Ohio EPA Total (Extracellular and Intracellular) Microcystins - ADDA by ELISA Analytical Methodology

OHIO EPA METHOD 701.0
Version 2.4

1. SCOPE AND APPLICATION

1.1. This method is used for the determination of total (extracellular and intracellular) Microcystins – ADDA in surface water, ground water and finished drinking water using enzyme-linked immunosorbent assay (ELISA), both manual and automated.

Reporting Limit (RL): 0.24 µg/L

2. SUMMARY OF METHOD

2.1. The Ohio EPA Total (Extracellular and Intracellular) Microcystins – ADDA by ELISA Analytical Methodology is an immunoassay for the detection of microcystins in water samples. This test is an indirect competitive ELISA allowing the congener-independent detection of microcystins and nodularins. It is based on the recognition of microcystins, nodularins and their congeners by specific antibodies. Microcystins, nodularins and their congeners when present in a sample and a microcystin-protein analogue immobilized on the plate compete for the binding sites of antibodies in solution. After a washing step and addition of the substrate solution, a color signal is generated. The intensity of the blue color is inversely proportional to the concentration of the microcystins present in the sample. The color reaction is stopped after a specified time and the color is evaluated using a microplate reader at 450 nm. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

3. SAMPLE COLLECTION AND PRESERVATION

3.1. A minimum of 100 mL should be collected in a glass or polyethylene terephthalate glycol (PETG) container.

> **NOTE:** Samples treated with chlorine or any other oxidizer (e.g. $KMnO_4$) must be quenched immediately after collection. 10.0 mg sodium thiosulfate added per 100 mL of sample is sufficient.

3.2. All samples must be protected from sunlight and cooled on ice at 0.0 – 10.0°C immediately after collection and maintained at 0.0 – 10.0º C until analysis.

3.3. Microcystin samples must be analyzed within 5 days from the time of collection.

Drinking water samples must be analyzed as soon as practical but no later than 5 days from the time of collection.

> **NOTE:** If the sample is not to be used for drinking water compliance, hold time can be increased by freezing the sample within 5 days of collection. The sample may be kept in this first freeze cycle indefinitely to be analyzed at a later date.

3.4. When freezing, allow adequate volume for expansion and place the glass sample container on its side to prevent breakage.

4. INTERFERENCES

4.1. Due to the high variability of compounds found in water samples, test interferences caused by matrix effects cannot be completely ruled out. Ohio EPA continues to work with U.S. EPA and other experts to identify and provide more guidance on potential interferences.

5. SAFETY

5.1. The toxicity or carcinogenicity of each reagent used in this method has not been fully established. Each chemical should be regarded as a potential health hazard and exposure should be as low as reasonably achievable. Cautions are included for known extremely hazardous materials or procedures.

5.2. A reference file of Safety Data Sheets (SDS) should be made available to all personnel involved in the chemical analysis. The preparation of a formal safety plan is also advisable.

6. APPARATUS

6.1. Glass or polyethylene terephthalate glycol (PETG) container: 100 mL

6.1.1. Cleaning of approved sample collection containers is acceptable as long as the laboratory can demonstrate effectiveness of the cleaning procedure by collecting and analyzing reagent water in 5% per batch of the cleaned containers. The reagent water results must be less than the reporting limit. The laboratory must document this procedure and must maintain these records.

6.2. Class 'A' Volumetric Flask: 500 mL

6.3. Bench sheets or logbooks

6.4. Micropipette: Capable of 10 - 100 µL

6.5. Multi-Channel Pipette: 50 - 300 µL

6.6. Stepping Pipette: 100 - 500 µL (optional)

6.7. Pipette Tips: Appropriate size for the pipette

6.8. Multi-Channel Pipette Reagent Reservoir: Minimum 50 mL capacity

6.9. Microplate Reader: Capable of analyzing at 450 nm

6.10. Glass Vials: 4.0 mL and 40.0 mL

6.11. Syringe Filters: 25 mm glass fiber, 0.45 µm or 1.2 µm pore size

6.12. Gastight Luer-Lock Syringes: 5.0 mL

6.13. ELISA Sealing Teflon Tape or equivalent

6.14. Freezer: Must maintain a temperature of ≤ 0.0ºC

6.15. Refrigerator: Must maintain a temperature of 4.0 ± 2.0ºC

6.16. Chlorine DPD Meter or Low-Range Chlorine Test Strips

6.17. pH Meter or pH Test Strips 0 - 14 pH units

6.18. Water Bath (optional): Must maintain a temperature of 35.0 ± 0.5ºC

6.19. Cyanotoxin Automated Assay System (CAAS) (optional)

7. REAGENTS

7.1. Liquid Disinfectant: Commercially prepared, Roccal® or equivalent disinfectant.

7.2. Analysis Kit (capable of analyzing all microcystin congeners with the ADDA structure): Store kit according to manufacturer's instructions. Standards and reagents may be used until the manufacturer's expiration date.

7.3. DPD-free Chlorine Reagent: Discard by manufacturer's expiration date.

7.4. Sodium Thiosulfate: De-chlorination agent (used for potable water). Discard by manufacturer's expiration date.

7.5. Reagent Water: Laboratory available deionized water. Quality of this must meet a minimum resistivity of 10.0 MΩ.

7.6. Ethanol/Dry Ice Mixture (optional)

7.7. Sodium Hydroxide Solution (0.1N): In a 1000 mL volumetric flask, dissolve 4.0 g sodium hydroxide (NaOH) in 800 mL reagent water. Stir to dissolve. Bring to volume with reagent water. Discard 1 year after date of preparation.

7.8. Hydrochloric Acid Solution (0.1N): In a 1000 mL volumetric flask, slowly and carefully add 8.3 mL concentrated hydrochloric acid (HCl) to 800 mL reagent water. Bring to volume with reagent water. Discard 1 year after date of preparation.

> **NOTE:** All reagents, standards and kits must be labeled with the received, opened and expiration dates. Prepared reagents must be labeled with content, date made, expiration date, and analyst initials. Prepared reagents must be discarded one year after preparation or the manufacturer's expiration date for items used, whichever comes first.

8. SAMPLE PREPARATION

> **NOTE:** Sample pH and chlorine levels must be checked upon receipt. An additional sample should be taken, or a portion of the sample should be poured off for these analyses.

8.1. Disinfect the work area.

8.2. Sample pH must be adjusted within the range of 5 – 11 pH units. Samples with pH levels outside of this range may produce inaccurate (falsely low) results and must be adjusted as necessary using 0.1N hydrochloric acid (HCl) or 0.1N sodium hydroxide (NaOH) solutions, prior to analysis.

8.3. Samples treated with chlorine: Check samples for residual chlorine. Any drinking water samples not sufficiently quenched (< 0.1 mg/L) must not be analyzed. Unquenched water samples must be recollected and appropriately quenched immediately after collection.

8.4. Samples treated with any other oxidizer must also be checked for sufficient quenching (< 0.1 mg/L). Insufficiently quenched raw water or treatment train samples may still be analyzed. Qualify the results with the appropriate qualifier (CL).

9. SAMPLE LYSING PROCEDURE BY FREEZE/THAW

9.1. Shake the sample and pour approximately 20.0 mL of the sample into two properly labeled, 40.0 mL vials to begin the three freeze/thaw lysing cycles. Keep the second vial in the third freeze cycle in case original sample vial cracks.

9.2. Place vials in the freezer until completely frozen (To speed up the process, vial(s) may be immersed in a saturated sodium chloride solution or dry ice/ethanol solution).

NOTE: Do not fully submerge the vial(s) if using either solution.

NOTE: Place sample vial(s) on its side in freezer to prevent vial(s) from cracking as the water freezes and expands.

9.3. Once sample is completely frozen, remove from freezer (or from sodium chloride solution) and thaw. To speed up the thawing process, vial(s) may be left at room temperature, placed in a container of lukewarm water or placed in a 35.0ºC water bath until completely thawed.

NOTE: Do not fully submerge the vial(s).

9.4. Repeat steps 9.2 and 9.3, two more times.

9.5. Once sample is completely thawed for the 3rd time, draw 5.0 mL of sample into the syringe and attach a filter.

9.6. Rinse the filter by passing a minimum of 5.0 mL sample through the filter and discard the filtrate.

9.7. Again, draw 5.0 mL of the sample into the syringe, re-attach the rinsed filter, and filter approximately 2.0 mL of sample into two, properly labeled, 4.0 mL vials. Samples are ready for immediate analysis.

10. INITIAL/ANNUAL DEMONSTRATION OF CAPABILITY

For both manual and automated analyses, to maintain the reporting limit set in the method, demonstration of the capability to achieve a Method Detection Limit (MDL) less than the reporting limit must be performed by each new analyst seeking certification or whenever there is a change in analytical performance (i.e., a change in instrument hardware or operating conditions).

MDLs must be established for microcystins using a standard with a concentration between one and ten times the reporting limit. To calculate the MDL value, during the same run, take seven replicate aliquots of the standard and process them through the entire analytical method. Once the results for the seven replicates have been obtained, calculate the MDL as follows:

$$MDL = (t)*(SD_R)$$

Where: t = Student's t value for a 99% confidence interval and a standard deviation estimates with n-1 degrees of freedom (t = 3.143 for the seven replicates)

SD_R = Standard deviation of the replicate aliquot analyses

The study will be valid if the resulting value of the MDL is no more than ten times lower than the replicate standard concentration level and does not exceed the replicate standard concentration level, and all QC requirements are met (see Section 12). Save and print a copy of each MDL study (including associated test data and calibration curves), adding the test kit lot number/expiration date and the analyst's initials as part of the laboratory's record maintenance protocol.

11. ANALYSIS

The accuracy of ELISA analysis is highly dependent upon analyst technique, adequate storage conditions of the test kit, pipetting sequence, accuracy of reagent volumes and maintenance of constant/optimum laboratory temperature during the analysis. The ELISA analysis is a time sensitive procedure. Care must be taken to ensure the reagent addition steps are completed in an efficient manner and incubation times are followed according to manufacturer's instructions.

11.1. Verify kit standards and reagents are used prior to the expiration date.

11.2. The assay procedure must be performed away from direct sunlight.

11.3. Bring samples and standards to room temperature prior to analysis.

11.4. Follow manufacturer's instructions provided with the individual Microcystins – ADDA kit for calibration, quality control (QC) and sample analysis procedures.

NOTE: If sample analysis results in a higher concentration than the highest standard in the calibration curve, the sample must be diluted and reanalyzed. If diluted, the sample must be diluted using the LRB to match the matrix and can be prepared in a 4.0 mL vial using the ratios in the table below.

Samples may not be diluted in the well plate.

Dilution	Sample µL	Laboratory Reagent Blank µL	Adjusted Reporting Limit µg/L	Maximum Microcystins µg/L
2	500	500	0.48	10
5	200	800	1.2	25
10	100	900	2.4	50
20	50	950	4.8	100

If a sample is diluted, the final values must be calculated by multiplying the result by the proper dilution factor. Report calculated values.

11.5. Save and print a copy of the calibration curve and sample results as part of the laboratory's record maintenance protocol. Record the analyst initials, date of analysis, and the kit lot number/expiration date on the results page.

12. QUALITY CONTROL AND DATA REPORTING

12.1. With each sample batch analysis, the following QC samples must be analyzed:

12.1.1. Laboratory Reagent Blank (LRB): An aliquot of reagent water that is lysed and filtered to match the sample processing procedure. An LRB must be analyzed with each batch of samples to verify laboratory, reagents and supplies are free of contaminants. The LRB must contain sodium thiosulfate if drinking water samples are included in the analysis batch. Values exceeding the reporting limit require corrective action and reanalysis of the sample batch.

NOTE: Do not use the LRB provided in the kit.

12.1.2. Low Calibration Range Check (LCRC): A LCRC must be analyzed with each batch of samples to verify accuracy of the calibration curve near the reporting limit. The LCRC may be one of the curve's calibration points and the concentration must be ≥ 0.24 µg/L and ≤ 0.50 µg/L. Acceptance limits must be within ± 40% of the true value. LCRC values exceeding the acceptance limits require corrective action and reanalysis of sample(s) with results below the concentration of an acceptable QCS in the same analytical batch. If reanalysis is not possible, all sample concentration results less than an acceptable QCS analyzed in the same analytical batch must be appropriately qualified with a J or UJ, based on the results and noted in the final report unless the requirements in the note below are met.

NOTE: If the LCRC is greater than upper control limit and sample result is less than the reporting limit (RL), no qualifier is required.

12.1.3. Quality Control Standard (QCS): A secondary source QCS must be analyzed with each batch of samples to verify the concentration of the calibration curve. If a QCS is already included in the kit, it may be used if it has a different lot number than the calibration standards and was prepared from a separate primary stock. Acceptance limits must be within ± 25% of true value. QCS values exceeding the acceptance limits require corrective action and reanalysis of sample(s) with results greater than the concentration of an acceptable LCRC in the same analytical batch. If reanalysis is not possible, all sample concentration results greater than an acceptable LCRC analyzed in the same batch must be appropriately qualified with a J or UJ, based on the results and noted in the final report.

NOTE: If both LCRC and QCS exceed acceptance limits and reanalysis is not possible, all sample results must be appropriately qualified with a J or UJ, based on the results.

12.2. Analyze all calibration standards, QC standards and samples in at least two well replicates. The mean of the well replicates must be used in all analytical calculations and reporting of sample results.

12.3. The curve generation must include a calibration concentration point less than or equal to the reporting limit.

12.4. Calibration curve Correlation Coefficient (R) must be ≥ 0.990 or calibration curve Coefficient of

Determination (R^2) must be ≥ 0.980 to be acceptable.

12.5. Coefficient of Variation (%CV) for well replicate absorbance values for calibration *standards* and QC *standards* must all be ≤ 10.0%. It is acceptable to have one calibration or QC standard > 10.0% as long as it is ≤ 15.0%. The zero standard is excluded from this requirement.

12.6. If %CV for more than one calibration or QC standard is > 10.0%, or if one is > 15.0%, the analytical run is not acceptable. Corrective action and reanalysis of the sample batch is required.

Calculate %CV as follows:

$\%CV = (SD_A/Mean_A) * 100$
$Mean_A$ = Mean of well replicate absorbances

Where: SD_A = Standard deviation of well replicate absorbances

12.7. %CV for replicate absorbance values for *MDLs* must be ≤ 15.0%. If the %CV value of any MDL is > 15.0%, the MDL study is not acceptable. Corrective action and reanalysis are required.

12.8. %CV for replicate absorbance values for *samples* must be ≤ 15.0%. If the value of any sample is > 15.0% then reanalyze or qualify the results with the appropriate qualifier (J or UJ) and note in the final report.

12.9. Samples not analyzed within the required holding time must be recollected.

13. QUALIFIERS

CL This qualifier is applied if residual chlorine of raw water or treatment train sample is > 0.1 mg/L.

J This qualifier is applied only if the sample result is greater than the reporting limit (RL) and any of the following conditions apply:

- Sample is collected in improper sample container.
- Sample is received warm (> 10°C).
- LCRC or QCS are out of acceptance criteria.

UJ This qualifier is applied only if the sample result is below the reporting limit (RL) and any of the following conditions apply:

- Sample is collected in improper sample container.
- Sample is received warm (> 10°C).
- Low LCRC recovery.

14. REVISIONS

14.1. Added Revision section; added UJ qualifier (04/2018).

14.2. Revised Sample Collection, Sample Lysing Procedure, Initial Demonstration of Capability, and LRB section (07/2018).

14.3. Reviewed and re-formatted (05/2019).

14.4. Reviewed (05/2020).

14.5. Reviewed. Changed Reporting limit (RL) from 0.30 µg/L to 0.24 µg/L, added detailed information how to make 0.1N NaOH and 0.1N HCl, added sample dilution table, clarified language for %CV acceptance, added qualifier definitions, re-worded to match Lab Cert SOP, added a note to clarify the condition when a qualifier is not required (11/2021).