INTERNATIONAL STANDARD

ISO 22104

First edition
2021-07

Water quality — Determination of microcystins — Method using liquid chromatography and tandem mass spectrometry (LC-MS/MS)

Qualité de l'eau — Dosage des microcystines — Méthode par chromatographie en phase liquide couplée à la spectrométrie de masse en tandem (CL-SM/SM)

Reference number
ISO 22104:2021(E)

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

Published in Switzerland

Contents

Page

Foreword ... **iv**

1 Scope ... **1**

2 Normative references ... **1**

3 Terms and definitions ... **1**

4 Principle ... **2**

5 Interferences ... **2**
5.1 Biases ... 3
5.2 Limitations ... 3

6 Reagents and standards ... **3**
6.1 General ... 3
6.2 Preparation of solutions ... 4

7 Apparatus ... **6**

8 Sampling ... **7**

9 Procedure ... **8**
9.1 Preparation of samples ... 8
9.1.1 General ... 8
9.1.2 Preparation of method blank sample ... 8
9.1.3 Preparation of laboratory control spike sample ... 8
9.1.4 Preparation of calibration control sample ... 8
9.1.5 Preparation of calibration standard solutions ... 8
9.1.6 Preparation of drinking water and freshwater sample ... 9
9.1.7 Sample preparation procedure with freeze/thaw cycles ... 9
9.2 Instrumental analysis by LC-MS/MS procedure ... 10
9.2.1 Instrument set-up parameters ... 10
9.3 Run processing and quality assurance ... 13
9.3.1 Run sequence ... 13
9.3.2 Run control operations / limits ... 13

10 Calibration ... **14**

11 Evaluation and calculation of results ... **15**
11.1 Identification and calculations ... 15
11.2 Calibration curve equation determination ... 15
11.3 Internal standard calculation ... 15
11.4 Internal standard recovery calculation ... 16

12 Expressing of results ... **16**

13 Test report ... **16**

Annex A (informative) **Use of high resolution mass spectrometry detectors (HRMS)** ... **17**

Annex B (informative) **Use of online solid phase extraction coupled to liquid chromatography for the automated analysis of microcystins** ... **19**

Annex C (informative) **Use of manual solid phase extraction prior to instrumental analysis for improved method detection limits** ... **25**

Bibliography ... **32**

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 2, *Physical, chemical and biochemical methods*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Water quality — Determination of microcystins — Method using liquid chromatography and tandem mass spectrometry (LC-MS/MS)

WARNING — Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices.

IMPORTANT — It is absolutely essential that tests conducted in accordance with this document be carried out by suitably qualified staff.

1 Scope

This document specifies a method for the quantification of twelve microcystin variants (microcystin-LR, -LA, -YR, -RR, -LY, -WR, -HtyR, -HilR, -LW, -LF, [Dha^7]-microcystin-LR, and [Dha^7]-microcystin-RR) in drinking water and freshwater samples between 0,05 µg/l to 1,6 µg/l. The method can be used to determine further microcystins, provided that analytical conditions for chromatography and mass spectrometric detection has been tested and validated for each microcystin. Samples are analysed by LC-MS/MS using internal standard calibration.

This method is performance based. The laboratory is permitted to modify the method, e.g. increasing direct flow injection volume for low interference samples or diluting the samples to increase the upper working range limit, provided that all performance criteria in this method are met.

Detection of microcystins by high resolution mass spectrometry (HRMS) as an alternative for tandem mass spectrometry (MS/MS) is described in Annex A.

An alternative automated sample preparation method based on on-line solid phase extraction coupled to liquid chromatography is described in Annex B.

When instrumental sensitivity is not sufficient to reach the method detection limits by direct flow injection, a solid phase extraction clean-up and concentration step is described in Annex C.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

3 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

4 Principle

This method is designed to identify and quantify total (free + intracellular) microcystins in water by direct flow injection liquid chromatography and tandem mass spectrometry (LC-MS/MS) with electrospray ionization[1],[2]. 12 microcystins (Table 1) are determined quantitatively by multi-point calibration using nodularin as internal standard.

Table 1 — Microcystin variants included in the method

Microcystin variant	CAS-RN[a]	Molecular formular
Microcystin-LR	101043-37-2	$C_{49}H_{74}N_{10}O_{12}$
Microcystin-RR	111755-374	$C_{49}H_{75}N_{13}O_{12}$
Microcystin-LA	96180-79-9	$C_{46}H_{67}N_7O_{12}$
Microcystin-YR	101064-48-6	$C_{52}H_{72}N_{10}O_{13}$
Microcystin-LY	123304-10-9	$C_{52}H_{71}N_7O_{13}$
Microcystin-WR	138234-58-9	$C_{54}H_{73}N_{11}O_{12}$
Microcystin-HtyR	913178-65-1	$C_{52}H_{72}N_{10}O_{13}$
Microcystin-HilR	N/A	$C_{50}H_{76}N_{10}O_{12}$
Microcystin-LW	157622-02-1	$C_{54}H_{72}N_8O_{12}$
Microcystin-LF	154037-70-4	$C_{52}H_{71}N_7O_{12}$
[Dha^7]-Microcystin-LR (dmLR)	120011-66-7	$C_{48}H_{72}N_{10}O_{12}$
[Dha^7]-Microcystin-RR (dmRR)	131022-02-1	$C_{48}H_{73}N_{13}O_{12}$

[a] CAS-RN: Chemical Abstracts System Registration Number.

Nodularin can be naturally occurring in brackish water samples. Blank levels should be checked before analysis for these samples. Alternatively, ^{15}N-labelled microcystin surrogates should be used if available.

NOTE Some microcystins (e.g. demethylated RR variants) have the same exact mass and a similar chromatographic behaviour. While some can be distinguished by their fragmentation (e.g. [Asp^3, Mdha^7] MC-RR and [MeAsp^3, Dha^7)] MC-RR), others even show the same fragmentation (e.g. Asp^3, Mdha^7] MC-RR and [Asp^3, Dhb^7] MC-RR).

Water samples are homogenized to disperse cell aggregates. A 5 ml aliquot is transferred to a 15 ml centrifuge tube, internal standard is added, and cells are lysed by three cycles of freeze/thaw. Solid particles and cell debris are centrifuged and syringe filtered directly into an autosampler vial. Quantification of microcystins is done by an internal standard method using LC-MS/MS or HRMS (Annex A).

Alternatively, lysed and filtered samples can be injected using an on-line SPE instrumental configuration (Annex B) or manual SPE (Annex C) for an increased sensitivity.

5 Interferences

This analysis was developed using liquid chromatography (LC) tandem mass spectrometry (MS/MS) with electrospray ionization (ESI), on a triple quadrupole mass spectrometer. Acquisition mode was based on multiple reaction monitoring (MRM). Isobaric interferences that are not resolved by chromatography or the unit mass resolution of the tandem quadrupoles may be present in some samples. These samples may require additional selectivity via additional sample clean-up and/or high-resolution mass spectrometry (HRMS). Some microcystins with the same exact mass might not be able to be distinguished by HRMS, but by their different fragmentation patterns, a few congeners cannot be distinguished by either of these approaches.

Variable instrument response and/or inconsistent retention times may be observed in the first gradient runs of the day. The column requires conditioning by running at least one gradient program prior to the first sample injection of the day.

5.1 Biases

All labware that contacts microcystins should have relatively inert surfaces; otherwise, compound losses may occur by adsorption onto the glass. Unscratched borosilicate glassware or polyethylene is recommended. To further minimize this effect, sample preparation should be carried out in a timely manner and quantification by matrix matched calibration standards is preferred.

Analytical results (method precision and accuracy) are calculated by internal standard quantitation methods and may be affected by differences in the recovery of the internal standard relative to that of the target compounds. When available, ^{15}N-labelled microcystins should be used for this purpose.

The concentration of on-site samples will vary greatly depending on the density of algae at each sampling point, and the concentration difference will also be large for each microcystins. Given this, the multi-point calibration curves for the microcystins, using a fixed amount of internal standard, are non-linear. Quantification is done by a second order (quadratic) curve-fitting procedure.

5.2 Limitations

The sample preparation method is restricted to water samples. Applicability of the method to samples with very high organic content, such as water containing high concentrations of humic materials, is unknown.

The working range of this method is 0,05 µg/l to 1,6 µg/l. If samples with a higher microcystin concentration than 1,5 µg/l are found or predicted, a smaller aliquot of sample should be taken, and a dilution factor applied to the final result. Surface waters containing thick cyanobacterial blooms may interfere with the instrumental analysis. In these cases, a smaller amount of sample can be diluted, and volume should be recorded for the final calculation of microcystins concentration.

Standards of specific microcystin variants are not always available on a continuous basis. Foreign suppliers are sometimes restricted by law and are not always able to export algal toxin standards to different countries. Before being used, newly prepared standards shall be compared to standards in current use. Purity of the different lots of standards should be checked against reference materials when available. Alternatively, purity can also be confirmed using universal detector like HPLC-UV (ISO 20179).

6 Reagents and standards

6.1 General

If available, reagents of purity grade “for analysis” or “for residue analysis” are used. The amount of impurities contributing to the blank value or causing signal interferences shall be negligible. This shall be checked regularly (see section for blank value measurements).

Solvent, water and reagents intended for use as elution agents shall be compatible with HPLC and mass spectrometry.

Microcystins are potent hepatotoxins. Laboratory safety measures should be strictly followed throughout the sample preparation (including lab gloves, labcoat, safety glasses) to prevent human exposure to these toxins.

NOTE 1 High purity grades of solvent applicable for use are available commercially.

NOTE 2 Reagents listed as “prepare as required” have an expiry date of one year from the moment they were prepared.

NOTE 3 Prepared standard solutions are stored at (5 ± 3) °C, with an expiry date of one year from the moment they were prepared.

Stock and intermediate standard solutions should be used as a reference, other stock and intermediate concentrations are acceptable to prepare the final working solutions.

6.1.1 Water, conforming with the requirements of ISO 3696, grade 1 or equivalent and without any interfering blank values.

6.1.2 Methanol, CH_3OH, LC-MS grade.

6.1.3 Acetonitrile, CH_3CN, LC-MS grade.

6.1.4 Formic acid, CHCOOH, LC-MS grade, mass fraction ≥98 %.

6.1.5 Electrospray tuning mixture, in accordance with the specification of the instrument manufacturer.

6.1.6 Sodium thiosulfate pentahydrate, $Na_2S_2O_3 \cdot 5H_20$, 99 % purity.

6.1.7 Concentrated phosphate-free detergent.

6.1.8 Internal standard substances like Nodularin, (CAS no 118399-22-7, ≥95 % purity determined by HPLC) or isotope labelled compounds of reference substances.

6.1.9 Reference Substances as listed in Table 1, with known mass fraction or purity ≥95 % determined by HPLC.

6.1.10 Microcystin-LR, 10 ng/µl certified reference standard.

6.2 Preparation of solutions

6.2.1 Tap water, quenched with sodium thiosulfate at 150 mg/l (for calibration standard solutions, QC samples and sample dilutions).

The method blank, calibration standard solutions, QC samples and sample dilutions (if necessary) are made with quenched laboratory tap water. This quenched water is made by taking 1 l of tap water and adding 1,5 ml of sodium thiosulfate preservative solution (i.e. 150 mg sodium thiosulfate) (6.2.2). Cap the bottle and shake vigorously to mix. This water is prepared as required before sample preparation in order to quench any residual chlorine in the tap water which would oxidize the microcystins. Store the reagent water at room temperature. Quenching is not necessary if it can be ensured that the used tap water is provided without chlorination.

NOTE Depending on the application, method blank, calibration standard solutions, QC samples and sample dilutions can be prepared with other matrices such as mineral water.

6.2.2 Sodium thiosulfate preservative solution, $Na_2S_2O_3$,100 mg/ml.

Into a 1 l volumetric flask put 157 g of $Na_2S_2O_3 \cdot 5H_2O$ (6.1.6), corresponding to 100 g of anhydrous $Na_2S_2O_3$. Dissolve in water (6.1.1), and make up to 1 l with pure water. Prepare as required. Store the preservative at room temperature.

6.2.3 Stock solution of internal standard substances

Prepare solutions with a mass concentration of, for example, 200 ng/µl.

For this use, for example, transfer 10 mg of an internal standard (6.1.8) to a separate 50 ml volumetric flask and dissolve it in methanol (6.1.2). Fill up to the 50 ml mark with methanol (6.1.2). The concentration of this solution is 200 ng/µl.

6.2.4 Internal standard solution (IS1)

Prepare a working solution with internal standard mass concentrations of, for example, 8,0 ng/µl each.

For this use, for example, transfer 1,0 ml of each internal standard stock solution (6.2.3) to a 25 ml flask and fill up to the mark with methanol (6.1.2).

6.2.5 Internal standard solution (IS2)

Prepare a working solution with internal standard mass concentrations of, for example, 80 pg/µl each.

For this use, for example, transfer 250 µl of each internal standard stock solution (6.2.4) to a 25 ml flask and fill up to the mark with methanol (6.1.2).

6.2.6 MCYST mix solution (S1)

Prepare a solution with microcystin mass concentrations of, for example, 4 ng/µl.

For this use, for example, transfer 100 µg [Dha^7] microcystin-LR (dmLR), 100 µg [Dha^7] microcystin-RR (dmRR), 100 µg microcystin-LF, 100 µg microcystin-LW, 100 µg microcystin-WR, 100 µg microcystin-LY, 100 µg microcystin-HtyR, and 100 µg microcystin-HilR to a 25 ml volumetric flask and dissolve it in methanol (6.1.2). Make up to 25 ml with methanol (6.1.2). The concentration of each microcystin is 4 ng/µl.

6.2.7 MCYST mix solution (S2)

Prepare a solution with microcystin mass concentrations of, for example, 400 pg/µl.

For this use, for example, transfer 2 500 µl of supplemental microcystin mix solution solution (6.2.6) to a 25 ml flask and fill up to the mark with methanol (6.1.2).

6.2.8 MCYST mix solution (S3)

Prepare a solution with microcystin mass concentrations of, for example, 40 pg/µl.

For this use, for example, transfer 250 µl of supplemental microcystin mix solution (6.2.6) to a 25 ml flask and fill up to the mark with methanol (6.1.2).

6.2.9 MCYST mix A solution

Prepare a solution with microcystin mass concentrations of, for example, 20 ng/µl.

For this use, for example, transfer 500 µg of microcystin-LR, 500 µg of microcystin-RR, 500 µg of microcystin-YR and 500 µg of microcystin-LA to a 25 ml volumetric flask and dissolve it in methanol (6.1.2). Make up to 25 ml with methanol (6.1.2). The concentration of each microcystin is 20 ng/µl.

6.2.10 MCYST mix B solution

Prepare a solution with microcystin mass concentrations of, for example, 2,0 ng/µl.

For this use, for example, dilute 2,5 ml of MCYST mix A solution (6.2.9) to 25 ml with methanol in a 25 ml volumetric flask. The concentration of each microcystin is 2,0 ng/µl.

6.2.11 MCYST mix C solution

Prepare a solution with microcystin mass concentrations of, for example, 200 pg/µl.

For this use, for example, dilute 250 µl of MCYST mix solution A (6.2.9) to 25 ml with methanol in a 25 ml volumetric flask. The concentration of each microcystin is 200 pg/µl.

6.2.12 MCYST mix D solution

Prepare a solution with microcystin mass concentrations of, for example, 20 pg/µl.

For this use, for example, dilute 25 µl of MCYST mix solution A (6.2.9) to 25 ml with methanol in a 25 ml volumetric flask. The concentration of each microcystin is 20 pg/µl.

6.2.13 Instrument check mix (high) solution

Into a 25 ml volumetric flask put 25 µl of MCYST mix solution A (6.2.9) and 250 µl of supplemental microcystin mix solution (6.2.6). Make up to 25 ml with pure water (6.1.1). The concentrations of microcystins -LR (6.2.10), -RR, -LA, -YR are 20 pg/µl. The remaining supplemental microcystins are at a concentration of 40 pg/µl.

6.2.14 Calibration control standard (CS1)

The calibration control standard is a reference substance solution produced independently of the other stock solutions (6.2.5 to 6.2.13), e.g. a solution from an alternative batch or manufacturer.

For this use, for example, a microcystin-LR, 10 ng/µl certified concentration standard can be purchased or prepared.

Other microcystins with certified concentration should also be used to validate standard mixture concentration when available.

6.2.15 Calibration control standard (CS2)

Prepare a solution with microcystin mass concentrations of, for example, 100 pg/µl.

For this use, for example, dilute 100 µl of calibration control standard CS1 (6.2.14) to 10 ml with methanol in a 10 ml volumetric flask. The concentration of each microcystin is 100 pg/µl.

6.2.16 **Mobile phase A**, water with 0,1 % formic acid.

Measure 1 l of pure water (6.1.1) using a graduated cylinder and pour into a 1 l amber bottle. Transfer 1 ml of formic acid (6.1.4) using a 1 ml pipette into the water. Cap the bottle and shake vigorously to mix. Store the reagent at room temperature. Prepare as required. Mobile phase A should be replaced at least on a weekly basis and should be degassed before the chromatographic run.

6.2.17 **Mobile phase B**, acetonitrile with 0,1 % formic acid.

Measure 1 l of acetonitrile (6.1.3) using a graduated cylinder and pour into a 1 l amber bottle. Transfer 1 ml of formic acid (6.1.4) using a 1 ml pipette into the acetonitrile. Cap the bottle and shake vigorously to mix. Store the reagent at room temperature. Prepare as required. Mobile phase B should be degassed before the chromatographic run.

7 Apparatus

NOTE Labware, reagents and equipment equivalent to those listed in this document are acceptable.

7.1 Bottles 500 ml, 1 l, amber glass, with polytetrafluoroethylene (PTFE) screw caps.

7.2 Cylinders, graduated, glass, 25 ml, 50 ml, 100 ml, 250 ml, 500 ml, 1 000 ml, 2 000 ml.

7.3 Microsyringes.

7.4 Centrifuge tubes, polypropylene, 15 ml, 17 mm diameter.

7.5 Centrifuge, suitable for 15 ml centrifuge tubes (7.4)

7.6 Pipette, 1 ml to 5 ml, adjustable.

7.7 Pipette tips, polypropylene, flextips, 1 ml to 5 ml.

7.8 Syringe, polypropylene, 5 ml.

7.9 Syringe filters, with low dead volume, GHP membrane, 13 mm, 0,2 µm.

7.10 Sample vials, appropriate for automated sample injection and with low adsorption, nominal volume 1,5 ml, clear glass, screw or crimp cap with PTFE/silicone septa with slit.

7.11 Freezer, capable of reaching −28 °C.

7.12 Temperature controlled water bath, capable of reaching 50 °C.

7.13 Ultrasonic bath

7.14 Homogenizer, capable of reaching 10 000 RPM.

7.15 Liquid chromatograph (LC)

The LC shall include a binary pump capable to run gradients from 95 % aqueous mobile phase A (6.2.16) and 5 % organic mobile phase B (6.2.17) to 5 % aqueous mobile phase A and 95 % organic mobile phase B, providing enough pressure to run at a constant flow of 0,35 ml/min using the analytical column described in 7.16. The instrument should also be equipped with an autosampler capable to accommodate enough samples to process an entire batch.

7.16 Analytical column, C_{18}, 2,1 mm ID × 150 mm length, 1,8 µm particle size, suitable for chromatography of the selected substances. Other columns showing similar performance can be used alternatively.

7.17 Mass spectrometer (MS)

The mass spectrometer should have a triple quadrupole (tandem MS/MS) configuration capable of performing collision induced dissociation (CID) experiments at different collision energies (CE) and acquire in multiple reaction monitoring (MRM) mode. The instrument should also be equipped with an ionization interface such as an electrospray ionization probe (ESI) with adjustable capillary voltage and a combination f pumps capable to provide sufficient vacuum for the correct operation of the system. The instrument shall also be supplied with the corresponding gases for the correct operation of the electrospray source (cone and desolvation gases, usually nitrogen) and collision cell (usually argon or nitrogen). Alternatively, a high-resolution mass spectrometer (HRMS) can be used instead of MS/MS, as described in Annex A.

8 Sampling

Collect the samples in 500 ml amber glass bottles (7.1). A minimum of 500 ml should be submitted for testing. Samples should be preserved with 150 mg/l of sodium thiosulfate (6.1.6) as a neutralizing additive to remove chlorine: add 0,75 ml of sodium thiosulfate preservative solution (6.2.2) to 500 ml sample. This is particularly important for treated drinking waters or those waters suspected of containing residual chlorine. Higher concentration of preservative can be employed in samples containing large amount of chlorine (ISO 5667-1, ISO 5667-3; ISO 5667-4, ISO 5667-5, ISO 5667-6).

For a sample preparation procedure with manual SPE (Annex C) a minimum volume of 1 l should be taken in amber glass bottles. Samples should be preserved by adding 1,5 ml of sodium thiosulfate preservative solution (6.2.2) to 1 000 ml sample.

Samples should be stored in the dark at (5 ± 3) °C, and should be extracted within 21 d of sampling.

9 Procedure

9.1 Preparation of samples

IMPORTANT — Allow all working standard solutions to warm to room temperature before opening the vials. Rinse syringes with methanol.

9.1.1 General

All glassware should be cleaned prior to the analysis (6.1.7).

9.1.2 Preparation of method blank sample

For each batch of samples, prepare a method blank sample by transferring 5 ml of quenched tap water (6.2.1) into a 15 ml centrifuge tube (7.4).

9.1.3 Preparation of laboratory control spike sample

For each batch of samples processed, prepare a laboratory control spike sample consisting of 5 ml of quenched tap water (6.2.1) in a 15 ml centrifuge tube (7.4) spiked with 8,0 µl of MCYST mix C solution (6.2.11) and 40 µl of supplemental microcystin mix solution S3 (6.2.8) using the appropriate volume microsyringes (7.3) to give a concentration of 0,32 µg/l for each target microcystin. This target concentration should be used as an example, the laboratory can modify the control spike concentration depending on the analysis expectations.

9.1.4 Preparation of calibration control sample

For each batch of samples processed, prepare a calibration control sample consisting of 5 ml of quenched tap water (6.2.1) in a 15 ml centrifuge tube (7.4) spiked with 16 µl of the control standard solution solution CS2 (6.2.15) to give a concentration of 0,32 µg/l for microcystin-LR, for example.

NOTE Other microcystins with certified concentration can also be used to validate standard mixture concentration when available.

9.1.5 Preparation of calibration standard solutions

The calibration standard solutions are used for calibration of the method including the whole sample preparation procedure.

Two different microcystin standard stock solutions are prepared: MCYST mix C and D solutions (6.2.11, 6.2.12) include those commercially available variants that are more commonly found in freshwater environments or regulated by guidelines (LR, LA, RR and YR[3],[4],[5]).

Supplemental microcystins solutions S2 and S3 (6.2.7, 6.2.8) include those variants that are also commercially available, but are usually not detected at high concentrations in freshwater (dmLR, dmRR, LF, LW, WR, LY, HtyR, HilR), even though some of them might be present in higher concentrations depending on the nature of the algal bloom (e.g. dmRR in Planktothrix blooms). Depending on standard availability or sample expectations, some laboratories might decide to spike the supplemental microcystins only for the lower range of the calibration curve, for example MR1 to MR5.

NOTE 1 Alternative mixtures of stock solutions including other microcystins can be prepared depending on expected local variants.

For each batch of samples processed, prepare the following method recovery (MR) samples consisting of 5 ml of quenched tap water (6.2.1) in 15 ml centrifuge tube (7.4). Table 2 gives the volume (µl) of each MCYST mix solutions and supplement mix solutions that is added to each 5 ml calibration standard solution to produce the required target compound concentrations.

NOTE 2 For freshwater samples, blank lake water can be used alternatively for the preparation of calibration standard solutions.

Table 2 — Calibration standard solutions preparation

MR No	Compound concentration µg/l	MCYST mix D (6.2.12) µl	MCYST mix C (6.2.11) µl	Supplemental mix S3 (6.2.8) µl	Supplemental mix S2 (6.2.7) µl
MR1	0	0	0	0	0
MR2	0,05	12,5	0	6,25	0
MR3	0,08	20	0	10	0
MR4	0,16	40	0	20	0
MR5	0,32	0	8	40	0
MR6	0,80	0	20	0	10
MR7	1,2	0	30	0	15
MR8	1,6	0	40	0	20

Not all MRs need to be prepared and processed. If sufficient information is known about the expected concentrations, fewer MRs are needed. The MRs selected are based on the concentrations that would sufficiently bracket the algal toxin concentrations in the samples. The working range of MR solutions can be modified to meet different guidelines values.

9.1.6 Preparation of drinking water and freshwater sample

Allow the sample bottles to warm to room temperature.

Shake samples well right before taking a 5 ml aliquot of the homogenized sample with a 5 ml pipette (7.6). Samples of fresh water samples having a high density of cyanobacteria (algal blooms) are homogenized using homogenizer (7.14) at 10 000 RPM for 5 min before aliquoting. For fresh water samples the preparation and analysis shall be performed in three replicates due to a possible inhomogeneous distribution of cyanobacteria with intracellular microcystin.

9.1.7 Sample preparation procedure with freeze/thaw cycles

9.1.7.1 Add 15,0 µl of the working internal standard solution IS2 (6.2.5) to the method blank (9.1.2), laboratory control spike sample (9.1.3), calibration control sample (9.1.4), calibration standard solutions (9.1.5) and samples (9.1.6) to give a internal standard concentration of 0,24 µg/l. Cap the centrifuge tubes and shake well to mix the samples.

9.1.7.2 Place all the 15 ml centrifuge tubes containing the samples (9.1.7.1) in tube rack(s) and put the rack(s) in the freezer (7.11) at −28 °C for 50 min. Make sure all samples are completely frozen by visual inspection.

9.1.7.3 Place the centrifuge tubes rack(s) with the frozen samples in the water bath (7.12) at 50 °C for 15 min. Make sure all samples are completely thawed by visual inspection.

9.1.7.4 Repeat steps 9.1.7.2 and 9.1.7.3 two more times to complete three freeze/thaw cycles.

9.1.7.5 Centrifuge all the 15 ml centrifuge tubes containing the samples (9.1.7.1) at 4 000 *r*/min for 5 min using a centrifuge (7.5).

9.1.7.6 Pour 4 ml of the supernatant into an opened plastic syringe (7.8) connected to a 0,2 µm GHP syringe filter (7.9). Discard the first 2 ml filtered to condition the membrane and collect around 1,5 ml filtered in a glass sample vial (7.10). Store the samples at (3 ± 2) °C until ready for analysis.

9.2 Instrumental analysis by LC-MS/MS procedure

9.2.1 Instrument set-up parameters

IMPORTANT — 9.2.1.1 and 9.2.1.2 require basic proficiency and familiarization of the analyst with the vendor's LC-MS/MS hardware and software. Set up conditions may vary depending on the different instrument models and the values detailed in the section below should be used as an example.

9.2.1.1 Typical liquid chromatograph (LC) parameters

Table 3 — Typical LC-parameters

Parameter	Value
Mobile phase - Reservoir A:	Water with 0,1 % formic acid
Mobile phase - Reservoir B:	Acetonitrile with 0,1 % formic acid
Gradient program:	Start at 95 % A until minute 3,75
	linear ramp to 5 % A until minute 8,25
	hold at 5 % A until minute 10
	linear ramp to 95 % A until minute 10,10
	hold at 95 % A until minute 13
Flowrate:	Constant flow, 0,35 ml/min
Back pressure:	6,8 MPa (typical at initial conditions)
Column compartment:	45 °C
Autosampler compartment:	5 °C
Injection volume:	50 µl
Filling speed:	10 µl/s
Injection speed:	5 µl/s
Stop time:	13 min
Maximum pressure:	11 MPa
Minimum pressure:	0 MPa

NOTE Changes in the instrumental conditions can be accepted as long as they offer similar chromatographic performance. Injection volume can be modified depending on instrumental sensitivity.

Operate the HPLC instrumentation in accordance with the instruction provided by the manufacturer.

Use a suitable HPLC column (7.16) for chromatographic separation and select the chromatograhic conditions in Table 3, for example.

Complete separation of the substances is not necessary provided that interferences of the quantitative determination does not occur during peak overlapping. Optimize the separation of the analytes with gradient elution if necessary.

9.2.1.2 Typical mass spectrometer (MS) conditions

Table 4 — Typical MS-conditions

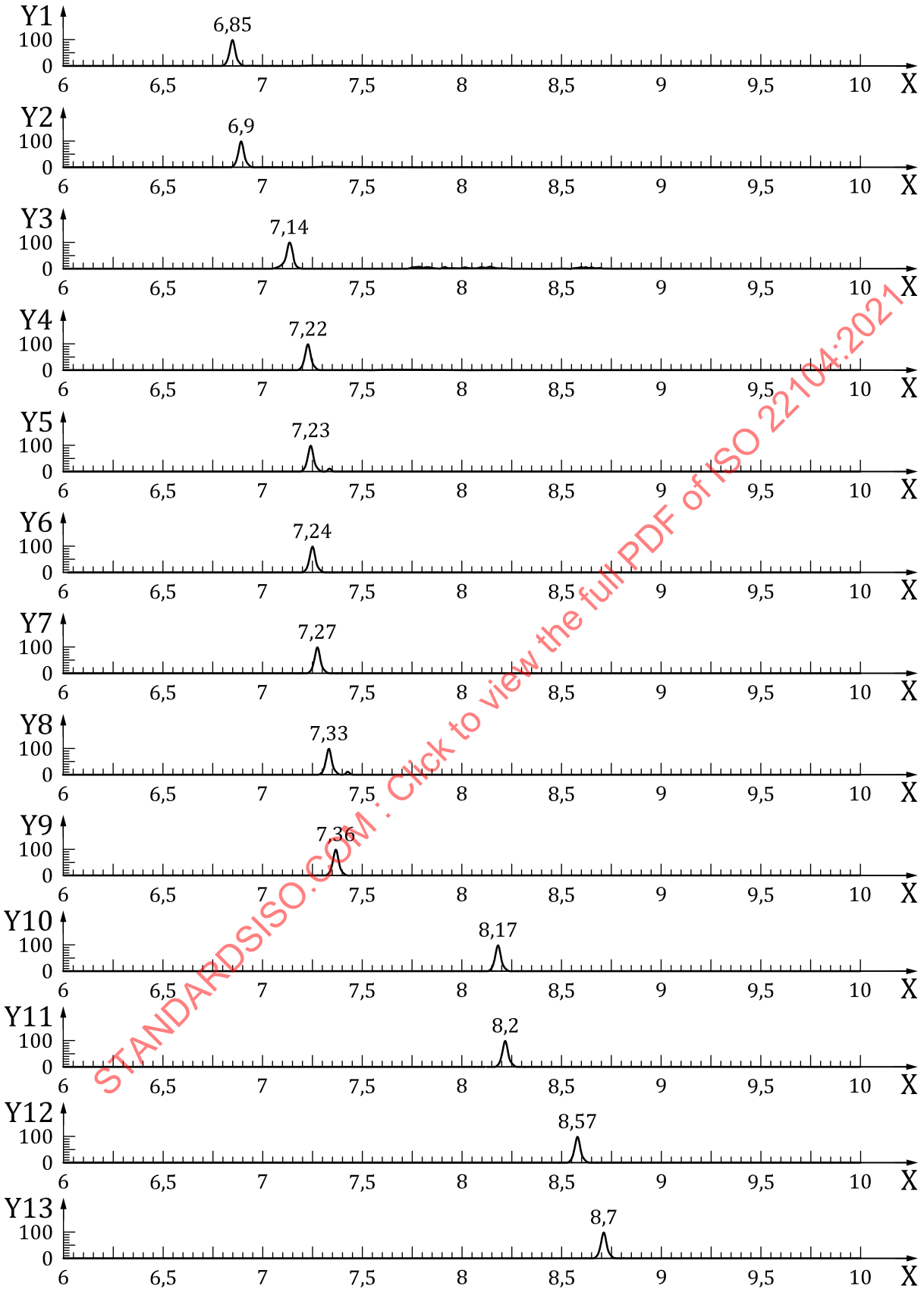
Parameter	Value
Scan type:	Multiple reaction monitoring (MRM)
Ion mode:	Positive ESI
Resolution Q1:	Unit
Resolution Q3:	Unit
MS/MS transitions:	see Table 5
Acquisition window:	± 30 s from retention time

Table 5 — Microcystins MS/MS acquisition conditions

Microcystin	Retention time (min)	Precursor ion	Quantifier product ion	Qualifier product ion	Collision energy (eV)
Microcystin-dmRR	6,85	512,8	135,1	199,1	45
Microcystin-RR	6,90	519,8	135,1	213,1	45
Nodularin (IS)	7,14	825,5	135,1	227,1	70
Microcystin-YR	7,22	1 045,5	135,1	213,1	102
Microcystin-dmLR	7,23	981,5	135,1	199,1	98
Microcystin-HtyR	7,24	1 059,6	135,1	213,1	105
Microcystin-LR	7,27	995,6	135,1	213,1	98
Microcystin-HilR	7,33	1 009,6	135,1	213,1	97
Microcystin-WR	7,36	1 068,6	135,1	213,1	105
Microcystin-LA	8,17	910,5	135,1	213,1	87
Microcystin-LY	8,20	1 002,5	135,1	213,1	97
Microcystin-LW	8,57	1 025,5	135,1	213,1	98
Microcystin-LF	8,70	986,5	135,1	213,1	92

NOTE Optimum collision energies to maximize the abundance of qualifying and quantifying ions can vary depending on the instrument.

Identify the optimal settings for ionization under the specified chromatographic conditions and set the method-specific settings for the source and MS parameters (Tables 4 and 5), for example. Figure 1 shows a typical chromatogram of the twelve microcystins and nodularin as internal standard under the instrumental conditions described in 9.2.1 (Tables 3, 4 and 5).



Key

Y1	microcystin-dmRR	Y8	microcystin-HilR
Y2	microcystin-RR	Y9	microcystin-WR
Y3	nodularin	Y10	microcystin-LA

Y4	microcystin-Yr	Y11	microcystin-LY
Y5	microcystin-dmLR	Y12	microcystinLW
Y6	microcystins-HtyR	Y13	microcystin-LF
Y7	microcystin-LR		

Figure 1 — LC-MS/MS chromatogram of the 12 microcystins and nodularin as internal standard (IS)

9.3 Run processing and quality assurance

9.3.1 Run sequence

A possible run sequence includes analysing, for example:

1) Instrument check mix (high) solution (6.2.13); minimum of two injections;
2) Method blank (9.1.2);
3) Calibration standard solutions (9.1.5);
4) Laboratory control spike sample (9.1.3);
5) Calibration control sample (9.1.4);
6) Samples, including replicates (9.1.6);
7) Calibration standard solution (MR5) every 20 samples to check for instrument drift during long sequences and at the end of the samples;
8) Instrument check mix (high) solution (6.2.13).

9.3.2 Run control operations / limits

9.3.2.1 Instrument check mix (high) solution

The instrument check mix (high) solution (6.2.13) is injected at least twice at the beginning of each instrument run. The two sample runs are used to stabilize the instrument system plus check chromatographic performance (peak shape, retention time, sensitivity) and general mass spectrometer sensitivity. Good chromatography is exemplified by Gaussian peaks with peak widths < 15 s at the baseline. If the chromatography is not adequate, the analytical run is stopped, and system maintenance is done in accordance with the instructions of the instrument manufacturer.

9.3.2.2 Low level method recovery (MR2) sample

A typical way to evaluate the chromatographic performance would be measuring the 0,05 µg/l method recovery (MR2) sample to ensure adequate instrument sensitivity and chromatographic peak shape. The microcystin-LR MRM transition 995,6 -> 135,1 is used to assess sensitivity and chromatographic peak shape. The microcystin-LR chromatographic peak should be less than 0,25 min (15 s) at the baseline. If the peak does not meet peak shape and sensitivity requirements the analytical run is stopped, and instrument maintenance is done in accordance with the instructions of the instrument manufacturer.

9.3.2.3 Method blank measurements

The method blank (9.1.2) is used to assess cleanliness of the method labwares, reagents and equipment. The method blank should not contain target compounds or interference peaks ≥0,025 µg/l. If interfering

blank values occur, identify the cause using systematic examination and eliminate the source of contamination.

9.3.2.4 Laboratory control spike sample measurements

The laboratory control spike sample (9.1.3) is a method spike used to assess method performance and check the validity of the calibration curve generated for the batch of samples analysed. The sample contains microcystins at 0,32 µg/l, for example. Results for target compounds should fall within ± 30 % of the expected value. If the laboratory control spike sample is out of control, the source of the problem is investigated.

9.3.2.5 Calibration control sample measurements

The calibration control sample (9.1.4) is a method spike, prepared from an independent reference standard, used to assess method performance and ensure the current standards and calibration curve generated are valid for the batch of samples analysed. The sample contains microcystins at 0,32 µg/l, for example. Results for microcystins in the calibration control standard should fall within ± 30 % of the expected value. If the calibration control sample does not meet the requirements, the source of the problem is investigated. If the problem is not related to the sample preparation stage, a new standard should be used for the calibration standard solutions.

9.3.2.6 Replicate samples measurements

The relative percent difference between results from replicate of fresh water samples (9.1.6) should be ≤30 %. If the difference is >30 % the source of the problem is investigated. Highest replicate value should be reported.

10 Calibration

10.1 Ensure that the instrument (liquid chromatograph and mass spectrometer) parameters described in 9.2.1 are selected.

10.2 Inject the calibration standard solutions (9.1.5) prepared with the batch of samples to be analysed and generate the calibration curve equation for quantitation of the samples using the instrument's processing software. Note all calibration standard solutions have a fixed amount of internal standard (IS), so relative area (analyte area/IS area) should be plotted against concentration.

10.3 A second order quadratic equation (origin excluded, 1/x weighting) is applied to the multi-level range of the calibration standard solutions. At least five concentration levels plus a blank is used to generate the calibration curve(s). Select a calibration range that covers the actual concentration of the target compound in samples processed.

10.4 The coefficient of determination (R^2) for the target compound calibration curves generated shall be > 0,995. If a point on the curve is an outlier (i.e. it deviates by > 30 % from the curve of best fit), this point may be excluded from the calibration curve equation calculation. If the excluded calibration point is the lowest or the highest, it shall be taken into account that the dynamic range for that analyte will be narrower.

11 Evaluation and calculation of results

11.1 Identification and calculations

The criteria for positive identification of a target compound are:

1) The target compound shows the specific MRM quantifier and qualifier transitions detailed in Table 5 (e.g. MCYST-LR, *m/z* 995,6→135,1 and 995,6→213,1).

2) Relative isotopic ratio between the quantifier transition and qualifier transition are equivalent to those in the calibration standard solutions within ± 30 % (9.1.5).

3) The relative retention times of the target compounds and internal standards are equivalent to those in the calibration standard solutions within ± 5 % of the relative retention time (9.1.5).

A calibration curve correlating microcystin relative area (microcystin area/internal standard) and microcystin concentration is adjusted using a second order quadratic equation (origin excluded, 1/x weighting) using the calibration standard solutions (9.1.5). Unknown microcystin concentration in samples and replicates (9.1.6) is determined using this calibration curve.

NOTE 1 If the instrument used has a linear dynamic range wide enough, a linear equation with 1/x weighing can be employed for quantification.

NOTE 2 Linear equation can be used alternatively for those instruments showing great linearity.

11.2 Calibration curve equation determination

As described in 10.3 a second order quadratic equation is adjusted correlating calibration standard solutions (9.1.5) relative peak area of each microcystin versus concentration, as shown in Formula (1):

$$\frac{Area_{\mathrm{MC}}}{Area_{\mathrm{IS}}} = a\left(\frac{Concentration_{\mathrm{MC}}}{Concentration_{\mathrm{IS}}}\right)^2 + b\left(\frac{Concentration_{\mathrm{MC}}}{Concentration_{\mathrm{IS}}}\right) + c \quad (1)$$

where

$Area_{\mathrm{MC}}$ is each of the individual microcystin (MC) integrated peak area in the calibration standard solutions;

$Area_{\mathrm{IS}}$ is the internal standard (IS) integrated peak area in the calibration standard solutions;

$Concentration_{\mathrm{MC}}$ is the concentration of each of the individual microcysins (MC) in the calibration standard solutions, in µg/l;

$Concentration_{\mathrm{IS}}$ is the concentration of the internal standard (IS), in the calibration standard solutions, in µg/l.

11.3 Internal standard calculation

Concentration of each microcystin in the sample is calculated by solving Formula (1) using the *a*, *b*, and *c* constants determined with the calibration standard solutions (Formula (2)).

$$Concentration_{MC} = Concentration_{IS} \times \left(\frac{-b \pm \sqrt{b^2 - 4a\left(c - \frac{Area_{MC}}{Area_{IS}}\right)}}{2a}\right) \quad (2)$$

where

$Area_{MC}$ is each of the individual microcystin (MC) integrated peak areas in the sample;

$Area_{IS}$ is the internal standard integrated (IS) peak area in the sample;

$Concentration_{MC}$ is the concentration of each of the individual microcysins (MC) in the sample, in µg/l.

$Concentration_{IS}$ is the concentration of the internal standard (IS), in the sample, in µg/l.

11.4 Internal standard recovery calculation

Internal standard recovery is calculated using Formula (3):

$$\text{Recovery}\,(\%)=\frac{Area_{IS\ sample}}{Area_{IS\ avg}}\times 100 \quad (3)$$

where

$Area_{IS\ sample}$ is the internal standard (IS) integrated peak area in the sample;

$Area_{IS\ avg}$ is the average internal standard (IS) integrated peak area of all calibration standard solutions.

If recovery of the internal standard falls outside the range 25 % to 150 %, microcystins results for that sample shall be flagged as out of control.

12 Expressing of results

The mass concentrations of the substances in accordance with Table 1 are indicated in micrograms per litre at two significant figures.

EXAMPLES

Microcystin-LR: 0,085 µg/l

0,15 µg/l

1,4 µg/l

13 Test report

The test report shall contain at least the following information:

a) the test method used, together with a reference to this document, i.e. ISO 22104:—;

b) identity of the sample;

c) expression of the results in accordance with Clause 13;

d) report of all circumstances that can have affected the result;

e) information regarding pretreatment, extraction methods and clean-up as well as any alterations in the method.

Annex A
(informative)

Use of high resolution mass spectrometry detectors (HRMS)

A.1 Scope

The use of alternate mass spectrometry detectors based on high resolution accurate mass spectrometry (HRMS) instead of tandem mass spectrometry (MS/MS) may be possible, provided they show comparable sensitivity and selectivity for the target analytes included in the method[6],[7].

A.2 Principle of the method

The procedure is identical to that specified in the main body of this document. The only difference is in the use of HRMS instead of MS/MS for the instrumental detection.

A.3 Interferences

There might be isobaric ions with similar exact mass as the target analytes that can interfere with the analysis. For this reason, HRMS should be operated at a resolution no less than 20 000 (full width at half maximum) with a mass accuracy no more that five parts per milion (ppm). Chromatograms should be extracted with a 0,02 *m/z* window. Some microcystin variants have the same exact mass and similar retention time, therefore can not be easily distinguished by LC-HRMS alone. In these cases, MS/MS is preferred.

A.4 Apparatus

There are mainly two different mass analysers that can meet the sensitivity and selectivity requirements to produce results comparable as those obtained by MS/MS: high resolution time of flight mass spectrometers (HRToF MS), and Orbitrap® [1] mass spectrometers. Both instruments are acceptable as long as they can meet the requirements specified in A.3.

A.5 Typical high resolution mass spectrometer conditions

Set up conditions can vary depending on the different instrument models. Conditions described in Table A.1 were optimized based on high resolution time of flight mass spectrometry.

Table A.1 — High resolution mass spectrometer conditions

Parameter	Value
Scan type:	Full scan MS
Mass range:	400 *m/z* to 1 200 *m/z*
Ion mode:	Positive ESI
Mass resolution:	> 20,000
Mass accuracy :	< 5 ppm
Chromatogram mass window :	0,02 Da

1) Orbitrap® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Table A.1 *(continued)*

Parameter	Value
Analyser mode:	Sensitivity
Dynamic range:	Normal
Target enhancement:	Off
Acquisition start time (min):	3
Acquisition end time (min):	10
Scan time:	0,25 s/scan (4 Hz)
Data format:	Centroided
Lock mass:	Leucine Enkephalin

A.6 Mass spectrometric acquisition

The HRMS acquisition conditions for all the analytes included in this document are detailed in Table A.2.

Table A.2 — Microcystins HRMS acquisition conditions

Compound	Adduct	Exact mass	Qualifier fragment 1	Qualifier fragment 2	Chromatogram mass window (Da)
Microcystin-dmRR	$[M+2H]^{2+}$	512,782 36	135,080 4	199,063 6	0,02
Microcystin-RR	$[M+2H]^{2+}$	519,790 18	135,080 4	213,087 0	0,02
Nodularin (IS)	$[M+H]^{+}$	825,450 52	135,080 4	213,087 0	0,02
Microcystin-LA	$[M+H]^{+}$	910,492 03	135,080 4	213,087 0	0,02
Microcystin-dmLR	$[M+H]^{+}$	981,540 38	135,080 4	199,063 6	0,02
Microcystin-LF	$[M+H]^{+}$	986,523 33	135,080 4	213,087 0	0,02
Microcystin-LR	$[M+H]^{+}$	995,556 03	135,080 4	213,087 0	0,02
Microcystin-LY	$[M+H]^{+}$	1 002,518 25	135,080 4	213,087 0	0,02
Microcystin-HilR	$[M+H]^{+}$	1 009,571 68	135,080 4	213,087 0	0,02
Microcystin-LW	$[M+H]^{+}$	1 025,534 23	135,080 4	213,087 0	0,02
Microcystin-YR	$[M+H]^{+}$	1 045,535 30	135,080 4	213,087 0	0,02
Microcystin-HtyR	$[M+H]^{+}$	1 059,550 95	135,080 4	213,087 0	0,02
Microcystin-WR	$[M+H]^{+}$	1 068,551 28	135,080 4	213,087 0	0,02

Annex B
(informative)

Use of online solid phase extraction coupled to liquid chromatography for the automated analysis of microcystins

B.1 Scope

Extraction, desorption and preconcentration steps during the sample preparation of water samples for microcystin analysis can be miniaturized and automated by using on-line solid phase extraction coupled to liquid chromatography. This approach substantially reduces sample preparation time, increasing the method's throughput and reducing the laboratory's response time in case of emergency, see References[5] to [8].

B.2 Principle

This method reproduces the same sample preparation steps as described in the main body of this document, with the difference that the most time-consuming steps are automated and instrumentally carried out by the liquid chromatograph (LC).

500 ml samples are collected (to ensure sample representativity) and homogenized. 5 ml aliquots are withdrawn and transferred to a 15 ml disposable polypropylene centrifuge tube. Cells are lysed by three cycles of freeze/thaw, and internal standard is added. 500 µl are directly injected in the LC, where microcystins are focused in the trapping column (extraction). More polar compounds and salts are not retained and are diverted to waste (clean-up). Using a six port valve, microcystins are eluted in opposite flow from the trapping column to the analytical column (elution), where the different variants are separated in time by chromatography using a gradient of aqueous-organic mobile phases prior to the MS detection (MS/MS or HRMS)[6],[7].

Interferences, biases and limitations of the method are the same as those described in the main body of this document.

B.3 Reagents and standards

NOTE Most of the reagents and reagent solutions are described in Clause 6 of the main body of this document.

B.3.1 General

B.3.1.1 Isopropyl alcohol, LC-MS grade.

B.3.1.2 Sodium tetraborate, $Na_2B_4O_7 \cdot 10H_2O$, 99,5 % purity.

B.3.1.3 Sodium hydroxide, NaOH, pellets, 97 % purity.

B.3.2 Preparation of solutions

B.3.2.1 Sodium tetraborate solution, 0,1 M.

In 1 l of pure water (6.1.1) dissolve 38,13 g of sodium tetraborate (B.3.1.2). Cap the bottle and shake to mix well. Final concentration is 0,1 M. Store the solution at room temperature in a nalgene (PP or PE) bottle. Prepare as required.

B.3.2.2 **Sodium hydroxide solution**, 0,4 M.

Dissolve 15,998 g of sodium hydroxide pellets (B.3.1.3) in 500 ml of pure water (6.1.1). Transfer to a 1 l volumetric flask and and fill up to the mark with pure water. Store the solution at room temperature in a Nalgene (PP or PE) bottle. Prepare as required.

B.3.2.3 **Borax buffer solution**, pH = 9,5.

Mix 1 l 0,1 M sodium tetraborate solution (B.3.2.1) with 176 ml of 0,4 M NaOH solution (B.3.2.2). Cap the bottle and shake to mix well. Store the buffer at room temperature. Prepare as required

B.3.2.4 **LC mobile phase αA,** acetonitrile, water 66:33, 0,3 % formic acid.

In a 2 l cylinder (7.2), combine 1,2 l of LC/MS grade acetonitrile (6.1.3) and 600 ml of pure water (6.1.1) and transfer to a 2 l bottle. Add 5,4 ml of pure formic acid (6.1.4). Roll bottle to mix well for 10 min.

B.3.2.5 **LC mobile phase αB,** water at pH 9,5.

In a 2 l bottle, add 30 ml of borax buffer (B.3.2.3) to 2 l of water (6.1.1). Cap the bottle and shake to mix well.

B.3.2.6 **LC mobile phase βA,** water, 0,1 % formic acid.

In a 1 l bottle, add 1 ml of pure formic acid (6.1.4) to 1 l of water (6.1.1). Cap the bottle and shake to mix well.

B.3.2.7 **LC mobile phase βB,** acetonitrile, 0,1 % formic acid.

In a 1 l bottle, add 1 ml of pure formic acid (6.1.4) to 1 l of LC/MS grade acetonitrile (6.1.3). Cap the bottle and shake to mix well.

B.3.2.8 **LC weak wash solvent**

In a 1 l bottle, mix 300 ml of acetonitrile (6.1.3), 300 ml of methanol (6.1.2), 300 ml of isopropyl alcohol (B.3.1.1) and 100 ml of water (6.1.1). Cap the bottle and shake to mix well.

B.3.2.9 **LC strong wash solvent**

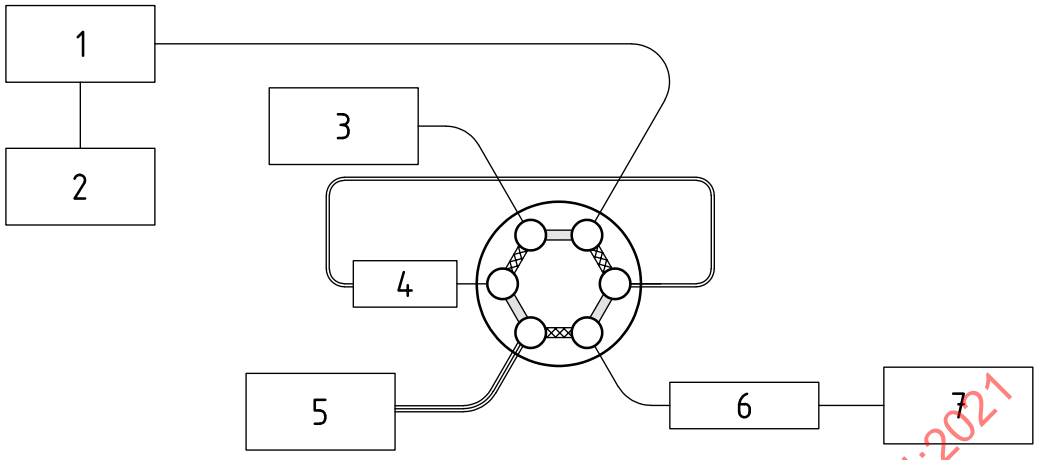
In a 1 l bottle, mix 900 ml of water (6.1.1) and 100 ml of acetonitrile (6.1.3). Cap the bottle and shake to mix well.

B.4 Apparatus

NOTE Most of the apparatus are described in Clause 7 of the main body of this document.

B.4.1 **Online solid phase extraction**

In order to perform on-line solid phase extraction coupled to LC, the chromatograph should be equipped with two binary pumps (one for the trapping stage and the other for the analytical separation stage), a column oven capable of fitting at least two columns, and a six-port valve capable to change the fluidic system from the trapping to the analytical separation stages. A diagram of the system is represented in Figure B.1 (see Reference [7]). The system shall also include an autosampler in order to automate large sequences of analyses.



Key

1 autosampler
2 loading pump
3 waste
4 trapping column
5 gradient pump
6 analytical column
7 MS
valve position: trap
valve position: elute

Figure B.1 — Diagram of a 2DLC in online solid phase extraction configuration

B.4.2 Trapping column, C8, 2,1 × 30 mm, 10 µm.

B.4.3 Analytical column, C_{18}, 2,1 × 150 mm, 1,8 µm.

B.4.4 Mass spectrometer

Mass spectrometric detection of the target analytes can be performed by MS/MS (9.2) or HRMS (Annex A), using the respective previously described detection conditions.

B.5 Procedure

B.5.1 Sample preparation procedure

The sample preparation procedure for drinking water and fresh water samples (9.1.6) and for calibration samples (calibration standard solutions (9.1.5)) and quality check samples (method blank (9.1.2), laboratory control spike sample (9.1.3), calibration control sample (9.1.4)) are the same as those described in Clause 9 of the main body of this document.

B.5.2 Automated instrumental analysis by online solid phase extraction coupled to liquid chromatography

B.5.2.1 Online SPE chromatograph set-up parameters

NOTE B.5.2.1 and B.5.2.2 require basic proficiency and familiarization of the analyst with the vendor's online SPE-LC hardware and software. Set up conditions can vary depending on the different instrument models. Conditions described in Table B.1 were optimized based on high resolution time of flight mass spectrometry.

Operate the on-line SPE chromatograph and mass spectrometer in accordance with the manufacturer's instruction and select the correct settings for the device. Identify the optimal settings for ionization under the specified chromatographic conditions and set the method-specific settings for the source and MS parameters (Table 4 and 5 in the main body or Table s A.1 and A.2), for example.

B.5.2.2 Online SPE liquid chromatograph set-up procedure

Check that there is sufficient solvent in the weak wash solvent (B.3.2.8), strong wash solvent (B.3.2.9) and in reservoirs αA (B.3.2.4), αB (B.3.2.5), βA (B.3.2.6) and βB (B.3.2.7). In the local instrument console, prime α and β pumps for 3 min, both channels.

B.5.2.3 Analysis procedure

Ensure that the instrument (liquid chromatography and mass spectrometer) parameters given in B.4.4 are correct and have been selected and the procedures listed in B.5.2 have been performed.

B.5.3 Run processing and quality assurance

B.5.3.1 Run sequence

A typical run format includes analysing:

1) Methanol injection;
2) Method blank (9.1.2.);
3) Calibration standard solutions (9.1.5);
4) Laboratory control spike sample (9.1.3);
5) Calibration control sample (9.1.4);
6) Methanol injection;
7) Samples including replicates (9.1.6);
8) Calibration standard solution MR5 (9.1.5) every 20 samples to check for instrument drift during long sequences
9) Methanol injection;
10) Calibration standard solution MR5 (9.1.5) to check for instrument drift at the end of the sequence.

Table B.1 — Online SPE liquid chromatograph set-up parameters

Parameter	Value
Mobile phase - Reservoir αA:	Acetonitrile: Water 66:33, 0,3 % formic acid
Mobile phase - Reservoir αB:	Water at pH 9,5 (borax buffer)
Mobile phase - Reservoir βA:	Water, 0,1 % formic acid
Mobile phase - Reservoir βB:	Acetonitrile, 0,1 % formic acid
1st dimension column (trapping):	C8 type column, 2,1 × 30 mm, 10 µm
2nd dimension column (analysis):	C18 type column, 2,1 × 150 mm, 1,8 µm

NOTE Changes in the instrumental conditions can be accepted as long as they offer similar online SPE and chromatographic performance.

[a] Xbridge® and UPLC® are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

Table B.1 *(continued)*

Parameter	Value
1st dimension flow (α-pump):	2 ml/min
1st dimension gradient (α-pump):	Start at 0 % A until minute 3,3
	Linear ramp to 95 % A until minute 3,4
	Hold at 95 % A until minute 6
	Linear ramp to 0 % A until minute 6,5
	Hold at 0% A until minute 13
Back pressure:	≈600 psi at initial conditions
Maximum pressure:	12 000 psi
Minimum pressure:	0 psi
Seal wash period:	5 min
2nd dimension flow (β-pump):	0,35 ml/min
2nd dimension gradient (β-pump):	Start at 95 % A until minute 3,75
	Linear ramp to 5 % A until minute 8,25
	Hold at 5 % A until minute 10
	linear ramp to 95 % A until minute 10,10
	hold at 95 % A until minute 13
Back pressure:	≈10 000 psi at initial conditions
Maximum pressure:	16 000 psi
Minimum pressure:	0 psi
Seal wash period:	5 min
Column compartment:	35 °C
Column manager	Position 2 at minute 0,01 (load in forward flow)
Left valve position:	Position 1 at minute 0,75 (elute in reverse flow)
	Position 2 at minute 13 (conditioning in forward flow)
Autosampler compartment:	5 °C
Syringe volume:	500 µl
Injection volume:	500 µl
Filling speed:	100 µl/min
Analysis/stop time:	13 min
NOTE Changes in the instrumental conditions can be accepted as long as they offer similar online SPE and chromatographic performance.	
[a] Xbridge® and UPLC® are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.	

B.5.3.2 Run control operations / limits

The control operation limits of quality check samples (method blank sample, low level recovery sample, laboratory control spike sample, calibration control sample and sample replicates) are the same as those described in 9.3.2 of the main body of this document.

B.5.3.3 Methanol injections

Methanol injections at the beginning of the sequence, before the samples and before the final calibration standard solution MR5 (9.1.5) are performed to ensure there is no contamination/carryover from previous injections above MDL.

If interfering blank values occur, identify the cause using systematic examination and eliminate the source of contamination.

Annex C
(informative)

Use of manual solid phase extraction prior to instrumental analysis for improved method detection limits

C.1 Scope

Depending on the performance of the instrument used, sometimes instrumental sensitivity alone might not be enough to reach the desired method detection limits (0,05 µg/l) by direct flow injection, even when using large volumes of injection (e.g. 100 µl). Annex C describes a manual dispersive solid adsorption phase step that allows reducing the method detection limits by preconcentrating the sample.

C.2 Principle

This method is designed to identify and quantify total (free + intracellular) microcystins in water by dispersive solid phase extraction using octadecyl-functionalized (C_{18}) silica gel, and analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) with electrospray ionization. Twelve microcystins (Table 1 of the main body of this document) are determined quantitatively by multi-point calibration using an internal standard. Water samples are filtered through a GF/C filter and the filtrate is saved. Cells are lysed by lyophilization, and intracellular toxins are extracted. This extract is combined with the original filtrate containing the extracellular toxins. The internal standard is added to the sample followed by of C_{18} silica gel as adsorbent. The sample is rolled for 1 h and then the C_{18} silica gel containing the adsorbed microcystins is filtered. The target compounds are desorbed with methanol. The methanol extract is evaporated to dryness and the residues redissolved in a 50 % methanol/50 % water solution prior to the instrumental analysis. Quantitation of microcystins is done by an internal standard method using LC-MS/MS or HRMS.

C.3 Reagents and standards

NOTE Most of the reagents and reagent solutions are described in Clause 6 of the main body of this document.

C.3.1 General

C.3.1.1 Octadecyl-functionalized (C18) silica gel, 200 mesh to 400 mesh, exent of labelling: 20 % to 22 % loading.

C.3.2 Preparation of solutions

C.3.2.1 Reagent solution 1, 75 % methanol / 25 % water.

Measure 750 ml of methanol (6.1.2) using a graduated cylinder (7.2) and pour into a 1 l bottle (7.1). Measure 250 ml of pure water (6.1.1) using a graduated cylinder (7.2) and pour into the same 1 l bottle (7.1). Cap the bottle and shake vigorously to mix well. Store at room temperature. Prepare as required.

C.3.2.2 Reagent solution 2, 50 % methanol / 50 % water.

Measure 500 ml of methanol (6.1.2) using a graduated cylinder (7.2) and pour into a 1 l amber bottle (7.1). Measure 500 ml of pure water (6.1.1) using a graduated cylinder and pour into the same 1 l amber bottle (7.1). Cap the bottle and shake vigorously to mix well. Store the reagent at room temperature. Prepare as required.

C.3.2.3 MCYST mix E solution

Prepare a solution with microcystin mass concentrations of, for example, 0,4 ng/µl.

For this use, for example, dilute 200 µl of MCYST mix A solution (6.2.9) to 10 ml with methanol (6.1.2) in a 25 ml volumetric flask. The concentration of each microcystin is 0,4 ng/µl.

C.4 Apparatus

NOTE Most of the apparatus are described in Clause 7 of the main body of this document.

C.4.1 Glassfibre filters, GF/C, 47 mm, Whatman™[2)].

C.4.2 Cryovials, polypropylene, 5 ml.

C.4.3 Filtration apparatus, glass, 1 l flask, for 47 mm and 55 mm diameter filter disks.

C.4.4 Squeeze and dropper bottle, low density polyethylene, 500 ml.

C.4.5 Filter paper, Whatman™[2)] No. 4, 55 mm diameter.

C.4.6 Spatula, stainless steel.

C.4.7 Aluminium weighing dish.

C.4.8 Centrifuge filters, Whatman™[2)], 0,45 µm, 47 mm diameter.

C.4.9 Centrifuge filter tubes, polypropylene, with 25 ml sample chamber and a 50 ml receiver tube with cap, polyethersulfone membrane with 10,000 Da molecular weight cutoff.

C.4.10 Pipette, 20 µl to 200 µl, adjustable.

C.4.11 Pipette tips, polypropylene, suitable for pipette 20 µl to 200 µl adjustable.

C.4.12 Centrifuge filter tubes, 1,5 ml, 0,2 µm.

C.4.13 Sample vials, appropriate for automated sample injection and with low adsorption, nominal volume 1,5 ml, clear glass, with wide openings suitable for inserts 0,35 ml flat bottom, with screw cap with PTFE/silicone septa with slit

C.4.14 Inserts, 0,35 ml, clear glass, flat bottom.

C.4.15 pH controls papers, pH 9 to 12.

C.4.16 Syringe, graduated, 1 ml.

C.4.17 Pipettes, class A, volumetric, 0,5 ml, 1 ml, 2 ml, 5 ml, 10 ml.

C.4.18 Flask, class A, volumetric, 10 ml, 25 ml, 50 ml, 100 ml.

2) Whatman™ is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.