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

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Foreword

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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).

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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 coumpounds 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 solution (6.2.5) 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, -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 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 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.

