Ohio EPA Amenable (Extracellular and Intracellular) Microcystins - ADDA in Sludge by ELISA Analytical Methodology
Ohio EPA DES 701.3
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1. SCOPE AND APPLICATION

This method is used for the determination of amenable (extracellular and intracellular) microcystins – ADDA in sludge samples using enzyme-linked immunosorbent assay (ELISA).

2. SUMMARY OF METHOD

The Ohio EPA Amenable (Extracellular and Intracellular) Microcystins – ADDA in sludge by ELISA Analytical Methodology is an immunoassay for the detection of microcystins in sludge 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.

3. SAMPLE COLLECTION AND PRESERVATION

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

NOTE: 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 maintain these records.

3.2 All samples must be protected from sunlight and cooled on ice at ≤10°C immediately after collection and maintained at ≤10°C until analysis. Samples must be analyzed as soon as practical but no later than 5 days from the time of collection. Holding time can be increased by freezing the sample within 5 days of collection. When freezing, allow adequate volume for expansion and place the sample container on its side to prevent breakage.

3.3 It is recommended to decant the sample only once before the start of any analysis. Allow the sample to settle for adequate period of time and decant excess water. Decanting may be performed at the time of sample receiving to maintain uniform consistency at every step of analysis.

4. INTERFERENCES

Due to the high variability of compounds found in sludge 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. Rocks, stones, and large woody material can significantly alter the amount of moisture in the sample and should be removed prior to initial weight being recorded.

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 Each laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. 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.

5.3 It is advised to exercise extreme caution while removing the hot dishes from drying ovens. They are hot enough to cause burns if not handled properly, without safety equipment.

6. APPARATUS

6.1 Class 'A' Volumetric Flask: 500 mL

6.2 Micropipette: capable of 10 to 100 µL

6.3 Multi-Channel Pipette: 50-300 µL

6.4 Stepping Pipette: 100-500 µL (optional)

6.5 Pipette Tips: appropriate for the pipette

6.6 Multi-Channel Pipette Reagent Reservoir: minimum 50 mL capacity

6.7 Microplate Reader: capable of analyzing at 450 nm

6.8 Glass Vials: 4.0 mL

6.9 Glass Vials: 40 mL

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

6.11 Filter manifold, with in-line vacuum or electrical vacuum pump

6.12 Glass Gastight Luer-lock Syringes: 5.0 mL

6.13 ELISA Sealing Teflon Tape

6.14 Freezer

6.15 Refrigerator: capable of 4ºC

6.16 pH Meter (pH Test Strips, pH 0 to 14, may be used upon demonstration of capability)

6.17 Sodium Hydroxide Solution (0.1N)

6.18 Hydrochloric Acid Solution (0.1N)

6.19 Water Bath: capable of 35ºC

6.20 Automated Assay Analysis System (optional)

6.21 Evaporating dishes of 100 mL capacity

6.22 Drying oven capable of maintaining a temperature of 103 to 105ºC

6.23 Analytical balance with a capability of measuring to at least 0.0001 g

6.24 Desiccator with indicator Drierite or silica gel

6.25 Weigh boats

7. REAGENTS

7.1 Liquid Disinfectant: Commercially prepared, Roccal® or any approved 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 Reagent Water: Water free of contaminants

7.4 Alcohol/Dry Ice mixture (optional)

8. SAMPLE LYSING PROCEDURE BY FREEZE/THAW

8.1 Homogenize sample by mixing sample in its container and pour 10 gram aliquot into a weigh boat. Record the weight in a log book to the nearest 10 mg. Use reagent water and transfer the sample into a 40 mL vial. The total volume of reagent water to transfer the sample is 20 mL. Test the pH of the sample then begin the three freeze/thaw lysing cycles.

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

8.2 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/alcohol solution).

8.3 Once sample is completely frozen, remove from freezer and thaw (To speed up the process, vial(s) may be immersed in a 35ºC water bath until it is completely thawed).

8.4 Repeat steps 8.2 and 8.3 two more times.

8.5 Rinse the filter by passing a minimum of 5 mL sample through the filter and discard the filtrate. Using same filter, filter approximately 2 mL of sample into two 4 mL vials. Samples are ready for immediate analysis. Freeze remainder of samples on their side, to be analyzed at a later date.

NOTE: If sample is frozen to extend hold time, once the sample is thawed it has undergone the first freeze/thaw cycle.

9. INITIAL DEMONSTRATION OF CAPABILITY

NOTE: The Method Detection Limit (MDL) study is satisfied by completion of the MDL study from Ohio EPA DES 701.0.

10. 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.

Laboratories using an automated assay analysis system must follow manufacturer's instructions.

NOTE: The assay procedure must be performed away from direct sun light.

10.1 Disinfect the work area.

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

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

10.4 Follow manufacturer's instructions provided with the individual Microcystins – ADDA kit for calibration and sample analysis procedures.

10.5 Sample analyses resulting in a higher concentration than the highest standard in the calibration curve must be diluted within the calibration range and reanalyzed to obtain accurate results. Samples may not be diluted in the well plate. If a sample is diluted, the final values must be calculated by multiplying the result by the proper dilution factor. Report calculated values.

10.6 Save and print a copy of the calibration curve and sample results as part of the laboratory's record maintenance protocol.

11. PROCEDURE FOR PERCENT SOLIDS DETERMINATION

11.1 The analytical balance calibration must be checked with standardized 0.01, 1.00 and 100.000 g weights each day of use and with 0.01, 0.100, 0.500, 1.000, 10.00, 50.00, and 100.00 g standardized weights once a week. Enter the results in the logbook.

11.2 Oven dry the evaporating dishes at 103 to105ºC for one hour and allow them to cool in a desiccator for at least one hour (preferably for two hours for consistent weights). Store the dishes in a desiccator until used.

11.3 Weigh the dishes to 0.1 mg and record the weights. The dishes should be labeled so that the markings do not evaporate or fade. Permanently marked dishes are preferred.

11.4 Homogenize sludge sample by mixing sample in its container and transfer a 25 gram aliquot to a labeled dried evaporating dish. Weigh the dish and sample to the nearest 10 mg.

NOTE**:** Do not decant the sample if decanting is done at the time of sample receiving.

11.5 Dry the sample overnight in a drying oven at 103 to 105ºC. Cool the sample in a desiccator for at least one hour and weigh (preferably for two hours for consistent

weights).

12. CALCULATIONS

12.1 Percent solids determination

The percent solids or percent residue of the sludge sample is calculated by dividing the weight of the dried residue by the initial weight of the sample. Accompanying units are % solids.

$$\% \text{ solids} = \frac{A - B}{C - B} \times 100\%$$

Where:
A = Weight of dish and dry sample residue (g)
B = Weight of evaporating dish (g)
C = Weight of dish and wet sample (g)

Report percent solids to three significant figures.

12.2 Microcystins-ADDA in Sludge

The result of microcystins-ADDA in the Sludge sample (µg/kg) is calculated as follows:

$$\text{Microcystins-ADDA in Sludge} = \frac{\text{ELISA Result (µg/L)} * \text{volume of reagent water added to sample (L)}}{\text{(Sludge weight (g)} * \text{(\% solids/100))/1000}}$$

13. QUALITY CONTROL (QC) AND DATA REPORTING FOR ELISA

The Ohio EPA requires at a minimum the following program specific analytical QC requirements be met.

13.1 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.

13.2 The curve generation must include a calibration concentration point ≤ 0.30 µg/L (RL).

13.3 Calibration curve Correlation Coefficient (R) must be > 0.990 or calibration curve Coefficient of Determination (R^2) > 0.980 to be acceptable.

13.4 Coefficient of Variation (%CV) for well replicate absorbance values for calibration standards and QC standards should be < 10%. If %CV for more than one calibration standard is > 10%, the analytical run is not acceptable. Corrective action and reanalysis of the sample batch is required. The zero standard is excluded from this requirement.

NOTE: An analytical run may be accepted if %CV for only one calibration standard is > 10% but < 15% as long as all other calibration standards in the analytical run are < 10%.

Calculate %CV as follows:

$$\%CV = (SD_A/Mean_A)*100$$

Where: SD_A = Standard deviation of well replicate absorbances
$Mean_A$ = Mean of well replicate absorbances

13.5 %CV for replicate absorbance values for samples must be < 15%, if the value is > 15% then reanalyze or qualify the results with the appropriate qualifier (J/UJ) and noted in the final report.

13.6 Laboratory Reagent Blank (LRB): For each Analysis Batch, an aliquot of reagent water that is lysed and filtered to match the sample processing procedure must be analyzed. Values exceeding the reporting limit require corrective action and reanalysis of the sample batch.

13.7 Low Calibration Range Check (LCRC): An 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 calibration points and the concentration must be ≥ 0.30 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 batch must be appropriately qualified (J/UJ) and noted in the final report.

13.8 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 (J/UJ) and noted in the final report.

NOTE: If both LCRC and QCS exceed acceptance limits and reanalysis is not possible all results must be appropriately qualified (J/UJ).

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

14. QUALIFIERS

J Analyte was positively identified; the associated numerical value is estimated.

UJ The analyte was not detected above the sample quantitation limit (QL). However, the reported QL is estimated.

15. REFERENCES

18.1 Ohio EPA Total (Extracellular and Intracellular) Microcystins - ADDA by ELISA Analytical Methodology, DES 701.0.

18.2 *Standard Methods for the Examination of Water and Wastewater*, 2540 G-2011

16. REVISIONS