Microcystins represent an emerging class of algal toxins of concern to the drinking water industry. Consequently, the World Health Organization, Australia, and Brazil have established guidelines for the amount of microcystins permissible in drinking water, and the United States has begun to evaluate the occurrence, health effects, and susceptibility of water treatments from these algal toxins. This article focuses on the initial development of a liquid chromatography–mass spectrometry method to screen for many of these toxins at low parts-per-billion levels.

Most of the world’s population centers rely upon surface freshwater as their primary source for drinking water. The drinking water industry constantly is challenged with surface water contaminants that must be removed to protect public health. Contaminants associated with cyanobacteria (blue-green algae) are called cyanotoxins. The mechanisms of the cyanotoxins’ toxicity are diverse and range from hepatotoxic, neurotoxic, and dermatotoxic effects to general inhibition of protein synthesis. One-third of the 50 genera of cyanobacteria can produce toxins, and approximately 60% of those genera are toxic. Toxic cyanobacterial blooms are an emerging issue in the United States because of increased source water nutrient pollution and subsequent eutrophication. The cyanotoxins are divided into three classes based upon chemical structure; these classes are cyclic peptides, alkaloids, and lipopolysaccharides.

Nodularin and microcystins are cyclic peptides that contain five and seven amino acids, respectively. Because nodularin is found primarily in marine and brackish water, it can serve as a good internal standard. At nearly neutral pH, microcystins, which are cyclic peptides, are very stable and can remain toxic even after being boiled (1). These compounds were named in 1988 from the cyanobacteria Microcystis aeruginosa (2), but they also can be produced by other genera including Anabaena, Oscillatoria, Nostoc, Anabaenopsis, and terrestrial Hapalosiphon (1). Approximately 60 variations of microcystins have been isolated from cyanobacterial blooms and cultures (1), and microcystin-LR is the most common (1,3). Microcystins are hepatotoxins that inhibit protein phosphatase enzymes in the liver. Carcinogenicity and epidemiological studies suggest that low-level chronic exposure to microcystins increases human health risk by carcinogenesis and tumor growth promotion of the liver. The World Health Organization (WHO, Geneva, Switzerland) guideline values for microcystin are based upon the typical daily intake of water, individual body weight, and the concentration of the toxin. The WHO drinking water guideline value for microcystin-LR is 1 mg/L for adults.

The alkaloid class of algal toxins contains both neurotoxins and hepatotoxins. Neurotoxins are divided into two major groups: anatoxins and saxitoxins. Anatoxins are low molecular weight secondary amines produced by Anabaena, Oscillatoria, Aphanizomenon, and Cylindrospermum. Researchers have reported three anatoxins: anatoxin-a, homoanatoxin-a, and anatoxin-a(S). Concentrations as high as 8 µg/L of anatoxin-a have been reported in Florida’s tap water (4). The anatoxin-a oral acute toxicity — the amount of a material, given all at once, that
causes the death of 50% of a test group (LD$_{50}$) — is greater than 5000 µg/kg body weight. Scientists have observed no subacute toxicity for anatoxin-a when administered orally to rats in the drinking water at 510 µg/L or 5100 µg/L, which suggests that chronic toxicity is unlikely. However, these studies did not include behavioral testing or receptor binding studies, which target the neurological response of the toxins.

Homoanatoxin-a is a potent neuromuscular blocking agent that causes severe paralysis, convulsions, and death by respiratory arrest. Anatoxin-a(S) is an organophosphate produced by *Anabaena*. This toxin blocks acetylcholinesterase activity in a manner analogous to organophosphate insecticides. *Cylindrospermopsis* is a cyanotoxin alkaloid analogous to organophosphate insecticides. *Cylindrospermopsis raciborskii* was identified as the agent causing an outbreak of acute hepatitis and renal damage among an aboriginal population in Australia (5). Animal toxicological studies with *cylindrospermopsis* show widespread and progressive tissue injury with cell necrosis in the liver, kidneys, adrenals, lungs, heart, spleen, and thymus. However, most of the *cylindrospermopsis* studies have been performed with dry cell extracts, leaving questions about whether *cylindrospermopsis* is the only active cyanotoxin present. *Cylindrospermopsis* concentrations greater than 80 mg/L have been reported in finished water at several drinking water utilities in Florida (4). The database for the alkaloid class is insufficient for estimation of a tolerable daily intake.

Cyanobacterial lipopolysaccharides are cell-wall components of gram-negative bacteria. They are pyrogenic and toxic (6). The few studies that have been performed on the cyanobacterial lipopolysaccharides suggest that they are less toxic than other bacterial lipopolysaccharides such as those produced by *Salmonella*. A lack of cyanobacterial cultures has limited the progress of research about the structures and toxicity of cyanobacterial lipopolysaccharides.

The increased awareness of the health risks associated with algal toxins has led the drinking water industry to investigate their potential threat further. The intracellular toxins are released after the cell is lysed. Cell lysis occurs through natural cell death, algal treatment, and the shear caused by pumping and treatment processes. Public health concerns require the understanding of whether standard drinking water treatment and disinfection deactivates cyanotoxins.

Analysts have used various analytical techniques — such as enzyme-linked immunosorbent assay (ELISA) (7); phosphatase inhibition assay (8); gas chromatography–mass spectrometry (GC–MS) (9,10); high-performance liquid chromatography with UV detection (HPLC UV) (11–14); and liquid chromatography–mass spectrometry (LC–MS) (15–18) — to analyze these toxins. LC–MS offers the advantage of providing specificity and good sensitivity. HPLC offers a powerful separation tool to separate specific toxins; however, the typical UV detection technique does not get near the sensitivity nor the specificity of LC–MS without extensive sample preparation or enrichment before analysis. ELISA methods offer a fast screening tool but can suffer from possible false positives depending upon the matrix. In addition, ELISA can confirm the presence of microcysts but does not identify which specific toxin is present. GC–MS offers good specificity but can require offline derivatization and cannot be used for the higher molecular weight, nonvolatile algal toxins such as microcystin. LC–MS previously has been shown to provide valuable molecular weight information and some of the target compounds have previously been analyzed using electrospray ionization (15–18).

Given the different molecular weights of the various toxins, LC–MS offers the advantage of monitoring only for those specific masses of interest as well as verifying the identification by retention times. The work presented in this article focused on trying to develop a simple and selective LC–MS method to analyze as many of the target toxins as possible using a single method. Figure 1 shows the structures of the various toxins studied in this article. One of the biggest challenges to monitoring these toxins is that very few of them are available as standards. For this reason, we analyzed only five of the toxins, which were the only ones commer-

![General structure of microcystins](image-url)

**Figure 1:** Structures and molecular weights for compounds studied.
cially available at the time. One of the toxins analyzed included microcystin-LR, the most common toxin found and the one specifically listed in WHO guidelines.

**Experimental**

**Materials:** Pure standards of the various microcystins and nodularin were purchased from Calbiochem (San Diego, California) and anatoxin-a was purchased from Sigma (St. Louis, Missouri). All standards initially were dissolved in methanol and kept in a −4 °C freezer when not being used to prepare standards. Working standards for quantification were prepared in high-purity water and kept at 10 °C in a refrigerated 4 °C freezer when not being used to prepare standards. Working standards for quantification were prepared in high-purity water and kept at 10 °C in a refrigerated 4 °C freezer when not being used to pre-

**Equipment and procedure:** The LC–MS system used consisted of a model 2695 solvent and sample manager, a model 996 photodiode-array detector and a ZQ single-quadrupole mass spectrometer (all from Waters). An electrospray probe operating in the positive mode was used for analysis. The cone voltages and masses monitored for analysis were optimized for each individual component studied. Selected-ion monitoring was performed for the specific masses of interest. The mobile phase was a binary gradient using a third solvent (1% formic acid) that was added at 10% during the entire run to enable consistent pH throughout the run. The two other solvents — water and acetonitrile — were mixed at 88% and 2% initially for 4 min followed by a step change in 1 min to 65% and 25% of each, respectively. After that gradient, a linear gradient to 29 min was performed for a final concentration of 33% and 55% of each solvent.

As mentioned previously, the standards were prepared by diluting the concentrated standards with water. Seven levels from 0.5 ppb to 250 ppb were prepared. Duplicate injections were made, and an external, linear calibration was performed to obtain a correlation coefficient (r²) of 0.997 or greater. Table I summarizes the masses monitored and retention times of each component. Before analysis, the standards and samples were filtered with a 0.45-μm GHP filter (Waters).

**Table I:** Compound names, retention time, and masses monitored for each component

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention Time (min)</th>
<th>Mass Monitored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anatoxin-a</td>
<td>2.5</td>
<td>166.2 (M + H)⁺</td>
</tr>
<tr>
<td>Microcystin-RR</td>
<td>12.2</td>
<td>520 (M + 2H)²⁺</td>
</tr>
<tr>
<td>Nodularin</td>
<td>14.9</td>
<td>825.5 (M + H)⁺</td>
</tr>
<tr>
<td>Microcystin-YR</td>
<td>16.6</td>
<td>1045.7 (M + H)⁺</td>
</tr>
<tr>
<td>Microcystin-LR</td>
<td>16.9</td>
<td>995.2 (M + H)⁺</td>
</tr>
</tbody>
</table>

**Results and Discussion**

Figure 2 shows an example of selected-ion chromatograms for the toxins studied at 2.5 ppb. In this instance, all toxins could be seen easily at a level of 2.5 ppb without any sample preparation. At the lower levels (0.5 and 1 ppb) all could be detected with the exception of microcystin-YR, which was not detected at 0.5 ppb. Using this method, we analyzed two lake water samples, and Table II shows the results.

Figure 3 shows the selected-ion chromatograms for the Kentucky lake water sample and illustrates the presence of the various microcystins, especially microcystin-LR and anatoxin-a.

Figure 4 is the selected-ion chromatogram for the Florida lake water sample, and it shows the presence of no microcystins and only a small amount of anatoxin-a. This sample also tested negative for microcysts using the ELISA test. In addition, the analysis for the presence of the toxin cylindrospermopsin was performed using two of the main masses known for the compound, mass 416.2 (molecular ion) and mass 336 (fragment ion). Harada and co-workers previously verified these two masses to be from cylindrospermopsin (19).

The two water samples were analyzed monitoring these masses using the same
HPLC conditions, and Figure 5 shows the results. As can be seen, the Kentucky lake water sample did not show the presence of cylindrospermopsin, but the other lake water sample did. The second lake water sample was from a Florida lake that had been suspected of containing cylindrospermopsin and was later confirmed, by biological testing, to contain *C. raciborskii*, which produces the toxin cylindrospermopsin. Because no standard material was available, we were unable to quantify cylindrospermopsin. We only could verify the likelihood of its presence.

**Conclusion**

Overall, the detection of the target microcystins, anatoxin-a, and nodularin was possible using LC–MS. Using this technique enabled easy quantification of the target compounds at low parts-per-billion levels with no sample preparation required beyond filtering of the water samples. Additional work will investigate the accuracy and precision of quantification of this method using spiked and unspiked samples. The detection of cylindrospermopsin also was possible using this method. Preliminary work on a solid-phase extraction (SPE)

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**Table II: Summary of the amounts of each component in the two lake water samples**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kentucky Lake Water Sample (ppb)</th>
<th>Florida Lake Water Sample (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anatoxin-a</td>
<td>3.2</td>
<td>20.1</td>
</tr>
<tr>
<td>Microcystin-RR</td>
<td>26.2</td>
<td>ND*</td>
</tr>
<tr>
<td>Nodularin</td>
<td>&lt;0.5</td>
<td>ND</td>
</tr>
<tr>
<td>Microcystin-YR</td>
<td>10.4</td>
<td>ND</td>
</tr>
<tr>
<td>Microcystin-LR</td>
<td>42.5</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ND = none detected.

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**Figure 3:** Selected-ion recording channels for the analysis of a Kentucky lake water sample (20 µL injected). Peaks: 1 = anatoxin-a (3.2 ppb), 2 = microcystin-RR (26.2 ppb), 3 = nodularin (<0.5 ppb), 4 = microcystin-YR (10.4 ppb), 5 = microcystin-LR (42.5 ppb).
method has shown promise for use in 10–100 fold sample enrichment. Using this technology probably would incorporate the use of an internal standard; with nodularin being one possible compound for use, given its similarity to the microcystins. The only challenge would be whether nodularin could be present in some samples of water if that strain of algae were present. A better standard would be an isotopically labeled microcystin; however, no known standards are commercially available. Further work will continue in our laboratories to look at developing an SPE method for lowering the detection limits of these target compounds as well as work on detection and quantification of the toxin cylindrospermopsin.

Acknowledgment
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References