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Water quality — Determination of microcystins — Method using solid phase extraction (SPE) and high performance liquid chromatography (HPLC) with ultraviolet (UV) detection

Qualité de l'eau — Dosage des microcystines — Méthode utilisant l'extraction en phase solide (SPE) et la chromatographie en phase liquide à haute performance (CLHP) avec détection dans l'ultraviolet (UV)

Reference number
ISO 20179:2005(E)

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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.

ISO 20179 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 2, *Physical, chemical and biochemical methods*.

Introduction

The user should be aware that particular problems could require the specification of additional conditions.

INTERNATIONAL STANDARD **ISO 20179:2005(E)**

Water quality — Determination of microcystins — Method using solid phase extraction (SPE) and high performance liquid chromatography (HPLC) with ultraviolet (UV) detection

WARNING — The method requires use of microcystin-containing solutions. Microcystins are highly hepatotoxic to humans. Laboratory wastes of microcystins shall be collected separately and disposed as highly toxic chemical waste. Long-term decontamination with concentrated sodium hypochlorite (NaClO) solution is also possible.

Persons using this International Standard should be familiar with normal laboratory practice. This standard 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 and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests conducted according to this standard be carried out by suitably trained staff.

1 Scope

This International Standard specifies a method for the determination and quantification of microcystins in raw water (containing biomass) and treated water, such as tap water. The method described is validated for MCYST-RR, MCYST-YR, and MCYST-LR. It is also applicable for the determination of several structure variants[1] of these microcystins, but an unambiguous identification cannot be made due to the lack of commercially available standards and due to co-elution.

The threshold value of 1 µg/l of MCYST-LR in water, proposed by the World Health Organization, can be followed after microcystin enrichment using solid phase extraction (SPE).

2 Normative references

The following referenced documents are indispensable for the application 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 3696:1987, *Water for analytical laboratory use — Specification and test methods*

ISO 5667-4, *Water quality — Sampling — Part 4: Guidance on sampling from lakes, natural and man-made*

ISO 5667-5, *Water quality — Sampling — Part 5: Guidance on sampling of drinking water from treatment works and piped distribution systems*

3 Abbreviated terms

For the purposes of this document, the following abbreviated terms apply.

APCI	atmospheric pressure chemical ionization
MCYST	microcystin
MCYST-LR	microcystin containing leucine (L) and arginine (R)

MCYST-RR	microcystin containing two arginine (R) units
MCYST-YR	microcystin containing tyrosine (Y) and arginine (R)
SIM	selected ion monitoring
SEC	size exclusion chromatography

4 Principle

Water samples containing cyanobacterial material (biomass) shall be filtered first. The biomass is extracted separately with a solvent (methanol/water). The extract is filtered, diluted and a solid phase extraction (SPE) is applied for sample clean-up. The filtrate is treated as a pure water sample (see below).

Pure water samples such as tap water are enriched using SPE. The microcystins are eluted from the SPE cartridges with methanol/water [90/10 by volume] containing 0,1 % by volume of trifluoroacetic acid (TFA).

Microcystins are quantified by reversed-phase high performance liquid chromatography (RP-HPLC) with ultraviolet/diode array detection at 238 nm.

5 Reagents

Use only reagents of recognized analytical grade and water complying with grade 3 as specified in ISO 3696:1987, unless otherwise specified.

5.1 **Methanol**, CH_3OH, HPLC grade.

5.2 **Acetonitrile**, CH_3CN, HPLC grade.

5.3 **Trifluoroacetic acid**, TFA, CF_3COOH.

5.4 **Standard dilution solution**, SPE rinsing solvent, and re-dissolving solvent.

Methanol/water [20/80 by volume].

5.5 Extraction solution

Methanol/water [75/25 by volume].

5.6 SPE elution solution

Methanol/water [90/10 by volume] containing 0,1 % by volume TFA.

5.7 **Sodium thiosulfate**, solution.

Dissolve 1 g of sodium thiosulfate $Na_2S_2O_3$ (anhydrous or with 5 H_2O) in 100 ml of water. The final concentration is ρ = 10 g/l (63 mmolar in case of anhydrous Na_2SO_3).

5.8 Ammonium hydroxide solution

Commercially available ~ 1 mol/l of ammonium hydroxide solution, NH_4OH.

5.9 Solid phase extraction cartridges (SPE) for microcystin enrichment

The column shall have a minimum capacity (amount of analyte to be retained by the column) of not less than 100 µg of each microcystin and shall give a recovery of not less than 80 % for MCYST-LR and not less than 70 % for MCYST-RR and MCYST-YR when applied as a standard solution in water containing 0,05 µg of each microcystin.

NOTE The recovery strongly depends on the SPE cartridge material/brand, material specifications such as carbon load, particle size etc. The recovery data are based on C-18 cartridges determined by a single measurement. The material should have the following material specifications: carbon load (16,9 %), particle diameter (54 µm), surface coverage (333 µg/m^2 based on % C) cartridge volume (3 ml), material per cartridge (500 mg). If the above required recovery values can not be reached, changing the brand of the SPE cartridge is recommended.

Disk-type SPE cartridges may also be used for the microcystin enrichment from water samples [2].

5.10 HPLC mobile phase solution (A)

To a 1 000 ml volumetric flask, add 800 ml of acetonitrile (5.2) and 500 µl of TFA (5.3) and bring to volume with acetonitrile. Transfer this solution in a HPLC-eluent bottle. Degas the solution before use.

This solution is stable at room temperature for about 3 weeks.

5.11 HPLC mobile phase solution (B)

To a 1 000 ml volumetric flask, add 800 ml of water and 500 µl of TFA (5.3) and bring to volume with water. Transfer this solution in a HPLC-eluent bottle. Degas this solution before use.

This solution is stable at room temperature for about 2 weeks.

5.12 HPLC mobile phase gradient (an example)

Table 1 — HPLC mobile phase gradient

Time min	**HPLC mobile phase solution (A) Acetonitrile with 0,05 % TFA** (5.10) %	**HPLC mobile phase solution (B); water with 0,05 % TFA** (5.11) %	**Total volume flow rate, depending on the column** ml/min
0	30	70	0,3 to 1,0
10	35	65	0,3 to 1,0
40	70	30	0,3 to 1,0
42	100	0	0,3 to 1,0
44	100	0	0,3 to 1,0
46	30	70	0,3 to 1,0
55	30	70	0,3 to 1,0

5.13 **Microcystins**, commercially available film in ampoules.

NOTE The quality of commercially available microcystins is very variable. Thus, it is important to follow the procedure given in 5.14.

5.14 Microcystin stock solutions

To determine the exact concentration of microcystins, dissolve in each stock solution the individual microcystin delivered from the supplier in pure methanol (5.1). Record the absorption curve between 220 nm and 250 nm in 1 cm quartz glass cells in a spectrophotometer with methanol (5.1) in the reference cell.

Calculate the mass concentration of each microcystin, ρ_i, in micrograms per millilitre, µg/ml, using Equation (1):

$$\rho_i = \frac{A_{max} \cdot M_i \cdot 1000}{\varepsilon_i \cdot d} \qquad (1)$$

where

A_{max} is the absorbance determined at the maximum of the absorption curve;

M_i is the molar mass of each microcystin, in grams per mol; g/mol;

ε_i is the molar absorptivity of each microcystin in methanol (5.1), in litres per (mole × centimetre), l/(mol × cm);

d is the optical path length of the cell, in centimetres, cm;

1 000 is a calculation factor to achieve the final unit micrograms per millilitre, µg/ml.

M_i and ε_i are tabulated in Table 2.

Table 2 — Molar mass and molar absorptivity of MCYST-LR, -YR, and -RR (in methanol, at 238 nm)

Microcystin	M_i g mol^{-1}	ε_i l mol^{-1} cm^{-1}
-LR	994	39 800
-YR	1 044	39 800
-RR	1 037	39 800
NOTE Data taken from Reference [1]. For further details refer to this reference.		

For further HPLC analysis, the solvent methanol/water ratio for the MCYST-LR, -YR, and -RR standards can be adjusted to 20/80 by volume (i.e. to the standard dilution solution described in 5.4), by adding water and allowing a concentration of 10 µg/ml for each microcystin.

5.15 Mixed microcystin stock solutions

Prepare a standard solution containing 2,5 µg/ml each of MCYST-LR, -YR, and -RR in the standard dilution solution (5.4). Store it below −16 °C. To avoid incorporation of water by condensation, do not open the vial until its contents have reached room temperature.

If the solution is to be stored for a long period, use a hermetic vial. In case of doubt, weigh the vial and record any changes in mass during storage.

5.16 Mixed microcystin standard solutions

Pipette the volumes of microcystin stock solutions (5.14) given in Table 3 into 1 ml vials.

To each vial, add the volume of the standard dilution solution (5.4) given in Table 3 to achieve a final volume of 1 000 µl, and shake well.

Table 3 — Pipetting scheme for the mixed microcystin standard solutions

Standard solution	Withdrawal volume from each microcystin stock solution [MCYST-LR, -YR, -RR (5.14)] µl	Volume to add from the standard dilution solution (5.4) to achieve a final volume of 1 000 µl µl	Mass concentration of standard solution µg/ml		
			MCYST-LR	**MCYST-YR**	**MCYST-RR**
1	20	940	0,2	0,2	0,2
2	40	880	0,4	0,4	0,4
3	100	700	1,0	1,0	1,0
4	200	400	2,0	2,0	2,0
5	300	100	3,0	3,0	3,0

5.17 Spiking solution for method control

Prepare a spiking solution by pipetting 200 µl of the mixed microcystin stock solution (prepared according to 5.15) into a 500 ml volumetric flask. Dilute it to the mark with water (tap-water or blank water from a natural lake), and shake well.

The concentration of this spiking solution is 1 µg/l for MCYST-LR, -YR, and -RR.

6 Apparatus

6.1 General

The laboratory glassware and equipment to be used is not specified in this International Standard, as the choice of apparatus will depend on the specific applications and circumstances.

Avoid the use of plastics whenever possible. This is necessary because the use of plastics (e.g. plastic pipettes, plastic tubing or plastic cartridges) may cause losses of microcystins through absorption on the surface walls.

6.2 Adjustable horizontal shaker, needed only for the analysis of samples containing phytoplankton.

6.3 Glass microfibre filter paper, retention size 1 µm to 2 µm.

The maximum diameter of the filter should be 47 mm.

Filtration is needed only for the analysis of samples containing phytoplankton.

6.4 SPE reservoir, 500 ml with connector for cartridges.

6.5 Vacuum pump for SPE

6.6 Laboratory centrifuge, $\geqslant$ 4 000 min^{-1}, relative centrifugal force (RCF) $\geqslant$ 10 000 g.

The use of an explosion-proof centrifuge is strongly advised due to the use of inflammable extraction solvents.

6.7 Ultrasonic probe, with characteristics of ~ 60 W, ~ 20 kHz.

6.8 Ultrasonic bath

6.9 Heating block with temperature control and nitrogen gas delivery unit, with the following characteristics: block-temperature 30 °C to 50 °C; gas temperature: ~ 20 °C; and gas-purity $\geqslant$ 99,996 %.

6.10 Disposable filter unit, pore size < 0,45 µm.

Prior to use, verify that no microcystin losses occur during filtration (recovery testing).

NOTE There is a possibility that various filter materials may retain microcystins. Optionally a microfuge may be used in order to avoid losses.

6.11 Sampling bottles, dark glassware, sterile and pre-cleaned.

6.12 HPLC system, consisting of the following.

6.12.1 Binary HPLC pump, suitable for volume flow rates between 0,3 ml/min and 1,0 ml/min.

6.12.2 HPLC column oven, with temperature control unit (35 °C).

6.12.3 Injection system, with injection volume range from 5 µl to 20 µl.

6.12.4 HPLC column (e.g. C_{18} column), packed with material of particle size 3 µm to 5 µm and inner diameter 2 mm to 4,6 mm, length 250 mm, to ensure baseline resolution of MCYST-LR, -YR, and -RR standards.

A suitable guard-column should be used. Pressure range should be 70,000 hPa to 200,000 hPa (1 015 psi to 2 900 psi).

6.12.5 UV/photo diode array (PDA) detector, with a wavelength of λ = 238 nm including background correction.

The wavelength range of the PDA shall be 200 nm to 300 nm.

The limit of detection (LOD) for the system should be $\leqslant$ 0,1 ng/µl (signal-to-noise-ratio = 3), and the limit of quantification (LOQ) should be $\leqslant$ 0,2 ng/µl (signal-to-noise-ratio = 6) for each microcystin (using a standard solution).

7 Procedure

7.1 Sampling and conditioning

Collect water samples as specified in ISO 5667-4 and ISO 5667-5 and store the samples (not longer than 48 h) in a cool (4 °C to 8 °C) and dark place.

Prior to conditioning, adjust the SPE cartridge (5.9) to room temperature.

For conditioning, refer to the suppliers´ recommendation. If this is not indicated, first pass 4 ml of methanol (5.1) through the cartridge. Then pass 4 ml of water through the cartridge. Let the solvents pass at a rate < 10 ml/min through the column, and make sure that a small portion of the solvent remains on top of the column until the sample solution is applied.

7.2 Sample preparation

7.2.1 Treated water/tap water

Concentrate microcystins in water samples using solid phase extraction (7.4).

7.2.2 Raw water containing phytoplankton

First pass the sample (recommended volume: 50 ml to 100 ml) through a filter (6.3) to separate the biomass from the liquid fraction. If floating layers of algae are present, one filter may be insufficient for the filtration of even 50 ml of water. In this case, immediately replace the clogged filter with a fresh one. Concentrate the microcystins in the filtrate by solid phase extraction (see 7.4).

Extract the biomass on the filter separately (see 7.3) followed by clean-up (see 7.5) of the extract prior to HPLC analysis (see 7.6).

NOTE If a gravimetric filter is used, the mass of the biomass may be determined and the microcystin content can be expressed in micrograms per gram (µg/g).

7.3 Extraction of microcystins from the cells on the filter

To extract the cells on the filter (if more than one filter is used, combine the filters), rinse the filter(s) three times with 3 ml of the extraction solution (5.5).

Sonicate the solution on ice for 2 min with an ultrasonic probe (6.7) or in an ultrasonic bath (6.8).

After that, centrifuge the solution at $\geqslant$ 4 000 min^{-1} for 10 min at room temperature.

After centrifugation, pool the supernatants and blow 1 ml of this solution to dryness under a nitrogen stream (40 °C).

Prior to clean-up, re-dissolve the extracts in 500 µl of standard dilution solution (5.4) and sonicate the sample in a bath for 5 min.

7.4 Solid phase extraction (SPE) for microcystin enrichment

To avoid losses, ensure that the pH of the water sample is in the range of 5,0 to 8,0. If the pH is outside of this range, adjust with either trifluoroacetic acid (5.3) or ammonium hydroxide solution (5.8), as appropriate.

Add 500 µl of sodium thiosulfate solution (5.7) to 500 ml of raw water filtrate or treated water sample. Shake well and allow the mixture to stand for 5 min.

Add 5 ml of methanol (5.1) and, after shaking, apply to the conditioned (see 7.1) cartridge at a flow rate of 10 ml / min or less (visible drops). Once the water sample has passed through the cartridge, rinse the cartridge with 4 ml of standard dilution solution (5.4).

Elute the enriched microcystins with either 2,0 ml of SPE elution solution (5.6) in a 4 ml glass vial (or follow the supplier's recommendation).

Evaporate the eluate to dryness with a nitrogen stream (40 °C).

Re-dissolve in 500 µl of standard dilution solution (5.4) and sonicate the sample for 5 min in a bath.

Analyse this extract directly with HPLC (7.6).

7.5 Solid phase extraction (SPE) for microcystin clean-up

Apply the extracted microcystins (7.3) to the top of the conditioned cartridge (7.1) and rinse the extract vial with an additional 500 µl of standard dilution solution (5.4) and apply it also on top of the cartridge.

After the liquid has passed through the cartridge, rinse it with 4 ml of standard dilution solution (5.4) and discard the eluate. When the rinsing solution has passed through the cartridge, elute the cleaned microcystins according to the supplier's recommendation, e.g. 2,0 ml of SPE elution solution (5.6) in a 4 ml glass vial.

Evaporate the eluate to dryness with a nitrogen stream (40 °C), re-dissolve in 500 µl of standard dilution solution (5.4) and sonicate the sample in a bath for 5 min.

If dilution of the sample is necessary, dilute 100 µl of the sample extract (7.3) with 900 µl of standard dilution solution (5.4).

If clean-up with cartridges does not reduce the co-elution, size exclusion chromatography (SEC)[3], clean-up with immunoaffinity columns[4] or polymeric materials may be used as an alternative.

NOTE For further literature, see also References [5] to [7].

7.6 High performance liquid chromatography (HPLC)

To guarantee maximum precision, inject the purified or enriched microcystins according to the instructions for the injection valve or injection port manufacturer.

Separate the microcystins by HPLC at 35 °C with a reversed phase column (6.12.4) using the gradient (5.12).

Adjust the volume flow rate and the injection volume according to the column dimensions (inner diameter, particle size) to obtain the optimal peak shape and resolution. The microcystins elute in the order MCYST-RR, MCYST-YR, and MCYST-LR and should be base-line resolved.

Acquire absorption spectra between 200 nm and 300 nm to confirm the identification. A typical chromatogram and typical absorption spectra of microcystins with PDA detection are included in Annex B.

7.7 Calibration curve

Measure the chromatographic signals at 238 nm (peak heights or areas) of each compound in the mixed microcystin standard solution (5.16).

Prepare the three calibration curves (MCYST-RR, MCYST-YR and MCYST-LR) by injecting an appropriate volume (5 µl to 20 µl) of the mixed microcystin standard solutions described (5.16). These solutions cover the range of 0,2 µg/ml to 3,0 µg/ml.

Set up the calibration curve (method of least squares) for each microcystin (area on y-axis and concentration on x-axis) using linear regression (Equation 2) and check the plot for linearity.

$$y = mx + b \tag{2}$$

where

b is the intercept with y-axis (area);

m is the slope in millilitres per micrograms, ml/µg;

x is the value from x-axis, in micrograms per millilitres, µg/ml;

y is the value from y-axis (area).

The working range is defined as the linear part of the calibration curve. If the contents of microcystin in the sample lie outside of the calibration range, adjust the calibration range according to the samples. Alternatively, the injection solution for HPLC analysis may be diluted with standard dilution solution (5.4) to a microcystin concentration appropriate for the established calibration curve. The varying methanol concentration of the injection solution may have an effect on quantification [7].

7.8 Spiking procedures

For the determination of the recovery, carry out the spiking procedure using the spiking solution (5.17). The spiking level shall be within the calibration range (preferably mean values).

7.9 Calculation of results for water samples

Calculate the mass concentration of each microcystin, in micrograms per litre, of the sample according to Equation (3) after solving Equation (2) for each microcystin:

$$\rho_{\text{MCYST, diss}} = \frac{(y-b)\cdot V_{\text{d}}}{m\cdot V_{\text{samp}}} \tag{3}$$

where

$\rho_{\text{MCYST, diss}}$ is the mass concentration of each individual microcystin in micrograms per litre, µg/l;

V_{d} is the re-dissolving volume of the sample after SPE, in millilitres, ml;

V_{samp} is the volume of water sample applied on SPE cartridge in litres, l;

y, b, m see Equation (2).

7.10 Calculation of results for biomass

Calculate the mass concentration of each microcystin in phytoplankton (procedure given under 7.3) in microgram per millilitre of the sample according to Equation (4) after solving Equation (2):

$$\rho_{\text{MCYST, part}} = \frac{(y-b)\cdot f\cdot V_{\text{ex}}}{m\cdot V_{\text{sam}}} \tag{4}$$

where

$\rho_{\text{MCYST, part}}$ is the mass concentration of each individual microcystin in particulate matter in microgram per millilitre, µg/ml;

V_{ex} is the extraction volume in millilitre, ml;

V_{sam} amount of filtered sample volume in millilitre, ml;

f dilution factor in the cell extraction step;

y, b, m see Equation (2).

NOTE Alternatively, a computer-aided quantification may be used.

7.11 Expression of results

Report the results of Equation (3) and Equation (4) separately. The results may be summed up for samples containing phytoplankton. Under natural conditions, the majority of microcystins are included in the particulate material, and usually less than 20 % is dissolved in the water.

Microcystins **other than** MCYST-RR, MCYST-YR, and MCYST-LR may be identified/recognized by their UV-spectra (Figure B.2 in Annex B). Their mass concentrations can be estimated using the MCYST-LR calibration curve. Report these results as MCYST-LR equivalents.

Report the mass concentrations of each microcystin in micrograms per litre (µg/l) to two significant figures. The analytical results obtained with this standard are subject to uncertainty which needs to be taken into account when interpreting the results. Methods have been elaborated for the evaluation of the uncertainty allowing the estimation of uncertainty from validation data within one laboratory, routine quality assurance data (range/mean control charts) as well as validation and accreditation data. Preferably, the uncertainty is stated as the extended uncertainty. For this purpose, the combined standard uncertainty – expressed as the standard deviation resp. the variation coefficient – is multiplied with an extension factor of 2. This corresponds to a confidence level of about 95 %.

In this International Standard, the uncertainty is estimated using the repeatability standard coefficients (CV_{R}) (see Table C.1), multiplied by 2. The derived extended uncertainty U of the method serves as an orientation, however, it cannot replace the estimation of the uncertainty from intra-laboratory data.

NOTE The uncertainty is concentration- and matrix-dependent, and its values increase when lowering the application range of the method.

8 Method performance characteristics and data

Details of the interlaboratory test of the precision of the method are summarized in Annex C. The values derived from the interlaboratory test may not be applicable to analyte concentration ranges and matrices other than those given in Table C.1.

9 Test report

This clause specifies which information is to be included in the test report. The clause shall require information to be given on at least the following aspects of the test:

a) reference to this International Standard (ISO 20179:2005);

b) identification of the water sample;

c) expression of results as specified in 7.11;

d) the data and type of sampling procedure (if known);

e) the test results and the units in which they have been expressed;

f) whether the repeatability has been verified;

g) any particular points observed in the course of the test;

h) if applicable, an indication of any circumstances that may have affected the results.

Annex A
(informative)

Mass spectrometry (MS) as an alternative detection

A.1 General

The unambiguous identification of the microcystin other than MCYST-RR, -YR, -LR cannot be guaranteed by using PDA detection. To obtain further information of the compounds of question, a mass spectrometric (MS) detector with atmospheric pressure ionization (ESI or APCI) should be applied. This detector may also be used optionally, in addition to the UV/PDA detector, to confirm the results obtained by UV/PDA detection.

A.2 Instrumental settings for MS

Operate in the selected ion monitoring (SIM) mode for MCYST-RR (m/z = 519,8, m = mass of the particle, z = number of charges on the particle), MCYST-YR (m/z = 1 045,5) and MCYST-LR (m/z = 995,6) or in scan mode [m/z = 500 to 600 (doubly charged MCYSTs) and 900 to 1 200 (singly charged MCYSTs)].

The instrument-settings (ion source, etc.) will depend on the particular instrument used.

A list MCYSTs with their relative molecular masses is given in Table A.1. Compounds identified as microcystins can be expressed in MCYST-LR equivalents, but the results are at best only approximations due to differences in ionization properties of different microcystins.

Table A.1 — Microcystins sorted according to their relative molecular masses (M_r)

No.	M_r	Microcystin
1	909	MCYST-LA
2	923	MCYST-Laba
2a	923	MCYST-Lbu
3	952	MCYST-AR
3a	952	MCYST-RA
4	959	MCYST-YA
5	965	MCYST-HilR
6	966	[D-Asp^3, Dha^7]MCYST-LR
7	969	[D-Asp^3,Dha^7]MCYST- EE(OMe)
8	971	MCYST-VF
8a	971	[D-Asp^3]MCYST-LF
9	980	[D-Asp^3]MCYST-LR
9a	980	[D-Asp^3,(E)-Dhb^7]MCYST-LR
9b	980	[D-Asp^3,(Z)-Dhb^7]MCYST-LR
10	980	[Dha^7]MCYST-LR
11	980	[D-Asp^3, (E)- Dhb^7]MCYST-LR
12	980	[DMAdda^5]MCYST-LR
12a	982	MCYST-AW

Table A.1 (*continued*)

No.	M_r	Microcystin
13	983	[D-Asp^3, Dha^7]MCYST- E(OMe)E(OMe)
14	983	[Dha^7]MCYST-EE(OMe)
15	985	MCYST-LF
16	994	MCYST-LR
17	994	[D-Asp^3, D-Glu-OCH$_3^6$] MCYST-LR
18	994	[D-Asp^3]MCYST-LHarg
19	997	[Dha^7]MCYST-E(OMe)E(OMe)
20	998	[L-Ser^7]MCYST-LR
21	1001	[D-Asp^3,L-Ser^7]MCYST- E(OMe)E(OMe)
22	1001	[L-Ser^7]MCYST-EE(OMe)
23	1001	MCYST-LY
24	1008	[D-Asp^3, ADMAdda^5, Dhb^7]MCYST-LR
25	1008	[D-Asp^3, ADMAdda^5]MCYST-LR
25a	1008	MCYST-LHar
26	1009	[D-Asp^3, Dha^7]MCYST-RR
26a	1010	Demethyl-MCYST-LW
27	1012	[L-MeSer^7]MCYST-LR
28	1015	[L-Ser^7]MCYST- E(OMe)E(OMe)
28a	1014	[D-Asp^3]MCYST-FR
29	1014	[Dha^7]MCYST-FR
30	1022	[ADMAdda^5]MCYST-LR
31	1022	[(6Z)-Adda^5]MCYST-LR
32	1022	[D-Asp^3, ADMAdda^5]MCYST-LHarg
33	1023	[D-Asp^3]MCYST-RR
34	1023	[Dha^7]MCYST-RR
34a	1023	[D-Asp^3,(E)-Dhb^7]MCYST-RR
35	1023	[D-Asp^3, Dhb^7]MCYST-RR
36	1024	MCYST-LW
37	1028	MCYST-M(O)R
38	1028	MCYST-FR
39	1028	[Dha^7]MCYST-HphR
40	1030	[D-Asp^3]MCYST-YR
41	1030	[Dha^7]MCYST-YR
42	1030	[D-Asp^3, Dha^7] MCYST-HtyrR
43	1036	[ADMAdda^5]MCYST-LHarg
43a	1036	[D-Leu^1]MCYST-LR
44	1037	MCYST-RR
45	1037	[(6Z)-Adda$_5$]MCYST-RR
45a	1037	[D-Asp^3,D-Glu(OCH$_3$)^6]MCYST-RR
46	1038	[D-Ser^1, ADMAdda^5]MCYST-LR
47	1040	[ADMAdda^5, MeSer^7]MCYST-LR

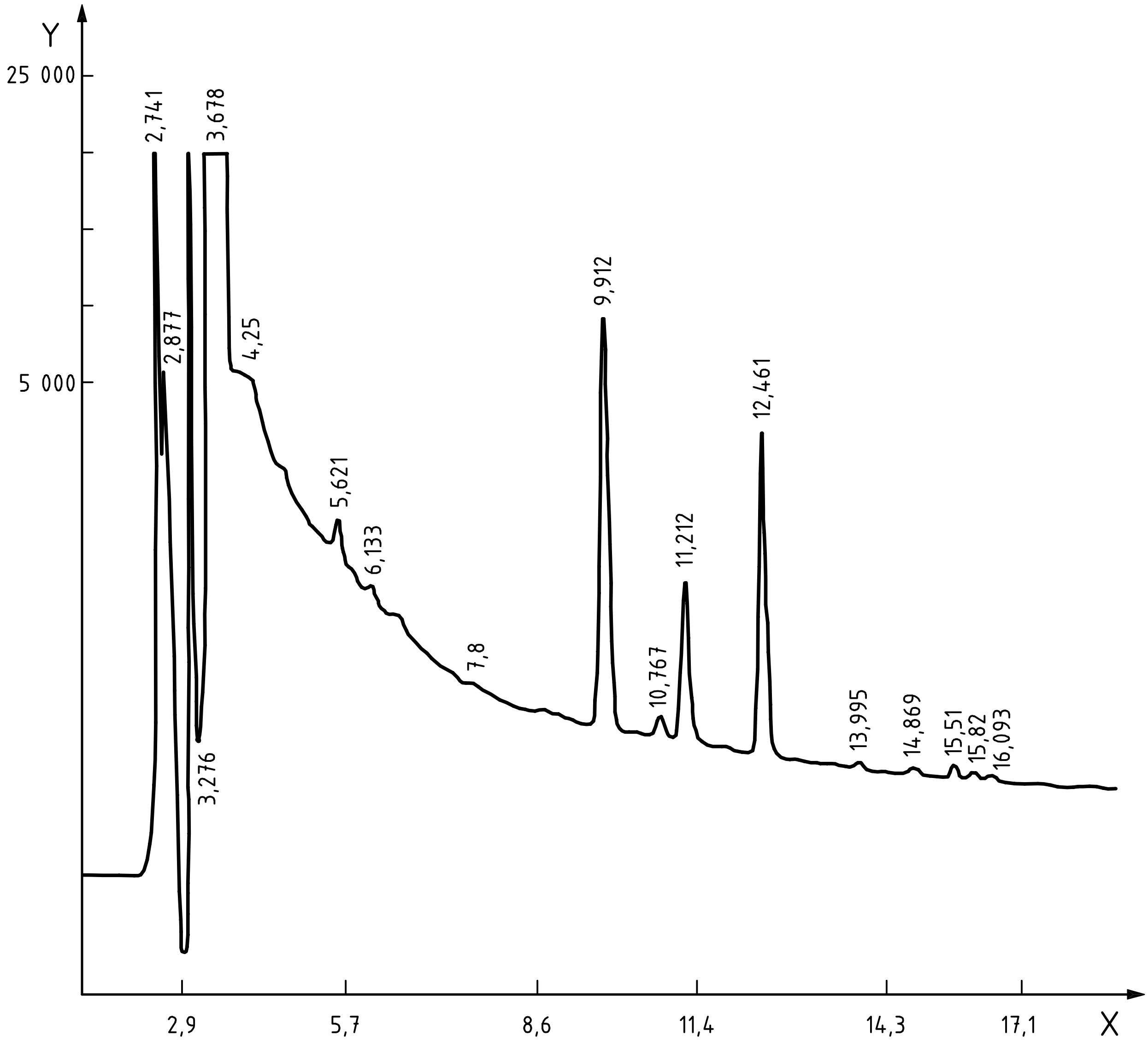
Table A.1 (*continued*)

No.	M_r	Microcystin
48	1041	[L-Ser^7]MCYST-RR
49	1041	[D-Asp^3, L-MeSer^7]MCYST-RR
49a	1041	*seco*[D-Asp^3]MCYST-RR
50	1044	MCYST-YR
51	1044	[D-Asp^3]MCYST-HtyrR
52	1044	[Dha^7]MCYST-HtyrR
53	1044	[D-Asp^3, (Z)Dhb^7]MCYST-HtyR
54	1044	[D-Asp^3, (E)- Dhb^7]MCYST-HtyR
55	1048	MCYST-(H_4)YR
56	1051	[D-Asp^3, ADMAdda^5, Dhb^7]MCYST-RR
57	1052	[D-Glu-($C_3H_7O^6$] MCYST-LR
57a	1053	[D-Asp^3]MCYST-WR
58	1055	MCYST-HtyrR
59	1059	[L-Ser^7]MCYST-HtyrR
60	1067	MCYST-WR

For further details about MS detection of microcystins, refer to References [10] and [11].

Annex B
(informative)

Typical chromatogram and absorption spectra



Key

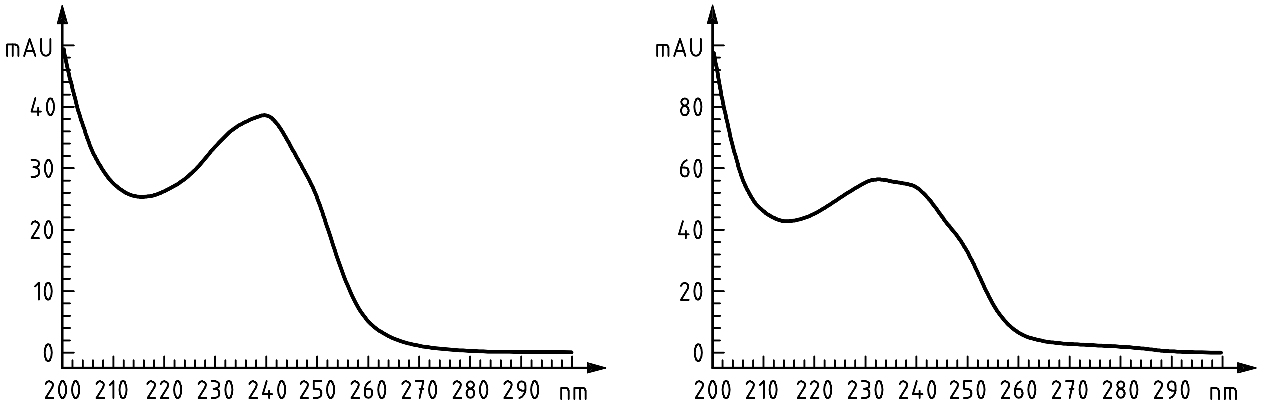
X retention time (min)
Y intensity (µV)

Operating conditions

Injection volume:	20 µl
Column:	Phenomenex, LUNA® (see Note), C18(2), 250 × 4,6 mm, 3 µm
Volume flow rate:	0,7 ml / min
Mobile phase:	Water containing 0,05 % TFA volume fraction (B) (5.11), acetonitrile containing 0,05 % TFA volume fraction (A) (5.10)
Detection:	PDA at 238 nm

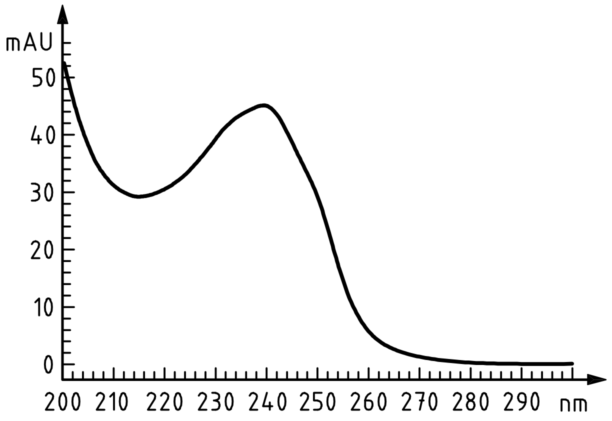
NOTE Phenomenex, LUNA® is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

Figure B.1 — Typical chromatogram of microcystin-RR (9,912 min), -YR (11,212 min), and -LR (12,461 min) in a spiked water sample after SPE (concentration in the water sample 1 000 ng/l)



a) Typical absorption spectra in an acetonitrile/water system of MCYST-RR

b) Typical absorption spectra in an acetonitrile/water system of MCYST-YR



c) Typical absorption spectra in an acetonitrile/water system of MCYST-LR

Operating conditions, taken from Reference [8]:

Detection	HPLC-PDA
Cuvette:	Quartz

NOTE Also see Reference [8] for spectra of other MCYSTs.

Figure B.2 — Typical absorption spectra in acetonitrile/water systems (MCYST-RR, MCYST-YR and MCYST-LR)

Annex C
(informative)

Precision data

The data in Table C.1 were obtained in an interlaboratory test carried out in accordance with ISO 5725-1 and ISO 5725-2.

Table C.1 — Precision data

Sample	Matrix	Parameter	l	n	n_{AP}	$\bar{\bar{x}}$	x_{ref}	η	s_R	CV_R	s_r	CV_r
A	spiked chlorinated drinking water, Jena, Germany	MCYST-RR	20	60	4,8	0,913	1,00	91,3	0,1773	19,4	0,0853	9,3
		MCYST-YR	15	45	28,6	0,896	1,00	89,6	0,0608	6,8	0,0270	3,0
		MCYST-LR	16	48	23,8	0,894	1,00	89,4	0,1344	15,0	0,0268	3,0
B	spiked surface water from the river Saale, Germany, containing dissolved and particulate organic material	MCYST-RR	21	63	0,0	0,770	1,00	77,0	0,3089	40,1	0,0699	9,1
		MCYST-YR	21	63	0,0	0,735	1,00	73,5	0,2741	37,3	0,0618	8,4
		MCYST-LR	20	60	4,8	0,782	1,00	78,2	0,2820	36,0	0,0632	8,1
C	spiked HPLC-water obtained from chemical industry	MCYST-RR	19	57	17,4	0,772	1,00	77,2	0,1351	17,5	0,0704	9,1
		MCYST-YR	23	69	0,0	0,761	1,00	76,1	0,1727	22,7	0,0793	10,4
		MCYST-LR	23	68	0,0	0,766	1,00	76,6	0,1801	23,5	0,0770	10,0

l is the number of laboratories after outlier rejection;

n is the number of analytical results after outlier rejection;

n_{AP} is the number of outliers, in percent;

$\bar{\bar{x}}$ is the total mean of results after outlier rejection, in micrograms per litre;

x_{ref} is the reference value, in micrograms per litre;

η is the recovery rate, in percent;

s_R is the standard deviation of the reproducibility, in micrograms per litre;

CV_R is the variation coefficient of the reproducibility, in percent;

s_r is the standard deviation of the repeatability, in micrograms per litre;

CV_r is the variation coefficient of the repeatability, in percent.

NOTE The intercalibration experiment was carried out in 2003 with 25 participating laboratories on an international basis (9 countries). Microcystins were enriched from the water samples with a unique SPE cartridge (Bakerbond, C-18). The high number of outliers (sample A: MCYST-YR and MCYST-LR) can be explained by unavoidable reaction of chlorination by-products with the analyte during the SPE enrichment.

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