fig. 2 (a) Typical absorption spectrum of most microcystins (for this example, microcystin-LR), and (b) typical absorption spectrum of microcystin variants containing tryptophan (for this example microcystin-WR).

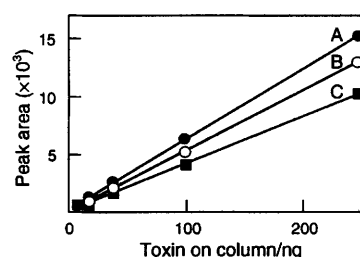


Fig. 3 Examples of linear calibration graphs by HPLC: A, nodularin; B, microcystin-LF; and C, microcystin-RR.

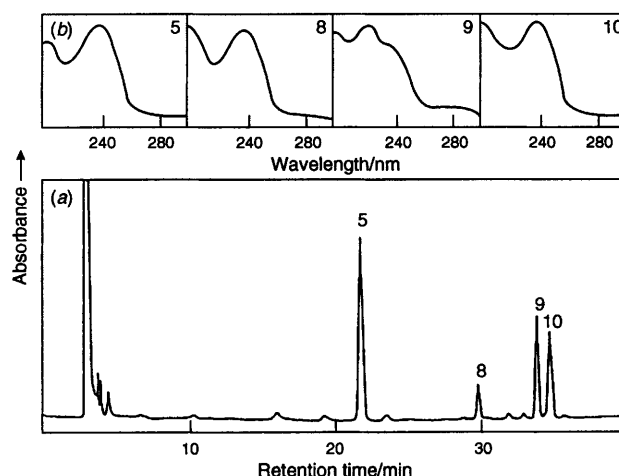


Fig. 4 (a) Chromatogram of microcystins present in *Microcystis aeruginosa* PCC7820 cells extracted with 100% methanol: 5, microcystin-LR; 8, microcystin-LY; 9, microcystin-LW; 10, microcystin-LF; and (b) corresponding absorption spectra determined by PDA-HPLC.

Recommended Procedure

Water samples were collected in glass bottles (2.5 l) and two 1 l portions were filtered immediately through 110 mm GF/C discs (Whatman, Maidstone, Kent, UK). The discs, which contain the cell-bound microcystin component, were folded with the upper surface innermost, placed in a suitable container and freeze-thawed prior to extraction. Filter discs were placed in glass beakers containing 20 ml of methanol and allowed to extract for 1 h at room temperature. The liquor was decanted into a pear-shaped rotary evaporation flask (50 ml) and the filter was gently squeezed with a spatula to ensure maximum transfer of the liquid. The extraction procedure was repeated a further twice. The sample was rotary evaporated at 40 °C *in vacuo* until dry, then the liquor from the second, and subsequently the third, extraction was added to the flask and dried as before. The residue was resuspended in 2 × 250 µl of methanol prior to analysis by photodiode-array HPLC.

To both of the 1 l sub-samples (filtrates) were added 100 µl of 1 g per 100 ml sodium sulfite solution, to eliminate residual free chlorine from the water sample; the solutions were shaken vigorously and allowed to stand for a few minutes. To one of the sub-samples were added 150 µl of microcystin-LR solution containing 5 µg of toxin per 150 µl as an internal standard (standard addition solution). Each sub-sample was measured into 500 ml portions, 5 ml of 10% TFA were added,

Table 3 Comparison of yields of four microcystin (MC) variants from *Microcystis aeruginosa* PCC7820 cells extracted from filter discs using three different liquid extractions. All extractions were carried out in triplicate with a cyanobacterial biomass equivalent to 160 µg l⁻¹ of chlorophyll *a*

Extraction method	MC yield/µg l ⁻¹			
	MC-LR	MC-LY	MC-LW	MC-LF
Methanol	23.46	1.61	2.01	3.75
Butan-1-ol-methanol-water (5 + 20 + 75)	20.90	1.42	1.72	3.29
Acetic acid (5% aqueous)	17.85	0.35	0.19	0.41

Table 4 Percentage of total microcystin-LR (sum of three extractions) found in each extraction of filter disc from 1 l cell suspensions of *Microcystis aeruginosa* PCC7820 (biomass indicated by concentration of chlorophyll *a*)

Chlorophyll <i>a</i> /µg l ⁻¹	No. of extractions		
	1	2	3
160	70	23	6
240	73	22	5
410	72	23	5

mixed and passed through a GF/C filter disc. The filtered samples were placed in 500 ml Pyrex bottles and 5 ml of methanol were added prior to solid-phase extraction. A Vac Elut SPS-24 vacuum manifold system (Varian Analytical Instruments, San Fernando, CA, USA) was set up with Isolute C₁₈ trifunctional, end-capped solid-phase extraction cartridges, 1 g in a 3 ml syringe (International Sorbent Technology, Mid-Glamorgan, UK). The cartridges were conditioned using 10 ml of methanol followed by 10 ml of water. Water samples were applied to the cartridges by means of poly(tetrafluoroethylene) (PTFE) tubing from sample bottles at a flow rate not exceeding 10 ml min⁻¹. The diameter of the cartridge was selected to prevent excessive flow rates. When all of the water sample had passed through the cartridge, the bottle and tubing were disconnected and the cartridge was washed with 10 ml of 10, 20 and 30% v/v aqueous methanol in series. Air was drawn through the cartridge for about 30 min to minimize the amount of water eluted with the sample.

The vacuum manifold system was then switched to the collect position and the cartridges were eluted with 3 ml of acidified methanol (0.1% v/v TFA). The tubes were removed from the vacuum station and placed in a hot block (45 °C) and blown with nitrogen until dry. Samples were resuspended in 2 × 100 µl of methanol, transferred into a micro-centrifuge tube (1.5 ml) and re-dried. Prior to HPLC analysis, samples were resuspended in 75 µl of 70% aqueous methanol.

Photodiode-array HPLC analysis was carried out with 25 µl injections of samples extracted from both cyanobacterial cells retained on the filter discs and from the water filtrates. Duplicate 25 µl injections of the standard addition solution were also analysed to allow calculation of the sample recovery.

Detection Limits and Calibration Graphs

Calibration and detection limits of a mixture of nine pure microcystins and nodularin were determined using a range of solutions containing between 0 and 250 ng per 25 µl. Injections (25 µl) were made in triplicate.

Calibration graphs for the experimental procedure were obtained over a range of concentrations (Table 2) for five microcystins and nodularin to determine the linearity range. Raw and treated water were used and were identical with those used in the subsequent precision testing.

Performance of Filter Extraction

As it was not possible to spike samples of cyanobacterial cells with free toxin standard(s) to determine the efficiency of recovery of a known amount of intracellular microcystin(s), the performance of the extraction procedure was assessed using toxin-containing cyanobacterial cells. Laboratory-grown cells of microcystin-containing *Microcystis aeruginosa*

Table 5 Recoveries of microcystins (MC) and nodularin (NOD) in spiked water samples

Water sample*	Amount of toxin/µg l ⁻¹	Recovery of microcystins/nodularin (%)†					NOD
		MC-RR	MC-LR	MC-LY	MC-LW	MC-LF	
R1	0.25	124 (5.7)	115 (2.1)	86 (0.7)	59 (2.1)	67 (2.8)	112 (2.8)
R1	0.50	111 (3.5)	129 (0.7)	95 (0.7)	53 (0.7)	73 (0.7)	116 (4.2)
R2	0.25	125 (14.1)	110 (3.5)	87 (0.7)	52 (8.4)	61 (7.1)	118 (7.1)
R2	0.50	103 (3.5)	107 (5.6)	83 (2.1)	52 (1.4)	68 (3.5)	100 (0.7)
T1	0.25	104 (3.5)	91 (6.3)	99 (8.4)	0	72 (8.4)	130 (24.7)
T1	0.50	80 (8.4)	100 (3.5)	118 (2.1)	33 (2.1)	88 (2.8)	147 (6.4)
T2	0.25	80 (23.3)	87 (10.6)	91 (0)	0	61 (4.2)	106 (3.5)
T2	0.50	92 (8.4)	81 (2.1)	103 (12.7)	8 (1.4)	70 (7.1)	105 (2.8)

* R1, Loch Leven raw water; R2, Loch Rescobie raw water; T1 Edinburgh tap water; T2 Glasgow tap water.

† Standard deviations (*n* = 2) in parentheses.

PCC7820 were diluted with culture medium to a concentration of cells equivalent to $160 \mu\text{g l}^{-1}$ of chlorophyll *a*. One litre volumes of culture were filtered as described, then freeze-thawed. Three commonly used extraction procedures for microcystins were compared: 100% methanol as proposed here, 5% acetic acid¹⁵ and 5% butan-1-ol-20% methanol in water.¹⁶ Triplicate filters were extracted in 20 ml of each of the test solutions for 1 h, after which the liquor was decanted into rotary evaporation flasks and dried *in vacuo* at 40 °C. Samples were resuspended and analysed by HPLC as described. The efficiency of extraction of cells was assessed further through repeated 1 h extractions in methanol and using differing biomass concentrations of 160, 240 and $410 \mu\text{g l}^{-1}$ of chlorophyll *a*.

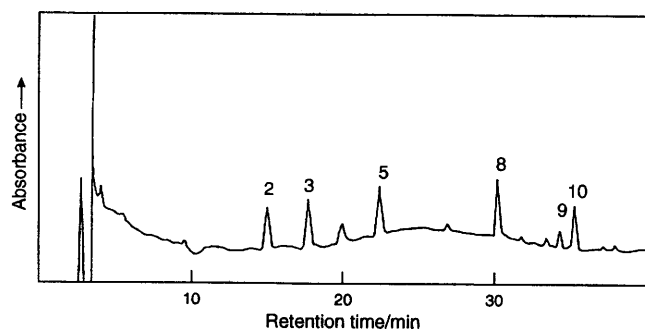


Fig. 5 Chromatogram of Edinburgh tap-water sample spiked with five microcystins and nodularin, recovered by the proposed method: 2, microcystin-RR; 3, nodularin; 5, microcystin-LR; 8, microcystin-LY; 9, microcystin-LW; and 10, microcystin-LF.

Table 6 Precision data for the extraction of 11 duplicate filters loaded with two concentrations of microcystin (MC)-containing cyanobacterial cells (biomass indicated by concentration of chlorophyll *a*)

Chlorophyll <i>a</i> /μg l ⁻¹	MC yield/μg l ⁻¹ *			
	MC-LR	MC-LY	MC-LW	MC-LF
50	7.5 (8.9)	0.5 (7.2)	1.0 (7.6)	1.3 (7.9)
500	78.1 (5.5)	5.2 (6.0)	9.5 (5.2)	12.4 (5.3)

* Relative standard deviations (%) (*n* = 22) in parentheses.

Table 7 Precision data for the recovery of microcystins (MC) and nodularin (NOD) in 11 duplicate spiked Loch Rescobie raw water samples

Microcystin/ nodularin	Amount of toxin/ μg l ⁻¹	Actual recoveries*		Adjusted recoveries*	
		μg l ⁻¹	% s _r (%) [†]	μg l ⁻¹	% s _r (%) [†]
MC-RR	4.30	3.52	82 6	3.78	88 14
	0.43	0.30	68 32	0.38	85 32
MC-LR	4.54	4.08	90 9	4.38	97 16
	0.45	0.36	75 10	0.45	98 9
MC-LY	1.60	1.42	89 11	1.53	95 15
	0.16	0.12	74 18	0.15	96 17
MC-LW	3.80	1.30	34 14	1.41	37 21
	0.38	0.07	20 16	0.09	24 20
MC-LF	4.78	3.40	71 12	3.55	74 20
	0.48	0.28	58 10	0.36	75 8
NOD	3.86	4.26	110 6	4.58	119 14
	0.39	0.48	130 17	0.66	152 16

* Actual recoveries are the observed values and adjusted recoveries are those calculated based on the recovery of microcystin-LR used as an internal standard.

[†] Relative standard deviation (*n* = 22).

Performance of Solid-phase Extraction

Two eutrophic raw waters [Loch Rescobie (Forfar) and Loch Leven (Kinross), Scotland] and two treated waters (domestic supplies, Edinburgh and Glasgow) were spiked with a mixture of five microcystins and nodularin (0.25 and $0.5 \mu\text{g l}^{-1}$ of each variant) to assess their recovery using the proposed method.

Intra-laboratory Precision Testing

Two cell densities of *M. aeruginosa* PCC7820 were prepared (50 and $500 \mu\text{g l}^{-1}$ of chlorophyll *a*) and 11 duplicate filter samples were extracted for both concentrations to assess the reproducibility of the proposed method.

Two water types (raw and treated) were spiked with five microcystins and nodularin at two concentrations, the high concentration being ten times greater than the low concentration. The total organic carbon for the waters after GF/C filtration was 7.0 and 1.7 mg l^{-1} for the raw and treated water, respectively. Eleven duplicate samples for each water type and concentration were processed to assess the reproducibility of the recommended procedure.

Results and Discussion

Microcystins are a large group of toxic peptides of differing hydrophobicity that can be readily chromatographed by reversed-phase HPLC. Fig. 1 shows the separation of nine microcystins and nodularin using the developed procedure. Microcystins show two typical spectra, one with an absorption maximum at 238 nm , which is exhibited by all except a few microcystins that contain tryptophan and that give an absorption maximum at 222 nm (Fig. 2). These characteristic spectra can be used in the identification of microcystins in naturally occurring samples in the absence of a wide array of standards.

A linear calibration for all the microcystins tested and nodularin was achieved over the specified range. Fig. 3 shows examples of typical calibration graphs for microcystin-RR, -LF and nodularin, which represented extremes within which the ultraviolet (UV) responses for all other microcystins tested were intermediate. The limit of detection for pure standards with the described HPLC system giving a consistently reproducible peak area was found to be 5 ng for all of the toxins tested. Linearity of the experimental procedure was achieved for all microcystins and nodularin over the range of concentrations tested (Table 2). The correlation coefficients

Table 8 Precision data for the recovery of microcystins (MC) and nodularin (NOD) in 11 duplicate spiked Dundee tap water samples

Microcystin/ nodularin	Amount of toxin/ μg l ⁻¹	Actual recoveries*		Adjusted recoveries*	
		μg l ⁻¹	% s _r (%) [†]	μg l ⁻¹	% s _r (%) [†]
MC-RR	4.30	2.92	68 14	3.52	82 19
	0.43	0.28	64 7	0.32	74 9
MC-LR	4.54	3.36	74 16	4.06	89 21
	0.45	0.37	79 6	0.43	94 6
MC-LY	1.60	1.28	80 13	1.54	95 18
	0.16	0.12	76 7	0.14	90 9
MC-LW	3.80	0.98	26 27	1.18	31 32
	0.38	0.09	23 19	0.10	27 19
MC-LF	4.78	2.96	62 14	3.54	75 19
	0.48	0.31	63 7	0.36	75 8
NOD	3.86	3.34	87 12	4.02	104 17
	0.39	0.41	105 6	0.48	126 7

* Actual recoveries are the observed values and adjusted recoveries are those calculated based on the recovery of microcystin-LR used as an internal standard.

[†] Relative standard deviation (*n* = 22).

for microcystin-LW in both treated and raw water were lower than those obtained for other microcystins; this is consistent with the instability of the compound. The limits of detection of the experimental procedure were lower in treated water than raw water, as might be expected owing to matrix interference. The lowest reproducible concentrations of all analytes examined were those determined for microcystin-LY, namely 34 and 170 ng l⁻¹ for treated and raw water, respectively. Increased interference in the raw water caused an over-all increase in the concentration of the toxin detectable. The use of amino-bonded cartridges has been suggested¹⁴ to reduce the level of co-extracted interference; however, evaluation during method development indicated that the use of such cartridges decreased the recovery and increased the variability.

M. aeruginosa PCC7820 produces at least four microcystins: microcystin-LR, -LY, -LW and -LF,¹⁷ with widely differing hydrophobicities (Fig. 4). The method described here advocates the use of methanol for the extraction of intracellular toxins as all microcystins are known to dissolve well in methanol and it allows rapid rotary evaporation of samples. Table 3 shows that both methanol and butan-1-ol-methanol-water extracted similar amounts of each of the microcystin variants, the yields being only slightly lower in the latter instance. However, the yield, particularly of the more hydrophobic variants, was poor with acetic acid extraction. Therefore, it appears that, of the extraction procedures examined, methanol provides a good recovery of variants with more rapid sample processing, as methanol dries considerably faster than a mixture with 75% water.

The effect of the amount of cyanobacterial cells present in a water sample on toxin recovery was investigated along with repeated sample extraction. Table 4 indicates that around 70% of the total microcystin-LR was present in the first extraction independent of cyanobacterial biomass. By the third extraction only 5% of the total microcystin-LR was found, suggesting that three extractions are sufficient to determine the amount of microcystin present in the intracellular component of a natural water sample up to a high level of cyanobacterial biomass indicated by 410 µg l⁻¹ of chlorophyll *a*.

Bloom samples collected from the water bodies can also be extracted with methanol using the method employed for the filter discs and can then be examined for the presence of microcystins. Where possible, it is recommended that cells are dried and a known mass is extracted with methanol to allow accurate determination. This method can be used as a rapid indicator of the presence of microcystins in water.

The recovery of microcystins from spiked water samples (Table 5), both raw and treated, was found to be acceptable for most of the variants, at both concentrations (0.25 and 0.5 µg l⁻¹). However, the recovery of microcystin-LW was poor in treated waters, although this was also low compared with the recoveries of other microcystins in the raw waters. No explanation for this has been found and the behaviour of this microcystin is the subject of further investigation. It is not known if other variants containing tryptophan, e.g., microcystin-WR, will also show low recoveries with this method.

The proposed sample extraction procedure was found to be highly reproducible between intra-sample replicates, with 75% having relative standard deviations less than 10% and over half (54%) of these were calculated to be <5%.

Fig. 5 shows a total chromatogram of one of the spiked treated waters (Edinburgh tap water), indicating the occurrence of several non-microcystin peaks. The use of the PDA detector allows the identification of microcystins from their characteristic spectra and enables other peaks to be eliminated. Earlier methods such as that of James and James¹⁴ rely entirely on retention data and single-wavelength detection to

identify nodularin and microcystin-LR. However, there is a high probability of co-elution in these complex matrices and confirmation is required. The use of a PDA detector and gradient separation helps to overcome these difficulties. We have used the procedure described here for the determination of microcystins and nodularin in a wide range of raw and treated waters and in laboratory-based studies on microcystin production by monocyanobacterial cultures. The identities of the toxin peaks detected by this PDA-based method have been confirmed by mass spectrometry.¹⁸

The results of the intra-laboratory precision testing are presented in Tables 6–8. The extraction of GF/C filters was found to be highly reproducible with all relative standard deviations less than 10% (Table 6). Precision data for the recovery of five microcystins and nodularin from both raw and treated waters indicate an acceptable degree of reproducibility for a method of this complexity. As previously observed (Table 5), the recovery of microcystin-LW was poor compared with the other microcystins studied. The actual recoveries and adjusted recoveries (that is, those calculated based on the recovery of microcystin-LR used as a form of standard addition) are reported in Tables 7 and 8 and indicate that, although the reproducibility tends to be slightly better in the actual recoveries, the adjusted figures give a more accurate indication of the level of toxins present.

Conclusions

The method presented here provides a rapid determination of the potent cyanobacterial microcystins and nodularin hepatotoxins in both raw and treated water within 24 h of the commencement of processing. It allows for the accurate determination of both intra- and extracellular toxins. The proposed method permits the identification of these toxins from their characteristic absorption spectra. Where further verification is required, samples prepared by this method are suitable for analysis by liquid chromatography-mass spectrometry. It is also envisaged that this method could be readily adapted to a laboratory robot system.

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