The Determination of Microcystins in Raw and Potable Waters (2022)

Methods for the Examination of Waters and Associated Materials

August 2022

The Determination of Microcystins in Raw and Potable Waters

Methods for the Examination of Waters and Associated Materials

This booklet contains three techniques for the determination of microcystins in waters

Method A using Online Enrichment Solid Phase Extraction (SPE) Liquid Chromatography-Mass Spectrometry (LCMS)

Method B using Direct Aqueous Injection (DAI) Liquid Chromatography-Mass Spectrometry (LCMS)

Method C preparation of intracellular and extracellular microcystin for analysis by Method A or B.

Each method has been validated in only one laboratory and consequently details are included for information purposes only as an example of the type of procedures that are available to analysts. Information on routine multi-laboratory use of these methods would be welcomed to assess their full capabilities.

Whilst this booklet may report details of the materials actually used, this does not constitute an endorsement of these products but serves only as an illustrative example. Equivalent products are available and it should be understood that the performance characteristics of the method might differ when other materials are used. It is left to users to evaluate methods in their own laboratories.

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About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on recommended methods of sampling and analysis for determining the quality of drinking water, ground water, river water and sea water, waste water and effluents as well as sewage sludges, sediments, soils (including contaminated land) and biota. In addition, short reviews of the most important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

Ideally, all methods should be fully evaluated with results from performance tests. These methods should be capable of establishing, within specified or pre-determined and acceptable limits of deviation and detection, whether or not any sample contains concentrations of parameters above those of interest.

For a method to be considered fully evaluated, individual results from at least three laboratories should be reported. The specifications of performance generally relate to maximum tolerable values for total error (random and systematic errors) systematic error (bias) total standard deviation and limit of detection. Often, full evaluation is not possible and only limited performance data may be available.

In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

Standing Committee of Analysts

The preparation of booklets within the series "Methods for the Examination of Waters and Associated Materials" and their continuing revision is the responsibility of the Standing Committee of Analysts (established 1972 by the Department of the Environment). At present, there are eight working groups, each responsible for one section or aspect of water quality analysis. They are

1 General principles of sampling and accuracy of results
2 Microbiological methods
3 Empirical, Inorganic and physical methods
4 Metals and metalloids
5 Solid substances
6 Organic impurities
7 Biological, biodegradability and inhibition methods
8 Radiochemical methods

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and main committee. The names of those members principally associated with these methods are listed at the back of this booklet.

Publication of new or revised methods will be notified to the technical press. If users wish to receive copies or advanced notice of forthcoming publications or obtain details of the index of methods then contact the Secretary on the SCA's web-page:- http://www.standingcommitteeofanalysts.co.uk/Contact.html

Every effort is made to avoid errors appearing in the published text. If, however, any are found, please notify the Secretary. Users should ensure they are aware of the most recent version they seek.

Andrew Fegan
Secretary
October 2021

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc. Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 2002 (SI 2002/2677). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted.

Numerous publications are available giving practical details on first aid and laboratory safety. These should be consulted and be readily accessible to all analysts. Amongst such publications are; "Safe Practices in Chemical Laboratories" and "Hazards in the Chemical Laboratory", 1992, produced by the Royal Society of Chemistry; "Guidelines for Microbiological Safety", 1986, Portland Press, Colchester, produced by Member Societies of the Microbiological Consultative Committee; and "Safety Precautions, Notes for Guidance" produced by the Public Health Laboratory Service. Another useful publication is "Good Laboratory Practice" produced by the Department of Health.

The Determination of Microcystins in Raw and Potable Waters

1 Introduction

Microcystins are a group of naturally occurring toxins produced by various genera of cyanobacteria, including Microcystis, Anabaena, and Oscillatoria (Planktothrix). Cyanobacteria, commonly referred to as blue-green algae, are photosynthetic prokaryotes that occur naturally in surface waters. Nutrient rich, eutrophic, warm and low turbulent conditions in freshwater bodies typically promote the dominance of cyanobacteria within phytoplankton communities. Excessive proliferation of cyanobacteria leads to blooms that disrupt ecosystems, adversely affect the taste and odour of water, and increase water treatment costs (Figure 1). When a cyanobacterium dies, its cell wall degrades and the toxins are released in the water. Microcystins are extremely stable in water and withstand chemical breakdown such as hydrolysis or oxidation. At typical conditions in the environment, the half-life of MC-LR is approximately 10 weeks.

Figure 1: Blue/green algae bloom

Microcystins are cyclic heptapeptides. They are composed of five common amino acids and pairs of L-amino acids as variants. The common ones are methyl aspartic acid, alanine, N-methyldehydro-alanine, glutamic acid, and a unique amino acid called Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6 dienoic acid). The structural differences among the toxins are related to the remaining two L-amino acids. In MC-LR these variable residues are L-leucine (L) and L-arginine (R) (Figure 2).

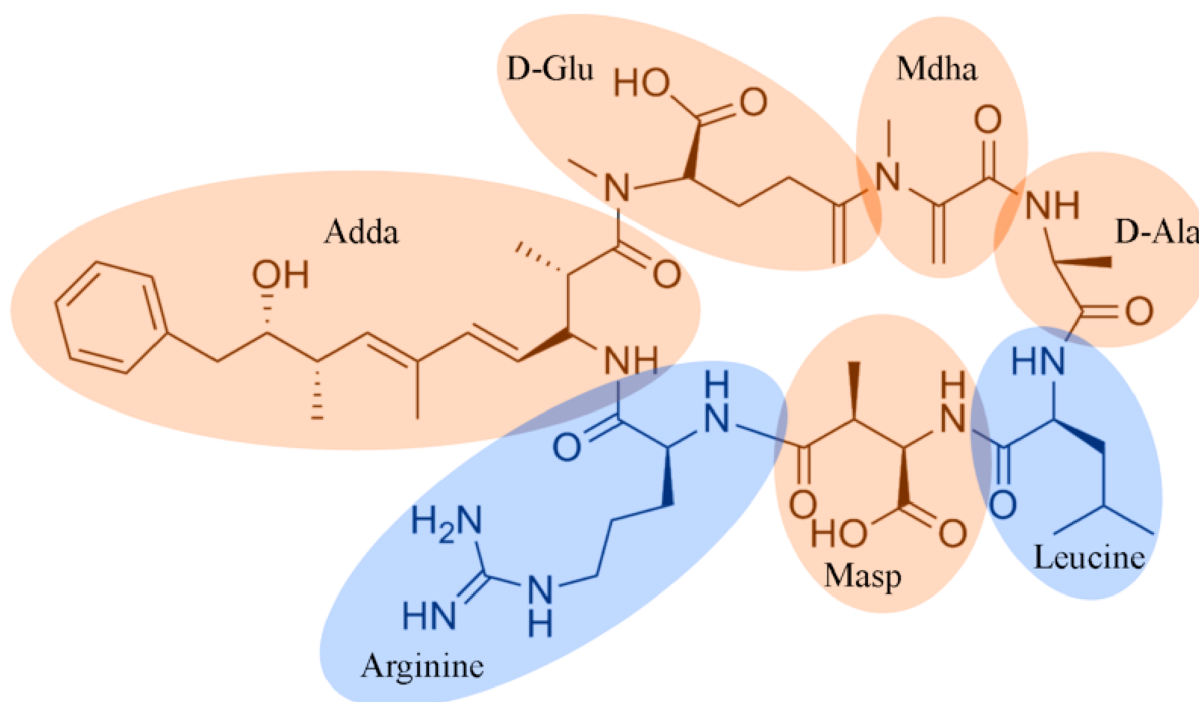


Figure 2: Structure of Microcystin-LR (C49H74N10O12, CAS: 101043-37-2).

Other L-amino acids include L-alanine (A), L-phenylalanine (F), L-tryptophan (W) and L-tyrosine (Y). Over 80 structural variants are known, differentiated by the two variable L-amino acids as well as by chain modifications.

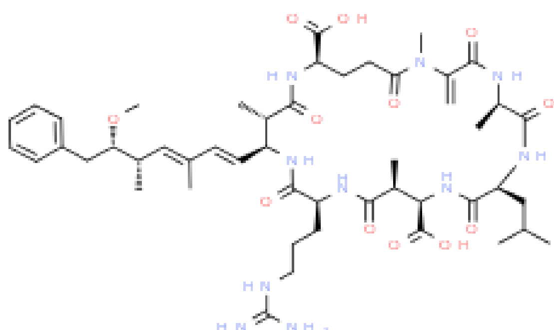
Microcystin-LR is one of the most prevalent and potent microcystins, it is designated as possibly carcinogenic to humans by the International Agency for Research on Cancer (IARC).

The need for a rapid, sensitive, and reliable analytical method for MC-LR has been emphasized by the awareness of toxic cyanobacteria as a human-health risk through drinking water.

2 Structures and description

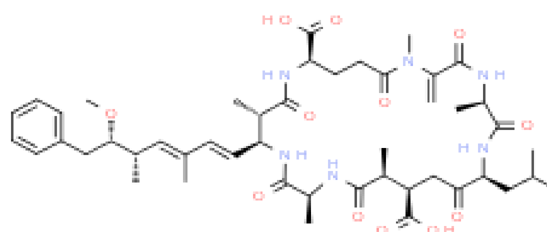
The following is a list of determinands contained in this document, their structures, CAS numbers and other relevant information sources to aid in the use of this document.

Microcystin-LR (MC-LR)



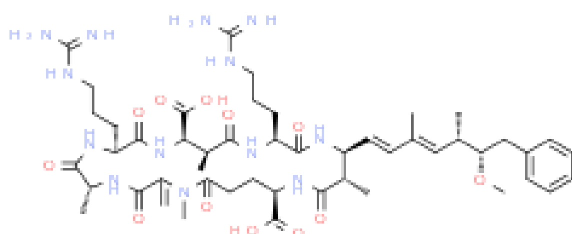
Molecular formula: $C_{49}H_{74}N_{10}O_{12}$
CAS: 101043-37-2
Molecular weight: 995.172 Da
Monoisotopic mass: 994.548767 Da

Microcystin-LA (MC-LA)



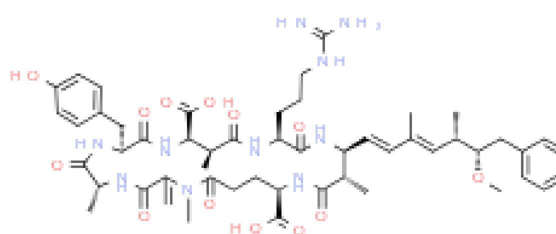
Molecular formula: $C_{47}H_{68}N_{6}O_{12}$
CAS: 96180-79-9
Molecular weight: 909.076 Da
Monoisotopic mass: 908.489502 Da

Microcystin-RR (MC-RR)



Molecular formula: $C_{49}H_{75}N_{13}O_{12}$
CAS: 111755-37-4
Molecular weight: 1038.200 Da
Monoisotopic mass: 1037.565796 Da

Microcystin-YR (MC-YR)



Molecular formula: $C_{52}H_{72}N_{10}O_{13}$
CAS: 101064-48-6
Molecular weight: 1045.187 Da
Monoisotopic mass: 1044.528076 Da

3 Sample stability

Compound	Matrix / Stability time (Days)			Sample Container	Preservative	Comments	Number of replicates
	Drinking Water	Surface Water	Ground Water*				
Microcystin RR	14	14	14	Glass (Amber)	Sodium thiosulfate	Headspace in the bottle is required at time of sampling	11
Microcystin YR	14	14	14	Glass (Amber)	Sodium thiosulfate	Headspace in the bottle is required at time of sampling	11
Microcystin LR	21#	14	14	Glass (Amber)	Sodium thiosulfate	Headspace in the bottle is required at time of sampling	11

* 14 days stability provided by Wessex Water based on data from groundwater study (worst case scenario matrix).

21 days stability for Microcystin LR in drinking water provided by ALS.

4 References

Water quality — Sampling Part 3: Preservation and handling of water samples (ISO 5667-3).

Standing Committee of Analysts. The Stability and Preservation of Drinking, Ground and Surface Water Samples 2018, November 2018.

United States Environmental Protection Agency, Single Laboratory Validated Method for Determination of Microcystins and Nodularin in Ambient Freshwaters by Solid Phase Extraction and Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS). November 2017. EPA document # EPA/600/R-17/344.

Standing Committee of Analysts. The Determination of Microcystin algal toxins in raw and treated waters by high performance liquid chromatography 1998.

Agilent application note. Analysis of microcystins and nodularin in drinking water using an Agilent Ultivo triple quadrupole LC/MS.

A The Determination of Microcystin-LR in Raw and Potable Waters by Online Solid Phase Extraction Liquid Chromatography Tandem Mass Spectrometry

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A1 Performance characteristics of the method

A1.1 Substances determined Microcystin-LR

A1.2 Type of sample Raw waters (ground and surface), potable waters.

A1.3 Basis of method Determinands are pre-concentrated on-line by the use of a re-useable solid phase extraction cartridge prior to analysis by liquid chromatography with mass spectrometric detection.

A1.4 Range of application Typically, up to 1.50 $\mu g\ L^{-1}$

A1.5 Standard deviation See Table A1.

A1.6 Limit of quantitation Typically, 0.030 $\mu g\ L^{-1}$, based on a low level standard (0.050 $\mu g\ L^{-1}$). See Table A1.

A1.7 Bias See Table A1.

A2 Principle

The method is a large volume injection (LVI) on-line solid phase extraction procedure. Samples are analysed by high performance liquid chromatography using a triple quadrupole mass spectrometer as a detector. The sample, after dilution if required, after the addition of internal standard and formic acid, is loaded onto a pre-conditioned online solid phase extraction column. The online SPE column is then switched in line with the analytical column and the determinands are back flushed on to the analytical column using a gradient LC method. Determinands are separated and then identified and quantified with tandem mass spectrometric detection (MSD) in selected reaction monitoring (SRM) mode. Quantitation is based on an internal standardisation procedure. The method is applicable to the analysis of extracellular microcystin-LR only. Intracellular microcystin-LR can be recovered following suitable disruption of algal cells.

A3 Interferences

Any co-extracted material which has a LC retention time similar to microcystin-LR and which gives a detector response at the monitored MS transition will interfere. However, none are known at the $^{m}/_{z}$ values selected.

A4 Hazards

Analysts using this method should familiarise themselves with the COSHH and risk assessments for the analysis. See Safety Data Sheet (SDS) provided by the chemical manufacturer/supplier.

A4.1 Methanol, CH_3OH, CAS No. 67-56-1

A4.2 Certified Reference Materials

Acute toxicity, oral and dermal. Skin irritation. Eye irritation. Skin sensitization

Note: All should be treated with caution as they may contain Microcystins.

A4.3 Formic acid, CH_2O_2, CAS No. 64-18-6

A4.4 Syringes

Adequate care should be taken when handling syringes in order to avoid injecting one-self. Damaged and broken syringes should be disposed of in a safe manner, preferably to a "sharps" canister.

A5 Reagents

All reagents should be of sufficient purity that they do not give rise to interferences during the analysis and distilled, deionised or similar grade water should be used throughout. A procedural blank should be run with each batch of samples to check for interferences. All solutions should be mixed well prior to use.

A5.1 Methanol LCMS grade

A5.2 Sodium thiosulfate pentahydrate AR grade

A5.3 Formic acid LCMS grade

A5.4 Water LCMS grade

Ultra-pure grade water should contain no measurable amounts of the compounds of interest. Millipore 'MilliQ SP' water has been found to be satisfactory. This water is used to prepare aqueous stock, intermediate and working solutions.

A5.5 Matrix Water, a treated water or equivalent

Matrix water should contain no measurable amounts of the compounds of interest. This 'real' water is used to prepare the calibration and AQC matrix water.

A5.6 Sodium thiosulfate solution, 1.8% $^{w}/_{v}$ or 18 g L^{-1}

Prepared by dissolving 9.00±0.01g of sodium thiosulfate pentahydrate in 500mL of ultra-pure water in a volumetric flask. This is used as matrix modifier for all samples and to produce the calibration and AQC matrix water. Store at ambient room temperature or under refrigeration. Shelf life: 1 month**.**

A5.7 Calibration/AQC matrix water

Prepared by measuring 1000mL of an appropriate 'analyte free' treated water into a 1L coloured glass bottle and using a suitable pipette add 1mL 18g L^{-1} sodium thiosulfate solution. This is used for the preparation of the calibration and AQC working standards, instrument sensitivity and calibration drift standards and to dilute samples that are over the calibration range. Store under refrigeration at 1-5°C. Shelf life: 1 month**.**

A5.8 Certified reference materials

These are stored in the laboratory freezer at less than -17°C. The expiry date is recorded on their certificate when supplied.
Calibration and AQC certified reference materials are sourced from separate independent suppliers where possible (<u>only</u> if this is not possible – then as a minimum – different lot numbers from the same supplier are used).

A5.9 Certified reference stock solution, calibration and AQC, Microcystin-LR, 10 mg L^{-1} in methanol

Certified reference stock solutions are purchased from approved analytical suppliers, e.g., LGC, part number MCLR-A and Supelco, part number 33893-1ML-R. Shelf life: as stated by supplier for both the calibration and AQC solutions. Store in a freezer at less than -17°C.

A5.10 Certified reference stock solution, internal standard, Microcystin-LR $^{15}N_{10}$, 10 mg L^{-1} in methanol:water (1:1)

Certified reference stock solutions are purchased from approved analytical suppliers, e.g., LGC, part number CIL-NLM-10295-1.2ML. Shelf life: as stated by supplier for the internal standard solution. Store in a freezer at less than -17°C.

A5.11 Certified reference stock solution, internal standard, Nodularin, 10 mg L^{-1} in methanol

Certified reference stock solutions are purchased from approved analytical suppliers, e.g., Supelco, part number 32539-1ML. Shelf life: as stated by supplier for the internal standard solution. Store in a freezer at less than -17°C.

A5.12 Calibration standard spiking solution, 0.20 mg L^{-1}, in methanol, used to prepare the calibration working standards 1 to 5

Using a micro-syringe or micro-pipette transfer 500µL of 10mg L^{-1} Microcystin-LR, LGC, part number MCLR-A, certified reference stock solution, to a 25mL volumetric flask and make up to volume with methanol, stopper and mix. Transfer contents to an amber hypo-vial and store in a freezer at less than -17°C. Shelf life: 6 months.

A5.13 Calibration working standards, 0 (Blank), 0.150, 0.300, 0.750, 1.200 and 1.500 µg L^{-1} in calibration/AQC matrix water

Remove spiking solutions from freezer and allow to equilibrate to room temperature. On each occasion a batch of samples is analysed prepare calibration working standards by adding the required volume of calibration spiking solution in the table below into 20mL of calibration/AQC matrix water in a 40mL glass vial. Then follow the analytical procedure from **A8.1.3**.

Calibration Working Standard	Volume of Calibration Standard Spiking Solution (µL)	Calibration Working Standard Concentration (µg L^{-1})
Cal Blank	0	0.000
Cal 1	15	0.150
Cal 2	30	0.300
Cal 3	75	0.750
Cal 4	120	1.200
Cal 5	150	1.500

A5.14 Internal standard spiking solution, 0.20 mg L^{-1}, in methanol

Using a micro-syringe or micro-pipette transfer 500µL of certified reference stock solution in A5.10, and 500µL certified reference stock solution in A5.11, to a 25mL volumetric flask and make up to volume with methanol, stopper and mix. Transfer contents to an amber hypo-vial and store in a freezer at less than -17°C. Shelf life: 6 months.

A5.15 AQC standard spiking solution, 0.20 mg L^{-1}, in methanol, used to prepare the AQC working standard

Using a micro-syringe or micro-pipette transfer 500µL of certified reference stock solution in A5.9, to a 25mL volumetric flask and make up to volume with methanol, stopper and mix. Transfer contents to an amber hypo-vial and store in a freezer at less than -17°C. Shelf life: 6 months.

A5.16 AQC working standards, AQC Blank and 1.000 µg L^{-1} in calibration/AQC matrix water

Remove spiking solution from freezer and allow to equilibrate to room temperature. On each occasion a batch of samples is analysed prepare AQC working standards by adding the required volume of AQC spiking solution in the table below into 20mL of calibration/AQC matrix water in a 40mL glass vial. Then follow the analytical procedure from **A8.1.3.**

AQC Working Standard	Volume of AQC Standard Spiking Solution (µL)	AQC Working Standard Concentration ($\mu g\ L^{-1}$)
AQC Blank	0	0.000
AQC	100	1.000

A6 Apparatus

In addition to normal laboratory glassware and apparatus the following may be required.

A6.1 Amber glass bottles, 500mL capacity, with PTFE lined screw caps containing 900µL 1g L^{-1} sodium thiosulfate solution, used for sample collection.

A6.2 Refrigerator and freezer, set at 1-5°C and less than -17°C, respectively with valid calibration status.

A6.3 Analytical Balance, four decimal place, for the preparation of stock solutions.

A6.4 Top Pan Balance, two decimal place.

A6.5 Micro-syringes, glass, and/or micro-pipettes with valid calibration status, various sizes.

A6.6 Electronic digital auto-pipette with valid calibration status, 20mL capacity.

A6.7 Flasks, volumetric, glass, various sizes.

A6.8 Measuring Cylinder, stoppered, 1000mL capacity, used for mobile phase preparation

A6.9 Hypo-vials, crimp top, amber glass, 50mL and caps.

A6.10 40mL clear glass vials and caps with PTFE lined septa, used for sample preparation.

A6.11 Pasteur pipettes, glass, disposable.

A6.12 Auto-sampler vials, amber glass, 2mL capacity, screw capped with 0.5mL graduations.

A6.13 Liquid Chromatograph/Mass Spectrometer

A high performance liquid chromatograph triple quadrupole mass spectrometer system fitted with binary and quaternary pumps, auto-sampler and column oven connected to a triple quadrupole mass spectrometer capable of unit mass resolution and operating in Selected Reaction Monitoring (SRM) mode, with an appropriate data station, e.g., Agilent, 1200 series liquid chromatograph and 6460 triple quadrupole mass spectrometer.

A6.14 The following instrument conditions have been used and found to be satisfactory.

Typical Operating Conditions for the Liquid Chromatograph

Quaternary Pump, On-line Solid Phase Extraction Conditions	
Quaternary Pump	Agilent 1260 Infinity Series
SPE Cartridge	Agilent Zorbax SB-C18, 30 mm long x 2.1 mm id, 3.5 µm particles, part number 873700-902 (part number 873700-932 for pack of 3)
Temperature	Ambient
Injection Volume	450 µL
Mobile Phase A Mobile Phase B	WATER, 0.10 % Formic Acid METHANOL, 0.10 % Formic Acid
Flow Rate (Loading)	0.60 mL $minute^{-1}$

Quaternary Pump, On-line Solid Phase Extraction Gradient Program		
Time	Flow Rate (mL $minute^{-1}$)	Mobile Phase B (%)
0.00	0.60	2
0.25	0.60	2
0.50	0.60	98
2.50	0.60	98
2.75	0.60	2
9.75	0.60	2
10.00	0.10	2
12.25	0.10	2
12.50	0.60	2
14.00	0.60	2

Auto-Sampler	
Injector Program	**Command**
DRAW	Draw 450 µL from sample with 150 µL $minute^{-1}$
WASH	Wash needle as specified in the method
VALVE	Switch valve to "Mainpass"
WAIT	Wait 2.50 minutes
REMOTE	Set remote line "Start" for 125 ms
WAIT	Wait 10.00 minutes
EJECT	Eject 450 µL to seat with 200 µL $minute^{-1}$

Binary Pump, Analytical	
Binary Pump	Agilent 1260 Infinity Series
Analytical Column	Agilent Zorbax Eclipse Plus C18, 150 mm long x 2.1 mm id, 3.5 µm particles, part number 830990-902
Temperature	60 °C
Mobile Phase A Mobile Phase B	Water, 0.10 % Formic Acid Methanol, 0.10 % Formic Acid
Flow Rate	0.40 mL $minute^{-1}$

Binary Pump, Analytical Gradient Program		
Time	Flow Rate (mL minute^{-1})	Mobile Phase B (%)
0.00	0.40	25
0.50	0.40	25
4.00	0.40	60
5.00	0.25	65
11.00	0.25	95
13.00	0.25	95
13.10	0.40	25
14.00	0.40	25

Typical Operating Conditions for the Tandem Mass Spectrometer

Triple Quadrupole Mass Spectrometer	
Mass Spectrometer	Agilent 6460
Ion Source	Jet Stream Electrospray Ionisation
Polarity	Positive
Source Parameters	
Drying Gas Temp.	300 °C
Drying Gas Flow	8 L minutes^{-1}
Nebulizer Pressure	40 psi
Shealth Gas Temp.	300 °C
Shealth Gas Flow	11 L minutes^{-1}
Capillary	3750 V
Charging Voltage	1000 V

MS Time Segments				
Time Segment #	Time	Div Valve	Delta EMV	Store
1	0.00	To Waste	0	No
2	5.00	To MS	200	Yes
3	13.00	To Waste	0	No

Time Segment 1 - 3:								
Compound Name	**ISTD**	**Prec. Ion**	**MS1 Res**	**Prod. Ion**	**MS2 Res**	**Frag (V)**	**CE (V)**	**Cell Acc (V)**
MC-LR	N	995.6	Widest	135.1	Widest	215	80	7
NODULARIN	Y	825.5	Widest	227.1	Widest	215	60	7
NODULARIN	Y	825.5	Widest	135.1	Widest	215	60	7
MC-LR 15N10	Y	503.4	Widest	135.1	Widest	90	4	7
MC-LR 15N10	Y	503.4	Widest	70.1	Widest	90	80	7
MC-LR	N	498.4	Widest	135.1	Widest	90	4	7
MC-LR	N	498.4	Widest	70.1	Widest	90	80	7

Typical Retention Times and SRM Transition Information

Compound Name	RT (min)	Quantifier SRM		Qualifier SRM(s)		Qualifier SRM Ratio (%)
		Precursor	Product	Precursor	Product	
NODULARIN	8.85	825.5	135.1	825.5	227.1	25
MC-LR $^{15}N_{10}$	9.55	503.4	135.1	503.4	70.1	35
MC-LR	9.55	498.4	135.1	498.4	70.1	35
				995.6	135.1	10

All compound retention times will vary depending on the condition of the analytical column therefore they should only be used as a guide. Compound elution order will remain unchanged.

A7 Sample collection and preservation

Samples are taken in 500mL amber glass vials with caps (lined with an appropriate inert material) containing 0.9mL 1g L^{-1} sodium thiosulfate. Drinking water samples may be stored for up to 21 days at 1-5°C.

A8 Analytical Procedure

A8.1 Remove the samples from storage and allow the samples to equilibrate to room temperature.

A8.2 Weigh 20.0±0.1g (approximately equivalent to 20mL) of sample into a 40mL glass vial, with a PTFE lined screw cap, using a top pan balance.

A8.3 Remove the internal standard spiking solution from freezer. Allow the hypo-vial, containing the spiking solution, to equilibrate to room temperature and use this to spike the internal standard spiking solution.

A8.4 Using a micro-syringe or micro-pipette, add 25µL of the internal standard spiking solution. This produces a MC-LR $^{15}N_{10}$ and Nodularin concentration of 0.250 µg L^{-1}. After returning the vial cap shake the vial to mix.

A8.5 Using a Pasteur pipette transfer standards and samples to 2 mL auto-sampler vials ready for instrumental analysis.

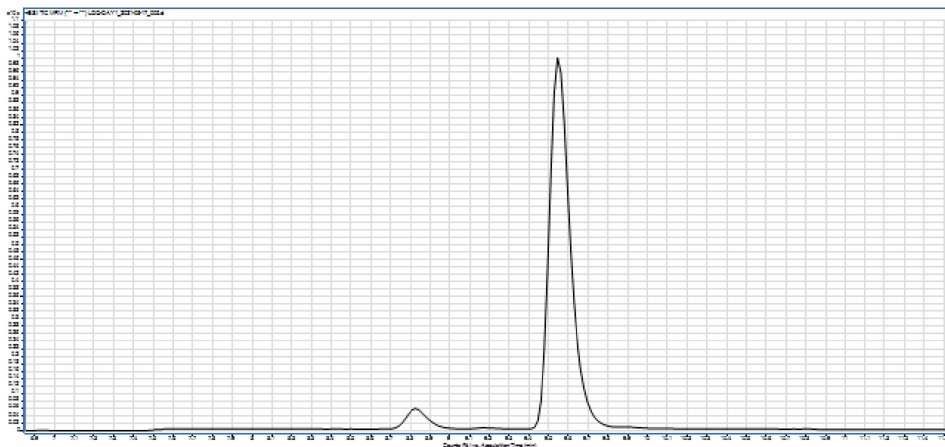
A9 Calculation

Results for target compounds are calculated using the following equation:-

$$\text{Concentration } (\mu g\ L^{-1}) = \frac{(\text{Sample Response})/(\text{Sample ISTD Response})}{(\text{Standard Response})/(\text{Standard ISTD Response})} \times \text{Standard Concentration } (\mu g\ L^{-1})$$

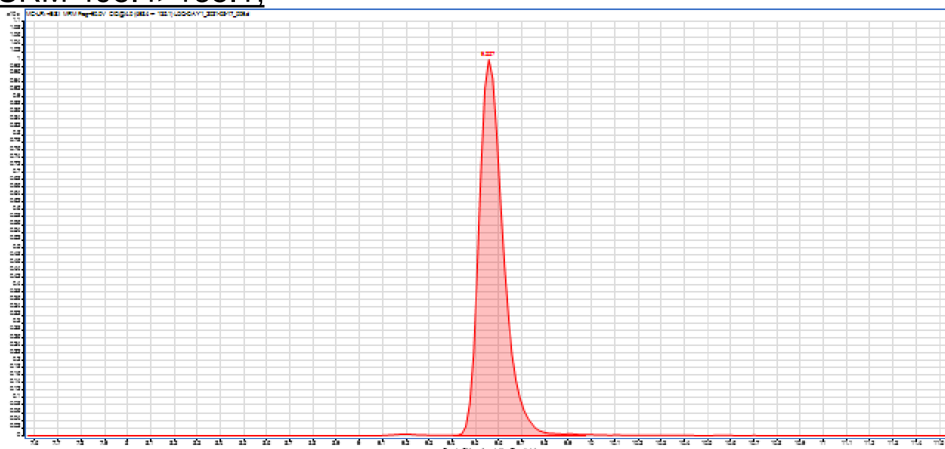
Figure A1 Typical chromatograms

Total Ion Chromatogram, Calibration Standard, L5, 1.500 µg L^{-1}

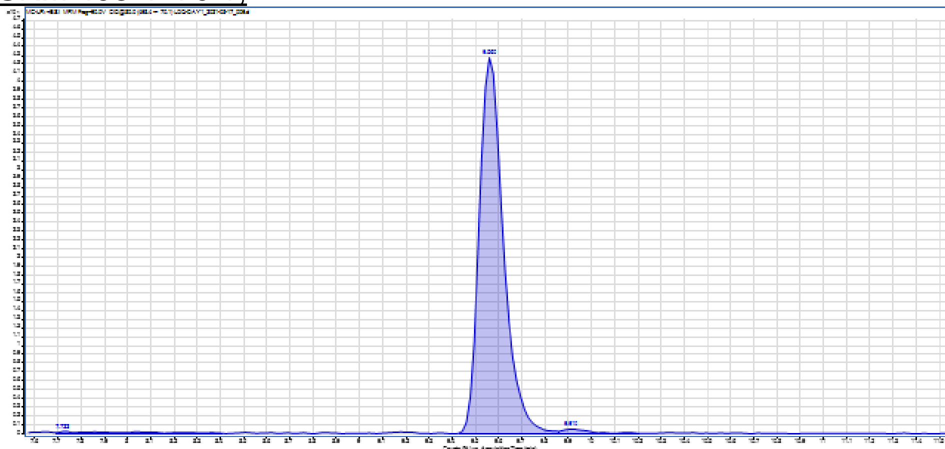


Selected Reaction Monitoring (SRM) transitions, Microcystin-LR, 1.000 µg L^{-1}

SRM 498.4>135.1,



SRM 498.4>70.1,



SRM 995.6>135.1

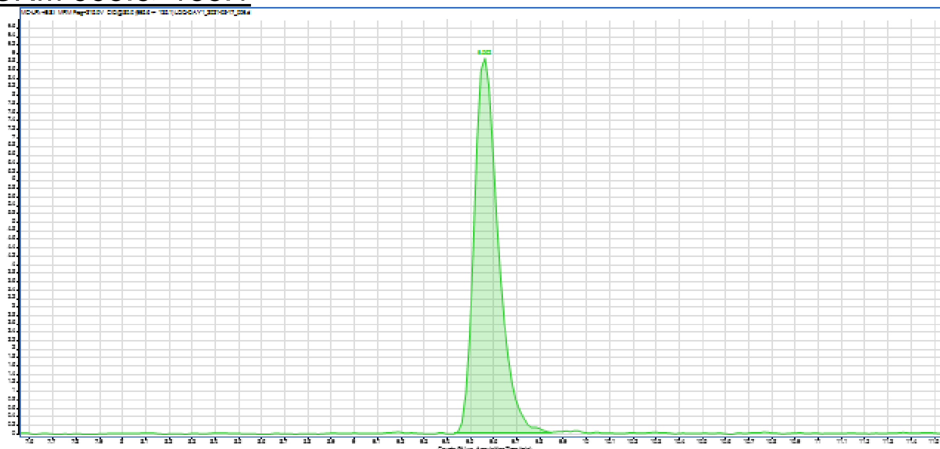


Table A1 Method performance data

Determinand	LOQ µg L^{-1}	Direct Standards				Elvington Treated Water (Hard Hardness)	
		Low Standard, 0.300 µg L^{-1}		High Standard, 1.200 µg L^{-1}		PCV Spike, 1.000 µg L^{-1}	
		Bias	RSD	Bias	RSD	Recovery	RSD
MC-LR	0.030	0.98%	4.47%	0.73%	3.46%	95.84%	4.78%

Method performance data is based on results produced with Nodularin used as the internal standard by ALS.

Note: Either Nodularin or MC-LR $^{15}N_{10}$ can be used as the internal for MC-LR.

B The Determination of Microcystins in Raw and Potable Waters by Direct Injection Liquid Chromatography Tandem Mass Spectrometry

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B1 Performance characteristics of the method

B.1.1	**Substances determined**	Microcystin-RR, Microcystin-YR and Microcystin-LR
B1.2	**Type of sample**	Raw waters (ground and surface), potable waters.
B1.3	**Basis of method**	Analysis on a liquid chromatograph with mass spectrometric detection by direct aqueous injection.
B1.4	**Range of application**	Typically, up to 1.50 µg L^{-1}
B1.5	**Standard deviation**	See Table B1**.**
B1.6	**Limit of quantitation**	Typically, 0.050 µg L^{-1}, based on a low level standard (0.075 µg L^{-1}). See Table B1.
B1.7	**Bias**	See Table B1.

B2 Principle

The sample, after dilution if required, is de-chlorinated and internal standard is added. The sample is transferred to a vial and injected on the LC system and the determinands are eluted on to the analytical column using a gradient LC method and detected by LC mass spectrometric detection.

B3 Interferences

Any co-extracted material which has a LC retention time similar to any of the above determinands and which gives a detector response at the monitored masses will interfere. However, none are known at the $^{m}/_{z}$ values selected.

B4 Hazards

Analysts using this method should familiarise themselves with the COSHH and risk assessments for the analysis. See Safety Data Sheet (SDS) provided by the chemical manufacturer/supplier.

B4.1 Methanol, CH_3OH, CAS No. 67-56-1

B4.2 Acetonitrile, CH_3CN, CAS No. 75-05-08

B4.3 Propan-2-ol, ($CH_3CHOHCH_3$), CAS No. 67-63-0

B4.4 Certified Reference Materials

Acute toxicity, oral and dermal. Skin irritation. Eye irritation. Skin sensitization

Note: All should be treated with caution as they may contain Microcystins.

B4.5 Formic acid, CH_2O_2, CAS No. 64-18-6

B4.6 Ammonium formate, NH_4CHO_2, CAS No. 540-59-2

B4.7 Ammonium fluoride, NH_4F, CAS No. 12125-01-8

B4.8 Syringes

Adequate care should be taken when handling syringes in order to avoid injecting one-self. Damaged and broken syringes should be disposed of in a safe manner, preferably to a “sharps” canister.

B5 Reagents

All reagents should be of sufficient purity that they do not give rise to interferences during the analysis and distilled, deionised or similar grade water should be used throughout. A procedural blank should be run with each batch of samples to check for interferences. All solutions should be mixed well prior to use.

B5.1 Water, LCMS grade, or deionised

B5.2 Acetonitrile, LCMS grade

B5.3 Propan-2-ol, HPLC grade

B5.4 Methanol, HPLC grade

B5.5 Ammonium formate (>99%)

B5.6 Ammonium fluoride, LCMS grade

B5.7 Formic acid, LCMS grade

B5.8 Sodium thiosulfate solution 1% w/v sodium thiosulfate solution (10g L^{-1})

Prepared by dissolving 5.00±0.01g of sodium thiosulfate in 500mL of ultra-pure water in a volumetric flask. This is used as matrix modifier for all samples and to produce the calibration and AQC matrix water. Store at ambient room temperature or under refrigeration at 1-5°C. Shelf life: 12 months.

B5.9 Matrix Water, a treated water or equivalent

Matrix Water should contain no measurable amounts of the compounds of interest. 500 mL of matrix water is dechlorinated using 1.2mL of 1% sodium thiosulfate solution. This ‘real’ water is used to prepare the calibration and AQC matrix water.

B5.10 Water, 0.1% Formic Acid, 5mM Ammonium Formate, 2mM Ammonium Fluoride (Eluent A)

Add 1mL formic acid, 0.315g ammonium formate and 0.074g ammonium fluoride to a 1L volumetric flask and make to the mark with HPLC grade water and mix thoroughly. The solution may be stored at ambient temperature for 1 week.

B5.11 80:20 Acetonitrile: Propan-2-ol (Eluent B)

Add 800mL of acetonitrile and 200mL of propan-2-ol to a 1 L glass bottle and mix thoroughly. The solution may be stored at ambient temperature for up to 6 months.

B5.12 Mixed Microcystin calibration stock solution, 5mg L^{-1}

This standard is bought in as a custom mix containing Microcystin RR, Microcystin YR and Microcystin LR, Supelco, part number 33578-1ML

B5.13 Mixed Microcystin calibration intermediate solution, 10µg L^{-1}

Add 20µL of mixed microcystin calibration stock solution to 10mL volumetric flask and make to volume with HPLC grade water. The solution is prepared on day of analysis.

B5.14 Mixed Microcystin AQC stock solution, 5mg L^{-1}

This standard is bought in as a custom mix containing Microcystin RR, Microcystin YR and Microcystin LR. Cyano Biotech GmbH Part No. MC-mas

B5.15 Mixed Microcystin AQC intermediate solution, 10µg L^{-1}

Add 20µL of mixed microcystin AQC stock solution to 10mL volumetric flask and make to volume with HPLC grade Water. The solution is prepared on day of analysis.

B5.16 Nodularin Internal standard stock solution, 10mg L^{-1}

Certified reference stock solutions are purchased from approved analytical suppliers, e.g., Sigma-Aldrich, part number 32539-1ML. Shelf life: as stated by supplier for the internal standard solution. Store in a freezer at less than -17°C.

B5.17 Internal standard intermediate solution, 1mg L^{-1}

Add 1mL of internal standard stock solution into a 10mL volumetric flask and make to volume with methanol. The solution may be stored between -20°C and has an expiry date of 6 months.

B5.18 Internal standard working solution, 4µg L^{-1}

Add 100µL of internal standard intermediate solution into a 5mL volumetric flask and make to volume with HPLC grade water. The solution is prepared on day of analysis.

B5.19 Calibration standards

Prepare by adding the required amounts of intermediate calibration solution in the table below to the designated volumetric flasks and fill with HPLC grade water.

Calibration Standard	Calibration intermediate solution to add (µL)	Volumetric flask volume (mL)	Concentration (µgL^{-1})
Cal 1	50	5	0.05
Cal 2	100	5	0.2
Cal 3	250	5	0.5
Cal 4	500	5	1.0
Cal 5	750	5	1.5
Cal Blank	0	5	0

B5.20 AQC working solution, 0.80µg L^{-1}

Add 400µL of AQC intermediate solution to a 5mL volumetric flask, fill with relevant matrix water. Treat as a sample from this point.

B6 Apparatus

In addition to normal laboratory glassware and apparatus the following may be required.

B6.1 Amber glass sample vials - 40mL capacity with PTFE-lined screw plastic cap, containing 100µL of 1% w/v sodium thiosulfate solution (10g L^{-1}) or equivalent.

B6.2 Refrigerator and freezer, set at 1-5°C and less than -17°C, respectively with valid calibration status.

B6.3 Analytical Balance, four decimal place, for the preparation of stock solutions.

B6.4 Auto-pipette with valid calibration status, 10-100µL & 100-1000µL capacity.

B6.5 Measuring Syringes, various sizes.

B6.6 Volumetric Flasks, glass, various sizes.

B6.7 Measuring Cylinder, 1000mL capacity.

B6.8 Reagent Bottles, amber glass, 50mL and caps.

B6.9 Pasteur pipettes, glass, disposable.

B6.10 Auto-sampler vials, amber glass, 2mL capacity, snap-capped and snap-caps with septa.

B6.11 Liquid Chromatograph/Mass Spectrometer

A high performance liquid chromatograph triple quadrupole mass spectrometer system fitted with a binary pump and column oven connected to a triple quadrupole mass spectrometer capable of unit mass resolution and operating in MRM mode, with an appropriate data station, e.g. Agilent, 1200 series liquid chromatograph and 6495 triple quadrupole mass spectrometer

The following instrument conditions have been used and found to be satisfactory.

B6.11.1 Typical Operating Conditions for the Liquid Chromatograph

HPLC ACQUISITION PARAMETERS				
Analytical Column	Agilent Poroshell 120 C18-EC, 50mm x 3.0mm, 2.7µm			
Injection Volume	95 µL			
Column Temperature	50°C			
Binary Pump	Agilent 1260 Infinity Series			
Mobile Phase A	Water, 0.1% Formic Acid, 5mM Ammonium Formate, 2mM Ammonium Fluoride			
Mobile Phase B	80:20 Acetonitrile: Propan-2-ol			
Gradient Program Timetable				
	Time (minutes)	**A (%)**	**B (%)**	**Flow rate (mL minute^{-1})**
Initial	0.00	90.0	10.0	0.400
1	2.00	90.0	10.0	0.400
2	3.00	50.0	50.0	0.400
3	7.00	5.0	95.0	0.400
4	8.00	5.0	95.0	0.400

B6.11.2 Typical Operating Conditions for the Mass Spectrometer

MS ACQUISITION PARAMETERS	
Acquisition parameters	AJS ESI mode, positive ionization polarity; MRM mode
Drying gas	Nitrogen
Drying gas temperature	250°C
Drying gas flow	11 L minute^{-1}
Nebulizer pressure	40 psi
Sheath Gas Flow	12 L minute^{-1}
Nebulizer voltage (V Charging)	500 V
Capillary (V)	3500

Retention time and LCMS/MS transitions									
Compound Name	**RT (min)**	**Mode**	**Prec. Ion**	**MS1 Res**	**Prod. Ion**	**MS2 Res**	**Frag. (V)**	**CE (V)**	**Cell Acc. (V)**
Microcystin RR	4.017	+ ESI	519.9	Unit	135.1	Unit	380	30	3
Microcystin YR	4.144	+ ESI	1045.5	Unit	135.4	Widest	380	60	3
Microcystin LR	4.189	+ ESI	995.6	Unit	135.2	Widest	380	60	3
Nodularin	4.724	+ ESI	825.5	Unit	135.2	Widest	380	60	3

All compound retention times will vary depending on the condition of the analytical column therefore they should only be used as a guide. Compound elution order will remain unchanged.

B7 Sample collection and preservation

Samples are taken in 40mL amber glass vials with caps (lined with PTFE) containing sodium thiosulfate. See B6.1. Samples may be stored for up to 14 days at 1-5°C.

B8 Analytical procedure

B8.1 Prepare calibration standards and AQCs as listed B5.19 and B5.20.

B8.2 Transfer 1mL to individual 2mL amber vials and add 50µL of working internal standard solution.

B8.3 Set up LC and MS as above.

B8.4 Run calibration standards and construct a calibration graph of response versus amount for each determinand.

B8.5 Analyse sample extracts and from the calibration graph calculate amount of the determinand in the sample.

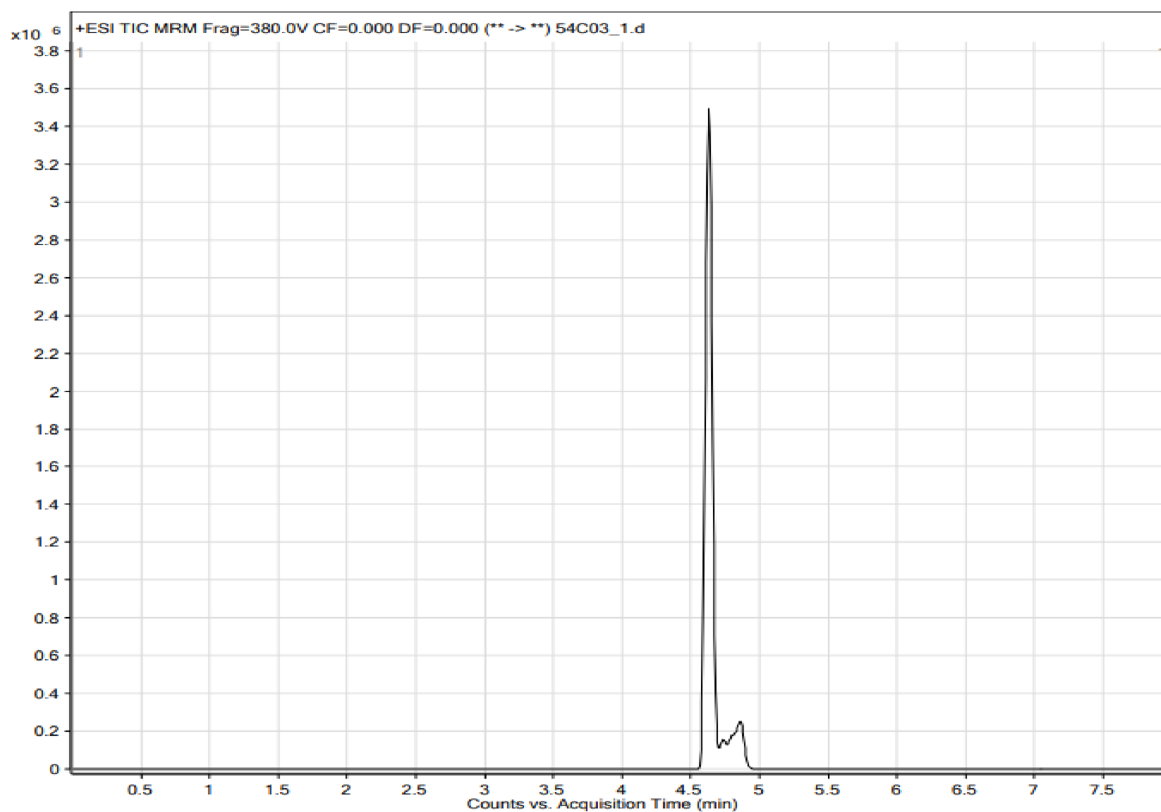
B9 Calculation

The concentration of each analyte is given by an internal standard procedure:

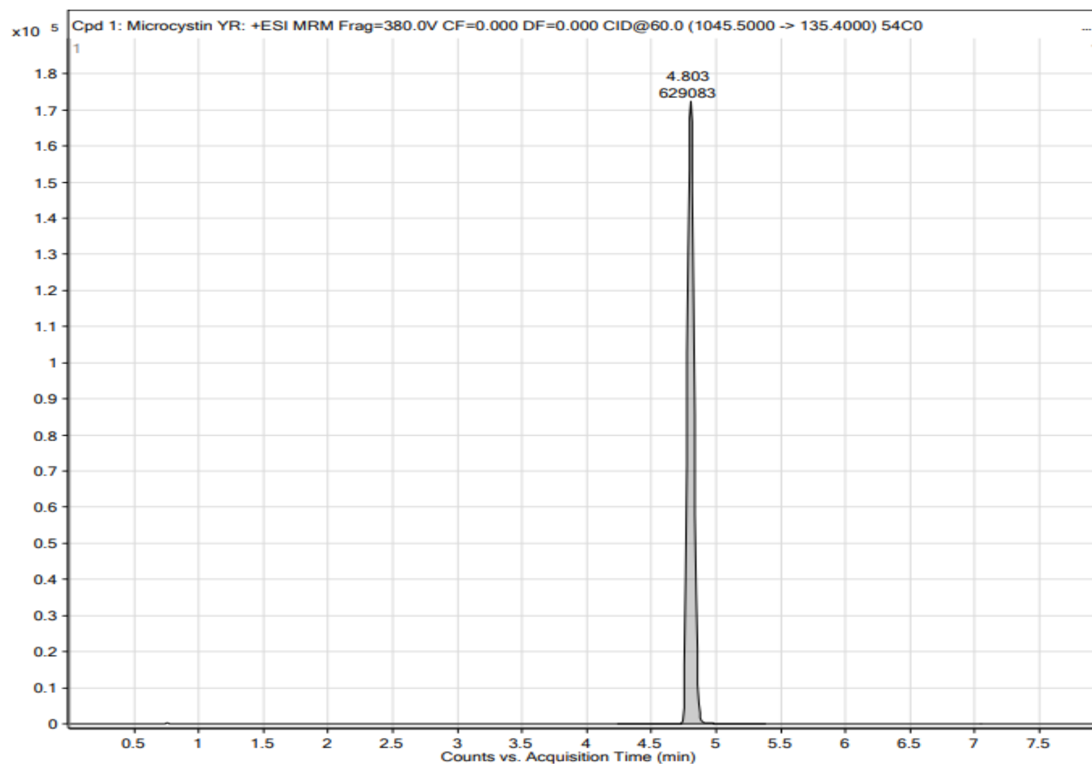
$$\text{Concentration } (\mu g\ L^{-1}) = \frac{(\text{Sample Response})/(\text{Sample ISTD Response})}{(\text{Standard Response})/(\text{Standard ISTD Response})} \times \text{Standard Concentration } (\mu g\ L^{-1})$$

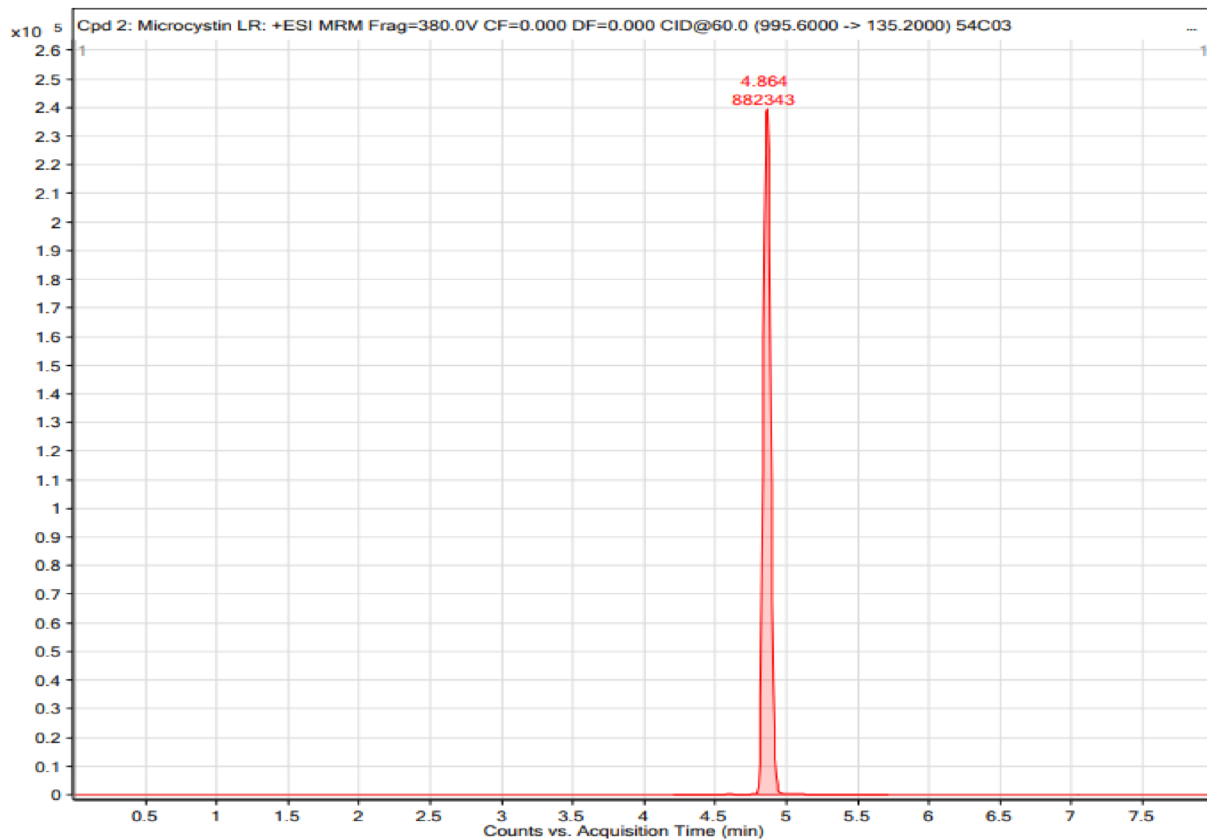
Figure B1 Typical chromatograms

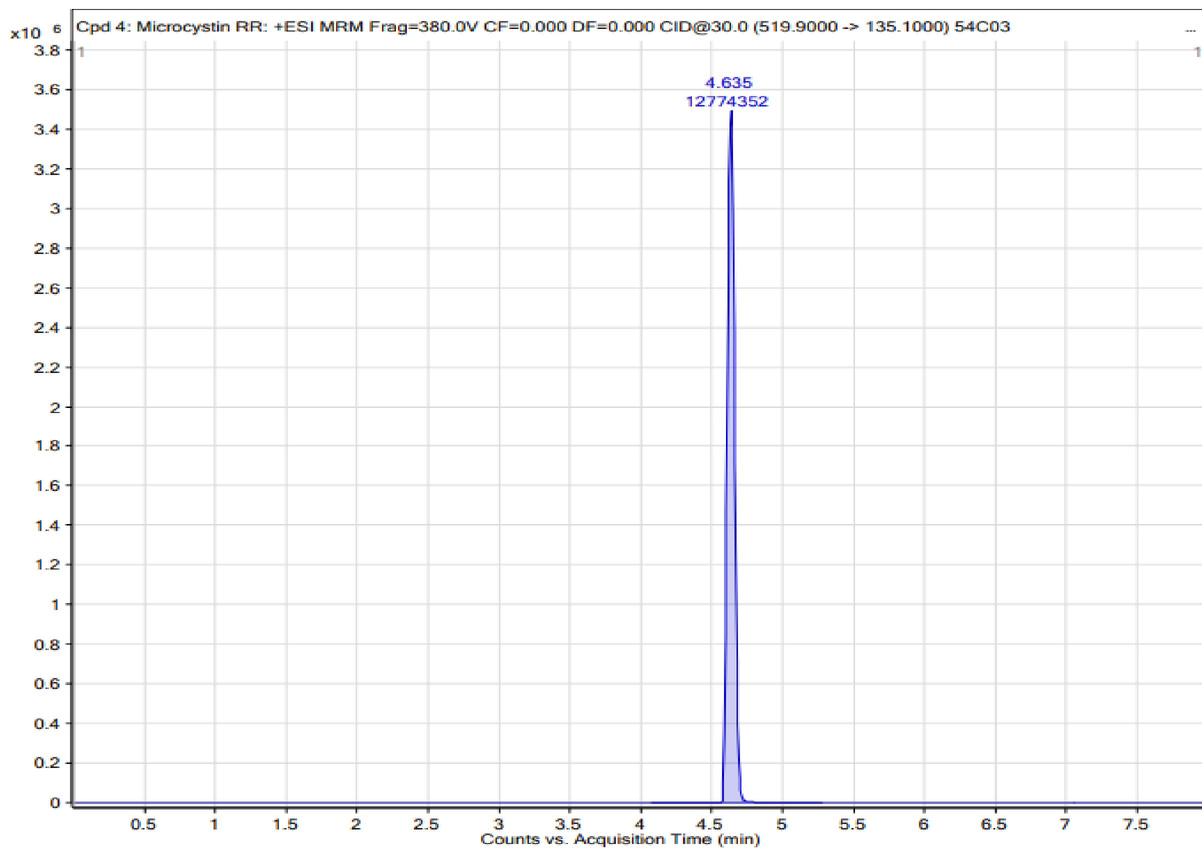
Calibration Standard, L5, 1.50 µgL^{-1}, Total Ion Chromatogram



Selected Reaction Monitoring (SRM) transitions, 1.50 µg L^{-1} Calibration standard




x10 5
Cpd 2: Microcystin LR: +ESI MRM Frag=380.0V CF=0.000 DF=0.000 CID@60.0 (995.6000 -> 135.2000) 54C03
4.864
882343
Counts vs. Acquisition Time (min)


x10 6
Cpd 4: Microcystin RR: +ESI MRM Frag=380.0V CF=0.000 DF=0.000 CID@30.0 (519.9000 -> 135.1000) 54C03
4.635
12774352
Counts vs. Acquisition Time (min)

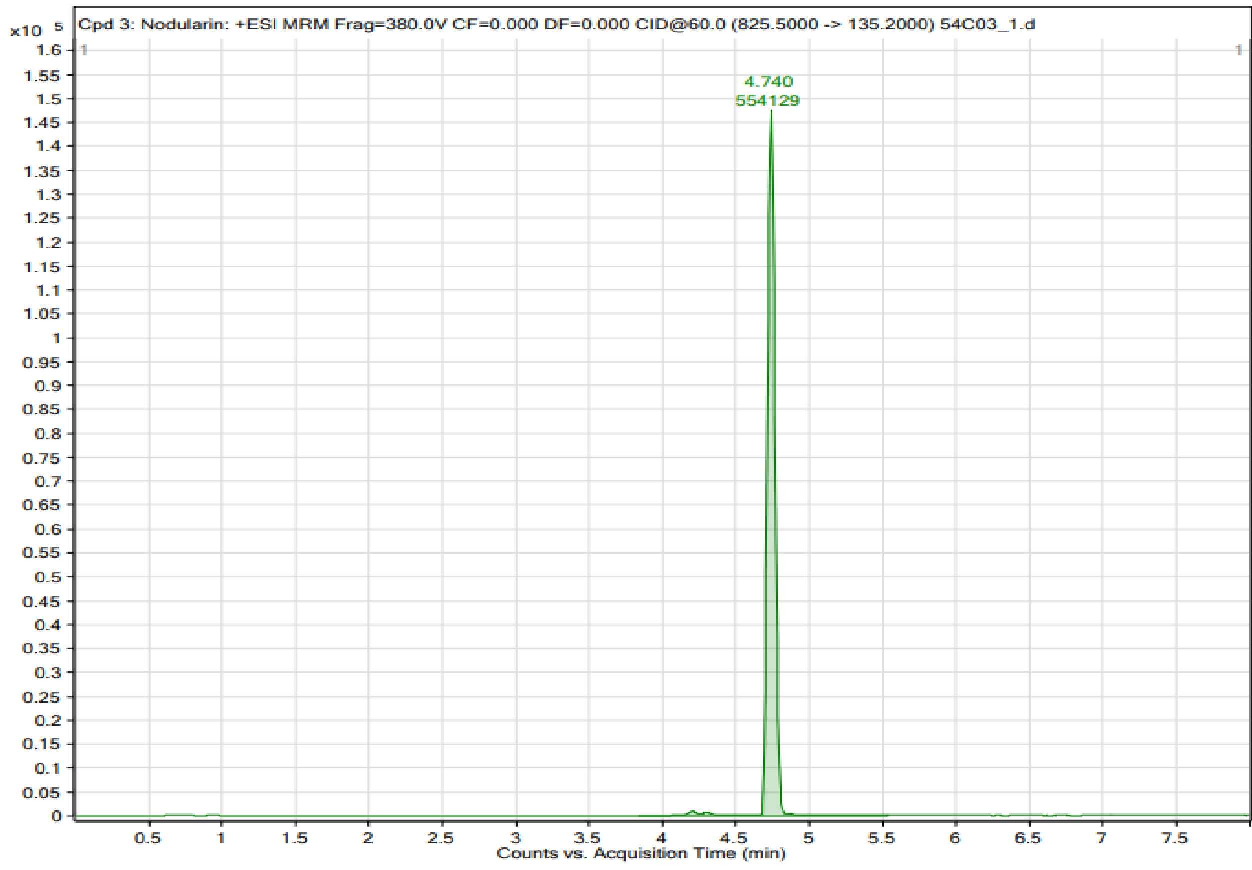

x10 5
Cpd 3: Nodularin: +ESI MRM Frag=380.0V CF=0.000 DF=0.000 CID@60.0 (825.5000 -> 135.2000) 54C03_1.d
4.740
554129
Counts vs. Acquisition Time (min)

Table B1 Method performance data

Determinand	LOQ	Direct Standards			
		Low Standard, 0.150 µg L^{-1}		High Standard, 1.350 µg L^{-1}	
	µg L^{-1}	Bias %	RSD %	Bias %	RSD %
Microcystin RR	0.014	-15.2	10	-1.8	8
Microcystin YR	0.040	-10.4	7	-2.7	7
Microcystin LR	0.050	-13.3	10	-4.3	7

Determinand	Recovery	Edinburgh Water (Soft water)			
		Low Spike, 0.300 µg L^{-1}		High Standard, 1.000 µg L^{-1}	
	%	Bias %	RSD %	Bias %	RSD %
Microcystin RR	103.2	-3.4	6	2.0	9
Microcystin YR	102.8	-8.3	7	-2.2	9
Microcystin LR	107.3	-8.0	7	-2.3	8

Determinand	Recovery	Newcastleton Water (Hard Water)			
		Low Spike, 0.300 µg L^{-1}		High Standard, 1.000 µg L^{-1}	
	%	Bias %	RSD %	Bias %	RSD %
Microcystin RR	108.8	-7.2	6	2	10
Microcystin YR	109.9	-6.5	8	3.1	13
Microcystin LR	107.3	-7.2	7	1.0	11

Determinand	Recovery	Howdenhaugh Water (Ground Water)			
		Low Spike, 0.300 µg L^{-1}		High Standard, 1.200 µg L^{-1}	
	%	Bias %	RSD %	Bias %	RSD %
Microcystin RR	105.3	-6.3	5	0.3	7
Microcystin YR	105.5	-7.2	7	0.2	5
Microcystin LR	103.5	-6.9	6	-1.3	4

Determinand	Recovery	Glencorse Water (Surface Water)			
		Low Spike, 0.300 µg L^{-1}		High Standard, 1.200 µg L^{-1}	
	%	Bias %	RSD %	Bias %	RSD %
Microcystin RR	105.8	-8.8	5	-7.6	7
Microcystin YR	101.7	-7.1	7	-2.6	5
Microcystin LR	102.9	-6.9	6	-1.7	4

Method performance data provided by Scottish Water.

C The Preparation of Intercellular and Extracellular Microcystins in Raw and Potable Waters by Direct Injection Liquid Chromatography Tandem Mass Spectrometry

C1 Performance characteristics of the method

C1.1	**Substances determined**	Preparation of Intercellular and extracellular Microcystin RR, Microcystin YR and Microcystin LR
C1.2	**Type of sample**	Raw waters (ground and surface), potable waters.
C1.3	**Basis of method**	**Extracellular Microcystin** Samples are filtered and filtrate is collected and analysis as per method A or B. **Intracellular Microcystin** The filtered material is treated with acetone or methanol to release the microcystin, diluted and analysis as per method A or B.
C1.4	**Range of application**	Typically, up to 1.50µg L^{-1}
C1.5	**Standard deviation**	Performance based on Method B
C1.6	**Limit of quantitation**	Typically, 0.050µg L^{-1}, based on a low-level standard (0.075µg L^{-1}). Performance based on Method B
C1.7	**Bias**	Performance based on Method B

C2 Principle

The intracellular and extracellular microcystin are separated by filtering the sample under vacuum using a glass fibre filter paper. The filtrate is transferred to a vial and analysed as per method A or B. This is the extracellular microcystin in the sample.

The glass fibre filter paper holds the algal cells, these must be broken (lysed) prior to extraction – the intracellular microcystin portion of the sample. The glass fibre filter paper portion of the sample is placed in a small test tube and a known amount of acetone or methanol is added to cover the full filter paper. This is heated to boil the solvent gently or left for 12 hours to allow the algal cell walls to break and release the intracellular microcystins. A portion of this solvent is then diluted in HPLC grade water and analysed as per method A or B.

Notes on typical sample types are given below.

Figure C1: Scum

Scums (Figure C1) generally are formed as algal blooms start to die, they tend to be gel-like/glutinous masses, the cells begin to collect together and wash ashore/collect at margins; they degrade releasing microcystins into the surrounding water – thus concentrations are similar, or may even become higher within the water body, depending on how old scums are. It is best to dilute an aliquot of the scum prior to attempting filtration. A typical dilution would be x100 with HPLC grade water.

Figure C2: Algal bloom

Algal blooms (Figure C2) are actively growing – the cells have far higher concentrations of toxins compared to surrounding water.

C3 Interferences

Any co-extracted material which has a LC retention time similar to any of the above determinands and which gives a detector response at the monitored masses will interfere. However, none are known at the $^{m}/_{z}$ values selected.

C4 Hazards

Analysts using this method should familiarise themselves with the COSHH and risk assessments for the analysis. See Safety Data Sheet (SDS) provided by the chemical manufacturer/supplier.

C4.1 Methanol, CH_3OH, CAS No. 67-56-1

C4.2 Acetone, (CH_3COCH_3), CAS No. 67-64-1

Refer to Hazards in Method B

C5 Reagents

All reagents should be of sufficient purity that they do not give rise to interferences during the analysis and distilled, deionised or similar grade water should be used throughout. A procedural blank should be run with each batch of samples to check for interferences. All solutions should be mixed well prior to use.

C5.1 Acetone, Analar grade or equivalent

C5.2 Methanol, HPLC grade

C5.3 Sodium thiosulfate Solution 10% w/v sodium thiosulfate solution (100g L^{-1})

Prepared by dissolving 25.00±0.01g of sodium thiosulfate in 250mL of ultra-pure water in a volumetric flask. This is used as matrix modifier for all samples and to produce the calibration and AQC matrix water. Store at ambient room temperature or under refrigeration at 1-5°C. Shelf life: 12 months.

C6 Apparatus

In addition to normal laboratory glassware and apparatus the following may be required.

C6.1 500mL glass sample bottles with PTFE-lined screw plastic cap containing 125µL of 10% w/v sodium thiosulfate solution (240mg L^{-1}) or equivalent.

C6.2 Refrigerator and freezer, set at 1-5°C and less than -17°C, with valid calibration status.

C6.3 Analytical Balance, four decimal place, for the preparation of stock solutions.

C6.4 Auto-pipette with valid calibration status, 10-100µL & 100-1000µL capacity.

C6.5 Volumetric Flasks, glass, various sizes.

C6.6 Measuring Cylinder, 500mL capacity.

C6.7 Pasteur pipettes, glass, disposable.

C6.8 Auto-sampler vials, amber glass, 2mL capacity, snap-capped and snap-caps with septa.

C6.9 1L glass Buchner funnel.

C6.10 Glass fibre filter paper, GF/B grade 70mm diameter or equivalent.

C6.11 Vacuum pump

C7 Sample collection and preservation

Samples are taken in 500mL glass bottles with caps (lined with PTFE) containing sodium thiosulfate. See C6.1. Samples may be stored for up to 14 days at 1-5°C.

C8 Analytical procedure

C8.1 500mL of sample is filtered using a glass fibre filter paper. The filtrate is collected. This portion of the sample is analysed via method A or B. This is the extracellular microcystin in the sample and this is analysed as described as in method B

C8.2 The filter paper is transferred to a 5mL stoppered test tube. 5mL of acetone or methanol is added to the test tube. This test tube is either left for 12 hours at 1-5°C or is boiled carefully in a hot water bath for 1-2 minutes.

C8.3 100µL of the solvent is transferred to 10mL volumetric flask and made to the mark with HPLC grade water. This part of the sample is analysed via method A or B. This is the intracellular microcystin in the sample and this is analysed as described in Method B. Further dilution can be done depending on sample matrix or suspected concentration.

C9 Calculation

The concentration intracellular and extracellular are analytes is given by an internal standard procedure:

$$\text{Concentration } (\mu g\ L^{-1}) = \frac{(\text{Sample Response})/(\text{Sample ISTD Response})}{(\text{Standard Response})/(\text{Standard ISTD Response})} \times \text{Standard Concentration } (\mu g\ L^{-1}) \times \text{sample dilution factor}$$

The individual total microcystin is the sum of the of the intracellular and extracellular fractions.

Note that at present due to the lack of availability of certified reference materials or proficiency testing schemes, there is no robust way to determine the efficiency of the procedure to extract intracellular microcystin.

Address for correspondence

However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts at the address given below. In addition, if users wish to receive advanced notice of forthcoming publications, please contact the Secretary.

Secretary
Standing Committee of Analysts
Environment Agency (National Laboratory Service)
56 Town Green Street
Rothley
Leicestershire
LE7 7NW

www.environment-agency.gov.uk/nls

Environment Agency
Standing Committee of Analysts

Members assisting with these methods

Without the good will and support given by these individuals and their respective organisations SCA would not be able to continue and produce the highly valued and respected blue book methods.

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Kenny Burnside	SEPA
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Kate Clark	Subadra Consulting
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