Methods for the Analysis of Microcystins in Water to sub-Parts Per Trillion Detection Levels

Douglas Stevens,
Marijn Van Hulle, Gordon Fujimoto
Waters Corporation
Introduction

- Microcystins are cyclic peptides produced by cyanobacteria.

- They are produced by overgrowth of algae, especially at higher water temperatures – Climate change, invasive species, global trade and agricultural practices can exacerbate the problem.

- Microcystins can be toxic for plants and animals including humans.

- Once ingested, microcystins travel to the liver, via the bile acid transport system, where most is stored. Some remains in the blood stream and may contaminate tissue. Microcystins bind covalently to protein phosphatases thus disrupting cellular control processes. Their hepatotoxicity may cause serious damage to the liver. Microcystins can strongly inhibit protein phosphatases type 1 (PP1) and 2A (PP2A), and are linked to pansteatitis.

- Over 80 toxic variants are known.
Introduction

- Microcystin-containing 'blooms' are a problem in countries worldwide including China, Brazil, Australia, the USA and much of Europe

- WHO action limit = **1000 ng/L (1ppb)** and methods are proposed including US EPA Methods 544 and 545 and European ISO 20179:2005(E) guideline

- For this reason sensitive detection is needed

- This presentation will discuss the various approaches to sample introduction and detection suitable for proposed regulatory limits as well as the lower limits often desired for research purposes
Experimental: UPLC conditions

- **Run Time:** 7.50 min
- **Inj Vol:** 50.00 µL
- **Column:** ACQUITY UPLC BEH C18 2.1x100mm, 1.7 µm
- **Solvent A:** 0.1% FA in 97/3 H2O/ACN
- **Solvent B:** ACN
- **Flow Rate:** 400 µL/min
- **Gradient:**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%A</th>
<th>%B</th>
<th>Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>100.0</td>
<td>0.0</td>
<td>Initial</td>
</tr>
<tr>
<td>0.75</td>
<td>100.0</td>
<td>0.0</td>
<td>6</td>
</tr>
<tr>
<td>5.00</td>
<td>20.0</td>
<td>80.0</td>
<td>6</td>
</tr>
<tr>
<td>6.00</td>
<td>0.0</td>
<td>100.0</td>
<td>1</td>
</tr>
<tr>
<td>7.50</td>
<td>100.0</td>
<td>0.0</td>
<td>1</td>
</tr>
</tbody>
</table>

- **Column Temp:** 50.0 C
Experimental: MS Method Settings

- Time segmented MRM with Dwells to give 15 pts across each peak
- 2 MRMs per analyte
- Most microcystins singly charged except for DE-M-RR & M-RR
Performance Criteria

- The following performance criteria were evaluated
  - Linearity
  - Robustness
  - Sensitivity
  - Repeatability
  - Accuracy
    - Standard Addition

Xevo TQ-S shown with UPLC and APGC
Mass Spectrometers
SQ, TQ, QTof, & QTof with Ion Mobility
Results: Typical Chromatogram

- Bottom to top: Cylindrospermopsin (CYL), Anatoxin-A (ANA), M-DE-RR, M-RR, Nodularin (NOD), M-LR, M-YR, M-LY, M-LW and M-LF at 100 ng/L
Results: Sensitivity

- All analytes detected with good S:N at the 25 ng/L

- YR: 23:1
- LW: 17:1
- LR: 40:1
- LF: 26:1
- Nod: 39:1
- RR: 60:1
- Cyl: *136:1
- Ana: 80:1
Results: Linearity from 10 to 1000ng/L

**Anatoxin**
- Compound name: Anatoxin
- Correlation coefficient: $r = 0.999714$, $r^2 = 0.999429$
- Calibration curve: $22.3124 \times x + -22.0103$
- Response type: External Std, Area
- Curve type: Linear, Origin: Include, Weighting: 1/x, Axis trans: None
- Residual: $-8.0$, $-6.0$, $-4.0$, $-2.0$, $0.0$, $2.0$, $4.0$

**Nodularin**
- Compound name: Nodularin
- Correlation coefficient: $r = 0.999806$, $r^2 = 0.999613$
- Calibration curve: $70.7322 \times x + 6.27616$
- Response type: External Std, Area
- Curve type: Linear, Origin: Include, Weighting: 1/x, Axis trans: None
- Residual: $-10.0$, $-5.0$, $0.0$, $5.0$, $10.0$

**CYL**
- Compound name: CYL
- Correlation coefficient: $r = 0.999861$, $r^2 = 0.999722$
- Calibration curve: $130.893 \times x + -183.659$
- Response type: External Std, Area
- Curve type: Linear, Origin: Include, Weighting: 1/x, Axis trans: None
- Residual: $-6.0$, $-4.0$, $-2.0$, $0.0$, $2.0$, $4.0$

**M-YR**
- Compound name: MC-YR
- Correlation coefficient: $r = 0.999714$, $r^2 = 0.999429$
- Calibration curve: $22.3124 \times x + -22.0103$
- Response type: External Std, Area
- Curve type: Linear, Origin: Include, Weighting: 1/x, Axis trans: None
- Residual: $-8.0$, $-6.0$, $-4.0$, $-2.0$, $0.0$, $2.0$, $4.0$
Results: Robustness

- Due to lack of IS, signal stability was evaluated
- First and last (5th) 100 ng/L std were compared. Time difference between samples approximately 15.5 hrs. Areas within 10% for 5 of the 8 compounds, and 31% for the other 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>100 ng/L (1)</th>
<th>100 ng/L (5)</th>
<th>ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-YR</td>
<td>2061</td>
<td>2215</td>
<td>107%</td>
</tr>
<tr>
<td>M-LW</td>
<td>1361</td>
<td>1660</td>
<td>122%</td>
</tr>
<tr>
<td>M-LR</td>
<td>2770</td>
<td>3353</td>
<td>121%</td>
</tr>
<tr>
<td>M-LF</td>
<td>1522</td>
<td>1990</td>
<td>131%</td>
</tr>
<tr>
<td>NOD</td>
<td>6848</td>
<td>7380</td>
<td>108%</td>
</tr>
<tr>
<td>M-RR</td>
<td>28133</td>
<td>29911</td>
<td>106%</td>
</tr>
<tr>
<td>CYL</td>
<td>12027</td>
<td>12074</td>
<td>100%</td>
</tr>
<tr>
<td>ANA</td>
<td>190364</td>
<td>183937</td>
<td>97%</td>
</tr>
</tbody>
</table>
Results: Repeatability

- Short term repeatability evaluated using 5 replicates from the same vial at 100 ng/L

- % CV values for DW and surface water SW

- Repeatability is good with % CV values below 5%

<table>
<thead>
<tr>
<th>Compound</th>
<th>% CV (n=5) DW</th>
<th>% CV (n=5) SW</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-YR</td>
<td>4.6</td>
<td>3.0</td>
</tr>
<tr>
<td>M-LW</td>
<td>3.2</td>
<td>2.2</td>
</tr>
<tr>
<td>M-LR</td>
<td>2.2</td>
<td>1.8</td>
</tr>
<tr>
<td>M-LF</td>
<td>4.2</td>
<td>4.6</td>
</tr>
<tr>
<td>NOD</td>
<td>1.9</td>
<td>3.0</td>
</tr>
<tr>
<td>M-RR</td>
<td>1.6</td>
<td>0.5</td>
</tr>
<tr>
<td>CYL</td>
<td>5.0</td>
<td>2.7</td>
</tr>
<tr>
<td>ANA</td>
<td>1.6</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Results: Accuracy

- Accuracy evaluated using spike/recovery experiments at 100, 200 and 400 ng/L in DW and SW

- DW recoveries are acceptable for compounds eluting in the middle of the run but too low for compounds eluting near the beginning and the end

- Suggests the need for IS to improve accuracy

<table>
<thead>
<tr>
<th>Name</th>
<th>DW 1</th>
<th>DW 2</th>
<th>DW 3</th>
<th>DW 4</th>
<th>DW 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYL</td>
<td>-54.7</td>
<td>-61.2</td>
<td>-59.6</td>
<td>-43.6</td>
<td>-40.8</td>
</tr>
<tr>
<td>Anatoxin-a</td>
<td>-2.4</td>
<td>-44.5</td>
<td>-47.5</td>
<td>-2.5</td>
<td>-59.3</td>
</tr>
<tr>
<td>MC-RR</td>
<td>7.9</td>
<td>5.0</td>
<td>7.4</td>
<td>3.7</td>
<td>3.0</td>
</tr>
<tr>
<td>Nodularin</td>
<td>4.0</td>
<td>3.5</td>
<td>5.9</td>
<td>3.8</td>
<td>6.6</td>
</tr>
<tr>
<td>MC-YR</td>
<td>-0.2</td>
<td>-1.8</td>
<td>2.5</td>
<td>2.5</td>
<td>9.4</td>
</tr>
<tr>
<td>MC-LR</td>
<td>2.7</td>
<td>-5.4</td>
<td>0.5</td>
<td>4.6</td>
<td>4.1</td>
</tr>
<tr>
<td>MC-LW</td>
<td>-20.6</td>
<td>-18</td>
<td>-29</td>
<td>-14.2</td>
<td>-19.6</td>
</tr>
<tr>
<td>MC-LF</td>
<td>-12.9</td>
<td>-14.9</td>
<td>-18</td>
<td>-13.1</td>
<td>-6.4</td>
</tr>
</tbody>
</table>
Results: Accuracy

- SW recoveries 30% - 150% and dependent on type of surface water
- Suggests the need for IS to improve accuracy

- In the absence of an IS, standard addition can be used
- Standard addition calculations can be done automatically in TargetLynx

- Following slides show quan of M-LR in Aquacheck Proficiency Test using double addition. Assigned value for M-LR, based on 14 results, is 720 ng/L

- Sample analyzed using double standard addition:
  - Sample
  - Sample + 200 ng/L
  - Sample + 400 ng/L
Results: Standard Addition

- 400 ng/L
- 200 ng/L
- Unspiked sample
Results: Standard Addition

- In the Sample List:
  - Use “Standard” for sample type
  - Add the spiked concentration
  - Assign a sample group for each set of samples

- In the TargetLynx processing method:
  - Check the option “Use Standard Addition?”
Results: Standard Addition

- TargetLynx calculates endogenous concentration
- Reported conc. **724 ng/L in good agreement** with known value
Summary of Direct Injection High Sensitivity TQ Method

- Sensitive and robust method for 10 microcystins was developed:
  - 25 ng/L or lower LOD for all analytes
  - Signal stable for 15 hours during study
  - Repeatability <5% RSD
  - Linearity and %deviation excellent over the studied range
- Run time 7.5 min
- 50 µL of drinking water or surface water was injected directly
- In the absence of IS, standard addition yields accurate quantitation
Beyond PPB and PPT: PPQ Detection
**Loading Pump:**

A pushes sample out of the autosampler.

B dilutes the flow coming from the autosampler with aqueous to improve trapping.

**Inject sample onto the 'Trap Column.'**

After the valve switch, flow from the column pump goes through the trap and onto the analytical column connected to the mass spec.

**Elute sample off 'Trap Column' onto Analytical Column and then to MS.**
Experimental Parameters

ACQUITY I-Class 2D-UPLC ACD Method

- Trap Column: Oasis HLB Direct Connect HP, 2.1 mm x 30 mm, 20 µm
- Analytical Column: Waters ACQUITY UPLC BEH C18, 130Å, 1.7 µm, 2.1 mm X 50 mm

UPLC System:
- ACQUITY I-Class Binary Solvent Manager (BSM) & ACQUITY Column Manager (CM-A)

250 µL Injections of SPE processed water sample

- Purge Solution = 90/10 H2O/MEOH
- Wash Solution = 30/30/30/10 ACN/IPA/MEOH/H2O

Sample Temperature = 7 °C
### Loading Pump

\[ A = 67/33 \text{ Acetonitrile/Water} \]
\[ B = \text{Water} \]
\[ A \& B \text{ have 0.3\% Formic Acid} \]

**Gradient (Flow=0.80 mL/min)**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A%</th>
<th>B%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>3.3</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>3.4</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>6.0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>6.5</td>
<td>15</td>
<td>85</td>
</tr>
</tbody>
</table>

**Switch at 2.25**

**Switch at 10.25**

### Column Gradient Pump

\[ A = \text{Water with} \]
\[ B = \text{Acetonitrile with} \]
\[ A \& B \text{ have 0.01\% Formic Acid} \]

**Gradient (Flow=0.45 mL/min)**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>A%</th>
<th>B%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>3.3</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>7.3</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>8.5</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>9.0</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

**Switch at 2.25**

**Switch at 10.25**

\[ t=0: \text{Flow from autosampler goes through trap column to waste} \]
\[ t=2.25: \text{valve switch to send flow from the column pump through the trap and onto the analytical column} \]
\[ t=10.25: \text{switch back to t=0 flow path} \]
Blank Injections 1 - 4

Water Extraction Blank 1  Acquired 16-Dec-2015 / 02:44:14
Test_MCLR_601 Sm (Mn, 1x2)
1: TOF MS ES+ 995.557 0.0200Da 1.06e3

Water Extraction Blank 3  Acquired 16-Dec-2015 / 03:12:23
Test_MCLR_603 Sm (Mn, 1x2)
1: TOF MS ES+ 995.557 0.0200Da 1.36e3

Water Extraction Blank 2  Acquired 16-Dec-2015 / 02:58:18
Test_MCLR_602 Sm (Mn, 1x2)
1: TOF MS ES+ 995.557 0.0200Da 1.62e3

Water Extraction Blank 4  Acquired 16-Dec-2015 / 03:26:27
Test_MCLR_604 Sm (Mn, 1x2)
1: TOF MS ES+ 995.557 0.0200Da 1.53e3
Blank Injection 5 and & RO Water Injection 1

Water Extraction Blank 5  Acquired 16-Dec-2015 / 03:40:32
Test_MCLR_605 Sm (Mn, 1×2)

RO 1 ppt Extraction 1  Acquired 16-Dec-2015 / 03:55:02
Test_MCLR_606 Sm (Mn, 1×2)

Retention Time & Peak Area

1ppt 65:1
RO Water Injections 2 & 3

RO 1 ppt Extraction 2
Test_MCLR_607 Sm (Mn, 1x2)
Acquired 16-Dec-2015 / 04:09:05
1: TOF MS ES+
995.557 0.0200Da
1.80e4
Area

Retention Time & Peak Area

1ppt 90:1

RO 1 ppt Extraction 3
Test_MCLR_608 Sm (Mn, 1x2)
Acquired 16-Dec-2015 / 04:37:37
1: TOF MS ES+
995.557 0.0200Da
1.79e4
Area

Retention Time & Peak Area

1ppt 76:1
RO Water Injections 3 & 4

Retention Time & Peak Area

1ppt 74:1

1ppt 68:1
## Instrument Detection Limits

Five Analyses of RO Water Samples Spiked at 1 ppt

<table>
<thead>
<tr>
<th>Injection</th>
<th>RT</th>
<th>Area</th>
<th>RMS S:N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.48</td>
<td>355.2</td>
<td>64.71</td>
</tr>
<tr>
<td>2</td>
<td>5.48</td>
<td>527.7</td>
<td>90.10</td>
</tr>
<tr>
<td>3</td>
<td>5.48</td>
<td>525.8</td>
<td>75.72</td>
</tr>
<tr>
<td>4</td>
<td>5.48</td>
<td>500.8</td>
<td>73.83</td>
</tr>
<tr>
<td>5</td>
<td>5.48</td>
<td>527.8</td>
<td>67.51</td>
</tr>
</tbody>
</table>

Average = 487.5  
Std Dev = 74.8  
RSD = 15.3%

Translates to LOD of approximately <100ppq
Spectrum for 1 ppt of Microcystin LR

Microcystin LR
C49 H74 N10 O12

Theoretical M+H
m/z = 995.5566

Mass Error = 3.6 mDa (3.6 ppm)
Why Use a QTof?

Because you need to know more.

Item name: Water_005
Channel name: 1: TOF MS(e) (50-1200) 6eV ESI+

©2015 Waters Corporation
Linearity for Microcystin LR

Compound name: Microcystin LR
Correlation coefficient: $r = 0.999008$, $r^2 = 0.998017$
Calibration curve: $197.411 \times x + -5.88617$
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: $1/x$, Axis trans: None

Calibration Curve for Microcystin LR
0.010ppb to 100ppb
Summary of 2D LC QTof Method

- Sensitive (PPQ) detection for MC-LR was achieved using 2D LC
  - 100 pg/L detection limit achieved
  - Linearity from 0.01 to 100ppb >0.99 R^2
  - Matrix effects were evaluated – minimal and acceptable
- Run time <15 min

Overall Summary

- Fit for purpose LOD can be achieved with high performance TQ through direct injection and little or no sample pretreatment
- 2D LC and SPE provide enrichment factors leading to fit for purpose LOD for lower sensitivity TQ or unparalleled sensitivity for research purposes when paired with high performance MS
Acknowledgements

- De Watergroep, Belgium
- Ministry of Environment and Climate Change, Toronto