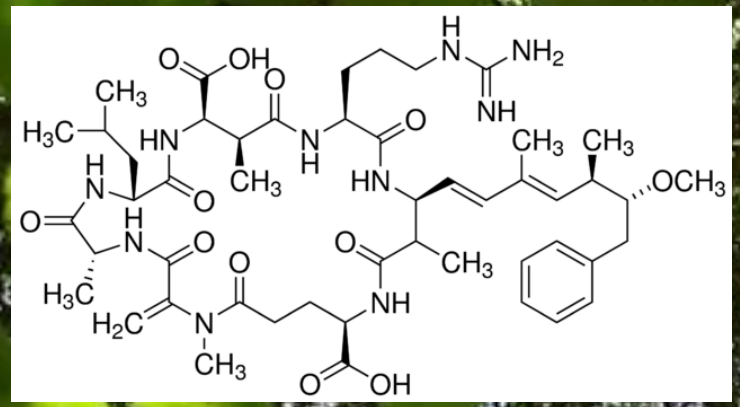

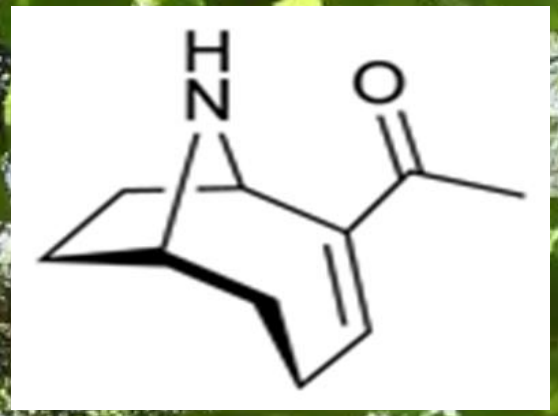

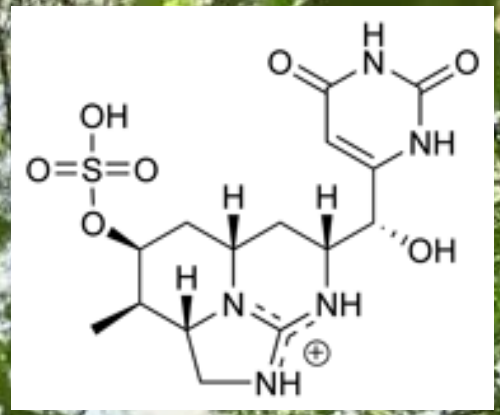

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Microcystins and the Challenges of the Unknown Analysis of Algal Toxins Using LC-MS/MS

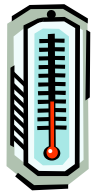
Dr. Judy Westrick and Dr. Johnna Birbeck
Wayne State University
Lumigen Instrument Center
October 8, 2019

Overview

- Why do freshwater Harmful Algal Blooms (HABS) happen?
- Regulation and Policy – The Road Traveled
- Anatoxin and Cylindrospermopsin, EPA Method 545
- Microcystin, EPA Method 544
- New Robust online concentration LC/MS/MS Methodology
    - Targeted MCs
    - Untargeted MCs
- Conclusion

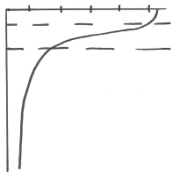
The Perfect Storm…

Nutrient Load ↑



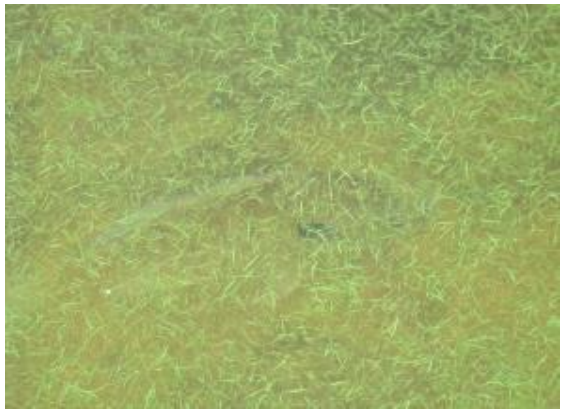
Water Temperature ↑

Flow ↓



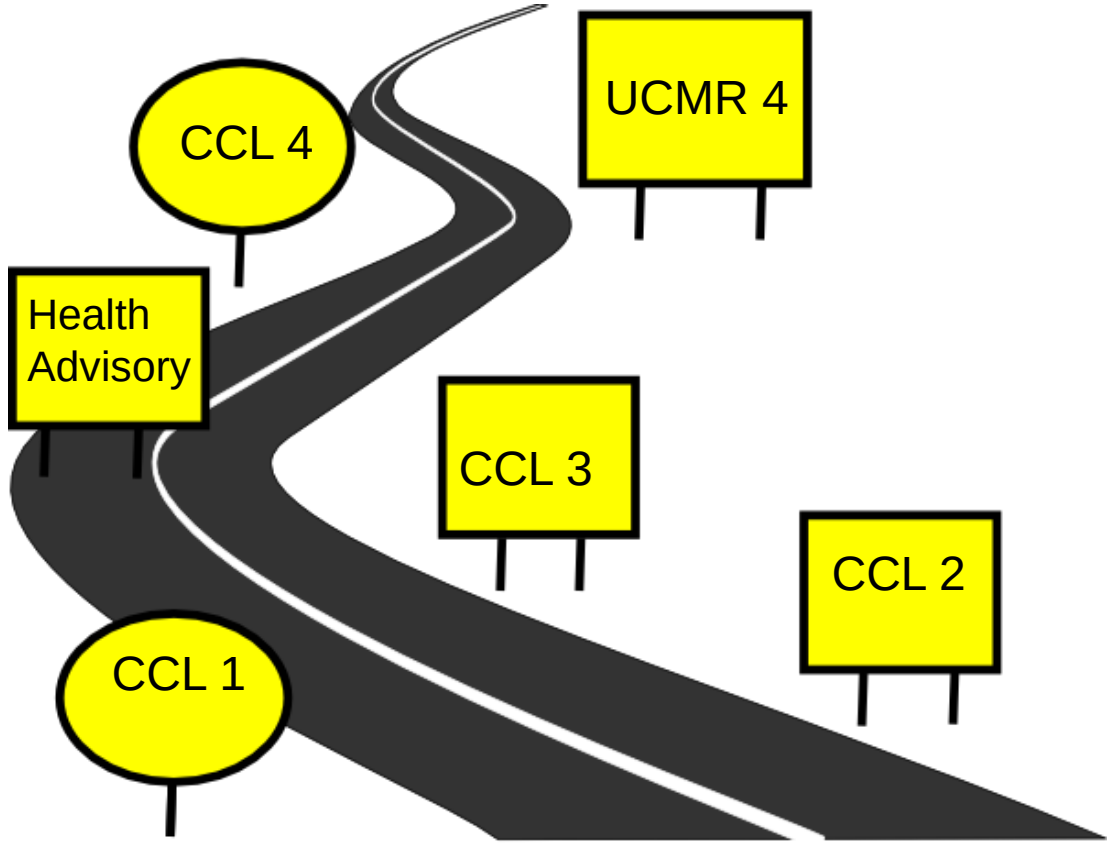
Thermal Stratification ↑

Rainfall ↑



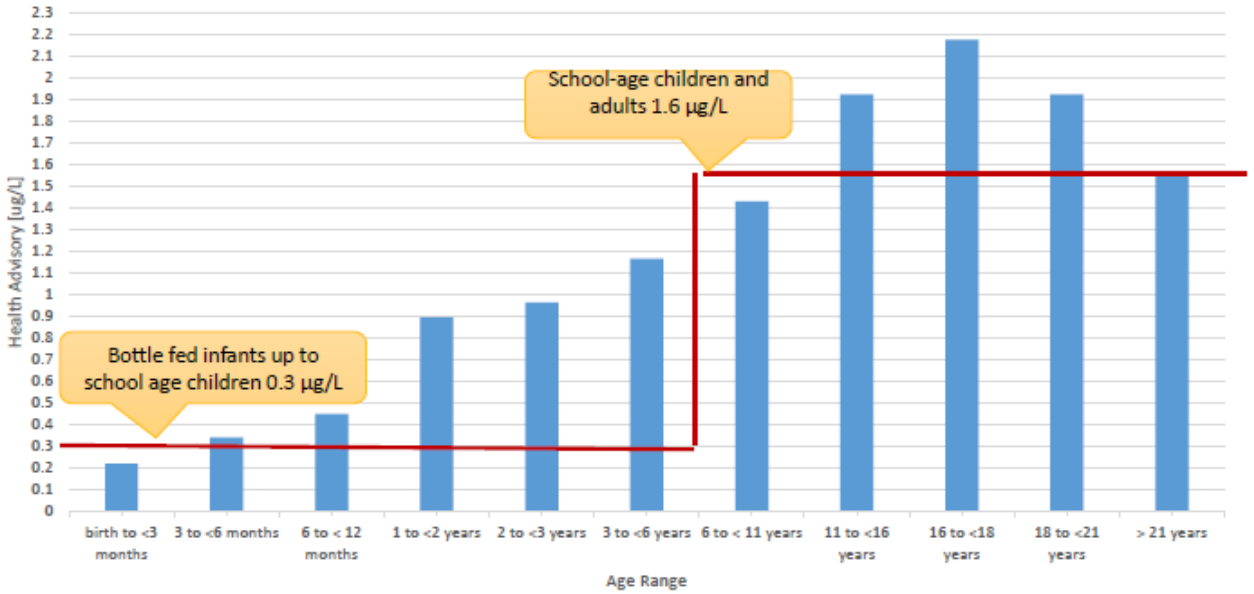
Regulation and Policy – The Road Traveled

- General Flow of the Safe Drinking Water Act
  - Contaminant Candidate List (CCL I, II, III, and IV)
  - Unregulated Contaminant Monitoring Rule (UCMR IV)
  - Determine if any contaminant needs a standard
  - Develop regulation
  - Six year review
- Drinking Water Microcystin and Cylindrospermopsin Health Advisories
- Informal non-regulatory guidance for unregulated drinking water contaminants to assist federal, state and local officials, and public water systems in protecting public health.



Microcystin Drinking Water Health Advisory

- Microcystin LR is considered a surrogate for all microcystin congeners (toxicology)
- Children < 6 years, drink 5x more water to body mass
- Most sensitive endpoint is liver damage
- Lowest Observed Adverse Effect Level (LOAEL) 50 µg/Kg/d
- Uncertainty Factor – 1000 (intra -10; inter -10; database -$10^{0.5}$; LOAEL-NOAEL $10^{0.5}$)
- Short term - 10 day (no lifetime or carcinogenic value derived)



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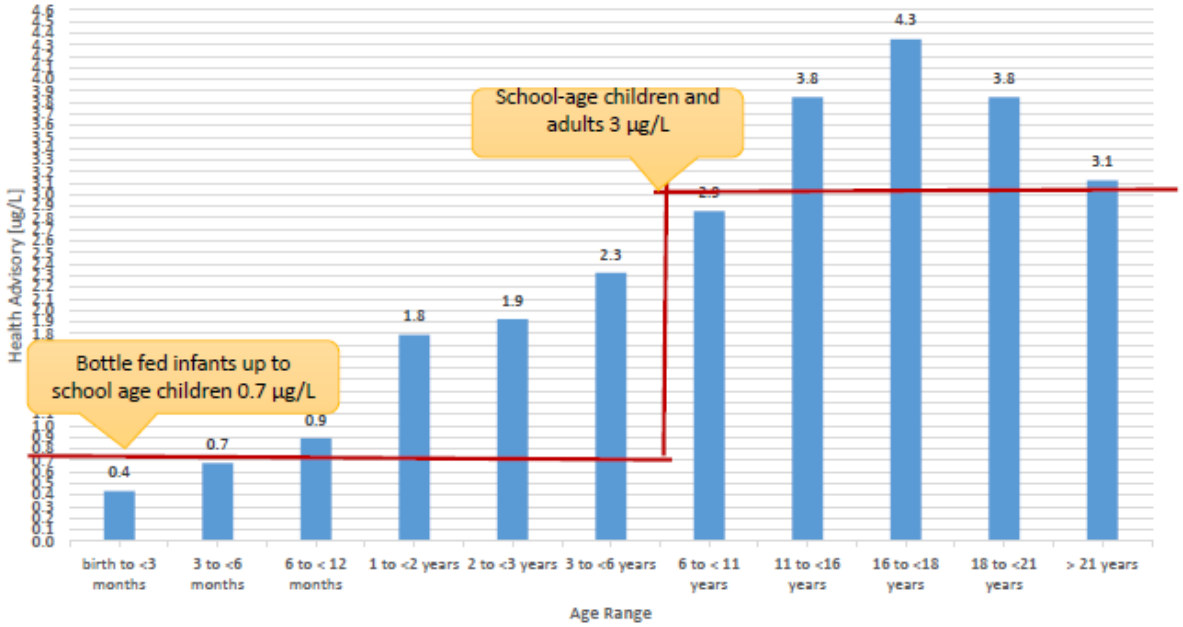
Cylindrospermopsin Drinking Water Health Advisory

- Cylindrospermopsin and does not include the two congeners.
- Children < 6 yrs, they drink 5xs more water to body mass.
- Most sensitive endpoint is kidney damage.
- No Observed Adverse Effect Level (NOAEL) – 30 ug/Kg/d
- Uncertainty factor – 300 (intra -10; inter – 10; and data $10^{0.3}$)
- Short term - 10 day (no lifetime or carcinogenic value derived)



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Recreational Waters Health Advisory for Microcystin and Cylindrospermopsin

- World Health Organization
  - Most countries use both cyanobacteria cell and microcystin-LR levels
  - Cells provide for the unknown…..(surrogate)

Relative Probability of Acute Health Effects	Cyanobacteria cell (cells/mL)	Microcystin-LR (ug/L)
Low	<20,000	<10
Moderate	20,00-100,000	10-20
High	100,000-10,000,000	20-2,000
Very High	>10,000,000	>2,000

US EPA
Approach to Recreational Health Advisory

- No cyanobacteria cells
- Swimming Advisory: not to be exceeded on any day.
- Recreational Criteria for Waterbody Impairment: not exceeded more than 10 percent of days per recreational season to one calendar year.

Microcystis (ug/L)	Cylindrospermopsin (ug/L)
8 ug/L	15 ug/L

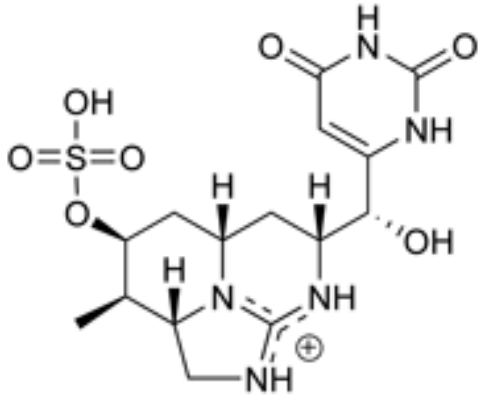
Cyanotoxin EPA methods used for UCMR monitoring

- EPA method 544 – Determination of Microcystins and Nodularin in drinking water by SPE and LC/MS/MS
  - SPE sample prep
  - Concentrated sample detected using LC/MS/MS
- EPA method 545 – Determination of Cylindrospermopsin and Anatoxin-a in Drinking Water by LC/ESI-MS/MS
  - Direct inject method using LC/MS/MS detection

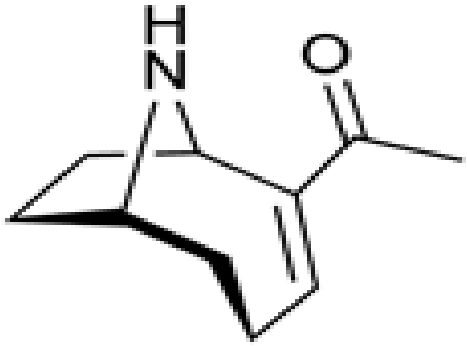
Cyanotoxin LC/MS/MS workflow

- Standards
  - How many standards are available?
  - Are any MCs certified standards available?
  - QA/QC?
- Chromatography
  - Microcystins
    - Degrees of hydrophilicity
    - Matrix effects
    - Mobile Phase
- Standard Methodology
  - EPA 544 – microcystins
  - EPA 545 – anatoxin and cylindrospermopsin
  - Sample preparation
  - Qualifier/quantifier ratio
- Measuring low ppt. levels of cyanotoxins
  - Concentration and clean-up
  - Optimizing chromatography
  - Online concentration

Cylindrospermopsin



Anatoxin-a



Cylindrospermopsis

Aphanizomenon

Dilochospermum

EPA Method 545

Pros

- Standard method
- Sample collection standardized
- Direct injection

Cons

- HPLC step method
- No quantifier/qualifier ratio
- No column wash in LC/MS/MS method
- Overloading column with sample

Goal: EPA Method 545 modification

- Modify EPA Method 545: Anatoxin-a and Cylindrospermopsin using LC/MS/MS.
  - HPLC method converted to UPLC method.
  - Include column wash step.
  - Smaller injection volumes.
  - Use quantifier/qualifier ion ratios for analyte verification.

EPA 545 Method development

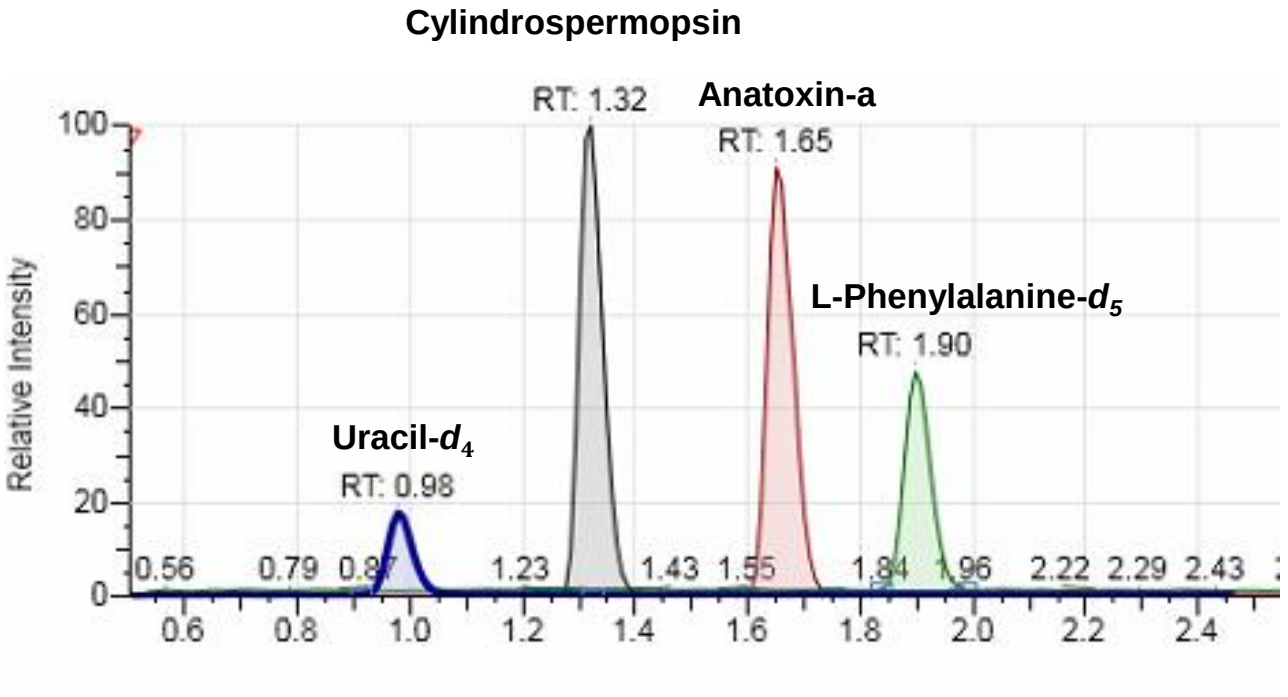
- Mobile Phases Loading and Eluting pump
  - 100 mM Acetic acid in water
  - 100 mM Acetic acid in methanol*
- Column
  - Hypersil GOLD aQ 100 x 2.1 mm, 1.9 µm particle size
- Injection volume
  - 5 µL*
- Flow rates
  - Analytical: 0.4 mL/min gradient
- Method Run time
  - 9 minutes

Method 545: Detection Settings and Limits

Analyte	Retention Time (min)	Quantifier Ions (m/z)	Qualifier Ions (m/z)
Uracil-d_4	0.98	98.00	n/a
Cylindrospermopsin	1.32	194.06	176.04
Anatoxin-a	1.65	149.00	131.00
L-phenylalanine-d_5	1.90	125.04	106.06

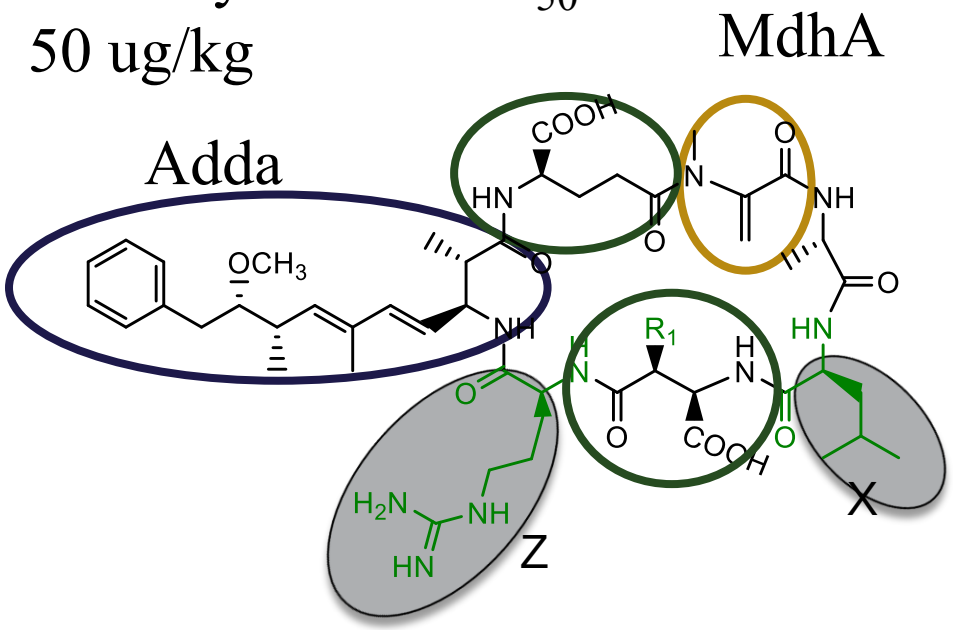
Analyte	Detection Limit (ppb)	Upper PIR	Low PIR	R^2
Cylindrospermopsin	0.0079	132	115	0.995
Anatoxin-a	0.032	134	66	0.998

10 ppb Standard Chromatogram



Microcystin

Microcystin LR LD_{50}
50 ug/kg



Dilochospermum

MC	X	Z	R1
MC-LR	L	R	CH_3
MC-RR	R	R	CH_3
MC-YR	Y	R	CH_3
MC-LF	L	F	CH_3
MC-WR	W	R	CH_3
D-Asp^3-RR	R	R	H
HtyR	Hty	R	CH_3

Microcystis

What are the most prevalent Microcystins in USA?

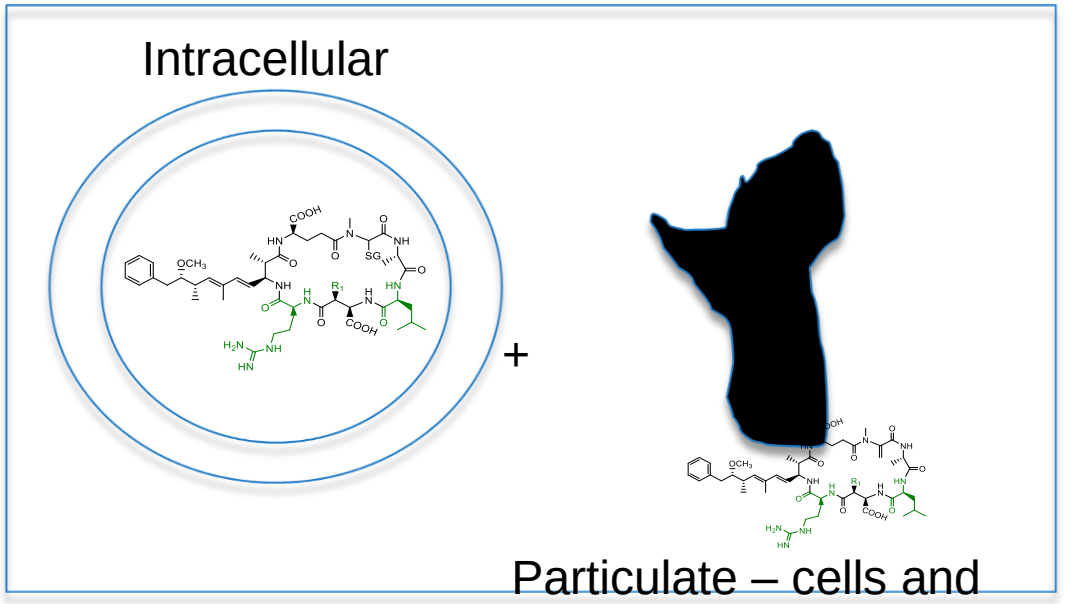
- Three types of publications
  - LC/MS/MS with all available microcystins
    - Foss and Abel, 2015 (Ohio, US)
    - Szlag et al, 2019 (Michigan, US)
  - High resolution mass spectrometry
    - Three studies – Green Lake, Seattle, WA and Homer Lake, IL, Poplar Island, Maryland
  - HPLC-PDA
    - California Studies investigating des-methyl MC-LR

The % of MC congeners detected by EPA Method 544	Number of Sites
0%-20%	6
21%-40%	0
41%-60%	2
61%-80%	3
81%-100%	11

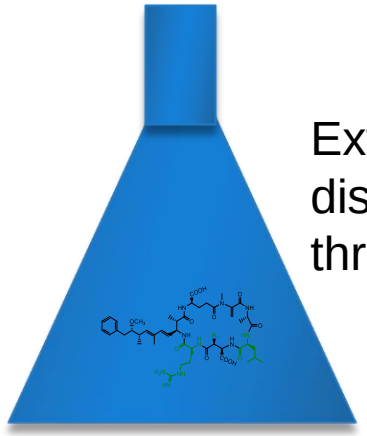
Citriglia: Water Research Project 4647:Evaluation of Methods for the Analysis of Cyanotoxins

Where are the cyanotoxins?

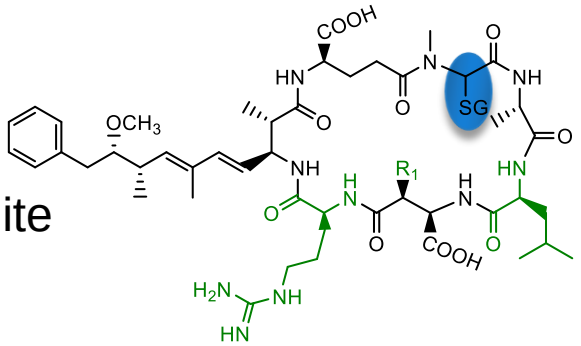
- Intracellular
- Particulate
- Dissolved
- Extracellular
- Conjugated



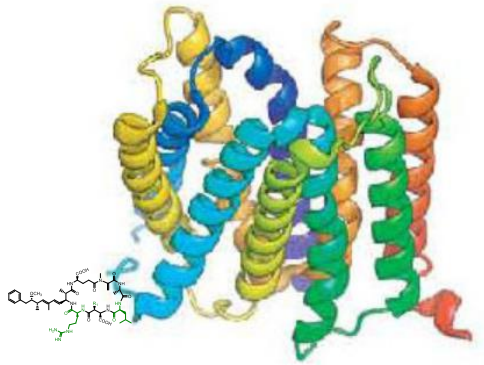
Particulate – cells and debris on the filter



Extracellular and dissolved – passed through the filter



Conjugated- metabolite or to protein



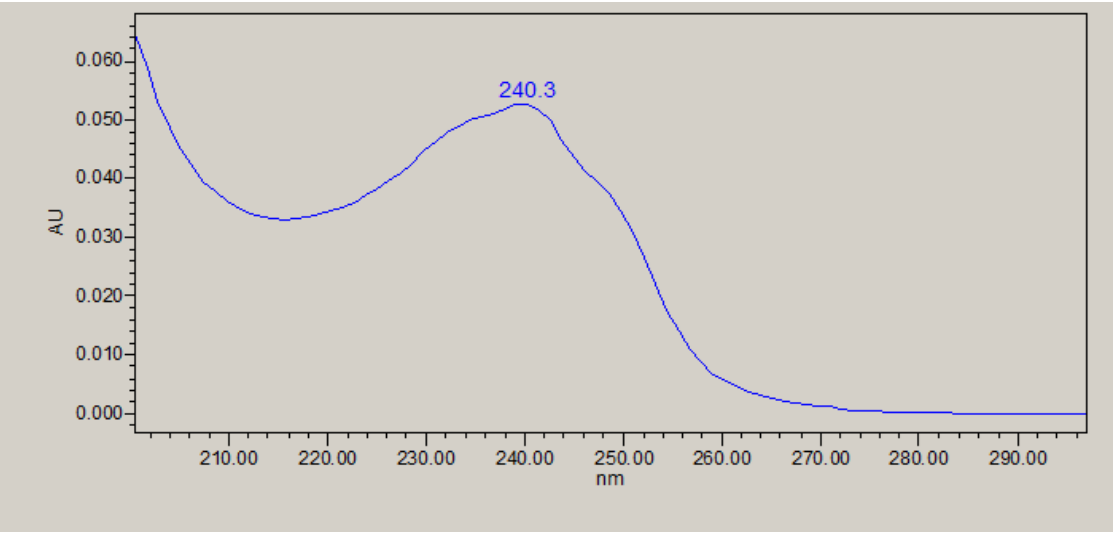
MC Concentration by Beer's Law

$$\text{Absorbance} = \varepsilon \times C \times l$$

ε: L mol^{-1} cm^{-1}

- ISO Method 170706 recommends using the Extinction Coefficient "ε" to calculate the concentration of the stock MC standard

MC	Referenced Extinction Coefficient	Reference	Extinction Coefficient Used
MC-LR	39800/36500	Harada et al., 1990 / Honkanen et al., 1990	*39800
MC-YR	38100/41100	Blom et al., 2001	*38100
MC-RR	39800	Harada et al., 1990	*39800
MC-LA	36500	unpublished data by Carmichael	36500
D-Asp3-LR	31600	Harada et al., 1990	31600
D-Asp3, E-Dhb7 MC-RR	50400	Blom et al., 2001	50400
[Dha7] - LR	46800	Harada et al., 1990	46800
MC-LW, MC-WR, MC-LF,MC-LY, MC-HtyR, D-Asp3-RR, C2D5 MC-LR			39800



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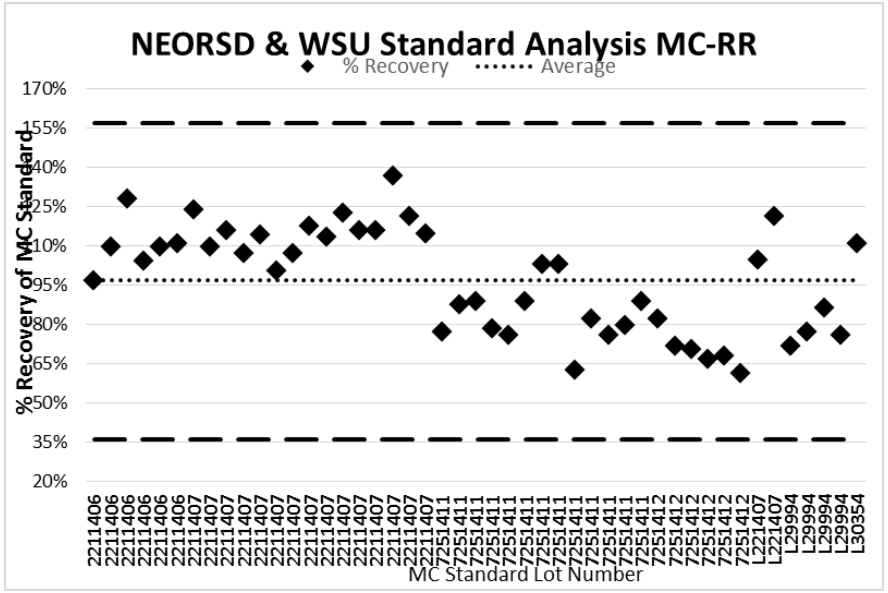
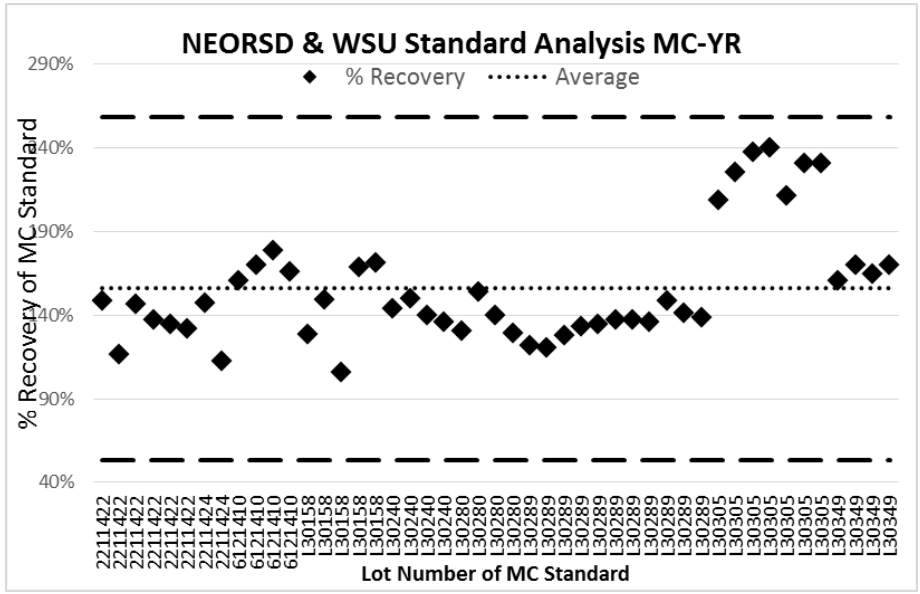
Commercial Standards: Buyer Beware (1)



Purity or concentration?

Variation within vial and between lots?



Citriglia: Water Research Project 4647:Evaluation of Methods for the Analysis of Cyanotoxins

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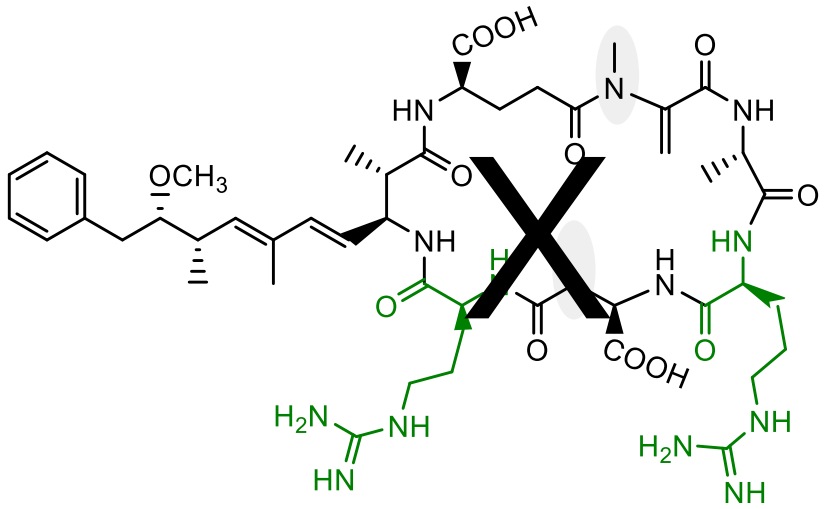
Commercial Standards: Buyer Beware (2)

Standard Purity Summary

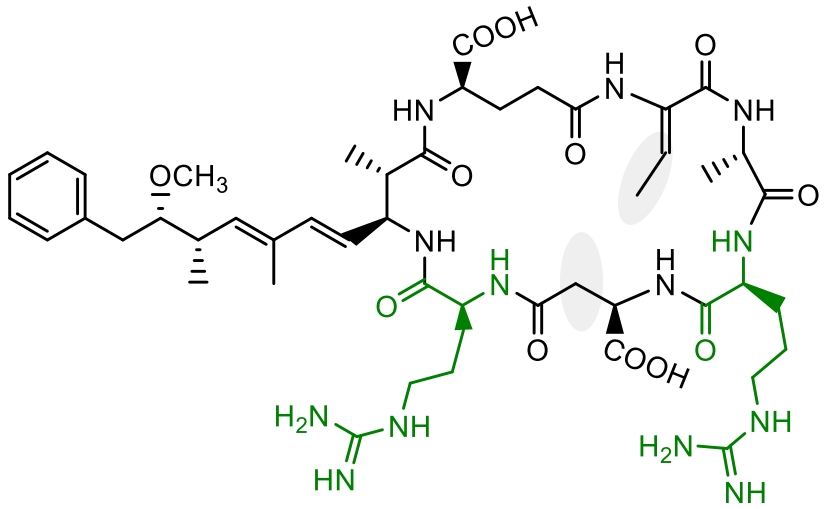
Standard	Lot Number	Observed Impurities	% Impurities
MC-LR	02211428	DAsp^{3}MC--RR	1%
MC-LW	L30332		
MC-YR	L30158	MC-HtyR	2%
DAsp^{3}MC-RR	L30311		
MC-HtyR	L30287	MC-YR, MC-LR, MC-D-Asp^{3}MC-LR	14%
Nodularin	0614420		
MC-LY	L30309		
DAsp^{3}MC-LR	L30336		
MC-LA	L30307		
MC-RR	0221407	MC-D-Asp^3-RR	2%
MC-LF	L30373	MC-LW	2%
MC-HilR	L30372		
MC-WR	L30310		

Citriglia: Water Research Project 4647

Commercial Standards: Buyer Beware (3)



[D-Asp^3]MC-RR



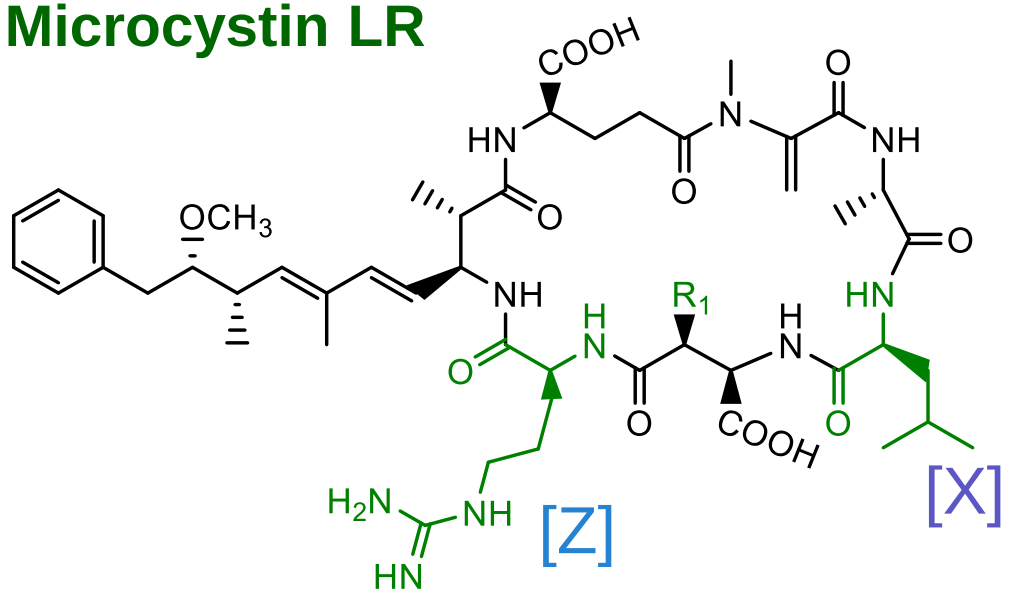
[D-Asp^3, Dhb^7]MC-RR

Birbeck et al. Submitted

Constitutional Isomer

EPA 544: Microcystin standards

Microcystin LR



Microcystin Congener	X	Z	R1
LR	L	R	CH_3
RR	R	R	CH_3
YR	Y	R	CH_3
LY	L	Y	CH_3
LA	L	A	CH_3
LF	L	F	CH_3
LW	L	W	CH_3
WR	W	R	CH_3
D-Asp3-LR	L	R	H
D-Asp3-RR	R	R	H
HtyR	Hty	R	CH_3
HilR	Hil	R	CH_3

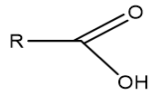
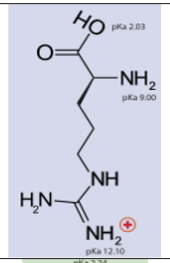
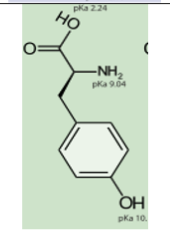
EPA Method 544

Pros

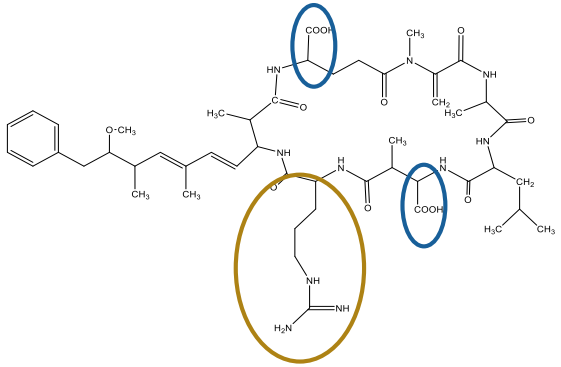
- Standard method
- Sample collection standardized
- Flexible chromatography

Cons

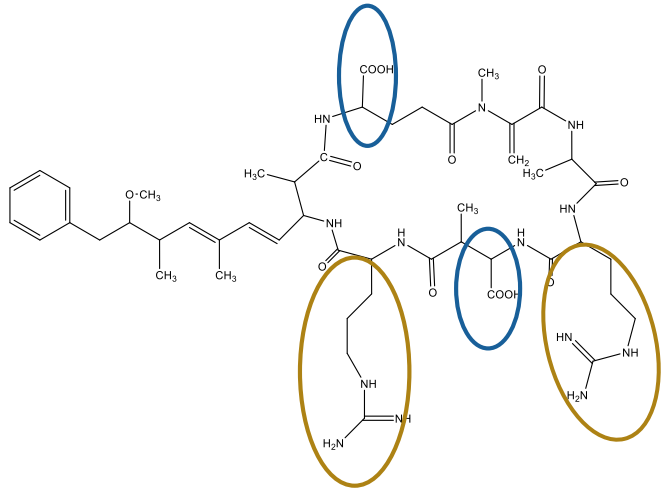
- Two day process
  - Solid Phase Extraction
  - LC/MS/MS analyses
- Loss of sensitivity
- Long method, 26 mins in which the first 10 mins no MCs are detected
- HPLC method
- Only six congeners
- No quantifier/qualifier ratio
- Large sample volumes to be shipped
- Increase chemical cost due to
  - SPE
  - HPLC method

Constituents	Structure	pKa's
Carboxylic Acid		4
Arginine, R		12.0
Tyrosine, Y		10.10

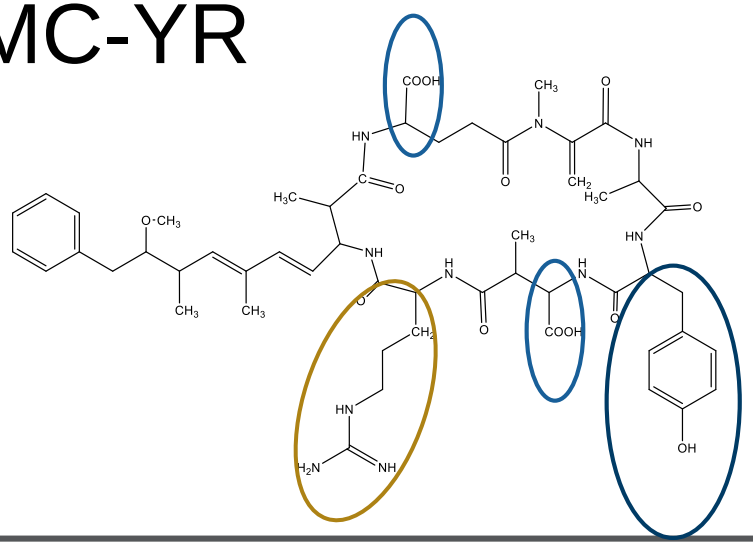
MC-LR



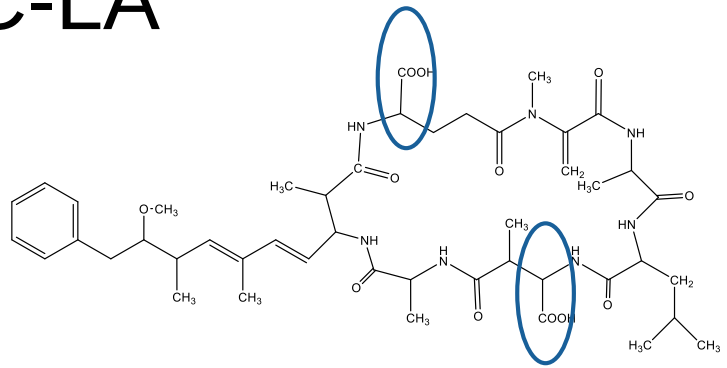
MC-RR



MC-YR



MC-LA



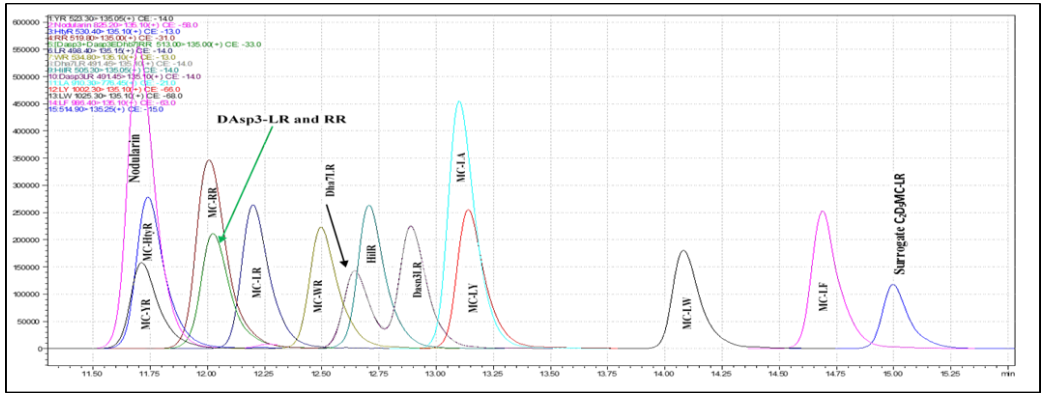
Mobile Phase affects the Speciation of MCs

Analyte	Formic acid mobile phase, pH = 2	Ammonium formate mobile phase, pH = 6.5
Microcystin-LR	+	-, -, +
Microcystin-RR	+, +	-, -, +, +
Microcystin-YR	+	-, -, +
Microcystin-LA		-, -

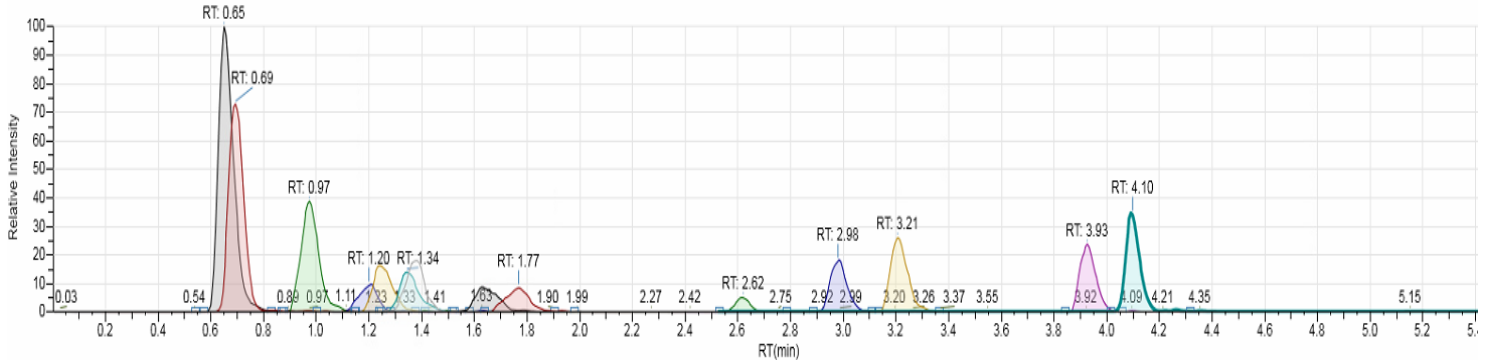
Comparison of LC/MS/MS- Method 544

Samples were extracted and prepared at NEORSD and UV_{238} correction for MC-LR, RR, and YR.

Method 544 - HPLC C_8 column; Water and ammonium formate/methanol



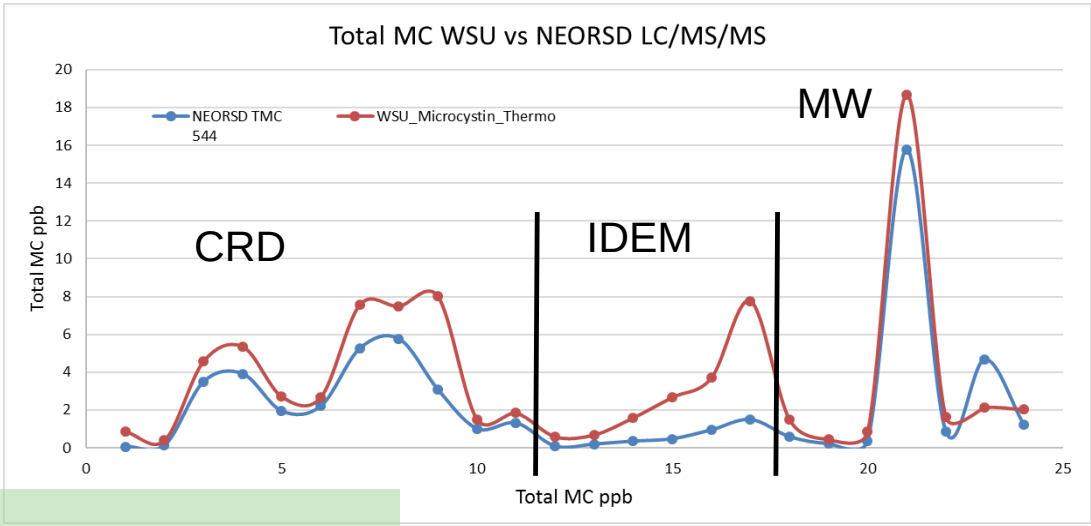
Method 544 “Method Flexibility: HPLC C_{18} column: Water/Acetonitrile with trace formic acid

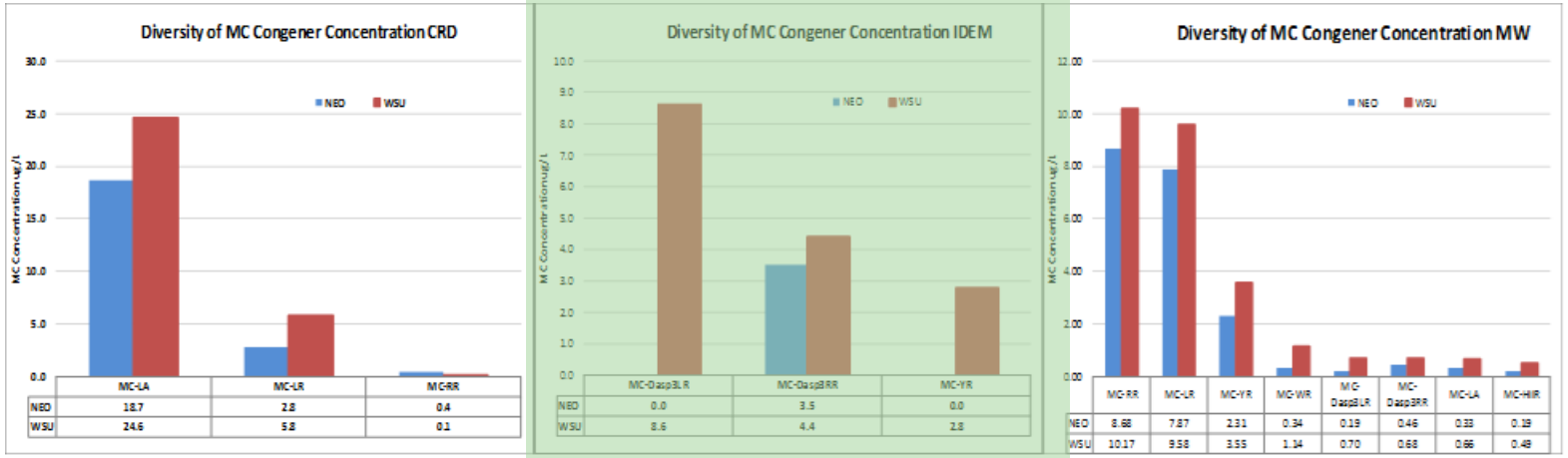


Citriglia: Water Research Project 4647:Evaluation of Methods for the Analysis of Cyanotoxins

Total MC by LC/MS/MS WSU and NEORSD?

- 24 Samples with positive ELISA results were analyzed by LC/MS/MS using two different LC/MS/MS methods.
  - EPA 544 (NEO-Blue)
  - Modified EPA 544 (WSU-Red)
- Both methods have more microcystin congeners





Citriglia: Water Research Project 4647:Evaluation of Methods for the Analysis of Cyanotoxins

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Conclusions

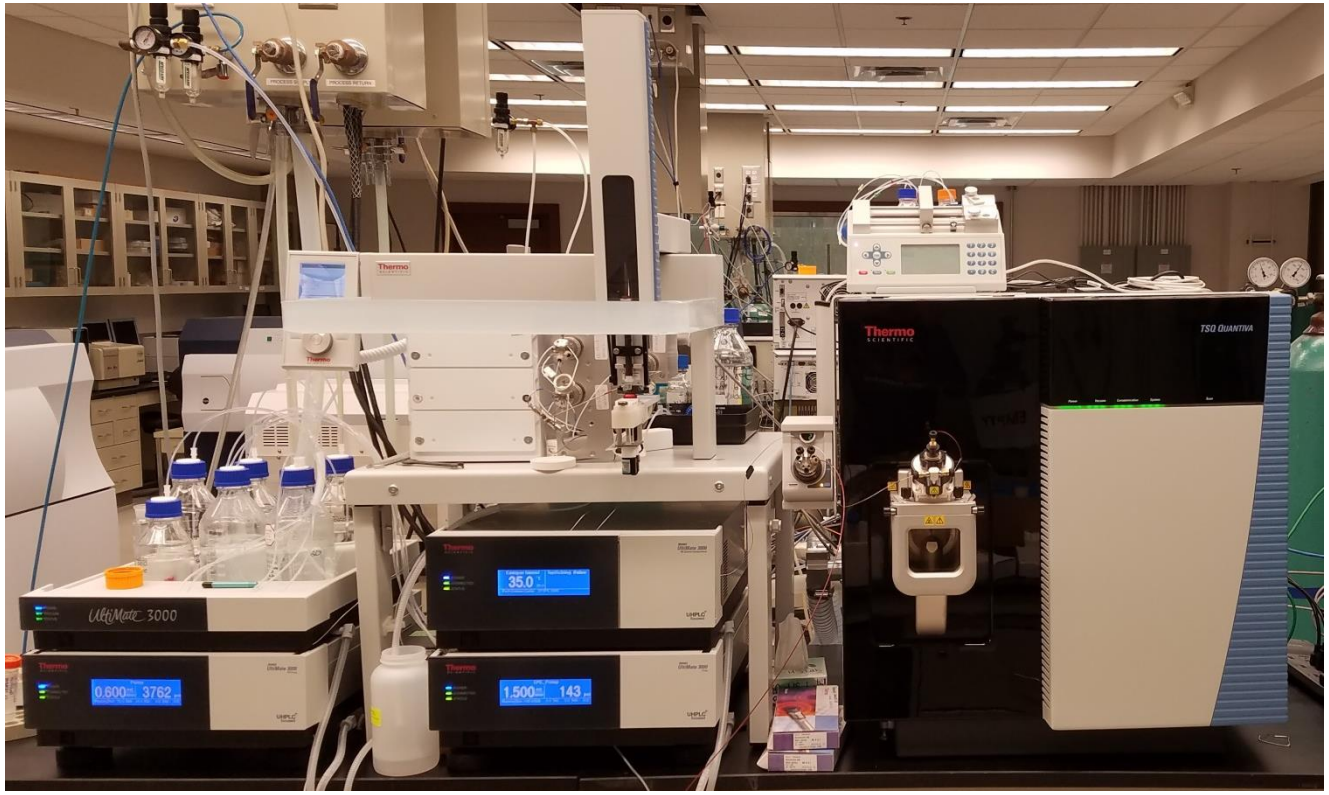
- Method 544
  - Standard Variation
  - Detection limits between 5 and 10 ppt
  - Concentrations appears to be mobile phase dependent.
  - Total run time for UHPLC is 5 minutes compared to 15.0 minutes HPLC.
- Method 545
  - Decreased run time by 4 minutes
    - Included a wash step in gradient
  - Changed mobile phase B to include 100mM acetic acid

Goal: EPA Method 544 modification

- To develop an online method for EPA Method 544: Microcystins LC/MS/MS.
  - Replace the Solid Phase Extraction step with an online concentrator.
  - Expand the method from six microcystin congeners and nodularin to twelve microcystin congeners and nodularin.
  - Use quantifier/qualifier ion ratios for analyte verification.

Instrument: Thermo Scientific™ EQuan MAX Plus™ LC-MS

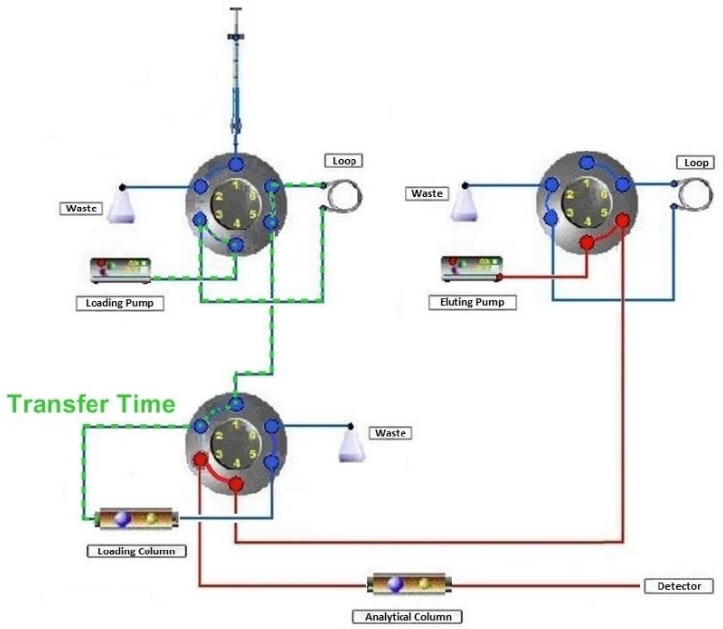
- Thermo Scienfitic™ TSQ Quantiva™ Triple Quadrupole mass spectrometer
- Two pump/column system
  - Low pressure pump for concentration on the first column
  - High pressure pump for separation on the second column



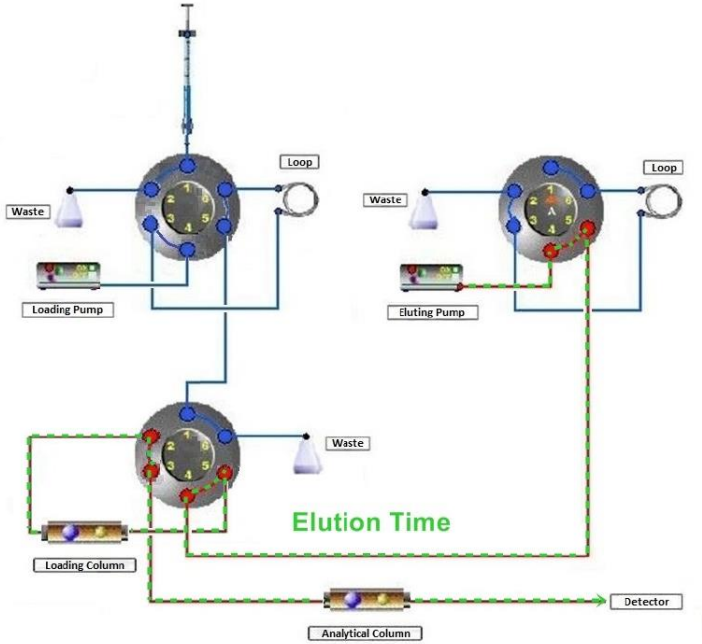
ThermoFisher SCIENTIFIC

Online concentration

Loading



Eluting



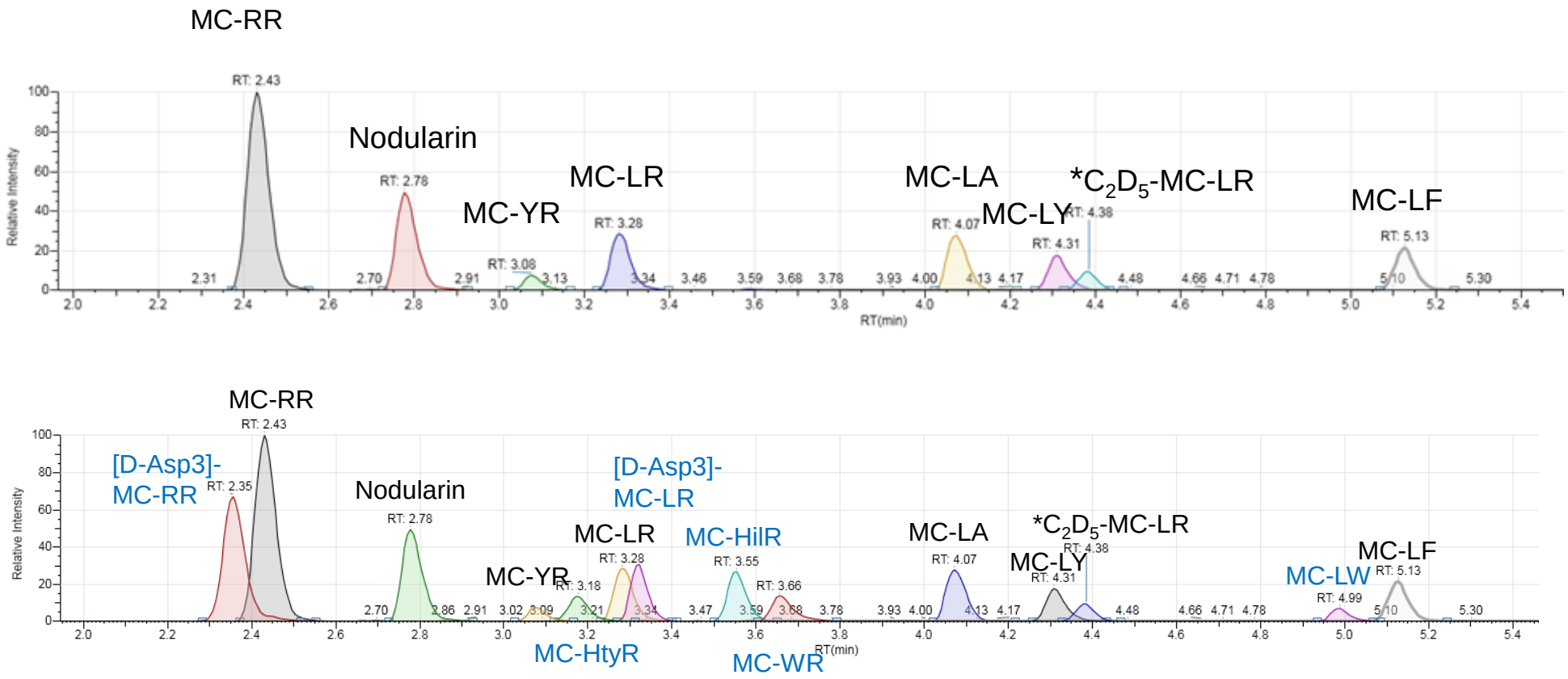
ThermoFisher SCIENTIFIC

Method development

- Mobile Phases Loading and Eluting pump
  - 0.1% Formic Acid in Water
  - 0.1% Formic Acid in Acetonitrile
- Columns
  - Concentrating
    - Hypersil GOLD aQ 20 x 2.1 mm, 12 µm particle size
  - Separating
    - Accucore aQ 50 x 2.1 mm, 2.6 µm particle size
- Injection volume
  - 1 mL
- Flow rates
  - Concentrator: 1.5 mL/min during loading, 0.1 mL/min after
  - Analytical: 0.6 mL/min gradient

Online Concentration Chromatograph

- 50 ppt standard



ThermoFisher SCIENTIFIC

Optimized Quantifier and Qualifier Ions

Analyte	Retention Time (min)	Quantifier Ions (m/z)	Qualifier Ions (m/z)
[D-Asp^3]-MC-RR	2.35	135	213
MC-RR	2.43	135	440
Nodularin	2.78	135	389
MCY-YR	3.06	213	135
MCY-HtyR	3.18	135	617
MC-LR	3.28	135	213
[D-Asp^3] MC-LR	3.31	135	375
MC-HilR	3.53	135	213
MC-WR	3.63	135	1040
MC-LA	4.07	402	375
MC-LY	4.31	494	375
C_2D_5-MC-LR	4.38	135	163
MC-LW	4.99	517	891
MC-LF	5.13	852	478

Method 544
Instrument Detection Limits and Linearity

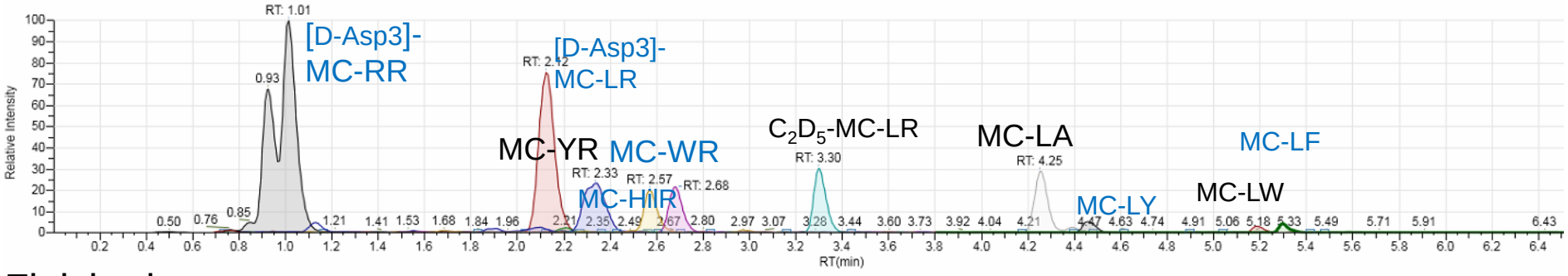
Analyte	Detection Limit (ppt)	Upper PIR	Lower PIR	R^2
[D-Asp^3]-MC-RR	0.65	119.55	84.98	0.9980
MC-RR	0.47	109.89	85.28	0.9981
Nodularin	0.99	124.52	72.14	0.9975
MCY-YR	2.46	137.81	72.88	0.9984
MCY-HtyR	1.41	147.60	73.24	0.9985
MC-LR	1.02	129.89	76.21	0.9984
[D-Asp^3] MC-LR	1.11	132.62	76.52	0.9975
MC-HilR	1.57	142.39	59.18	0.9977
MC-WR	4.26	150.88	50.61	0.9975
MC-LA	1.38	140.43	67.72	0.9981
MC-LY	2.78	137.55	64.03	0.9976
MC-LW	2.81	143.48	69.13	0.9963
MC-LF	2.70	138.63	67.26	0.9989

Comparison of Online method DL to EPA method 544 DL

Cyanotoxin	EPA 544 DL (ppt)	Online DL (ppt)
MC-YR	4.6	2.5
Nodularin	1.8	1.0
MC-RR	1.2	0.5
MC-LR	4.3	1.2
MC-LA	4.0	1.4
MC-LY	2.2	2.8
MC-LF	3.4	2.7

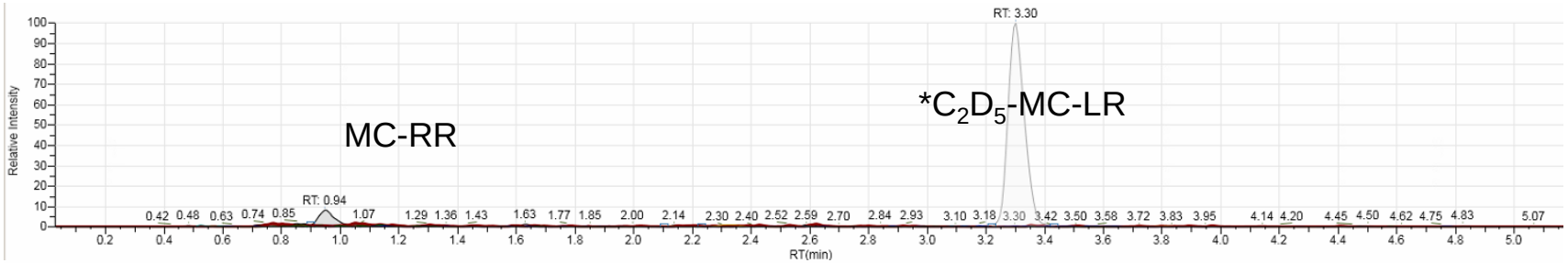
Through plant study: online concentrator vs SPE

Raw Water

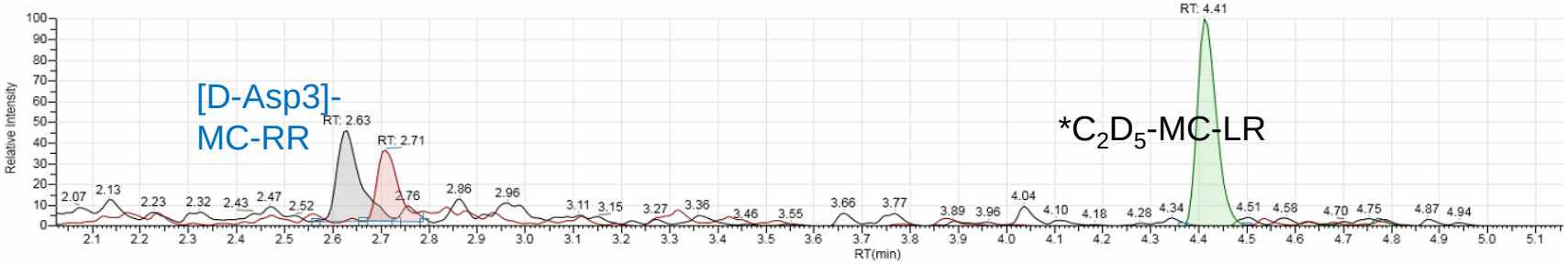


SPE

Finished



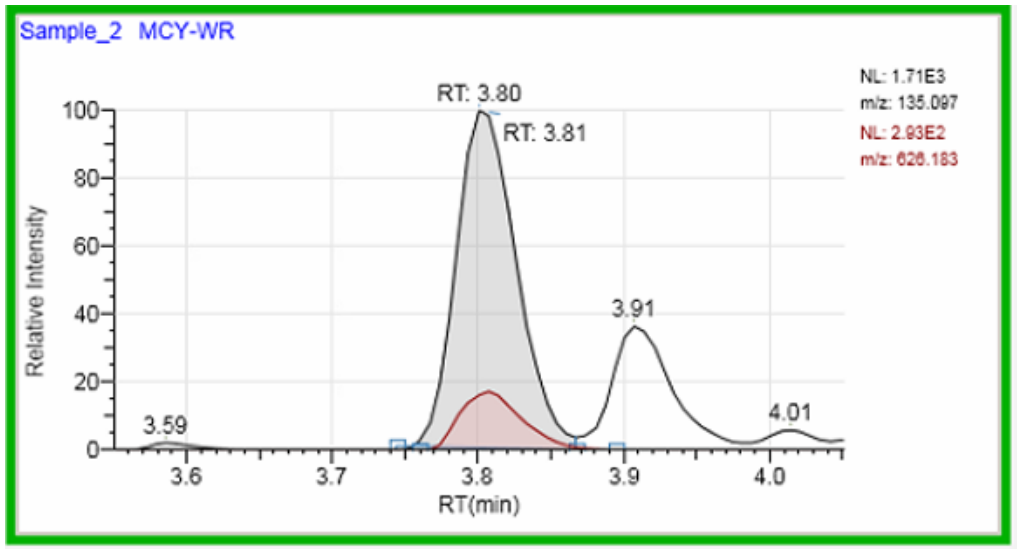
SPE

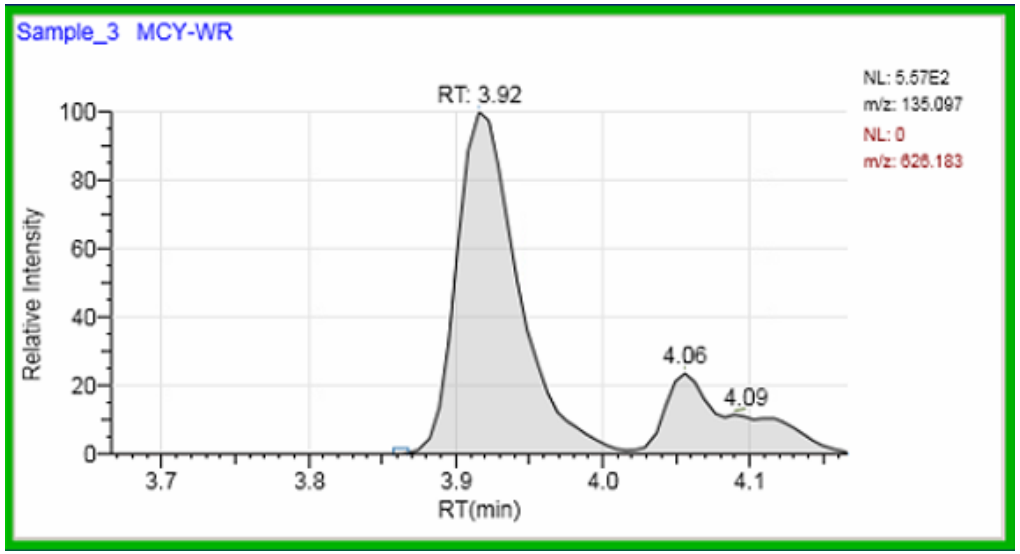


Online
Concentration

ThermoFisher SCIENTIFIC

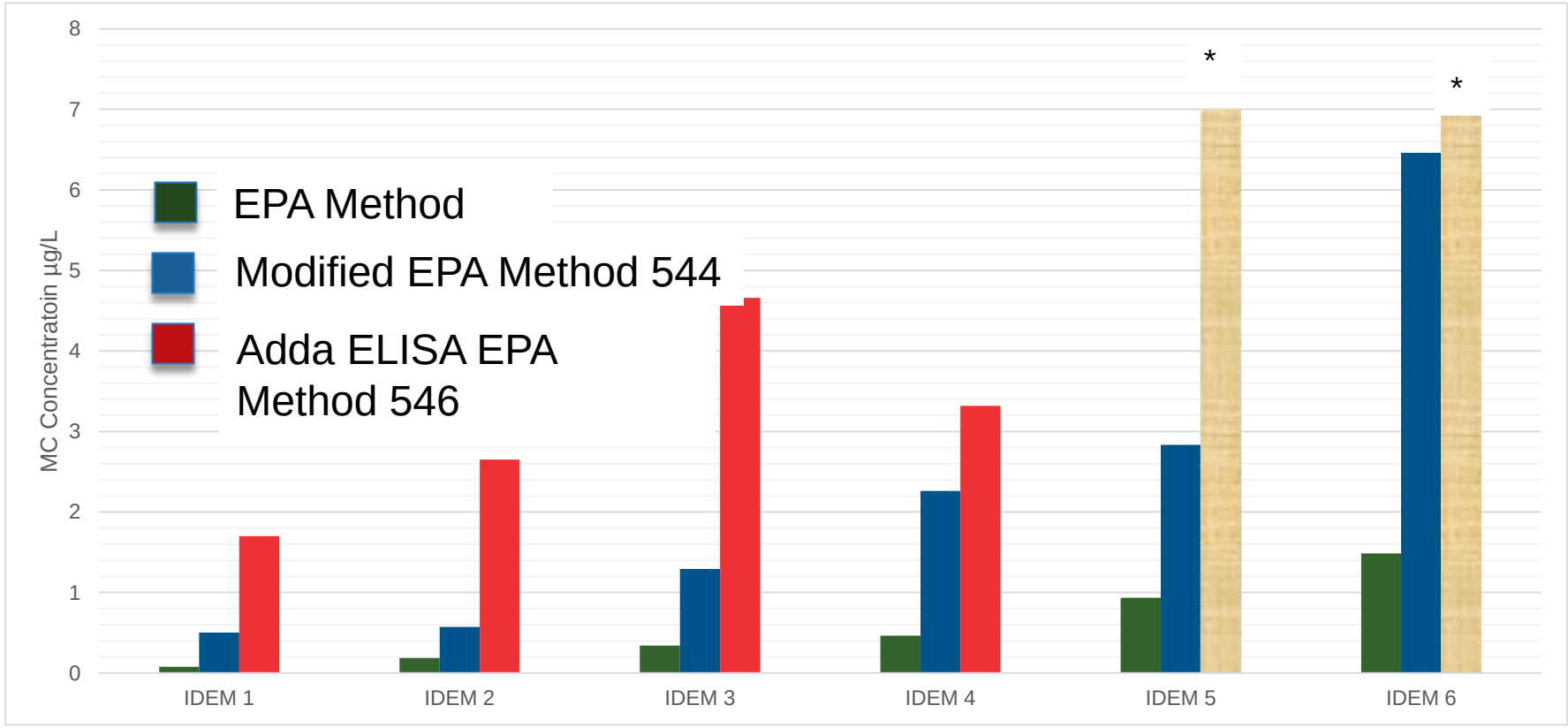
Why Quantifier and Qualifier Ions are Important





- Sample 2 is a sample spiked with 250 ppt Microcystin WR.
- Sample 3 is a sample that does not contain Microcystin WR, but, the software is recognizing it as WR.
  - There is a 0.12 minute shift in retention and the qualifier ion is not present.

Indiana Samples Quantified by ELISA and LC/MS/MS

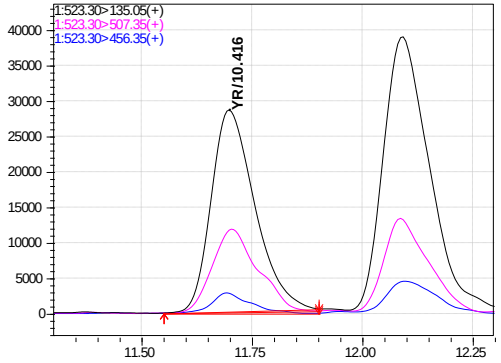


* ELISA run on these samples, but readings came back high. Dilutions provided no reproducible results.

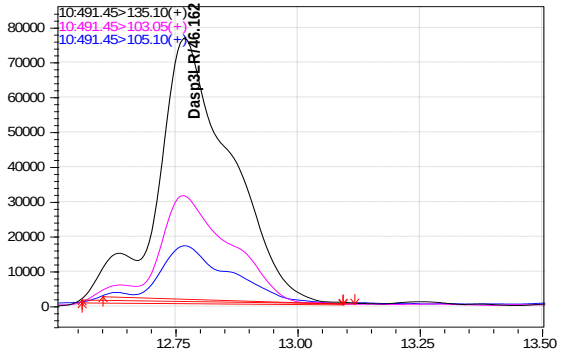
Chromatography - Indiana Sample

EPA 544 Method

MC-YR

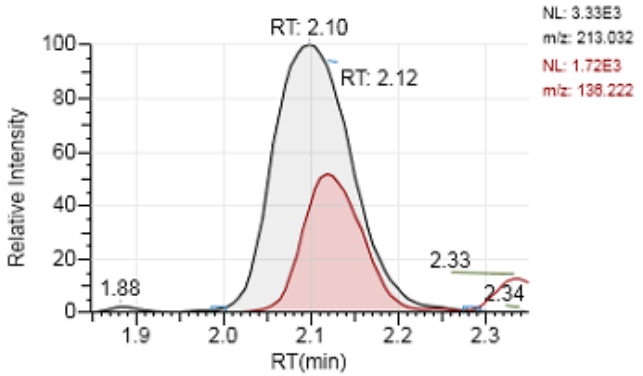


[D-Asp3]MCLR

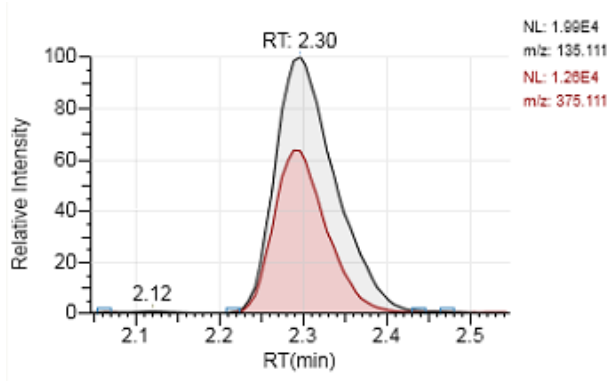


Modified EPA 544 Method

MC-YR



[D-Asp3]MCLR



Based on HRMS; the MCs are either MC-YR and [Asp3]MC-LR or are isomers of MC-YR and [Asp3]MCLR

Table 1. Comparison of standard and sample ion ratio percent and retention times.

Analyte	Standard Ion Ratio %	Sample Ion Ratio %	Standard Retention time (min)	Sample Retention time (min)
[D-Asp³]-MC-RR	12	14	2.56	2.50
MC-YR	68	28	3.34	3.40
[D-Asp³]-MC-LR	63	26	3.60	3.59
MC-LY	75	84	4.64	4.64

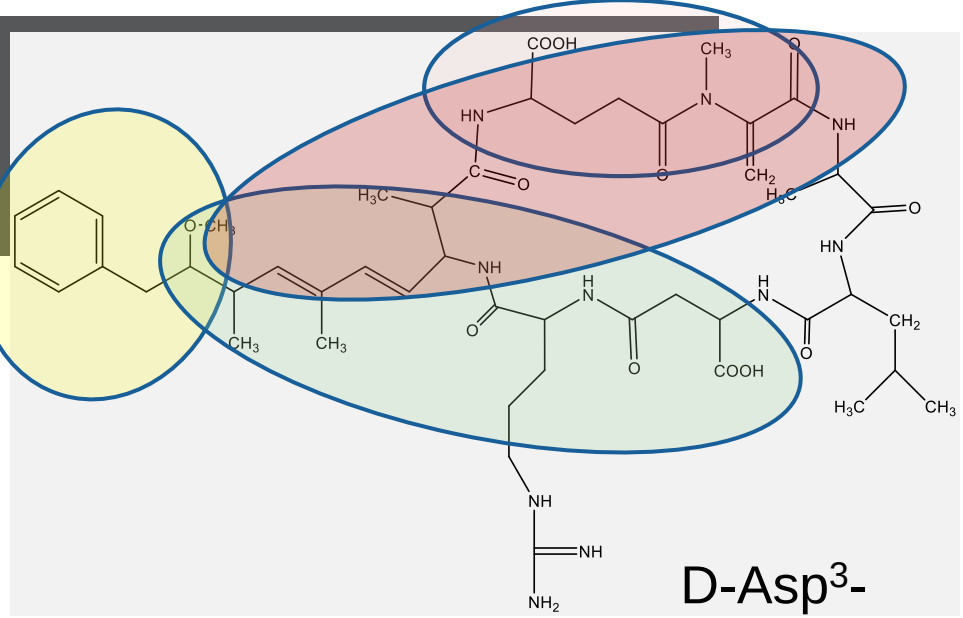
Table 2. HRMS results of the three prominent MC found in the impoundment sample and the corresponding standard.

Standard Analyte	Calculated Mass (m/z)	Found Mass (m/z)	Charge	Δ ppm
[D-Asp³]-MC-RR	512.7824	512.7816	2	-1.5
MC-YR	523.2713	523.2715	2	0.4
[D-Asp³]-MC-LR	491.2738	491.2738	2	0

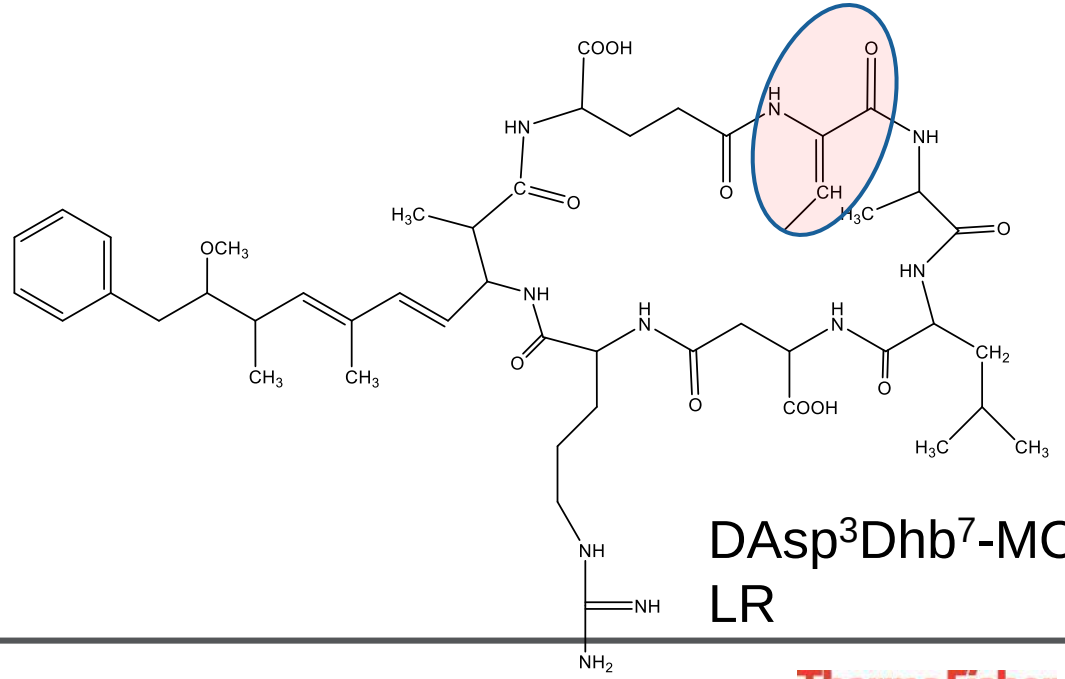
Sample Analyte	Calculated Mass (m/z)	Found Mass (m/z)	Charge	Δ ppm
[D-Asp³]-MC-RR	512.7824	512.7825	2	0.2
MC-YR	523.2713	523.2717	2	0.8
[D-Asp³]-MC-LR	491.2738	491.2740	2	0.4

DAsp3-MCLR

Ion Structure	[D-Asp3]-LR	IDEM 981
[Ph-CH2-CHOMe]+	135	135
[H+ Mdha-Ala]+	155	155
[C11H14O+ H]+	163	163
[Arg-NH2+ 2H]+	174	
[H+ Glu-Mdha]+	213	213
[H+ CO-Glu-Mdha]+	239	
[H+ Mdha-Ala-X]+	268	
[H+ Arg-Asp]+	272	272
[2H+ NH2-Arg-Asp]+	289	
[C11H14O-Glu-Mdha+ H]+	375	375
[H+ Glu-Mdha-Ala-X]+	397	397
[H+ Leu-Asp-Arg]+	385	
[H+ Arg-Adda]+	470	470
[M+ 2H]2+	491	491
[H+ Mdha-Ala-X-Asp-Z]+	539	539
[H+ Asp-Arg-Adda]+	585	585
[M+ 2H–135]+	847	847
[M+ H–CO]+	953	953
[M+H]+	981	981



D-Asp^3-MCLR

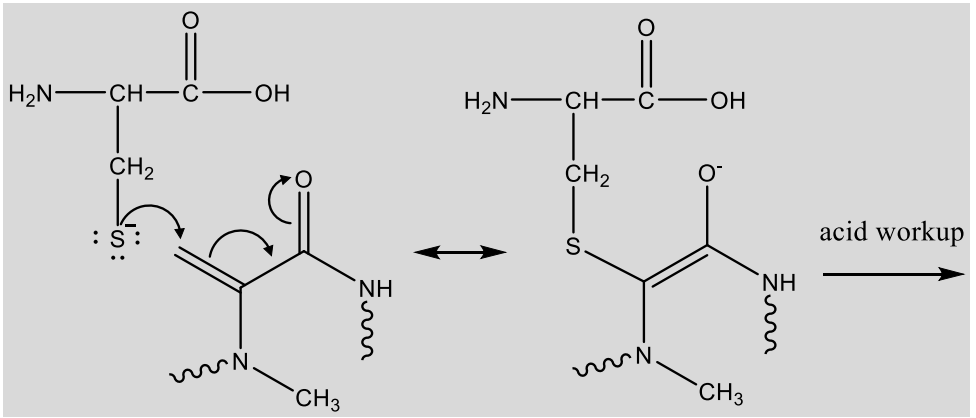


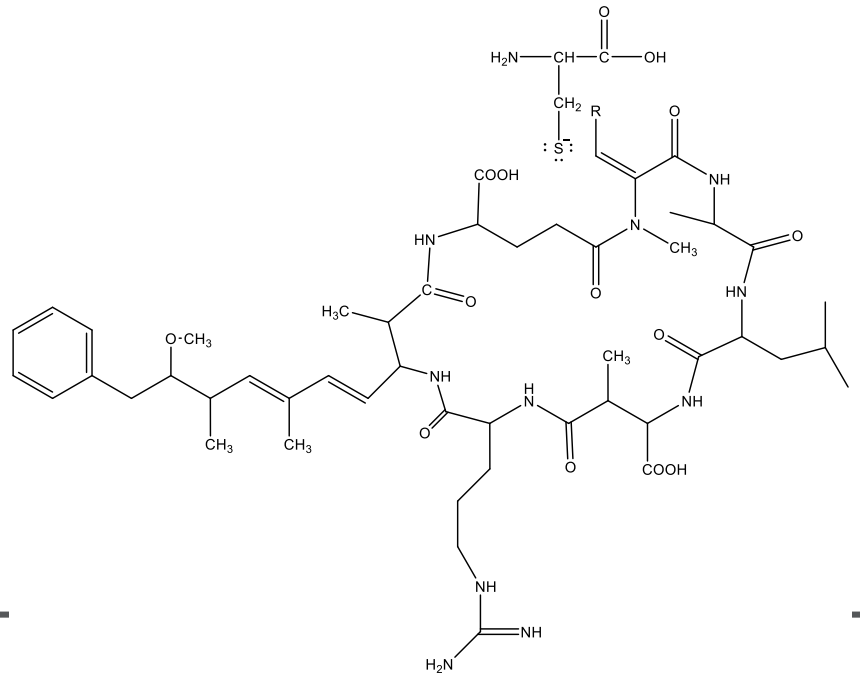
DAsp^{3}Dhb^7-MCLR

Kubwabo et al., 2005
Krishnamurthy et al., 1989
Sano et al., 1998

Michael Addition

- Cysteine (C) – nucleophile
- α-β unsaturated ketone – electrophile
- Basic condition

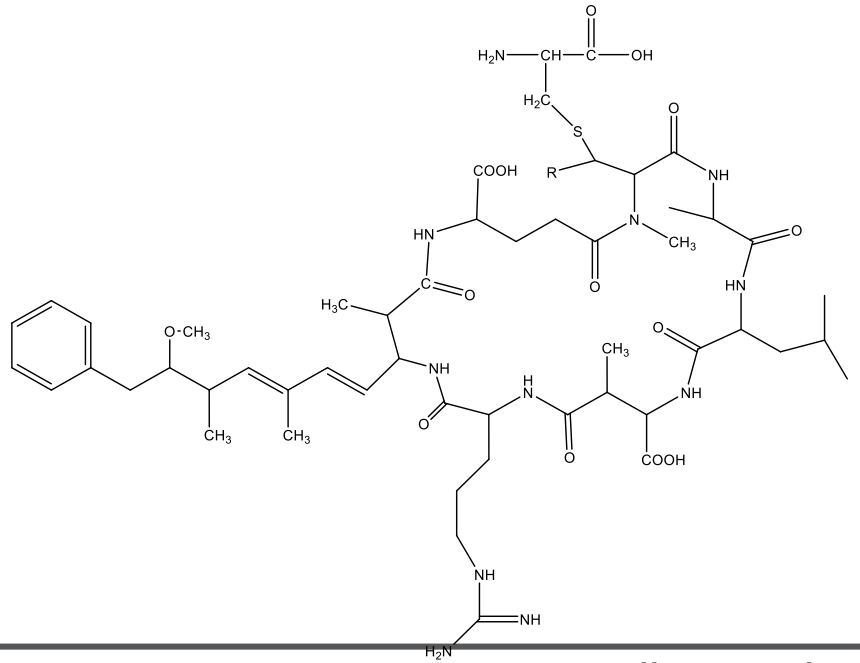




R=H, MDha

R=CH3 Dhb

X



Miles et al. 2012

Standards

[DAsp^3] MCRR, MCYR and [DAsp^3]MC-LR

BUT

[DAsp^3] MCRR not present

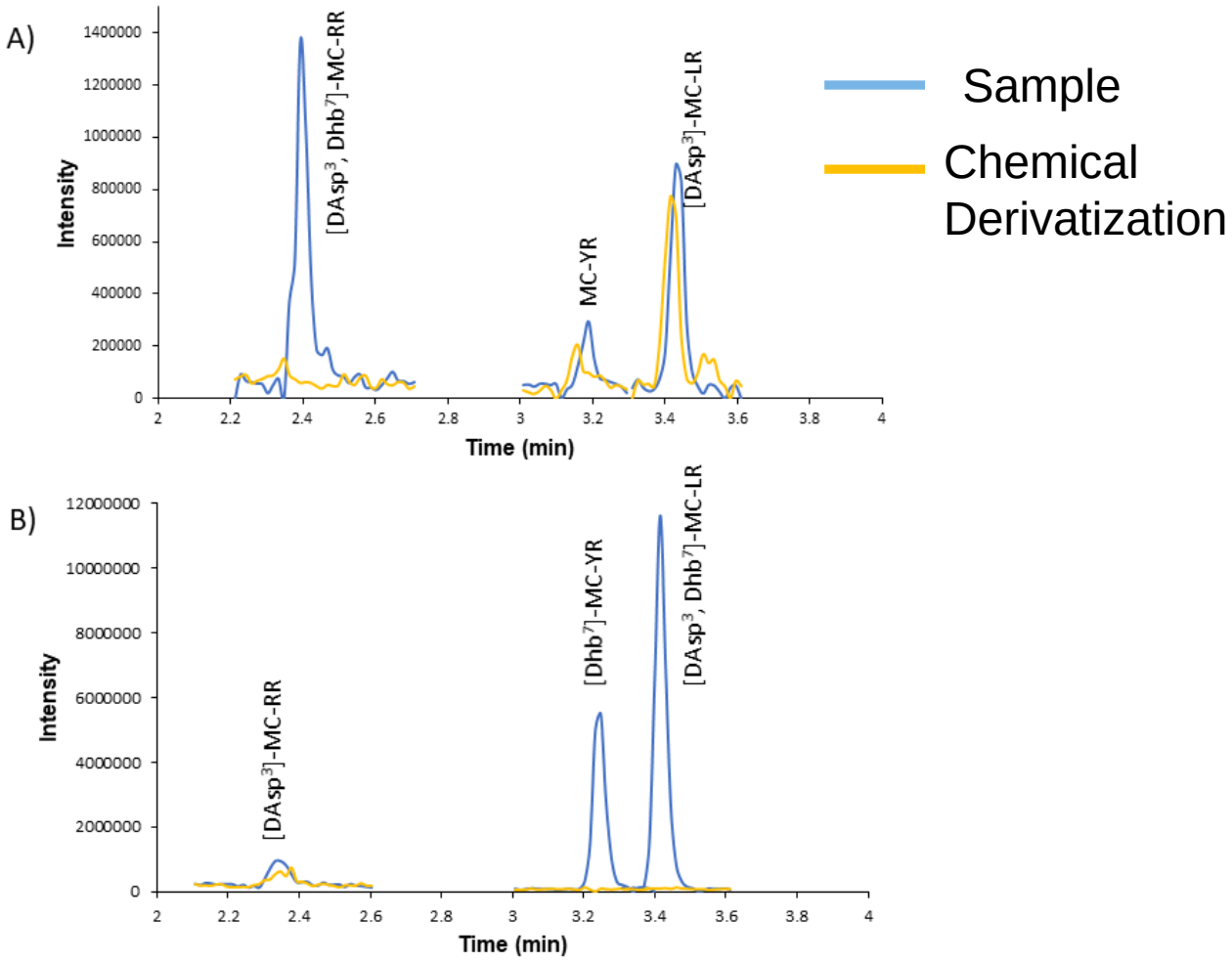
[DAsp3,Dhb7]MC-RR

Sample

[DAsp^3] MCRR

[Dhb^7]MCYR

[DAsp^3, Dhb^7]MC-LR



Conclusion

- Standards must be checked.
- First time Dhb MCs has been reported in USA
- First time [Dhb^{7}] MC-YR has been reported
- Chromatography (Retention Time Windows)
- Qualifier/Quantifier ion ratio

- MOST IMPORTANTLY
- All experiments can be performed with online concentration LC/MS/MS.
- NO solid-phase extraction step
- Pieces can be identify without high resolution mass spectrum

Outstanding analytical methodology needs.

- Standards
- Chromatography
- Qualifier and quantifier ions.
- MC prevalence survey.
- MS Untargeted MC methodology

Acknowledgments

P30 ES020957