HPLC Method Development to Analytically Quantify Microcystin in Laboratory and Environmental Water Systems

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Introduction

In freshwater environments, cyanobacterial harmful algal blooms (cHABs) occur when blue-green algae (cyanobacteria) grow under eutrophic conditions and begin to produce hepatic toxins, also known as cyanotoxins**.** cHABs cause extensive economic and ecological damage when they occur, costing the United States over $1 billion in the last 10 years. Eutrophication of freshwater environments across America coupled with global warming and increased atmospheric carbon dioxide is expanding the occurrence and intensity of cHAB events every year. The environmental conditions that promote cHAB occurrences are complex challenges on their own. Directly addressing these conditions may not immediately produce a solution soon enough to save animals, ecosystems, and humans. Methods that control cHAB growth or toxin production are currently underdeveloped.

Microcystis aeruginosa is one of the major contributors to cHABs in fresh and brackish waters. This microorganism, and others like it, are of specific concern because they produce toxin called microcystin. The physiological importance of this compound to the cyanobacteria is unknown, though the toxin poses a major threat to eukaryotic life. The threat of these toxins produced from HABs have subsequently closed beaches, lakes, and ponds, greatly impacting human society. Detection of the presence of the toxin in water systems becomes strategically necessary for early warning and laboratory experimentation.

The goal of this study is to develop a quick and inexpensive method using a HPLC instrument for detection and quantification of microcystin. This study aims to increase the sensitivity of HPLC detection to meet the standard limit of 1 µg/L in water set by the World Health Organization. Method development included improvements to how water samples were collected/filtered, cyanobacteria cells concentrated/lysed, gradient vs isocratic HPLC mobile phases **(Figure 2)**, and standardization of laboratory and environmental vs. control samples.

Problem and Instruments

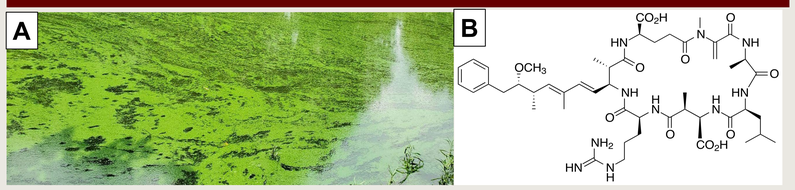

Figure 1. cHAB event and microcystin. A) cHAB event captured at SIUC Campus Lake in June 2021. B) Structure of microcystin.

The frequency and severity of cHABs increases every year **(Figure 1A)** and are exacerbated by the build up of excess nutrients due to agricultural run-off, stagnant water, and high temperatures. *M. aeruginosa* and other cyanobacteria can produce the heptapeptide toxin microcystin, a phosphatase inhibitor that affects the liver. **(Figure 2B)** Microcystin is responsible for the deaths of humans, animals, and livestock all around the world. The production of this toxin poses an ecological risk that must be managed. By developing a method for monitoring toxin production, steps can be taken to prevent the growth of cHABs. One cost effective method for toxin detection is by HPLC analysis. A biphenyl column **(Figure 2A)** should be selective enough to accurately detect microcystin while separating it from other compounds within the matrix. Using this column, a gradient elution method **(Figure 2B)** was determined to be ideal for detection and quantification.

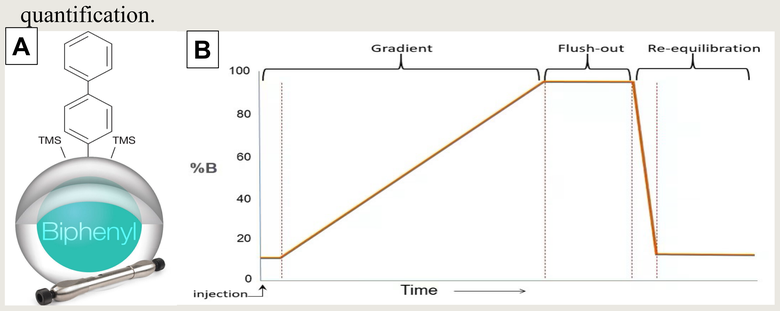

Figure 2. Depiction of gradient elution curve and column. A) Depiction of biphenyl column and chemical structure. B) Displays the purpose of each step of the method. Sourced from Shimadzu website.

Method Development Process

Filtration method and application of QuikLyse procedure

M. aeruginosa cultures were grown to a density of 10^7 cells/mL **(Figure 3A).** Glass fiber filters (**Figure 3B)** housed within filter casing were prepared and attached to a 10 mL syringe **(Figure 3C)**. Using the prepared syringe, 5 mL of culture (**Figure 3A)** was passed through the filter **(Figure 3D)**. The filter was removed from the casing and placed within a 4 mL glass bottle containing 1 mL of distilled water **(Figure 3E)**. QuikLyse solution (acquired from Eurofins Abraxis) was added to the bottle in accordance with the procedure provided with the QuikLyse kit. Lysed samples were extracted and prepared in HPLC vials for analysis.

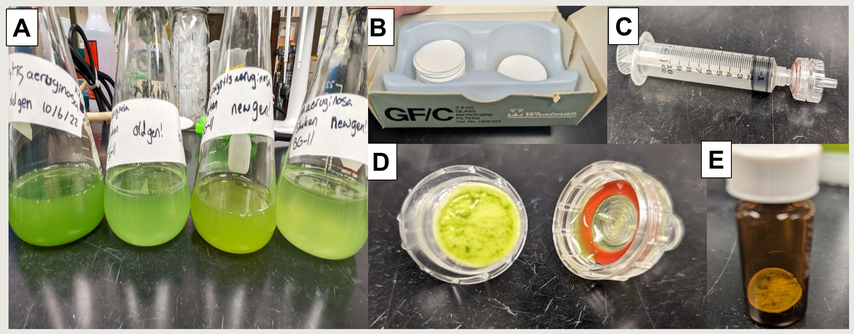

Figure 3. Filtration method and QuikLyse procedure. A) *M. aeruginosa* live culture. B) GF/C filters used to capture *M. aeruginosa* cells. C) 10 mL syringe attached to filter casing housing the GF/C filter. D) Filter after culture has been passed through it. E) Filter submerged in QuikLyse solution.

HPLC method for microcystin quantification

Two separate methods were developed over the course of this study. Used analytical column was a Kinetex biphenyl (150 x 4.6 mm) with 5 µm particle size. Volume of injected samples were 20 µL. HPLC system was Shimadzu Prominence-i LC-2030 equipped with a UV array detector set at 238 nm for detection of microcystin. The column temperature was 30 °C. Two mobile phases were applied including: A) 25 mM KH_2PO_4 and B) 100% acetonitrile. The first applied analytical procedure was gradient run as follows: 0-10 min 75% A and 25% B, 10-11.1 min 25% A and 75% B, 11.1-20 min 75% A and 25% B. The flow rate was kept in the range of 1 mL/min. **(Figure 4)**

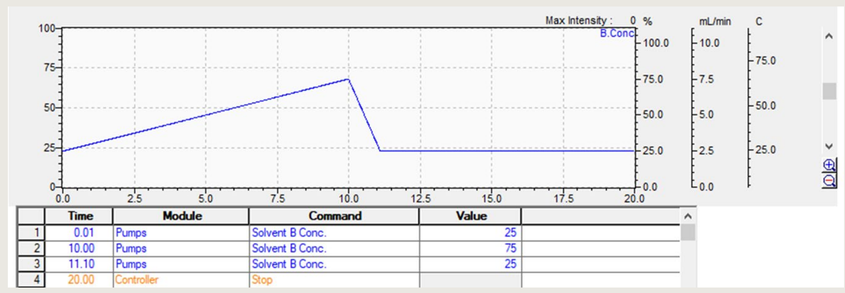

Figure 4. First HPLC Method. Curve representing gradient method.

The second applied analytical procedure was also a gradient method designed to emulate an isocratic method. In this procedure, the column, temperature, flow rate, UV settings, and mobile phases remained the same. The gradient run was edited as follows: 0-15 min 70% A and 30% B, 15-20 min 25% A and 75% B, 20-21 min 25% A and 75% B, 21-25 min 70% A and 30% B, 25-30 min 70% A and 30% B. **(Figure 5)**

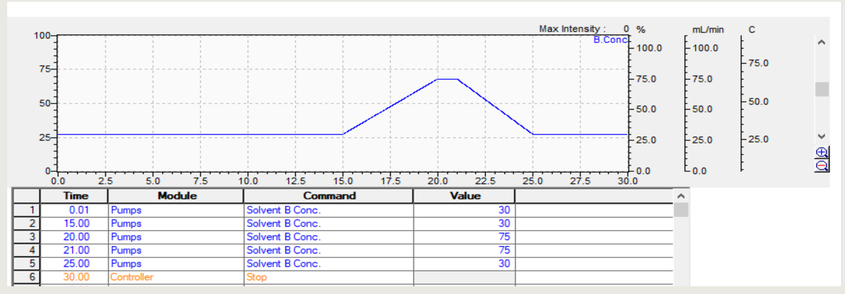

Figure 5. Second HPLC Method. Curve representing isocratic method within a gradient method.

Results

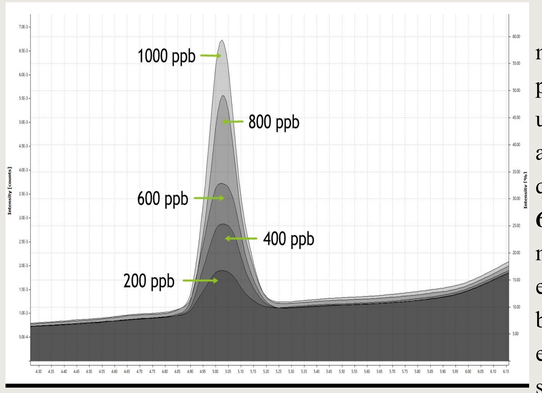

Figure 6. Concentrations of microcystin ranging from 200ppb-1000ppb. Peak area underneath curves used to generate standard equation.

Concentrations of microcystin ranging from 200 ppb – 1000 ppb were analyzed under both methods to generate a standard equation for quantifying microcystin **(Figure 6)**. Pairing these analytical methods with the standard equations allows us to analyze both lab-grown and environmental cyanobacterial samples to detect the precise levels of microcystin within them.

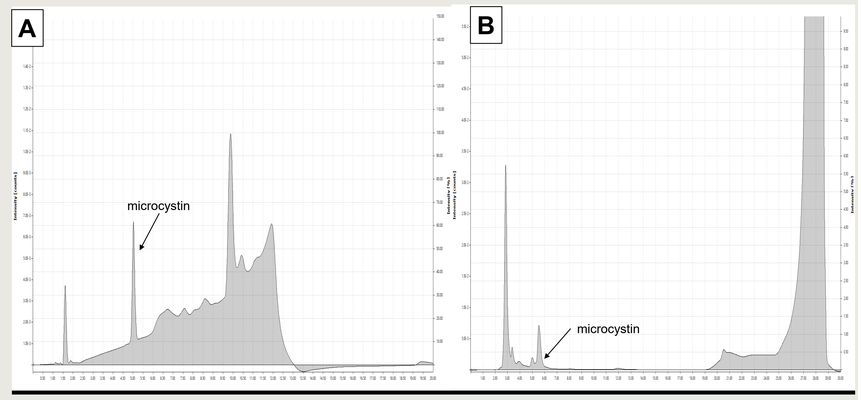

Figure 7. Chromatogram results from both methods. A) Comparison of MC-LR standard against live culture analyzed with first method. B) MC-LR standard analyzed under second method.

While both methods were capable of detecting microcystin, there was a lot of noise present in the baseline of the first method **(Figure 7A)**. The second method was developed in attempt to stabilize the baseline. The resulting chromatogram shows that the baseline was sufficiently stabilized. This resulted in a more accurate and precise quantification of microcystin **(Figure 7B)**.

Conclusion and Future

- The method development process is necessary to optimize microcystin quantification
- Implementing an isocratic method within the gradient stabilized the baseline
- A stable baseline allows for a more precise reading of analytes
- HPLC analytical methods provides a cheaper alternative to quantify microcystin than ELISA methods
- Using this method, studies will be developed to compare microcystin production with cell growth

Acknowledgements

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