# Reduced Injection Volume Applied to the Quantitation of Cylindrospermopsin and Anatoxin-a in Drinking Water According to EPA Method 545

Ali Haghani[1], Andy Eaton[1], Neloni Wijeratne[2], Claudia Martins[2], [1]Eurofins Eaton Analytical, Monrovia, CA, [2]Thermo Fisher Scientific, San Jose, CA

To demonstrate a sensitive, accurate, and reliable LC-MS/MS workflow to quantify anatoxin-a and cylindrospermopsin in drinking water according to EPA Method 545, performed at a reduced injection volume to increase method robustness while maintaining the desired levels of sensitivity.

## Introduction

Cyanobacteria naturally occur in surface waters. Under certain conditions, such as in warm water containing an abundance of nutrients, they can rapidly form harmful algal blooms (HABs). HABs can produce toxins known as cyanotoxins, which can be harmful to humans and animals. Anatoxin-a (also known as Very Fast Death Factor) is a neurotoxin with acute toxic effects and therefore subjected to monitoring and regulation efforts in several countries, including the United States. Cylindrospermopsin is toxic to liver and kidney tissues. As a result, the United States Environmental Protection Agency (EPA) has developed EPA Method 545[1] for the Unregulated Contaminant.

Monitoring Rule 4 (UCMR 4) program, which collects data for contaminants suspected to be present in drinking water but that do not have health-based standards set under the Safe Drinking Water Act (SDWA)[2]. This study demonstrates the performance of the new Thermo Scientific™ TSQ Quantis™ triple quadrupole MS platform performance using EPA Method 545: Determination of cylindrospermopsin and anatoxin-a in drinking water by liquid chromatography electrospray ionisation tandem mass spectrometry (LC/ESI-MS/MS).

## Experimental

### Sample preparation and LC/ESI-MS/MS conditions

The sample preparation was based on EPA Method 545. A triple freeze and thaw process was used on 2 mL of sample preserved with 0.1% sodium bisulphate and 0.01% ascorbic acid. The sample was filtered through a 0.2 µm pore size PVDF disposable filter to address the potential presence of intact algal cells in finished water samples. Then, 1 mL of sample was mixed with the phenylalanine-d5 and uracil-d4 internal standards and measured by direct injection-LC/ESI-MS/MS. Mobile phases used were 100 mM acetic acid in water and methanol.

Compounds were detected using the Thermo Scientific™ UltiMate™ 3000 LC system in conjunction with a Thermo Scientific™ TSQ Quantis™ triple quadrupole mass spectrometer equipped with a heated electrospray ionisation source. LC conditions were as stated in the EPA 545 method, and MS experimental conditions are listed in *Tables 1* and *2*.

*Table 1. MS source conditions.*

| Ion Source Parameter | Value |
|---|---|
| *Spray Voltage* | *3500 V* |
| *Sheath Gas* | *45 Arb* |
| *Aux Gas* | *10 Arb* |
| *Sweep Gas* | *0 Arb* |
| *Ion Transfer Tube Temperature* | *325 °C* |
| *Vaporizer Temperature* | *275 °C* |

## Requirements

The EPA has strict requirements that should be met before the analysis of any sample, referred to as the Initial Demonstration of Capability (IDC). These requirements include the demonstration of low background noise, precision by analysing four laboratory fortified reagent water blanks (LFB) at mid-level, the demonstration of accuracy and, finally, the demonstration of capability necessary to meet the minimum reporting limit (MRL). The percent relative standard deviation (%RSD) of the results of the replicate analyses must be ≤ 20%. The average percent recovery for each analyte must be within ± 30% of the true value. All IDC samples need to be treated as field samples going through the entire method process. For comparability of results using the TSQ Quantis triple quadrupole MS, 5 µL, 10 µL, and 25 µL injections are reported.

*Table 2. Optimised SRM conditions.*

| Compound | Polarity | Precursor (m/z) | Product (m/z) | Collision Energy (V) | RF Lens (V) |
|---|---|---|---|---|---|
| *Anatoxin-a* | *Positive* | *166* | *131* | *18* | *100* |
| *Cylindrospermopsin* | *Positive* | *416* | *176* | *35* | *120* |
| *Phenylalanine-d5 IS for Anatoxin-a* | *Positive* | *171* | *125* | *12* | *80* |
| *Uracil-d4 IS for Cylindrospermopsin* | *Positive* | *115* | *98* | *10* | *55* |

## Results and Discussion

### Calibration

The initial calibration was validated by calculating the concentration of each analyte as an unknown against its regression equation. For calibration levels that are ≤ MRL, the result for each analyte should be within ± 50% of the true value. All other calibration points must calculate to be within ± 30% of their true value. Results for all three injections met the calibration criteria. *Figure 1* shows representative calibration curves for all compounds using a 5 µL injection.

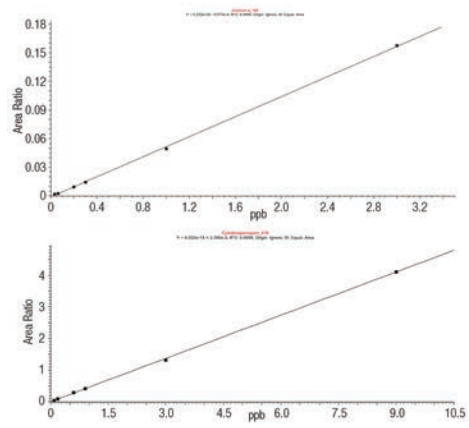


*Figure 1A. Calibration curves for (top) anatoxin-a and (bottom) cylindrospermospin.*

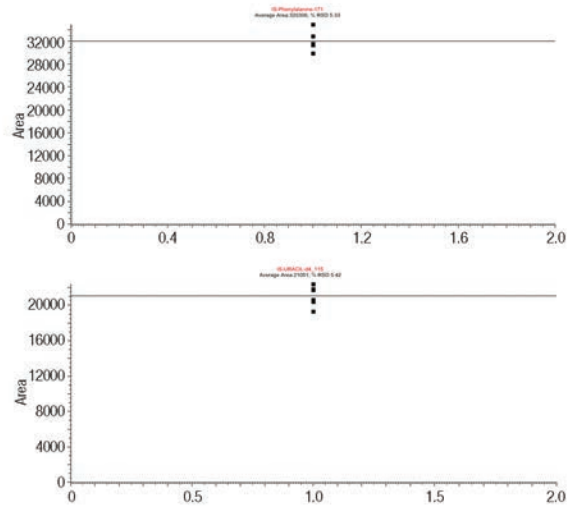


*Figure 1B. Calibration curves for reproducibility (%RSD) of internal standards with a 5 µL injection.*

*Table 3. Low background noise for all EPA Method 545 analytes.*

| Analyte | MRL (µg/L) | 1/3 MRL (µg/L) | 5 µL | 10 µL | 25 µL |
|---|---|---|---|---|---|
| *Anatoxin-a* | *0.03* | *0.01* | *NF* | *NF* | *NF* |
| *Cylindrospermopsin* | *0.09* | *0.03* | *NF* | *NF* | *NF* |

*NF= Not found*

## System Background

All method blanks exhibited less than 1/3 MRL contamination or carryover (*Table 3, Figure 2*).

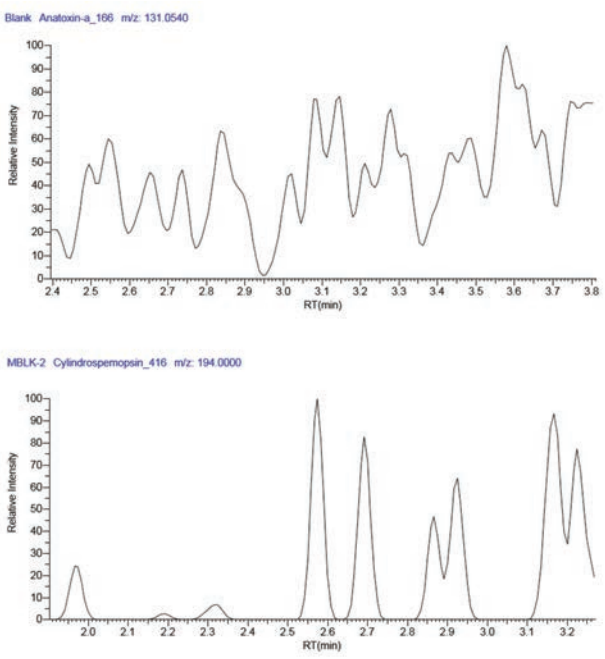


*Figure 2. 5 µL blank injection, (A) anatoxin-a, (B) cylindrospermopsin.*

*Table 4. Precision and accuracy at 10x MRL for all EPA Method 545 analytes.*

| Analyte | Actual (µg/L) | LFB1 (µg/L) | LFB2 (µg/L) | LFB3 (µg/L) | LFB4 (µg/L) | %Rec | %RSD |
|---|---|---|---|---|---|---|---|
| *5 µL Injection* | | | | | | | |
| *Anatoxin-a* | *0.3* | *0.315* | *0.326* | *0.286* | *0.29* | *101%* | *6%* |
| *Cylindrospermopsin* | *0.9* | *1.011* | *0.952* | *1.182* | *1.12* | *118%* | *10%* |
| *IS-Phenylalanine-d5* | | *90%* | *96%* | *123%* | *117%* | | |
| *IS-Uracil-d4* | | *122%* | *119%* | *127%* | *119%* | | |
| *10 µL Injection* | | | | | | | |
| *Anatoxin-a* | *0.3* | *0.297* | *0.306* | *0.309* | *0.309* | *102%* | *2%* |
| *Cylindrospermopsin* | *0.9* | *1.09* | *0.994* | *0.992* | *1.02* | *114%* | *4%* |
| *IS-Phenylalanine-d5* | | *90%* | *99%* | *100%* | *98%* | | |
| *IS-Uracil-d4* | | *89%* | *101%* | *93%* | *83%* | | |
| *25 µL Injection* | | | | | | | |
| *Anatoxin-a* | *0.3* | *0.347* | *0.319* | *0.324* | *0.315* | *109%* | *4%* |
| *Cylindrospermopsin* | *0.9* | *0.956* | *0.899* | *1.072* | *1.055* | *111%* | *8%* |
| *IS-Phenylalanine-d5* | | *88%* | *94%* | *96%* | *94%* | | |
| *IS-Uracil-d4* | | *93%* | *101%* | *80%* | *78%* | | |
| *IS criteria* | *50–150%* | | | | | | |
| *%Recovery* | *70–130%* | | | | | | |
| *%RSD* | *<20* | | | | | | |

## Precision and Accuracy

The initial demonstration of precision and accuracy was met by analysing four extracted LFBs spiked at 10x MRL level, which showed less than 20% RSD and ± 30% difference (*Table 4 and Figure 3*).

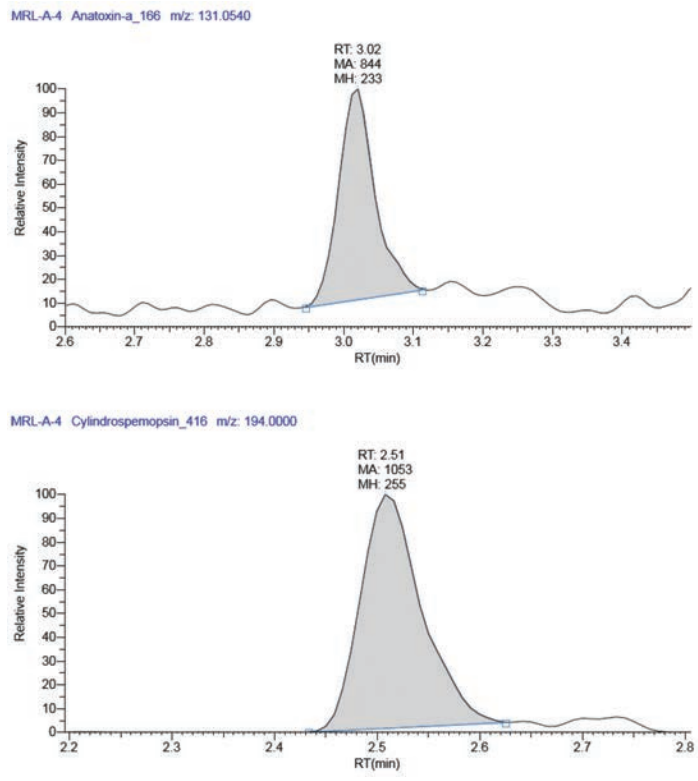


*Figure 3. 5 µL injection volume chromatograms, (A) anatoxin-a, (B) cylindrospermopsin.*

## MRL confirmation

MRL confirmation was evaluated by fortifying, extracting, and analysing seven replicate LFBs at the proposed MRL concentration. The mean and the half range (HR) was then calculated. The Prediction Interval of Results (PIR) is defined as

$PIR = Mean + HR_{PIR}$

where $HR_{PIR} = 3.963s$; *s* is the standard deviation and 3.963 is a constant value for seven replicates.

The upper and lower limits for the PIR meet the recovery criteria (upper PIR - 150%; lower PIR - 50%) (*Table 5*).

## Tap water analysis

A Monrovia, CA tap water sample (comprised of ground and surface water) was extracted and analysed using the methodology developed. Results are shown in *Table 6*.

*Table 5. Minimum reporting limit confirmation for all EPA Method 545 analytes.*

| Analyte | Actual (µg/L) | LFB1 (µg/L) | LFB2 (µg/L) | LFB3 (µg/L) | LFB4 (µg/L) | LFB-5 (µg/L) | LFB-6 (µg/L) | LFB-7 (µg/L) | Lower PIR (%) | Upper PIR (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| 5 µL Injection | | | | | | | | | | |
| Anatoxin-a | 0.03 | 0.038 | 0.03 | 0.031 | 0.035 | 0.031 | 0.029 | 0.032 | 66% | 149% |
| Cylindrospermopsin | 0.09 | 0.087 | 0.104 | 0.1 | 0.099 | 0.106 | 0.098 | 0.113 | 77% | 148% |
| IS-Phenylalanine-d5 | | 134% | 133% | 129% | 125% | 127% | 131% | 127% | | |
| IS-Uracil-d4 | | 122% | 119% | 127% | 119% | 126% | 125% | 118% | | |
| 10 µL Injection | | | | | | | | | | |
| Anatoxin-a | 0.03 | 0.034 | 0.033 | 0.035 | 0.036 | 0.036 | 0.037 | 0.035 | 99% | 135% |
| Cylindrospermopsin | 0.09 | 0.076 | 0.091 | 0.09 | 0.093 | 0.096 | 0.084 | 0.08 | 65% | 129% |
| IS-Phenylalanine-d5 | | 111% | 130% | 129% | 130% | 129% | 129% | 129% | | |
| IS-Uracil-d4 | | 105% | 112% | 111% | 110% | 115% | 113% | 116% | | |
| 25 µL Injection | | | | | | | | | | |
| Anatoxin-a | 0.03 | 0.039 | 0.039 | 0.041 | 0.04 | 0.04 | 0.042 | 0.041 | 120% | 149% |
| Cylindrospermopsin | 0.09 | 0.091 | 0.1 | 0.099 | 0.106 | 0.102 | 0.096 | 0.1 | 89% | 131% |
| IS-Phenylalanine-d5 | | 105% | 105% | 105% | 104% | 101% | 105% | 101% | | |
| IS-Uracil-d4 | | 103% | 101% | 102% | 103% | 106% | 111% | 110% | | |
| IS criteria | 50–150% | | | | | | | | | |

*LFB stands for Fortified Laboratory Blank. PIR stands for Prediction Interval of Results.*

*Table 6. Monrovia water sample analysed using the TSQ Quantis triple quadrupole MS*

| Analyte | Actual (µg/L) | FS | LFSM | LFSMD | %Rec | %RSD |
|---|---|---|---|---|---|---|
| 5 µL Injection | | | | | | |
| Anatoxin-a | 0.3 | 0 | 0.337 | 0.367 | 117% | 6% |
| Cylindrospermopsin | 0.9 | 0 | 0.954 | 0.966 | 107% | 1% |
| IS-Phenylalanine-d5 | | | 120% | 111% | | |
| IS-Uracil-d4 | | | 142% | 130% | | |
| 10 µL Injection | | | | | | |
| Anatoxin-a | 0.3 | 0 | 0.347 | 0.352 | 117% | 1% |
| Cylindrospermopsin | 0.9 | 0 | 1.162 | 1.085 | 125% | 5% |
| IS-Phenylalanine-d5 | | | 119% | 109% | | |
| IS-Uracil-d4 | | | 89% | 113% | | |
| 25 µL Injection | | | | | | |
| Date Analyzed | | | 4/7/2017 | 4/7/2017 | | |
| Anatoxin-a | 0.3 | 0 | 0.35 | 0.341 | 115% | 2% |
| Cylindrospermopsin | 0.9 | 0 | 0.788 | 0.843 | 91% | 5% |
| IS-Phenylalanine-d5 | | | 96% | 87% | | |
| IS-Uracil-d4 | | | 122% | 107% | | |
| IS criteria | 50–150% | | | | | |
| %Recovery | 70–130% | | | | | |
| %RSD | <30 | | | | | |

*FS stands for Field Sample. LFSM stands for Laboratory Fortified Sample Matrix. LFSMD stands for Laboratory Fortified Sample Matrix Duplicate.*

## Conclusion

- The TSQ Quantis triple quadrupole MS proved to be sensitive, accurate, reproducible, and reliable in the quantitation of cylindrospermopsin, and anatoxin-a in drinking water according to EPA Method 545.
- Adequate sensitivity was obtained with 5 µL, 10 µL, and 25 µL injection volumes for drinking water matrices. This represents an up to 10-fold reduction in the injection volume than what the EPA is recommending and results in less matrix injected, thereby reducing maintenance of the LC-MS system.

## References

1. *U.S. EPA Method 545: Determination of Cylindrospermopsin and Anatoxin-A in Drinking Water by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC/ESI-MS/MS), version 1.0, April 2015.*

2. *U.S. EPA. Monitoring Unregulated Drinking Water Contaminants. https://www.epa.gov/dwucmr/fourth-unregulated-contaminant-monitoring-rule (accessed April 23, 2017).*