Comprehensive Microcystins Analysis and Identification Using Innovative Sample Preparation and Novel LC/MS Techniques

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Introduction

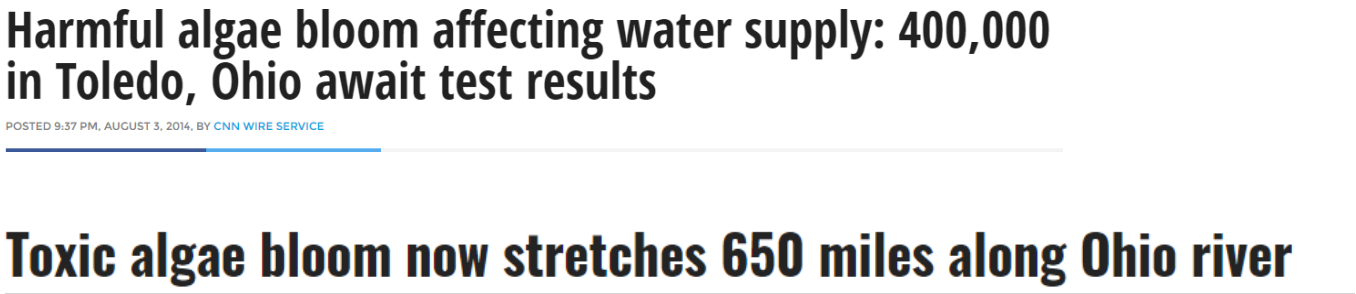
Cyanobacteria are naturally occurring in most lakes and rivers

In warm temperatures and presence of nutrients, they can form harmful algal blooms (HABs) and release cyanotoxins

Cyanotoxins can be classified into 3 categories:

- Cyclic peptides, including **microcystins (MCs)** and nodularins
- Alkaloids
- Lipopolysaccharides

MCs are the most commonly occurring in water and potent hepatotoxins. (100s of variations)



Microcystins

Global Guidelines

Organization	Compound	Drinking Water (ug/L)
World Health Organization	LR	1.0
EU Drinking Water Directive (proposed)	LR	1.0
Canada	LR*	1.5
USEPA	total	8.0
Ohio		0.3 µg/L bottle-fed infants and preschool age children 1.6 µg/L schoolage children and adults
Oregon		0.3 µg/L age 5 and younger 1.6 µg/L age 6 and older
Minnesota		0.1 ug/L

* “maximum acceptable concentrations (MAC); protective of total microcystins”

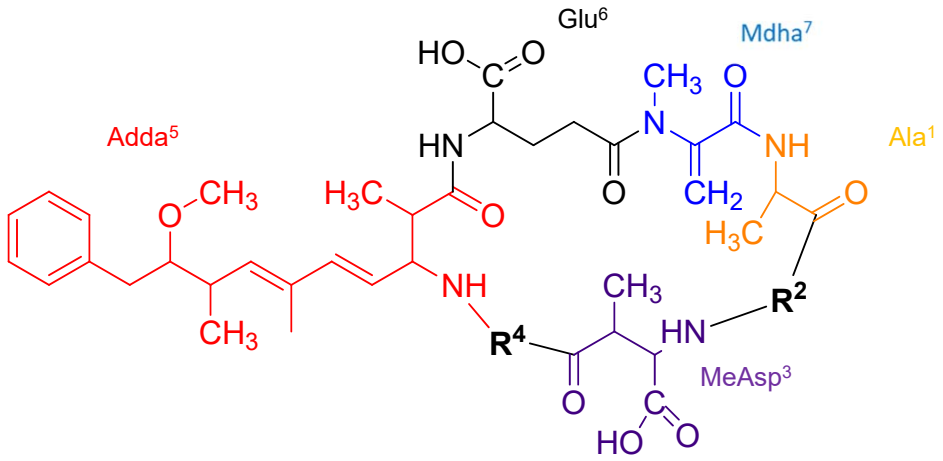
EPA Method 544:DETERMINATION OF MICROCYSTINS AND NODULARIN IN DRINKING WATER BY SOLID PHASE EXTRACTION AND LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY (LC/MS/MS)

General Microcystins Structure

- 7 amino acids in cyclic structure
- Most variations differ in R^2 & R^4 location

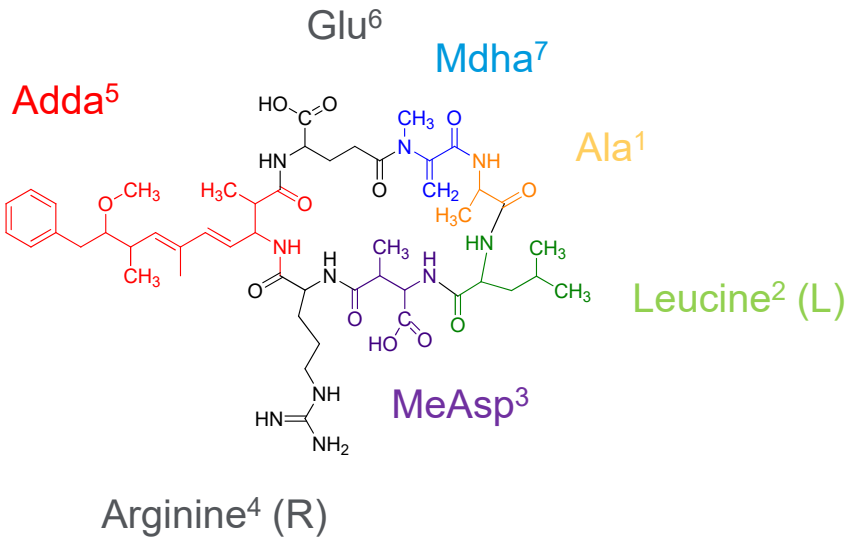
  Naming convention uses amino acid abbreviations for these positions

  Superscript number indicates amino acid order within molecule



Microcystin	R^2	R^4	Formula	Neutral Mass
LR	Leucine	Arginine	$C_{49}H_{74}N_{10}O_{12}$	994.5488
Desmethyl LR	Leucine	Arginine	$C_{48}H_{72}N_{10}O_{12}$	980.5331
RR	Arginine	Arginine	$C_{49}H_{75}N_{13}O_{12}$	1037.5658
YR	Tyrosine	Arginine	$C_{52}H_{72}N_{10}O_{13}$	1044.5280
LA	Leucine	Alanine	$C_{46}H_{67}N_7O_{12}$	909.4848
LW	Leucine	Phenylalanine	$C_{54}H_{72}N_8O_{12}$	1024.5270
LF	Leucine	Tryptophan	$C_{52}H_{71}N_7O_{12}$	985.5161
HtyR	Homotyrosine	Arginine	$C_{53}H_{74}N_{10}O_{13}$	1058.5437

Microcystin-LR Structure



$C_{49}H_{74}N_{10}O_{12}$
Mass = 994.5488

Position	Abbreviation	Amino Acid
R^1	Ala^1	Alanine
R^2	Leu^2 (L)	Leucine
R^3	$MeAsp^3$	Methylaspartic acid
R^4	Arg^4 (R)	Arginine
R^5	$Adda^5$	3-amino-9-methoxy-2,6,6-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid
R^6	Glu^6	Glutamic acid
R^7	$Mdha^7$	N-methyldehydroalanine

Optimizing Microcystin Analysis: Considerations

Sample Preparation

- Offline SPE
- Online SPE
- Direct Injection

LC Separation

- Separation of MCs

MS Detection

- Quantification only
- Quant + Screening + Unknowns ID

EPA Method 544
Analysis of Microcystins & Nodularin by Offline SPE + LC/MS/MS

Collection and preservation: 0.5 L

- TRIZMA, 2-chloroacetamide, ascorbic acid, EDTA

Add surrogate (C_2D_5-LR)

Filter; rinse bottle with 10% MeOH

- Soak filter in 80% MeOH; freeze 1 – 16 hours
- Rinse filter and combine with filtered water

Offline SPE to extract MC's

- Elute with 10 mL of 90% MeOH
- * Evaporate to dryness
- * Reconstitute with 1 mL of 90% MeOH

* "the laboratory is permitted to modify the evaporation technique, separation technique, LC column, mobile phase composition, LC conditions and MS and MS/MS conditions"

Sample Preparation for this study

- Collection and preservation: 0.5 L (well water + tap water)
  - TRIZMA, 2-chloroacetamide, ascorbic acid, EDTA
- Add surrogate (C_2D_5-LR)
- Filter; rinse bottle with 10% MeOH
  - Soak filter in 80% MeOH; freeze 1 – 16 hours
  - Rinse filter and combine with filtered water
- Offline SPE to extract MC's
  - Elute with 10 mL of 90% MeOH
  - *Evaporate to ~~dryness~~ 2-3 mL
  - *Reconstitute ~~with 1 mL of 90% MeOH~~ to 5.0 mL with MeOH

* "the laboratory is permitted to modify the evaporation technique, separation technique, LC column, mobile phase composition, LC conditions and MS and MS/MS conditions"

MRM Parameters[a]

1260 Infinity II LC + Ultivo LC/MS/MS

Microcystin	Precursor [b] (m/z)	Product Ion (m/z)	Collision Energy (V)
LR	995.6	135.2 213.2	80 80
Desmethyl-LR	981.5	135.2 213.2	80 80
RR	520.0	135.2 213.2	30 40
YR	1045.5	135.2 213.2	80 70
LA	910.5	135.2 213.2	70 70
LY	1002.5	135.2 213.2	80 70
LW	1025.5	135.2 213.2	80 60
LF	986.5	135.2 213.2	70 50
HtyR	1059.5	135.2 213.2	80 70

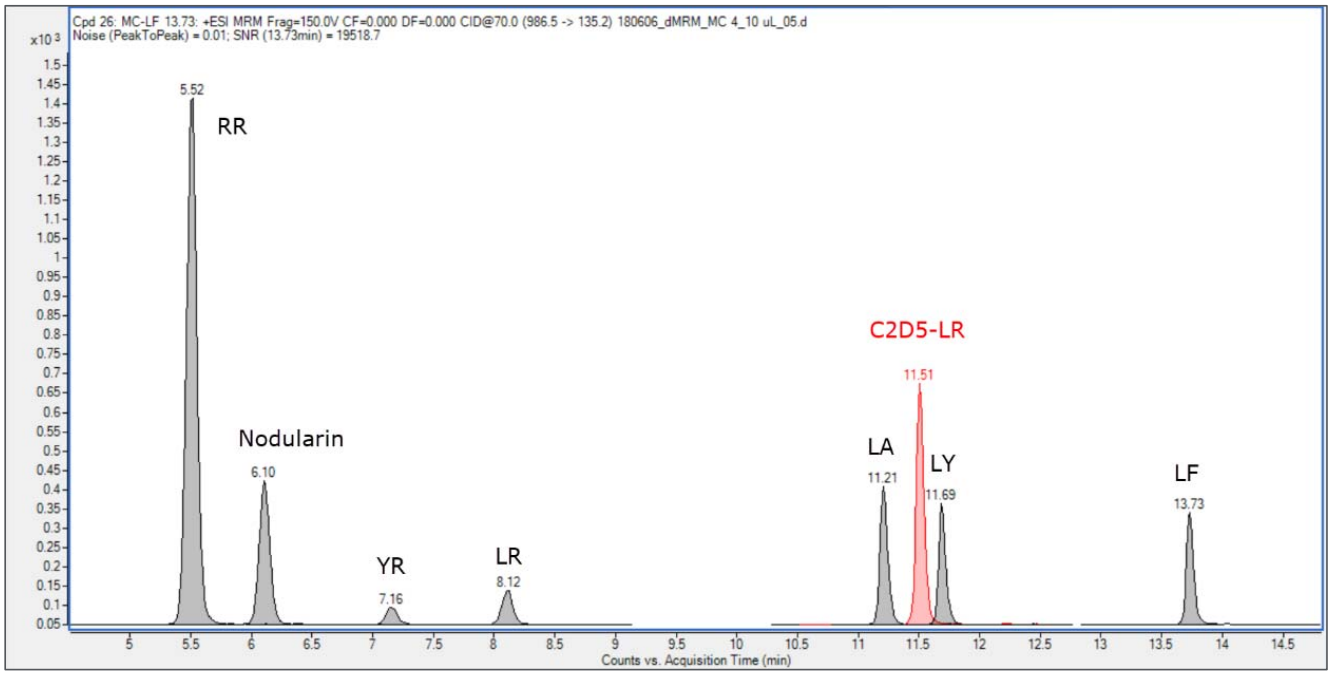
1260 Infinity II LC + 6470 MS/MS

[a] The fragmentor and cell acceleration voltages were 150 V and 2 V, respectively, for all transitions; minor differences on Ultivo LC/MS

[b] All precursors are singly charged, except RR which is doubly charged.

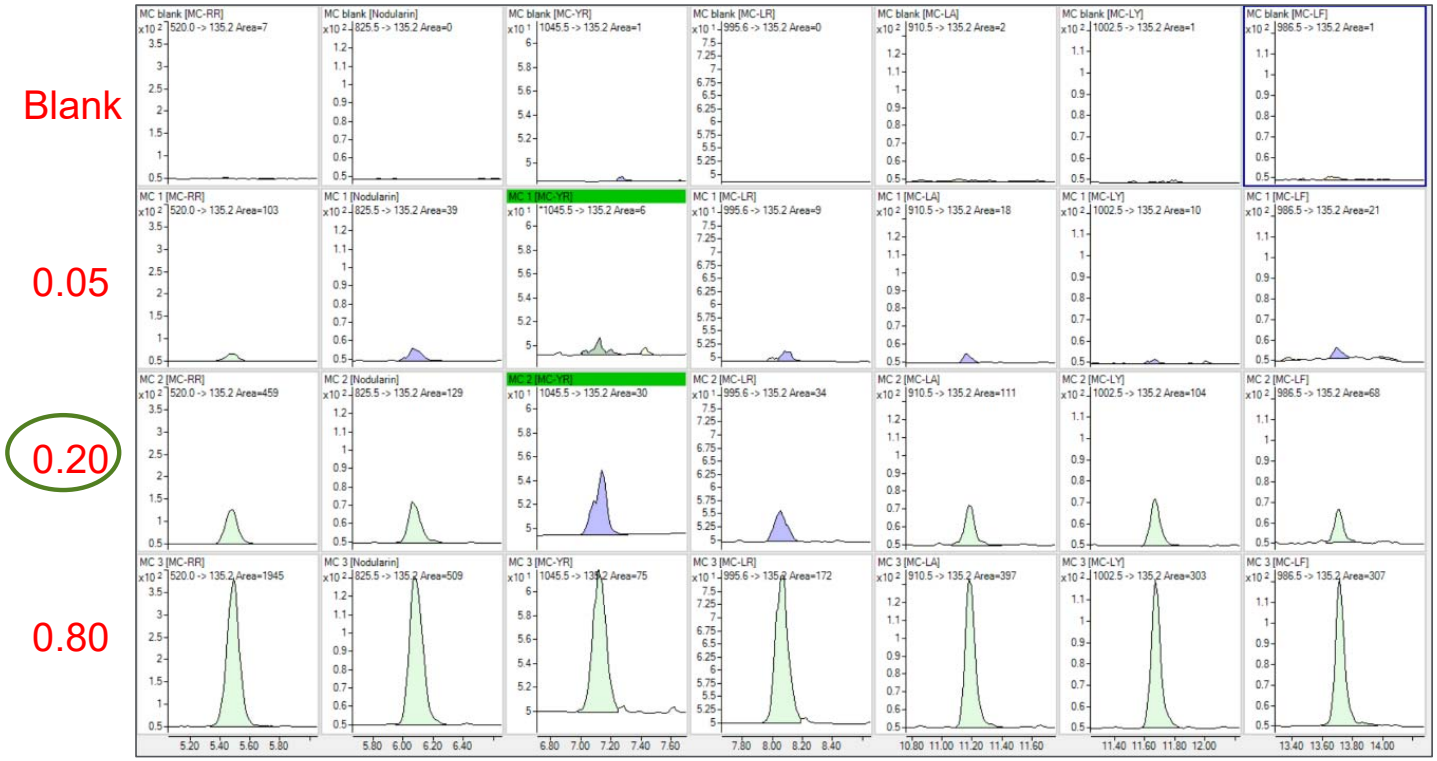
Chromatography

EPA 544 Method Flexibility (Sec. 1.6): "Analytes must be adequately resolved chromatographically in order to permit the mass spectrometer to dwell on a minimum number of compounds eluting within a retention time window."

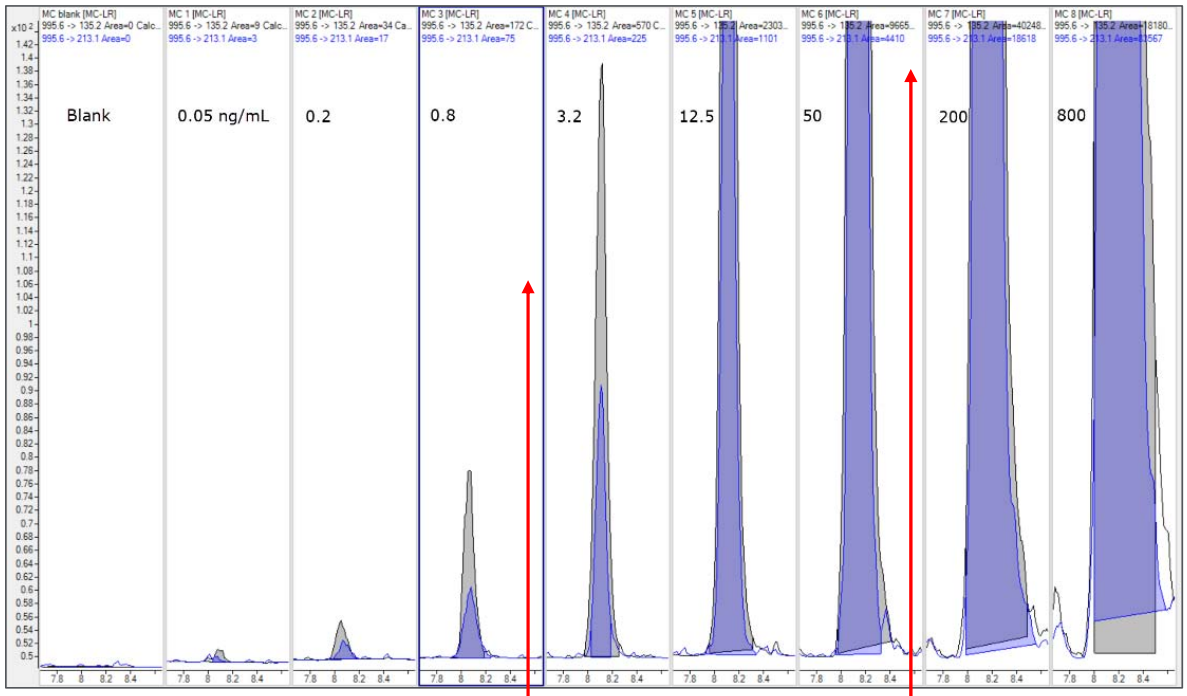


Agilent Poroshell SB-C18
2.1 x 50 mm 2.7 µm LC column

Low Calibrators (ng/mL in vial)
10-uL injections



MC-LR vs Drinking Water Guideline Levels
0.5 L SPE; reconstitute in 5 mL (100x conc)

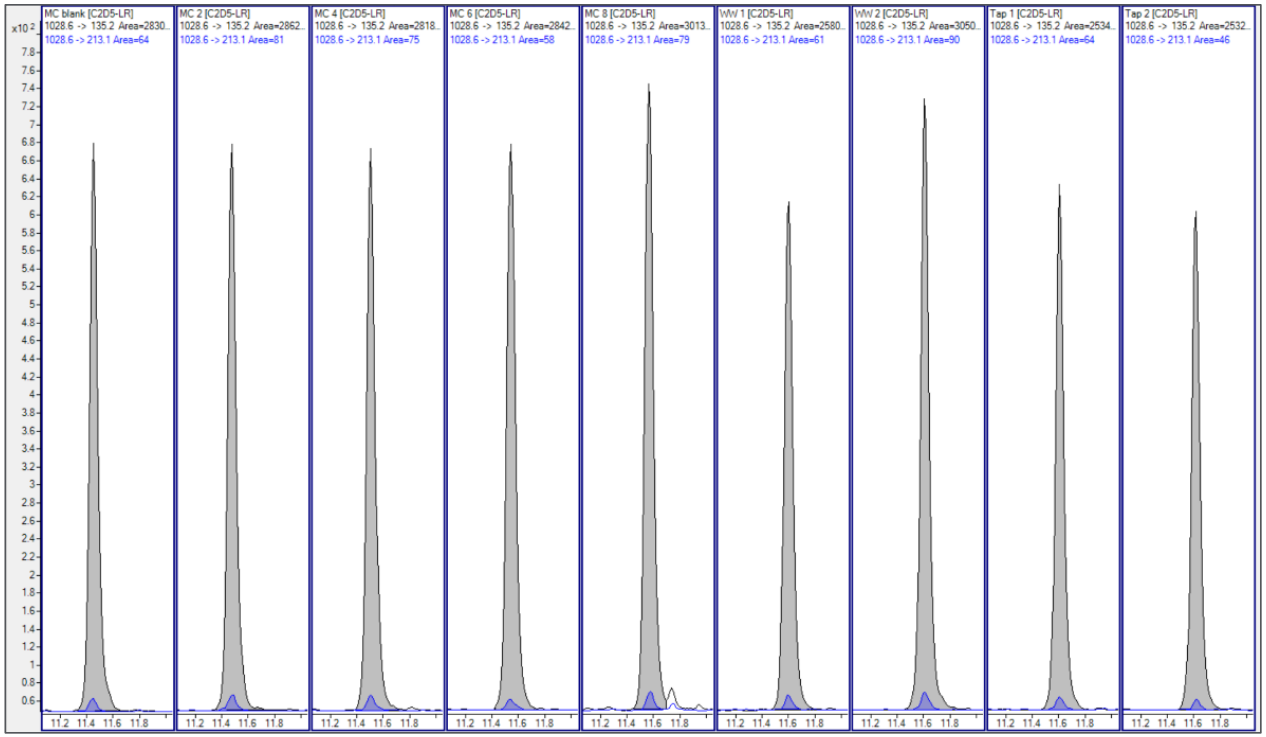


Guidelines for World Health Organization: Microcystin-LR = 1 ug/L in water

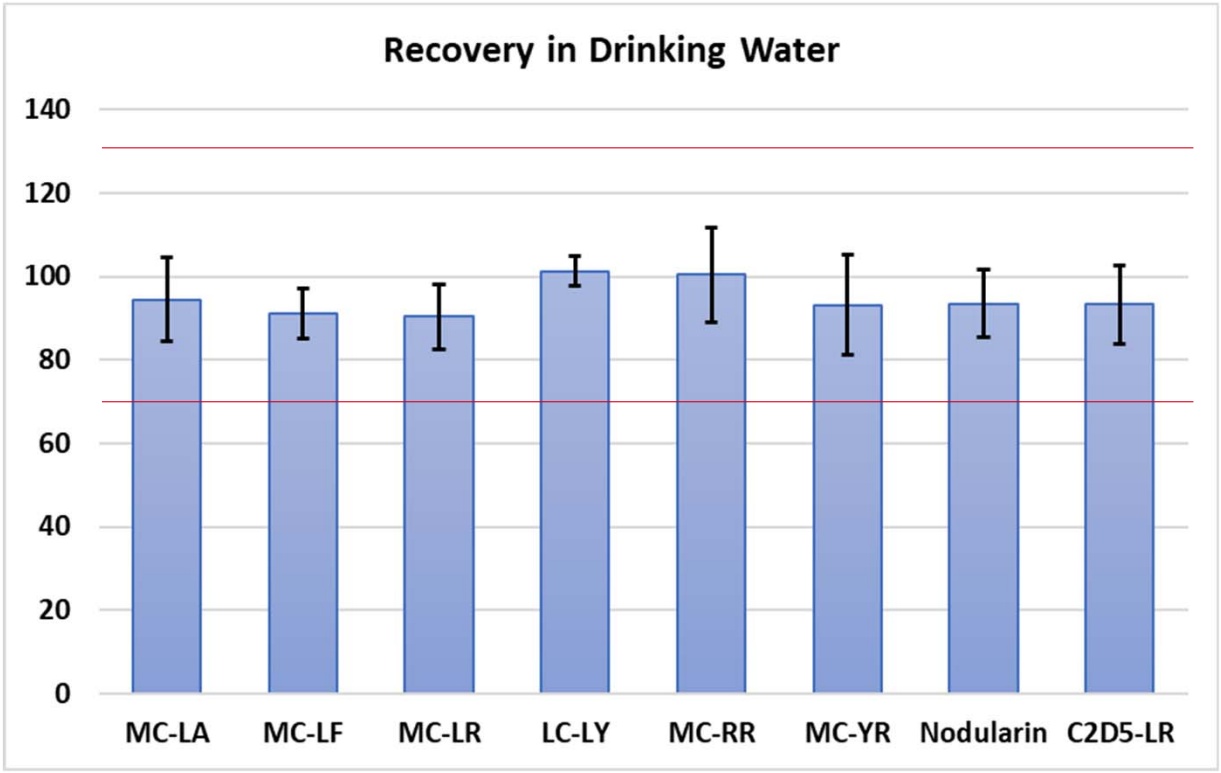
(1 ug/L * 0.5 L) in 5 mL final volume = 0.1 ug/mL = 100 ng/mL in vial

Ion Suppression Evaluation

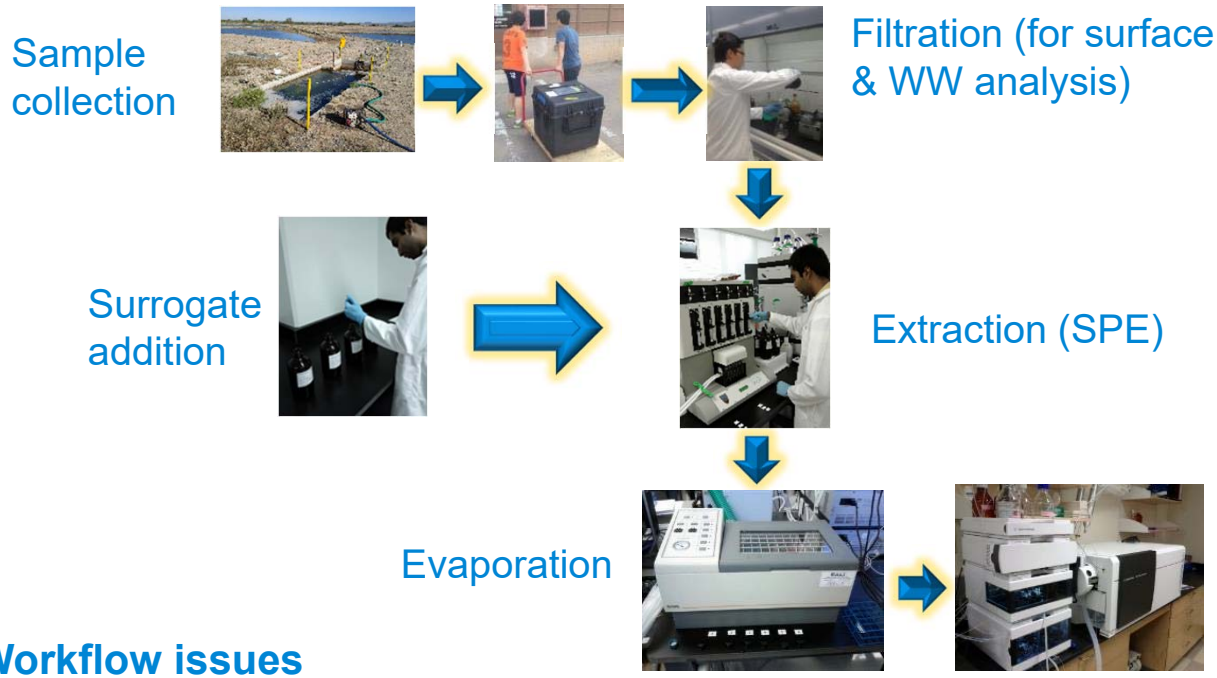
C_2D_5-LR surrogate in solvent blank, calibrators, and water samples



Quantitative Spike Recovery Results
Tap water spikes @ 0.02 ug/L (n=4)



Offline SPE vs Online SPE



Benefits of Online SPE

Lower sample collection & transport

More reproducibility due to automation

Much lower labor cost

Faster time to results

Sensitivity

Lower solvent usage

Lower internal std usage

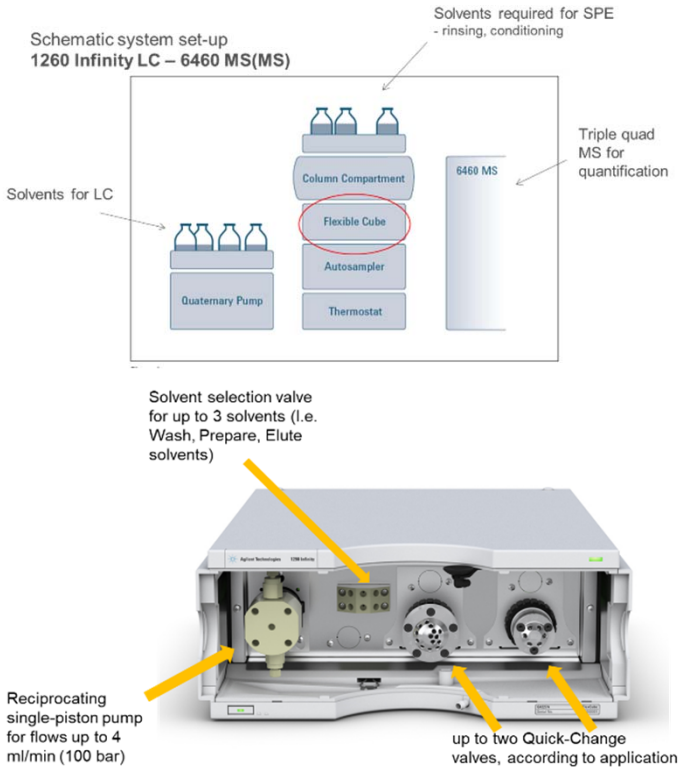
Workflow issues

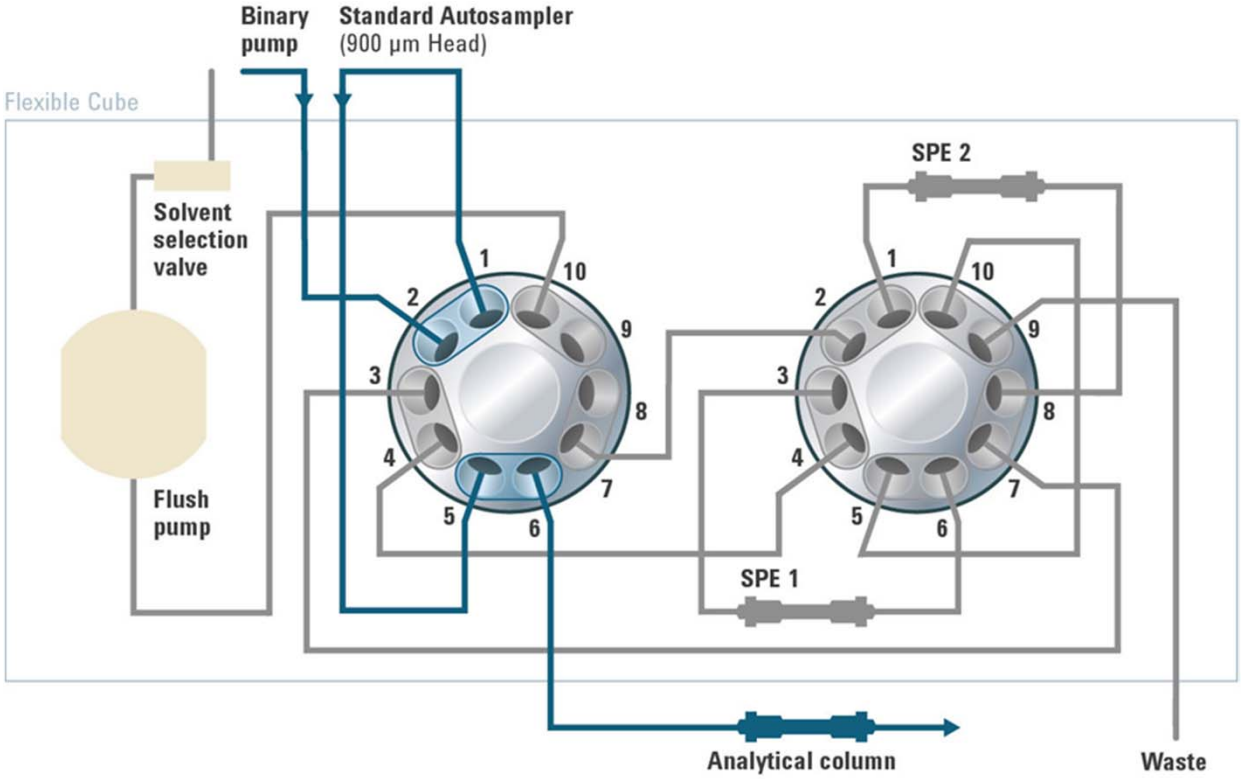
Risk of contamination between samples

Inter-operator consistency

Reproducibility & Accuracy

Online SPE Setup & Configuration





Online SPE & Direct Injection Conditions

Online SPE

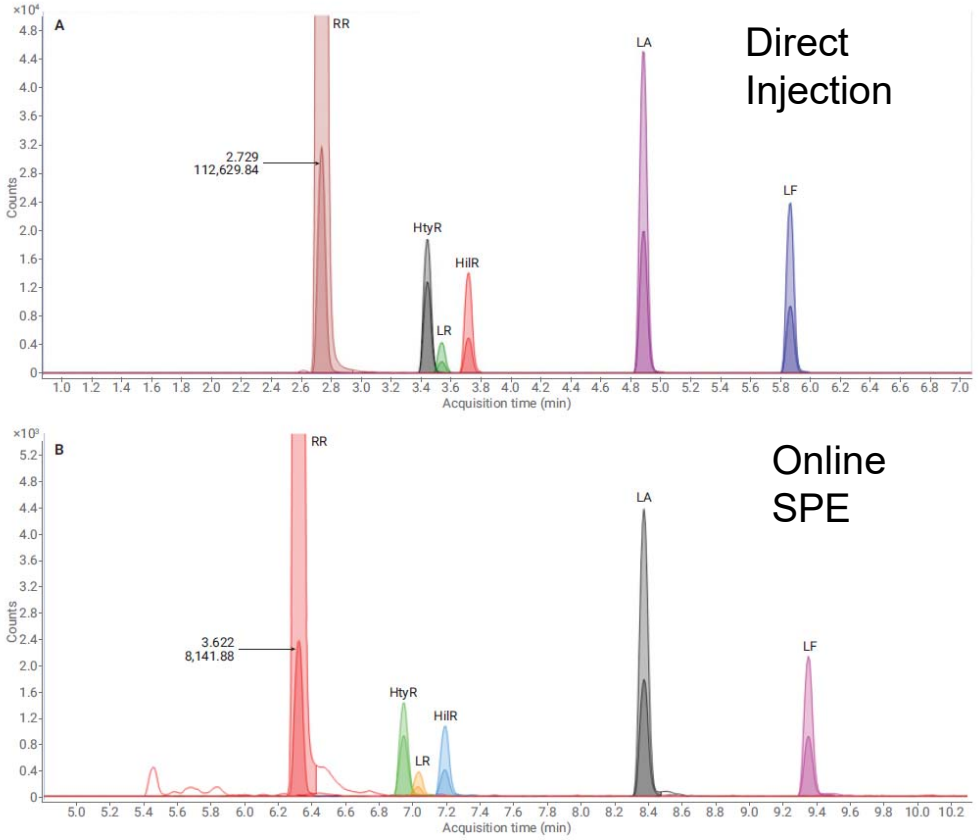
Parameter	Value
Analytical column	Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm (p/n 695975-302)
Column temperature	45 °C
Trapping columns	2 × PLRP-S cartridges, 2.1 × 12.5 mm (p/n 5982-1271); 6 mL screw top vials (p/n 9301-1377), screw caps (p/n 9301-1379), preslit septa (p/n 5188-2758)
Injection volume	1,800 µL
Draw and eject speed	1,000 µL/min; 4,000 µL/min
Needle wash	MeOH, 5 seconds
Mobile phases	A) 0.1 % FA + 1 mM NH_4F in H_2O B) 0.1 % FA in ACN
Stop time	14 minutes
Post time	3 minutes
Flow rate	0.4 mL/min
Gradient	Time (min) %B 0 30 4 30 12 95 14 95

Direct Injection

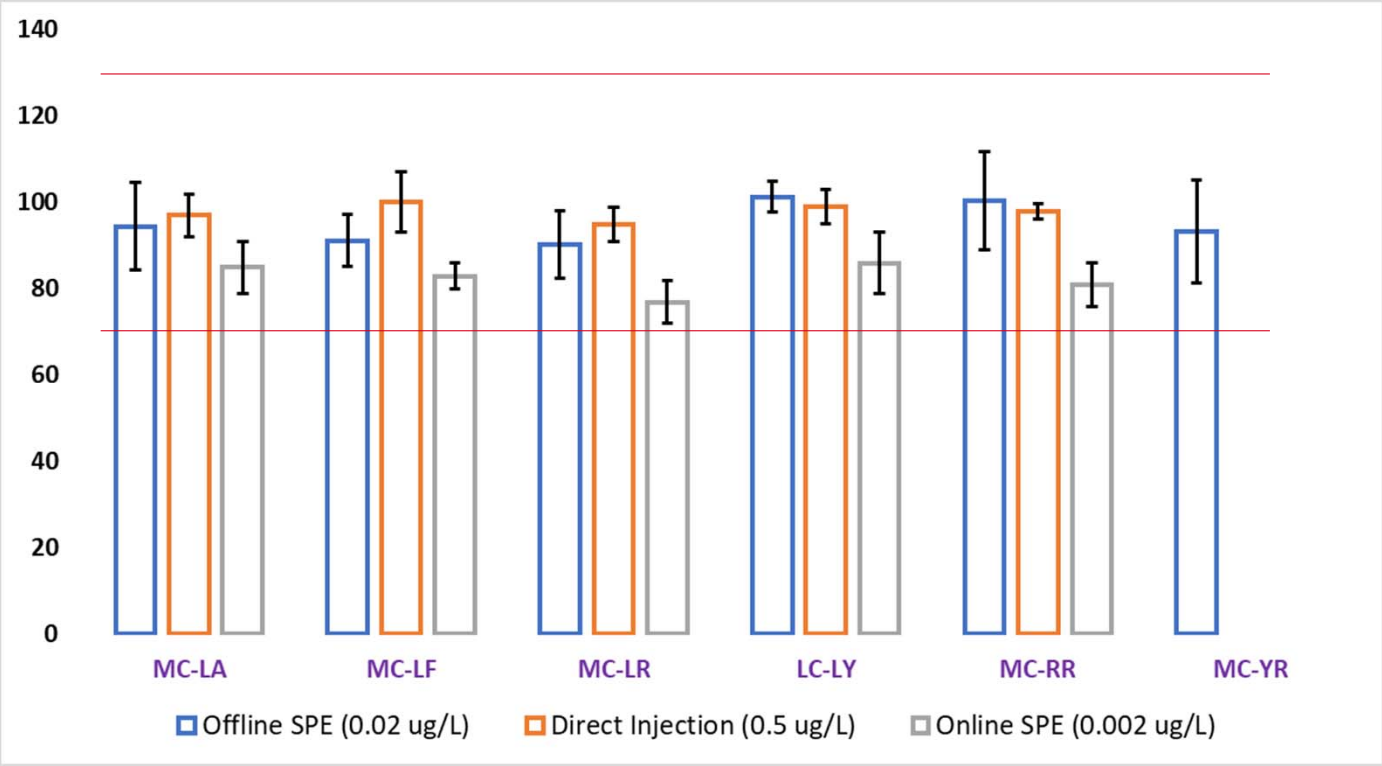
Parameter	Value
Analytical column	Agilent InfinityLab Poroshell 120 EC-C18, 3.0 × 100 mm, 2.7 µm (p/n 695975-302)
Column temperature	45 °C
Injection volume	10 µL
Draw and eject speed	100 µL/min; 400 µL/min
Needle wash	Methanol (MeOH), 5 seconds
Mobile phases	A) 0.1 % formic acid (FA) + 1 mM ammonium fluoride (NH_4F) in water (H_2O) B) 0.1 % FA in acetonitrile (ACN)
Stop time	10 minutes
Post time	3 minutes
Flow rate	0.4 mL/min
Gradient	Time (min) %B 0 30 8 95 10 95

Method Performance for Online SPE & Direct Injection

MCs	Direct injection		Online SPE	
	LOD (ppt)	LOQ (ppt)	LOD (ppt)	LOQ (ppt)
RR	10	50	0.05	0.2
HtyR	50	100	0.2	1
LR	100	500	0.5	2
HilR	50	100	0.2	1
LA	50	100	0.2	1
LF	50	100	0.2	1



Spike Recovery Experiments for Online SPE & Dir. Injection

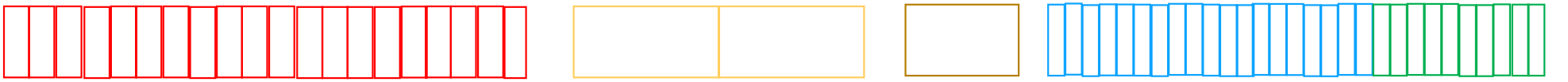


Avg. RSD (%)

Offline SPE: 8.7%
Online SPE: 5.1%
Dir. Injection: 4.3%

Offline SPE vs Online SPE time-savings
Assuming a batch of 20 samples

Offline Extraction



Sample Filtration plus Isotope Spiking
20 min/sample (500 mL)
= 400 min

400 min

Sample extraction with two batches of 10 samples taking 2 hrs each = 240 min

640 min

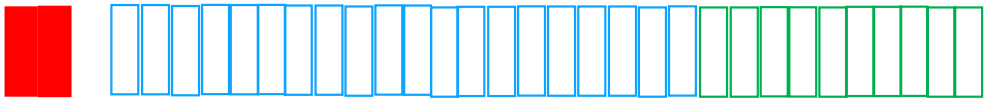
Evaporation = 90 min

730 min

13 min/analysis plus 10 QA/QC samples = 390 min

1120 min

Online Extraction



40 min

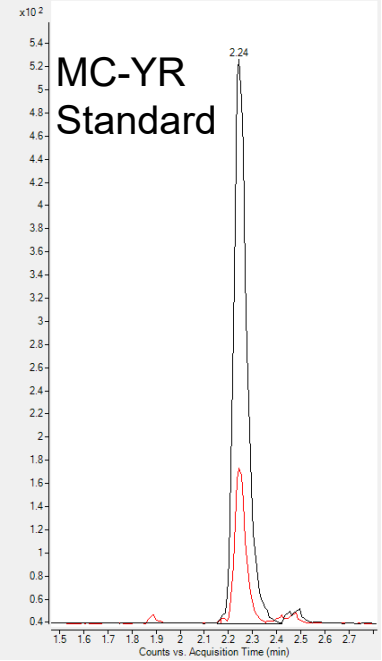
17 min/analysis plus 10 QA/QC samples = 510 min

550 min

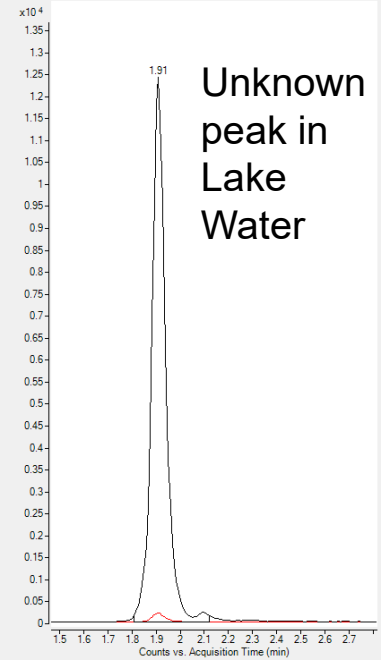
Analysis done in less than half the time!

Suspect peaks can show up on LC/MS/MS that can be misidentified

MC-YR identification in lake water sample



RT = 2.24 min
1045.5 -> 135.2
1045.5 -> 213.2
Qual Ratio = 24%

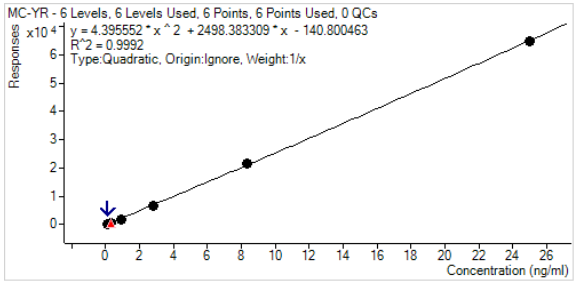


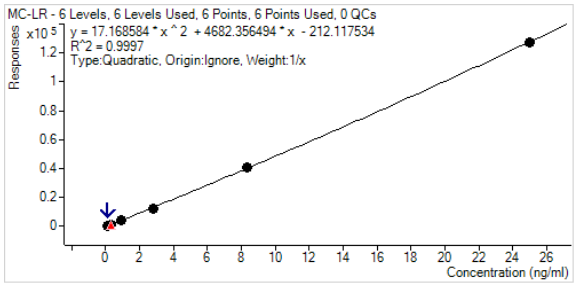
RT = 1.91 min
1045.5 -> 135.2
1045.5 -> 213.2
Qual Ratio = 2%

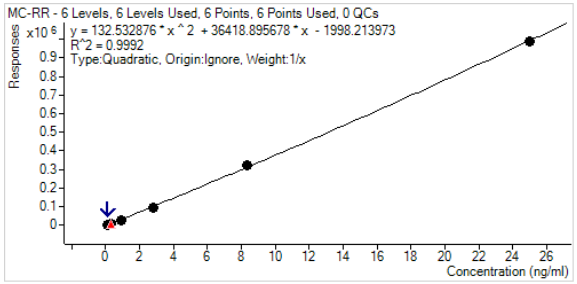
- Is this MC-YR?
- Is this another MC?
- Can it be something else with same precursor mass of 1045.5?
- How do we find out?

LC-Q/TOF

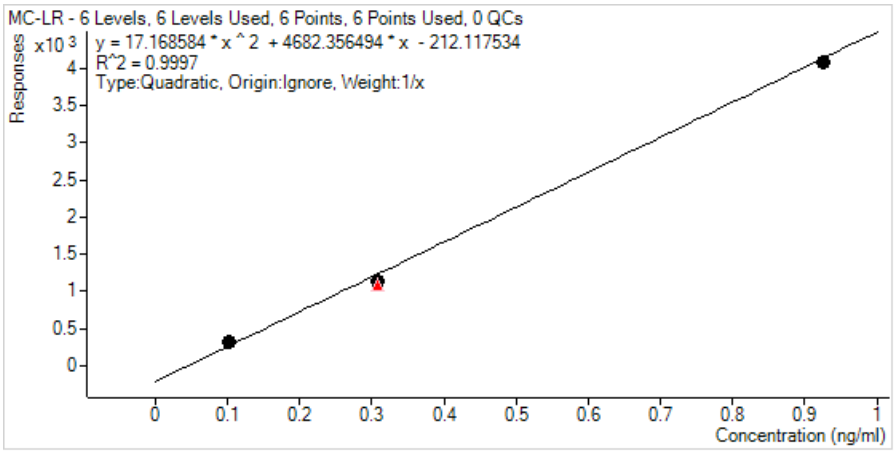
Calibration Curves for Quantification by LC-Q/TOF
0.1 – 25 µg/L





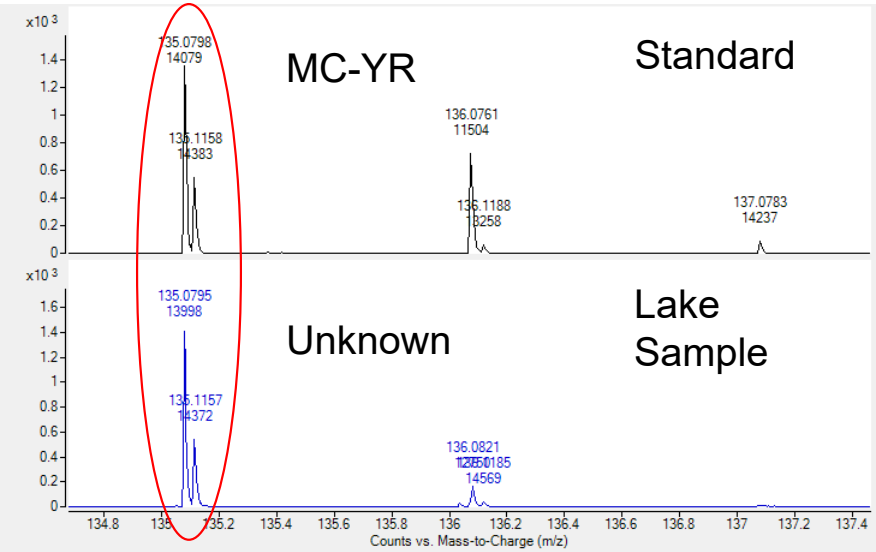


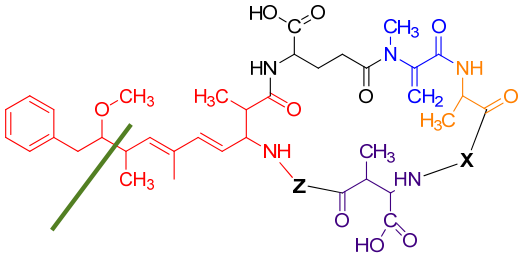
Cpd	R^2	Cal 1 Accuracy
YR	0.9993	118 %
LR	0.9997	110 %
RR	0.9992	119 %



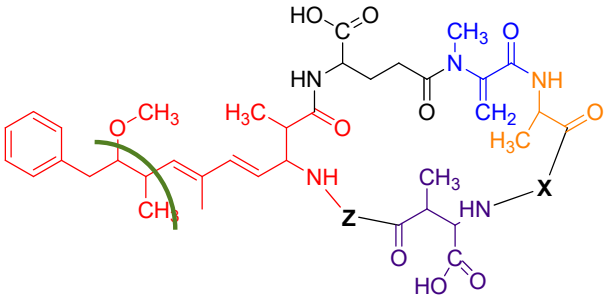
Is it a Microcystin?

Fragmentation – m/z 135





135.0804 = $C_9H_{11}O^+$

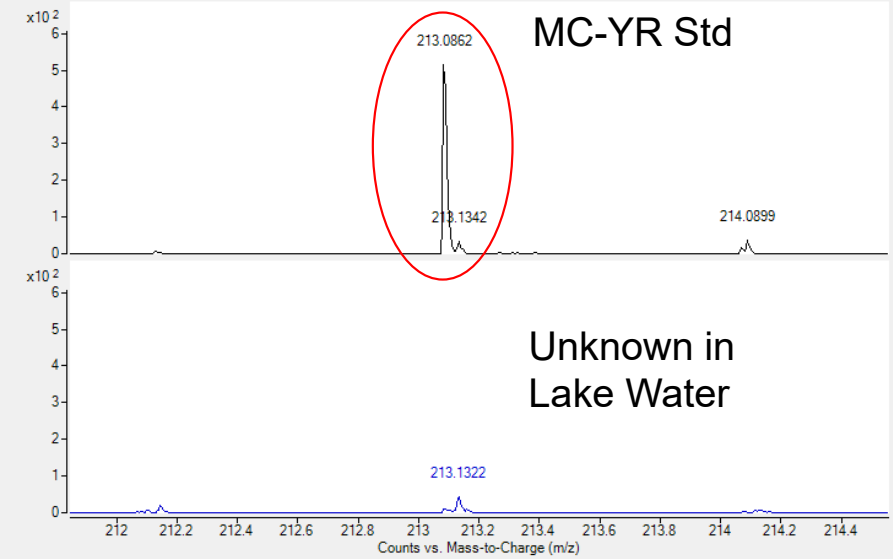


135.1168 = $C_{10}H_{15}^+$

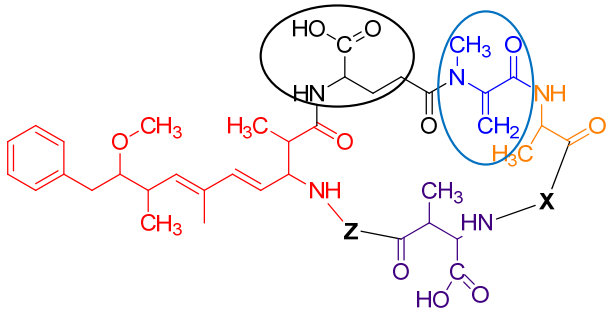
- The characteristic ion for microcystins, often used for quantitation, is nominal m/z 135
- There are actually two product ions at m/z 135 that are formed in microcystins from the Adda[5] group
- The presence in the lake sample indicates this is an unknown microcystin

Is it a Microcystin?

Fragmentation – m/z 213



Glu^6 + $Mdha^7$ = $C_9H_{13}N_2O_4^+$
m/z = 213.0870 vs 213.0862

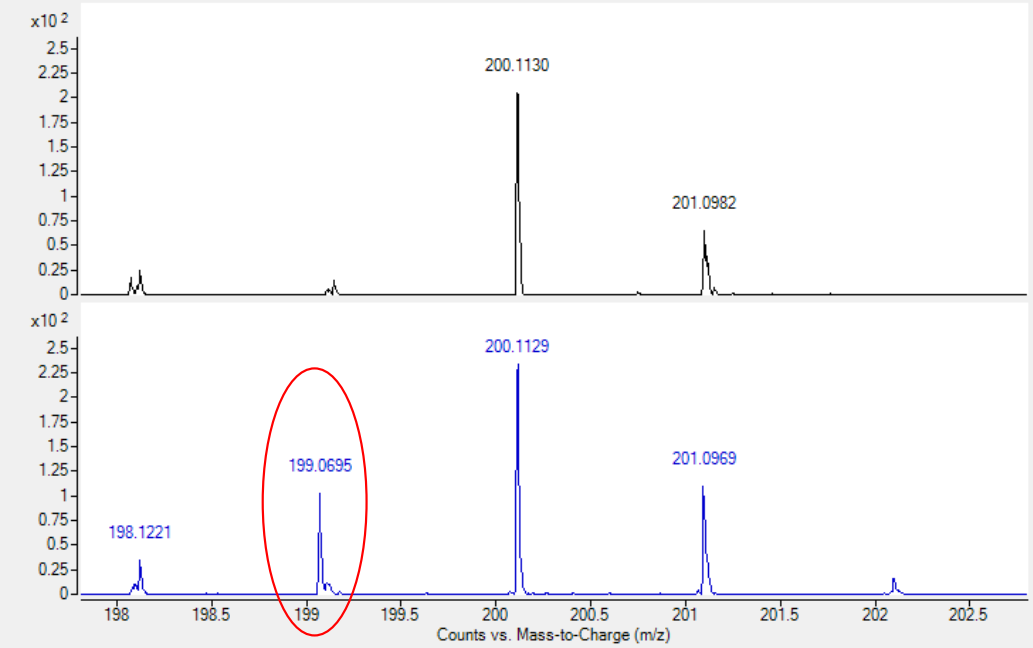


- m/z 213.0870 corresponds to the sequence Glu^6 + $Mdha^7$, as shown in the spectrum for YR (top)
- This ion is absent in the unknown spectra in lake

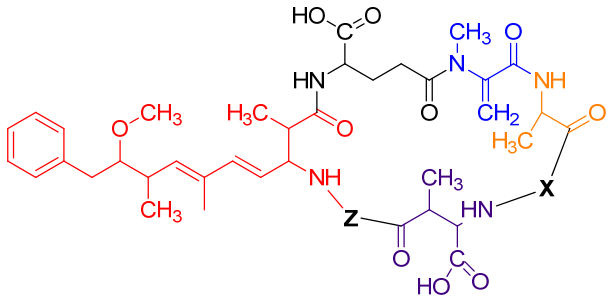
The Unknown is NOT MC-YR

What else is different in the Unknown Spectra?

Fragment at m/z 199



Glu^6 + $Mdha^7$ –CH_2 = $C_8H_{11}N_2O_4^+$
m/z = 199.0713 vs 199.0695

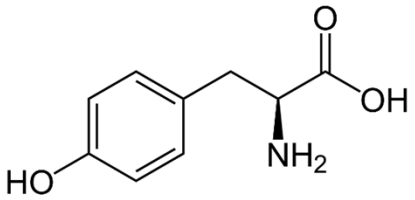


- Desmethylation of $Mdha^7$ would result in a loss of CH_2 (-14.0157), producing an ion at m/z 199.0713
- The lake spectrum is consistent with a desmethylated microcystin from $Mdha^7$, and the compound is not YR

What is generating such fragments from 1045.5 m/z?

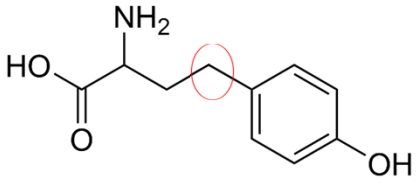
How can a methyl group be added elsewhere to a MC-YR like backbone?

Amino Acid substitutions at the R2 & R4 positions result in most MC analogues

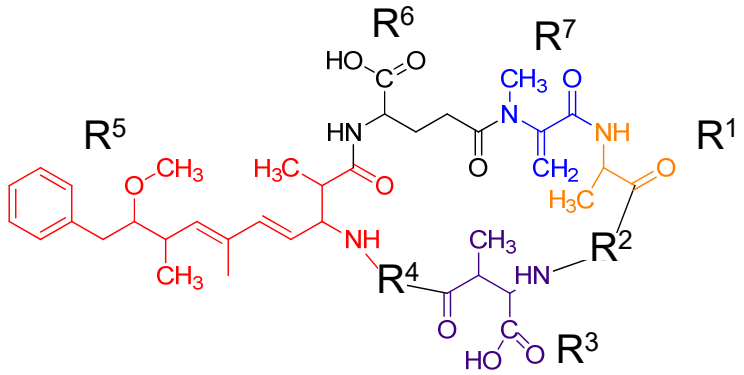


Tyrosine

Homotyrosine



Is the unknown a demethylated HtyR?



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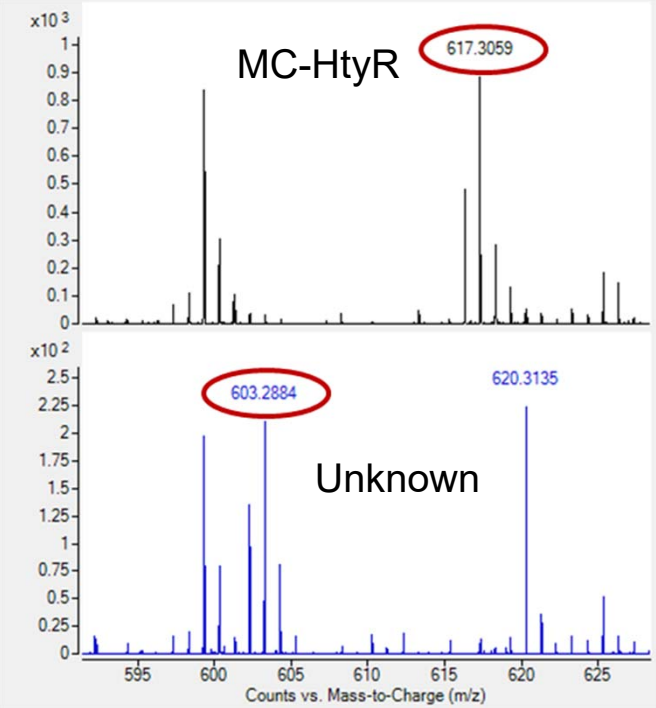
RR	Arginine	Arginine	$C_{49}H_{75}N_{13}O_{12}$	1037.5658
YR	Tyrosine	Arginine	$C_{52}H_{72}N_{10}O_{13}$	1044.5280
LA	Leucine	Alanine	$C_{46}H_{67}N_7O_{12}$	909.4848
LW	Leucine	Phenylalanine	$C_{54}H_{72}N_8O_{12}$	1024.5270
LF	Leucine	Tryptophan	$C_{52}H_{71}N_7O_{12}$	985.5161
HtyR	Homotyrosine	Arginine	$C_{53}H_{74}N_{10}O_{13}$	1058.5437

Is the unknown demethylated HtyR?

Look for fragments that include R2 (Hty)

Compare ions from authentic standard of HtyR (top) to the unknown (bottom), to show the presence of Hty in the R^2 position and desmethylation in R^7 position



- Fragment *Ion* : R^7 – *Ala*^{1} – R^2 – *MeAsp*^{3} – *Arg*^{4}

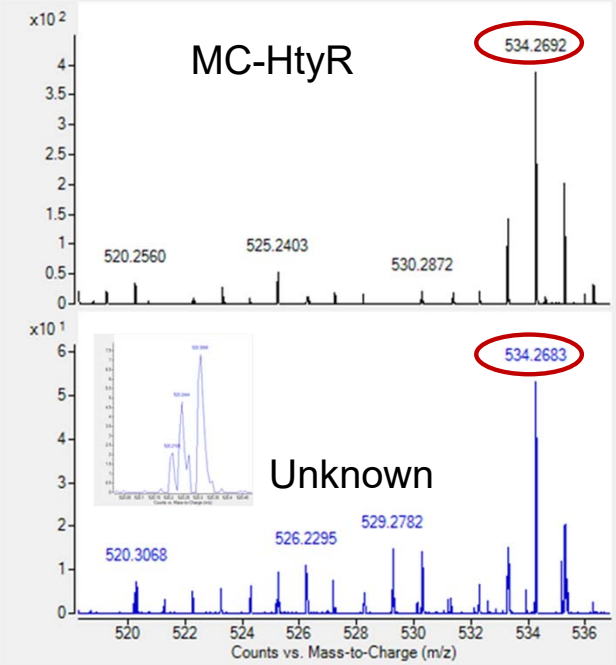
  - HtyR (from 1059.5 m/z)
  - R^7 = Mdha; R^2 = Hty
  - Calc m/z : 617.3042
  - Measured: 617.3059 (2.8 ppm mass error)

  - dm-HtyR (1045.5 m/z)
  - R^7 = Dha; R^2 = Hty
  - Calc m/z : 603.2885
  - Measured: 603.2884 (0.2 ppm mass error)

Is the unknown demethylated HtyR?

Look for fragments that include R2 (Hty)

No Demethylation occurring in R^3 and no contribution of R^7 so fragments should be same



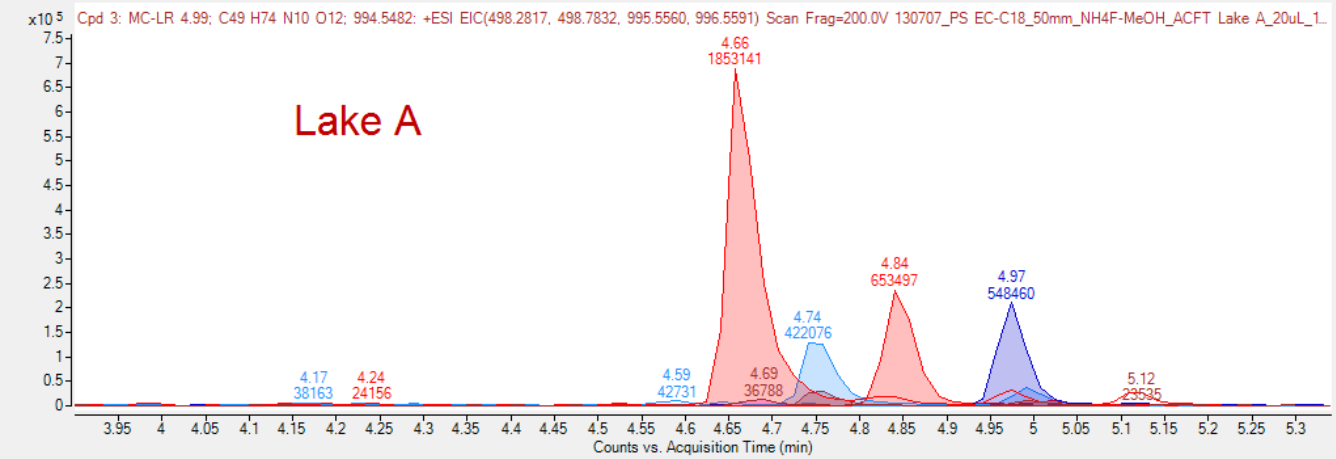
- Fragment *Ion* : $Ala^1 – R^2 – MeAsp^3 – Arg^4$
  - HtyR: R^2 = Hty
  - Calc m/z : 534.2671
  - Measured: 534.2692 (2.3 ppm mass error)

  - dm-HtyR: R^2 = Hty
  - Calc m/z : 534.2671
  - Measured: 534.2683 (4.0 ppm mass error)

Confirmation that unknown is MC-DesMe-HtyR

Additional unknowns detected – Lake A

7 additional new MCs found in lake sample without commercially available standards



RT	Name	Formula	Mass	m/z	Area	Score	Diff (Tgt, ppm)	Ions	Flags (Tgt)	Flag Severity (Tgt)
4.66	MC-YR	C52 H72 N10 O13	1044.5273	1045.5346	1853141	98.48	-0.65	5	multiple IDs	Information
4.69	MC-LR	C49 H74 N10 O12	994.5487	995.5549	36788	90.41	-0.09	3	multiple IDs	Information
4.74	MC-HtyR-DAsP3-Dha7	C51 H70 N10 O13	1030.5117	1031.5185	422076	96.79	-0.65	8	multiple IDs	Information
4.76	MC-HtyR	C53 H74 N10 O13	1058.5434	1059.5501	94974	98.43	-0.26	4		Pass
4.84	MC-DesMe-LR	C48 H72 N10 O12	980.533	981.5405	653497	99.12	-0.07	5	multiple IDs	Information
4.97	MC-HphR-Dha7	C52 H72 N10 O12	1028.533	1029.54	548460	99.56	-0.15	8	multiple IDs	Information
4.99	MC-LR-DAsp3-Dha7	C47 H70 N10 O12	966.516	967.5236	116668	94.3	-1.5	4		Pass
4.99	MC-LR	C49 H74 N10 O12	994.5482	995.5559	32185	92.69	-0.62	4	multiple IDs	Information

- A library of 100+ microcystins was built
- MS domain confirmation
- Auto searching possible using isotope pattern

Conclusions

LC/TQ

- Excellent tool for the targeted analysis of microcystins when analytical standards are available
- Quantification down to sub µg/L easily possible

Online SPE

- Reduces labor and significantly increases throughput with ng/L quantification possible.

LC/QTOF

- Complimentary tool that can confirm suspect compounds using accurate mass of the molecular ion adducts, as well as MS/MS fragments.
- Quantification of all MCs upto 0.1 µg/L possible

Databases

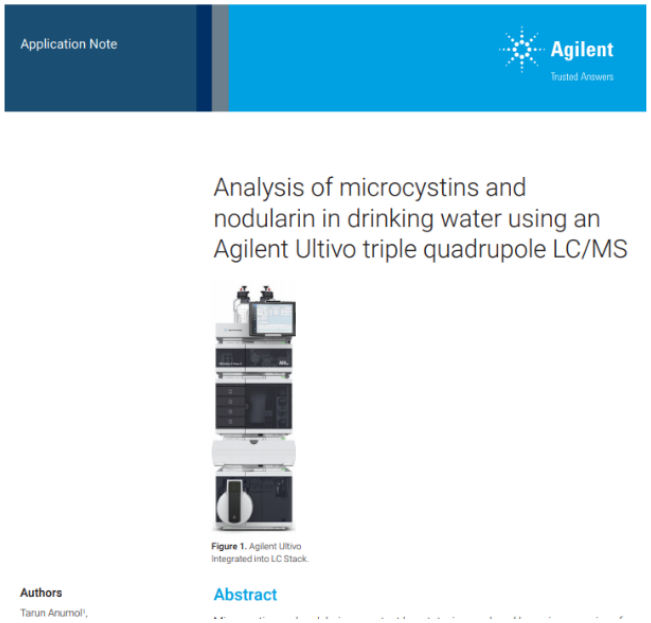
- Can be compiled to include the chemical formula for additional microcystins based on reported analogues in the literature

Acknowledgements & Resources

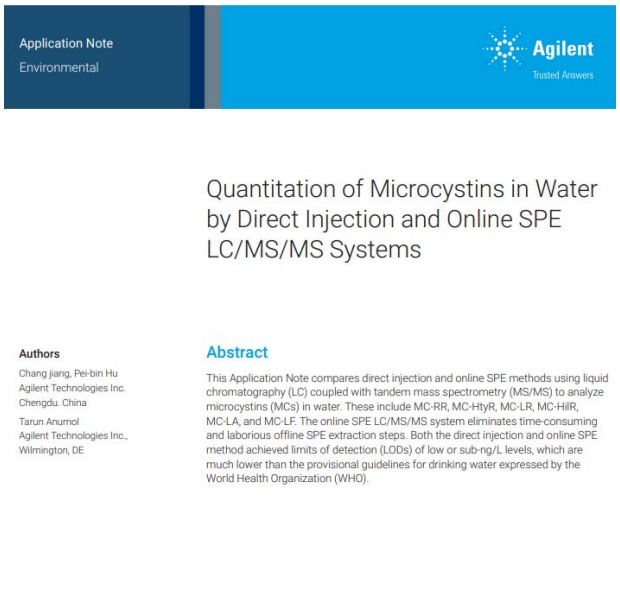
Ralph Hindle

Kathy Hunt

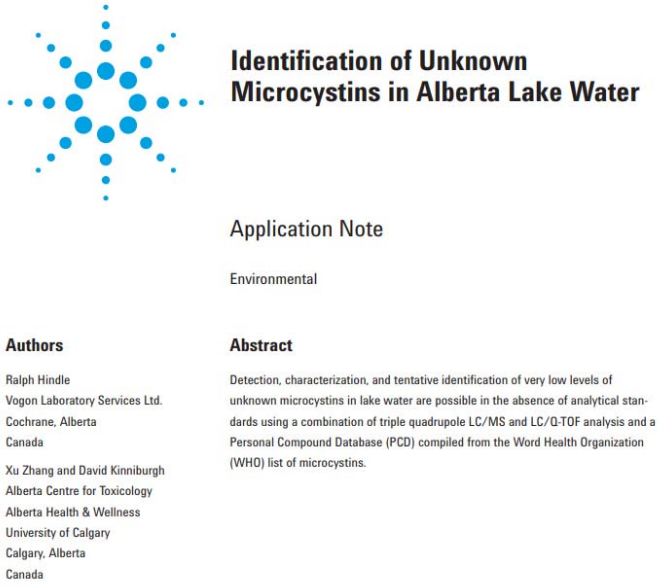
Chang Jiang



5991-9087EN



5994-0007EN



5991-4444EN

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Trusted Answers