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

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Introduction

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- **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

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- 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

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- **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:

Time (min)		%A	%B	Curve
0.00	100.0	0.0	Initial	
0.75	100.0	0.0	6	
5.00	20.0	80.0	6	
6.00	0.0	100.0	1	
7.50	100.0	0.0	1	

- Column Temp: 50.0 C

Experimental: MS Method Settings

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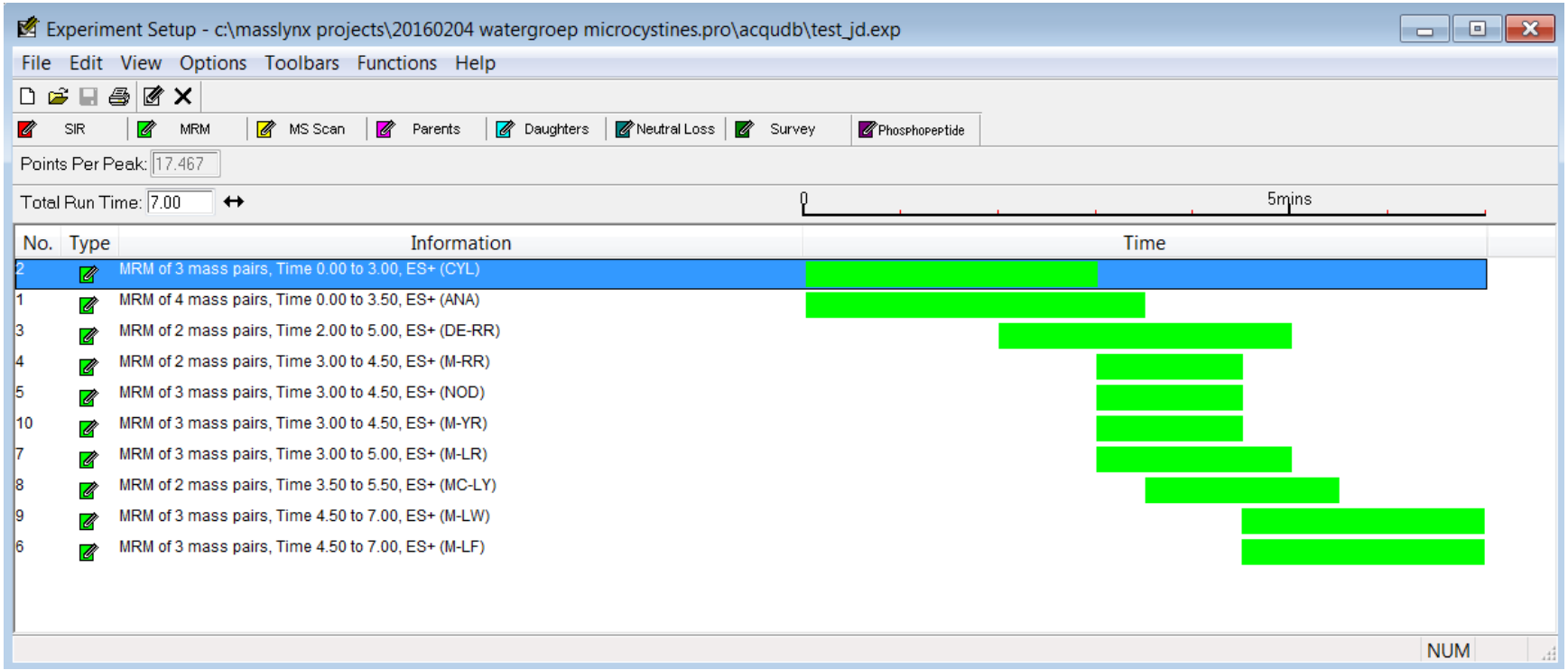
- Time segmented MRM with Dwells to give 15 pts across each peak
- 2 MRMs per analyte
- Most microcystins singly charged except for DE-M-RR & M-RR



Performance Criteria

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- 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

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QDa

SQD2

Xevo TQD

Xevo TQ-S micro

Xevo TQ-S

Xevo G2-XS Tof

Xevo G2-XS QTof

Vion

Synapt G2-Si

Results: Typical Chromatogram

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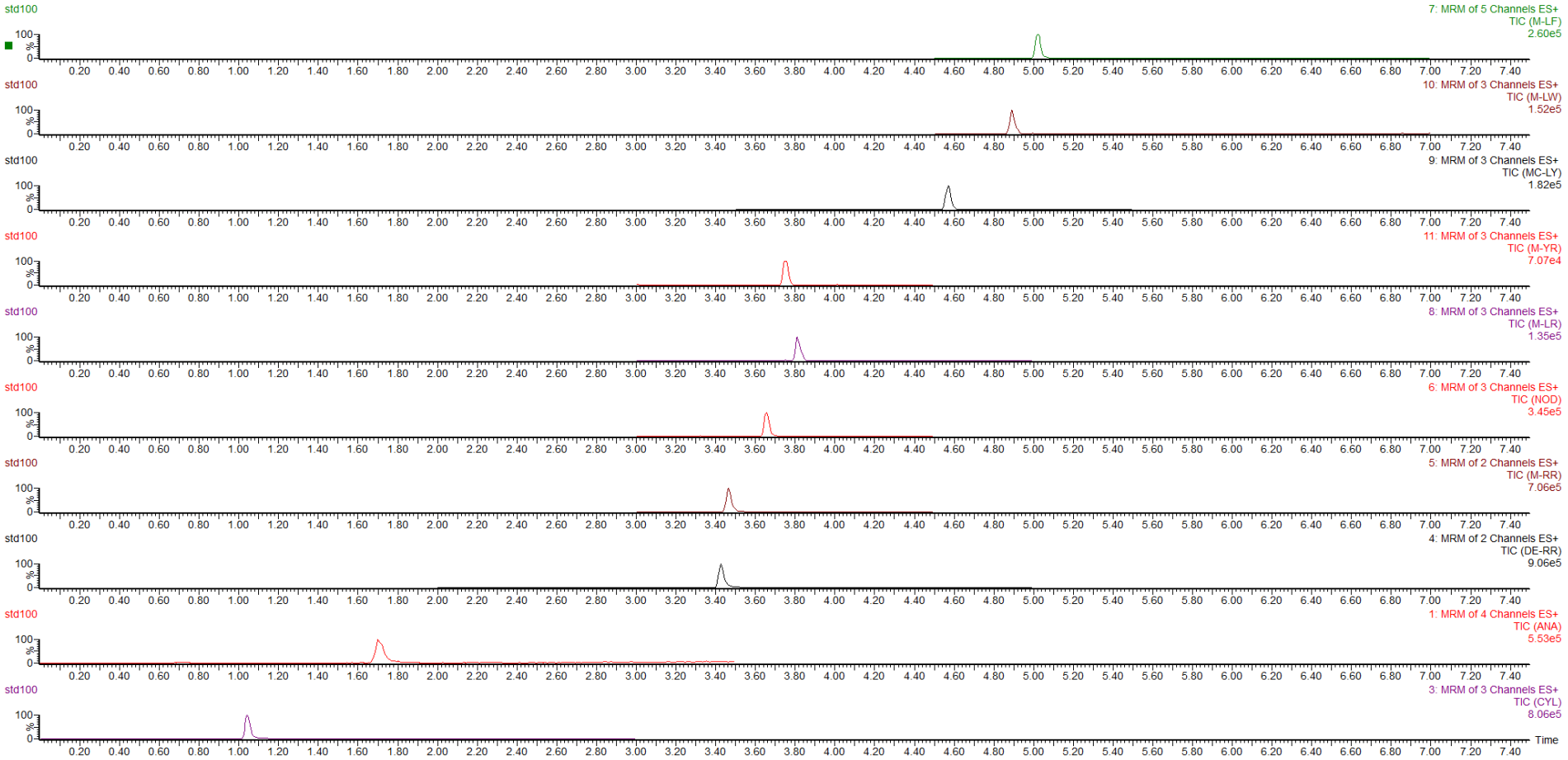
- Bottom to top: Cylindrospermopsin (CYL), Anatoxin-A (ANA), M-DE-RR, M-RR, Nodularin (NOD), M-LR, M-YR, M-LY, M-LW and M-LF at 100 ng/L



Results: Sensitivity

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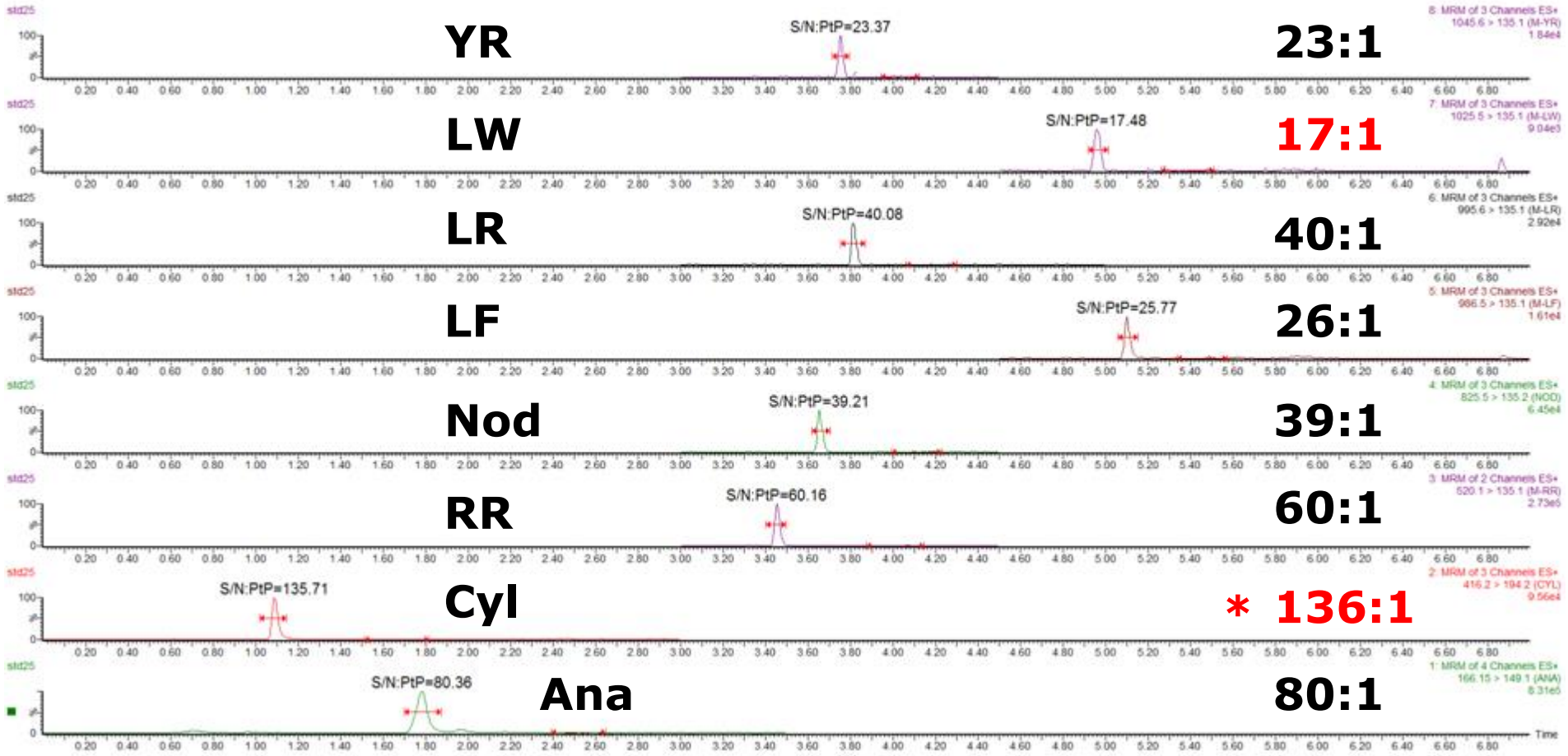
- All analytes detected with good S:N at the 25 ng/L



Results: Linearity from 10 to 1000ng/L

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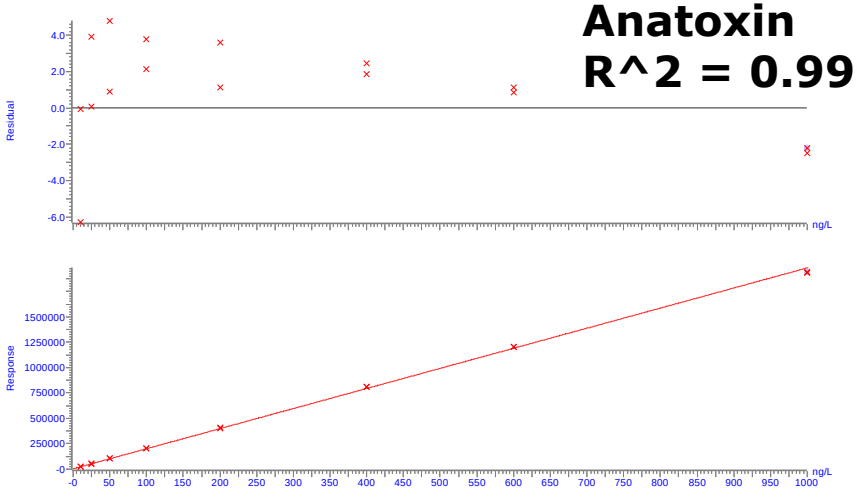
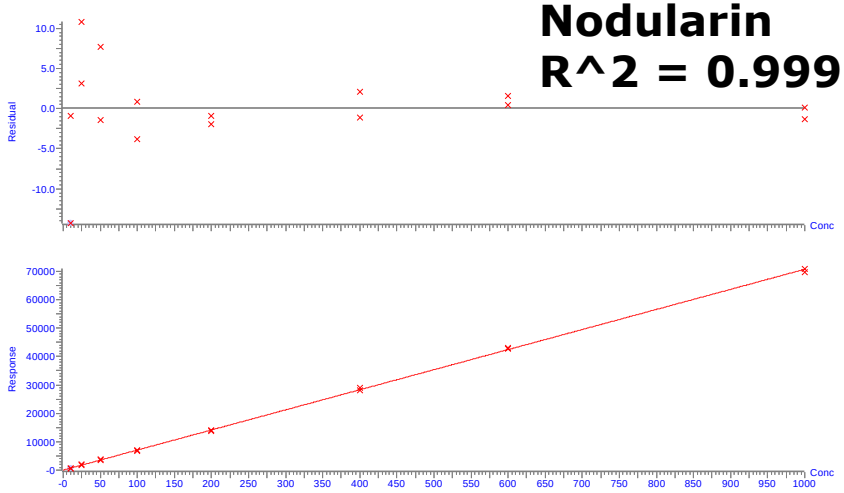
Compound name: Anatoxin-a
Correlation coefficient: r = 0.999701, r^2 = 0.999402
Calibration curve: 1978.2 * x + 3045.67
Response type: External Std, Area
Curve type: Linear, Origin: Include, Weighting: 1/x, Axis trans: None

Anatoxin
R^2 = 0.9994



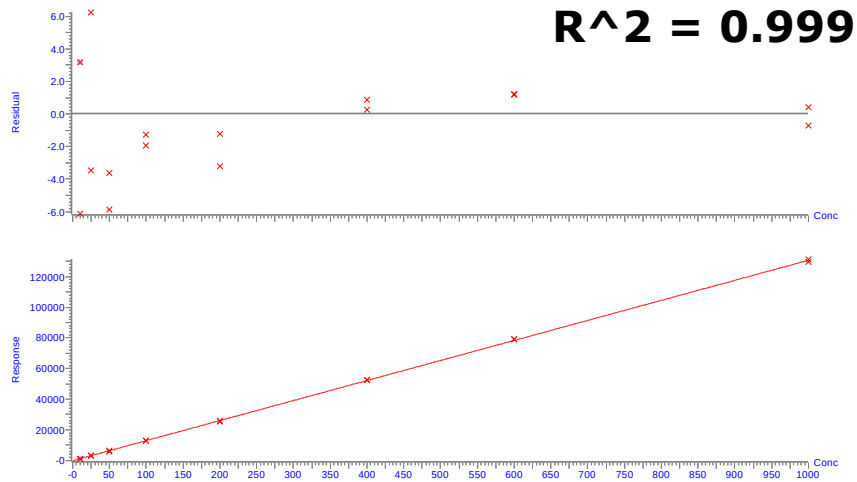
Compound name: Nodularin
Correlation coefficient: r = 0.999806, r^2 = 0.999613
Calibration curve: 70.7322 * x + 6.27616
Response type: External Std, Area
Curve type: Linear, Origin: Include, Weighting: 1/x, Axis trans: None

Nodularin
R^2 = 0.9996



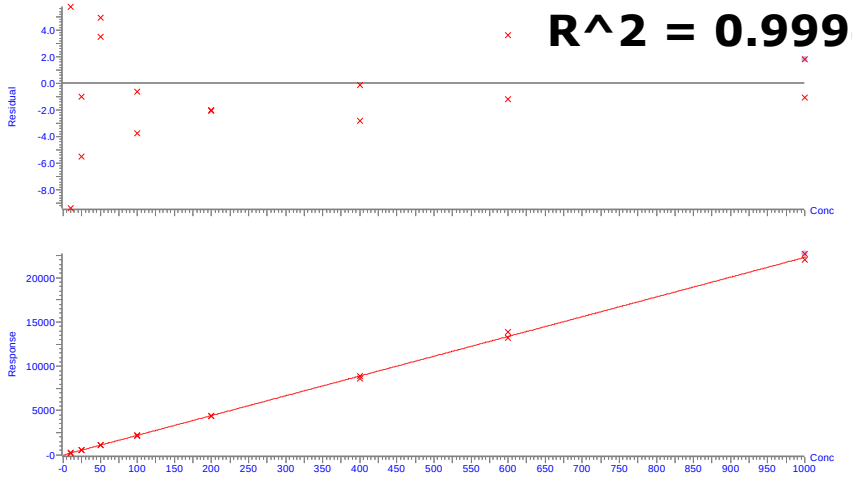
Compound name: CYL
Correlation coefficient: r = 0.999861, r^2 = 0.999722
Calibration curve: 130.893 * x + -183.659
Response type: External Std, Area
Curve type: Linear, Origin: Include, Weighting: 1/x, Axis trans: None

CYL
R^2 = 0.9997



Compound name: MC-YR
Correlation coefficient: r = 0.999714, r^2 = 0.999429
Calibration curve: 22.3124 * x + -22.0103
Response type: External Std, Area
Curve type: Linear, Origin: Include, Weighting: 1/x, Axis trans: None

M-YR
R^2 = 0.9994



Results: Robustness

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- 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

Compound	100 ng/L (1)	100 ng/L (5) +15.5h	ratio
M-YR	2061	2215	107%
M-LW	1361	1660	122%
M-LR	2770	3353	121%
M-LF	1522	1990	131%
NOD	6848	7380	108%
M-RR	28133	29911	106%
CYL	12027	12074	100%
ANA	190364	183937	97%

Results: Repeatability

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- 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%

Compound	% CV (n=5)	
	DW	SW
M-YR	4.6	3.0
M-LW	3.2	2.2
M-LR	2.2	1.8
M-LF	4.2	4.6
NOD	1.9	3.0
M-RR	1.6	0.5
CYL	5.0	2.7
ANA	1.6	0.5

Results: Accuracy

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- 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

% Spike Recovery Values

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

Results: Accuracy

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- 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

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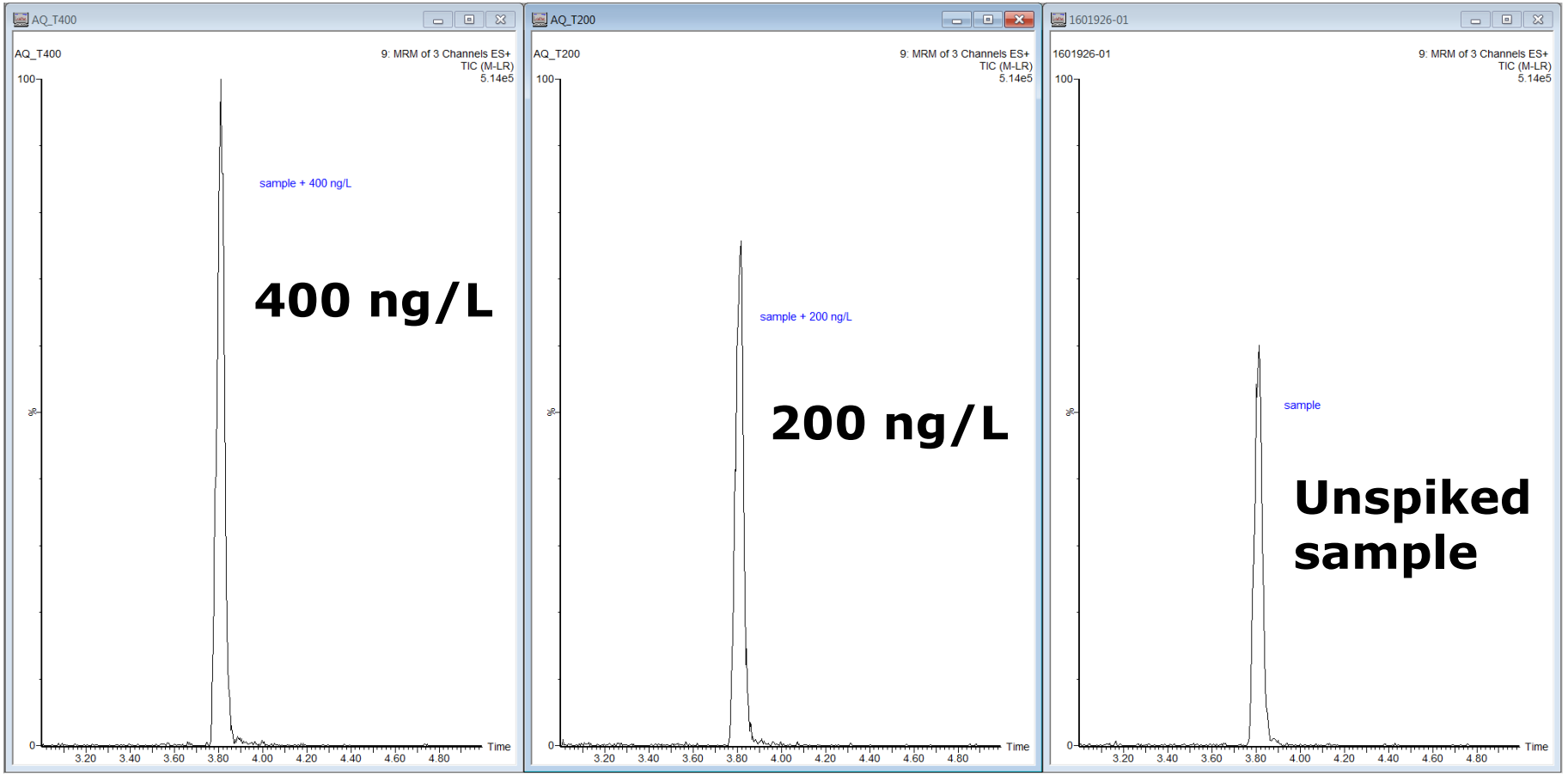

Results: Standard Addition

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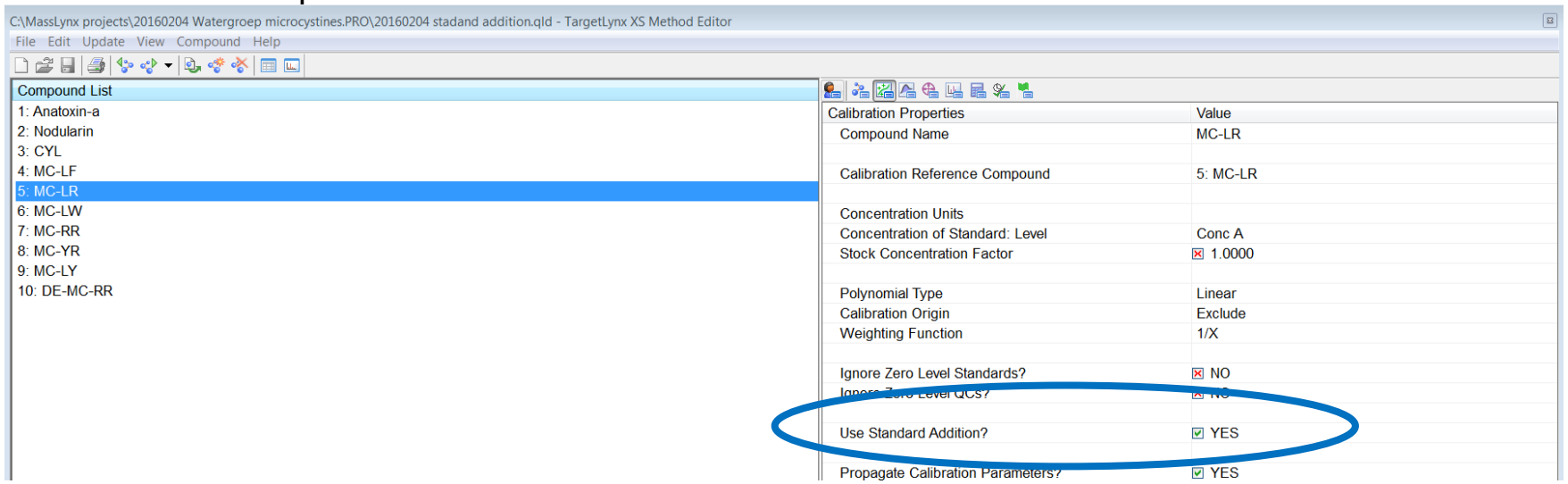
- In the Sample List:
  - Use "Standard" for sample type
  - Add the spiked concentration
  - Assign a sample group for each set of samples

Spectrum Chromatogram Map Edit Samples

	File Name	File Text	MS File	Inlet File	Bottle	Inject Volume	Sample Type	Conc A	Sample Group
1	1601926-01	AQ500-groep S32	test_JD	Microcysteines A1-B1	1:10	50.000	Standard		aquacheck
2	AQ_T200	AQ_T200	test_JD	Microcysteines A1-B1	1:11	50.000	Standard	200	aquacheck
3	AQ_T400	AQ_T400	test_JD	Microcysteines A1-B1	1:12	50.000	Standard	400	aquacheck

- In the TargetLynx processing method:
  - Check the option "Use Standard Addition?"



Results: Standard Addition

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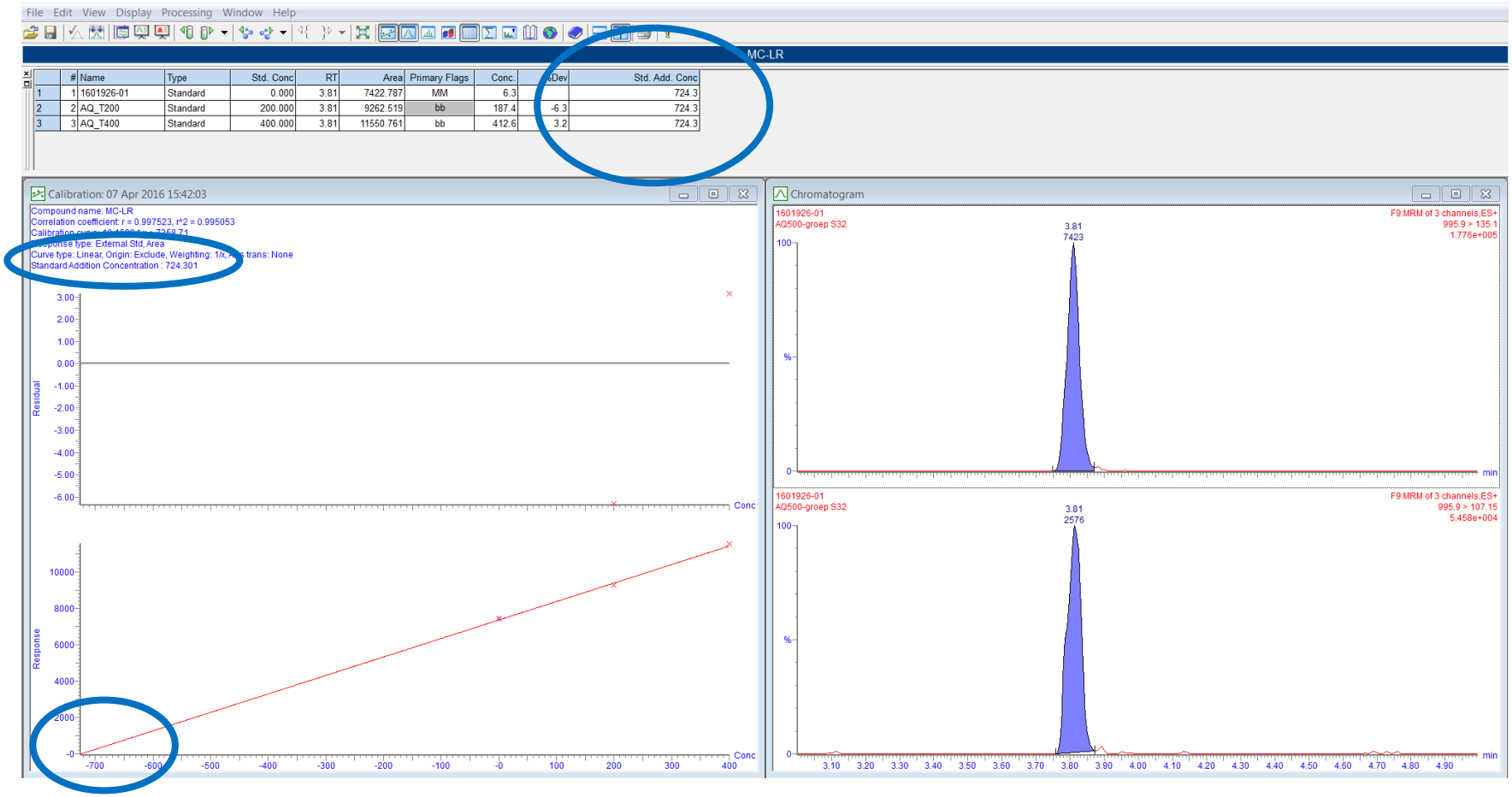
- TargetLynx calculates endogenous concentration
- Reported conc. **724 ng/L in good agreement** with known value



Summary of Direct Injection High Sensitivity TQ Method

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- 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

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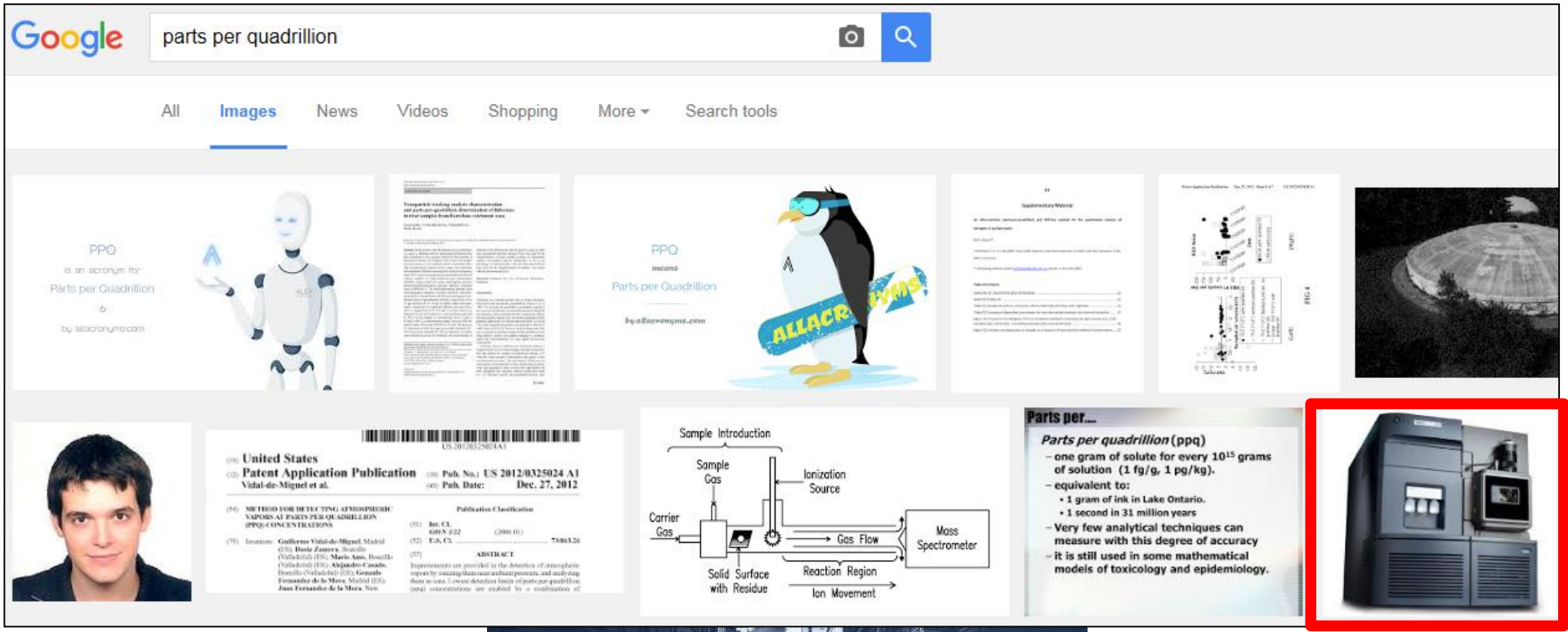
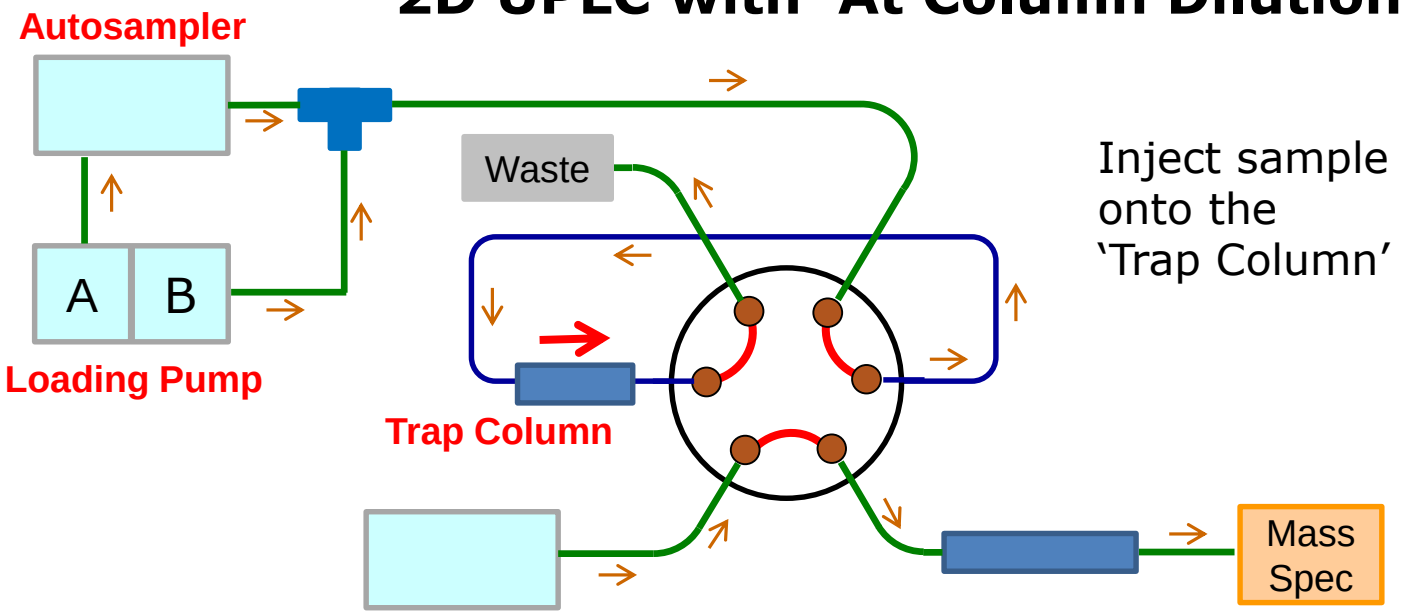


2D UPLC with 'At Column Dilution'

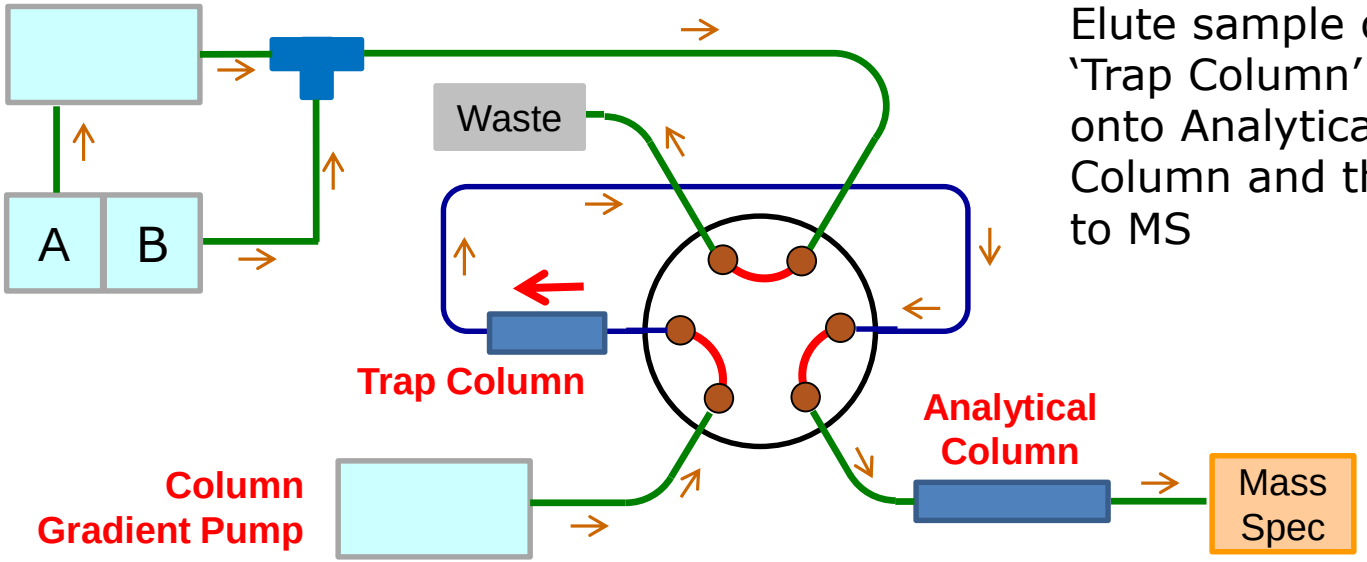
Loading Pump:
A pushes sample out of the autosampler

B dilutes the flow coming from the autosamper 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

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ACQUITY I-Class 2D-UPLC ACD Method

Trap Column
Oasis HLB Direct Conn
2.1 mm x 30 mm, 2(

Analytical Columr
Waters ACQUITY UPLC E
130Å, 1.7 µm, 2.1 mm X 50

UPLC System:
ACQUITY I-Class Bir
Solvent Manager (B
ACQUITY Column Manage

250 µL Injections of
processed water sample

Purge Solution =
90/10 H2O/MEOH

Wash Solution =
30/10 ACN/ IPA/MEOH/H2O

ple Temperature = 7 °C

Loading Pump

A = 67/33 Acetonitrile/Water
B = Water
A & B have 0.3% Formic Acid

Gradient (Flow=0.80 mL/min)

Time	A%	B%
0.0 min	15	85
3.3 min	15	85
3.4 min	95	5
6.0 min	95	5
6.5 min	15	85
11.0 min	15	85
11.5 min	15	85

Column Gradient Pump

A = Water with
B = Acetonitrile with
A & B have 0.01% Formic Acid

Gradient (Flow=0.45 mL/min)

Time	A%	B%
0.0 min	90	10
3.3 min	90	10
7.3 min	5	95
8.5 min	5	95
9.0 min	90	10
11.5 min	90	10

Switch at 2.25

Switch at 10.25

Loading Pump:
A is used to push the sample out of the autosampler
B is used to dilute the flow coming from the autosamper with aqueous before it gets to the trap column

t=0: Flow from autosampler goes through trap column to waste
t=2.25: valve switch to send flow from the column pump through the trap and onto the analytical column
t=10.25 switch back to t=0 flow path

Blank Injections 1 - 4

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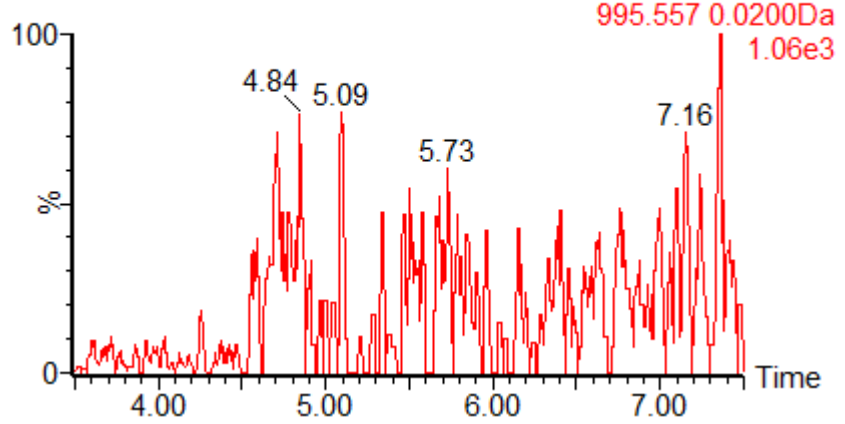
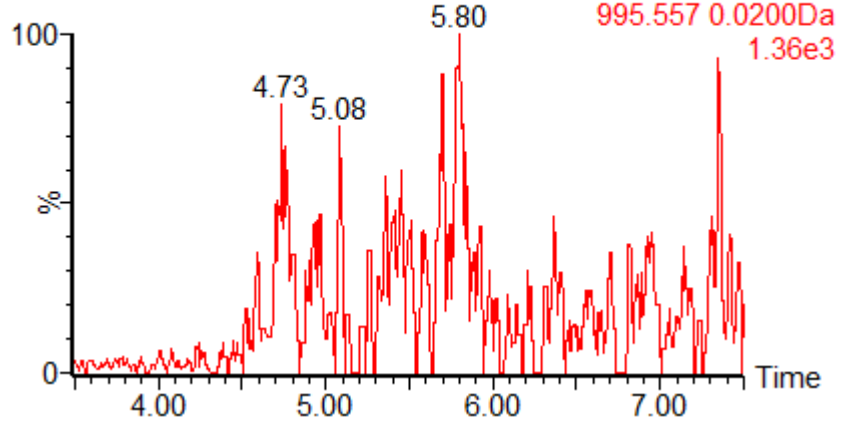
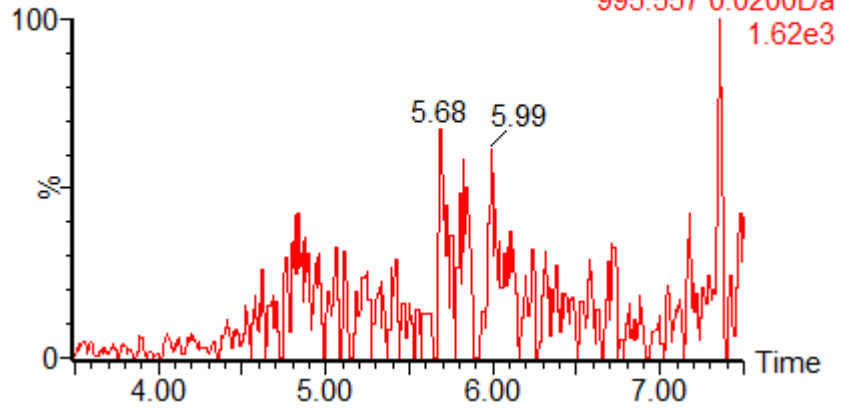
Water Extraction Blank 1 **Acquired 16-Dec-2015 / 02:44:14**



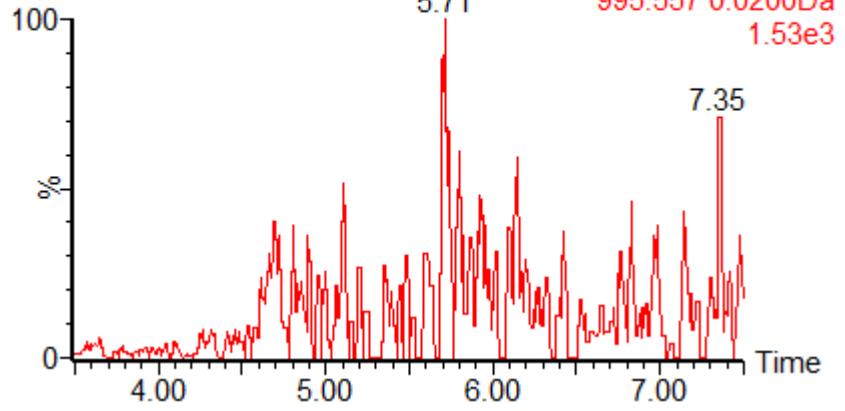
Water Extraction Blank 3 **Acquired 16-Dec-2015 / 03:12:23**



Water Extraction Blank 2 **Acquired 16-Dec-2015 / 02:58:18**



Water Extraction Blank 4 **Acquired 16-Dec-2015 / 03:26:27**



Blank Injection 5 and & RO Water Injection 1

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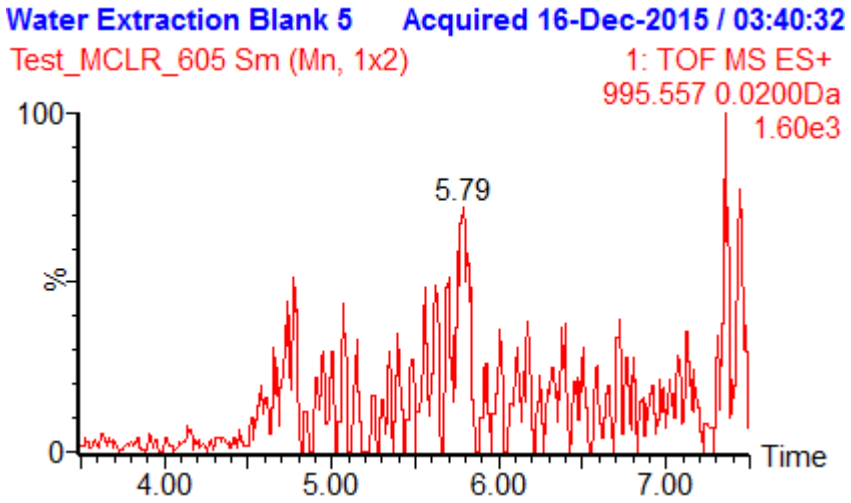

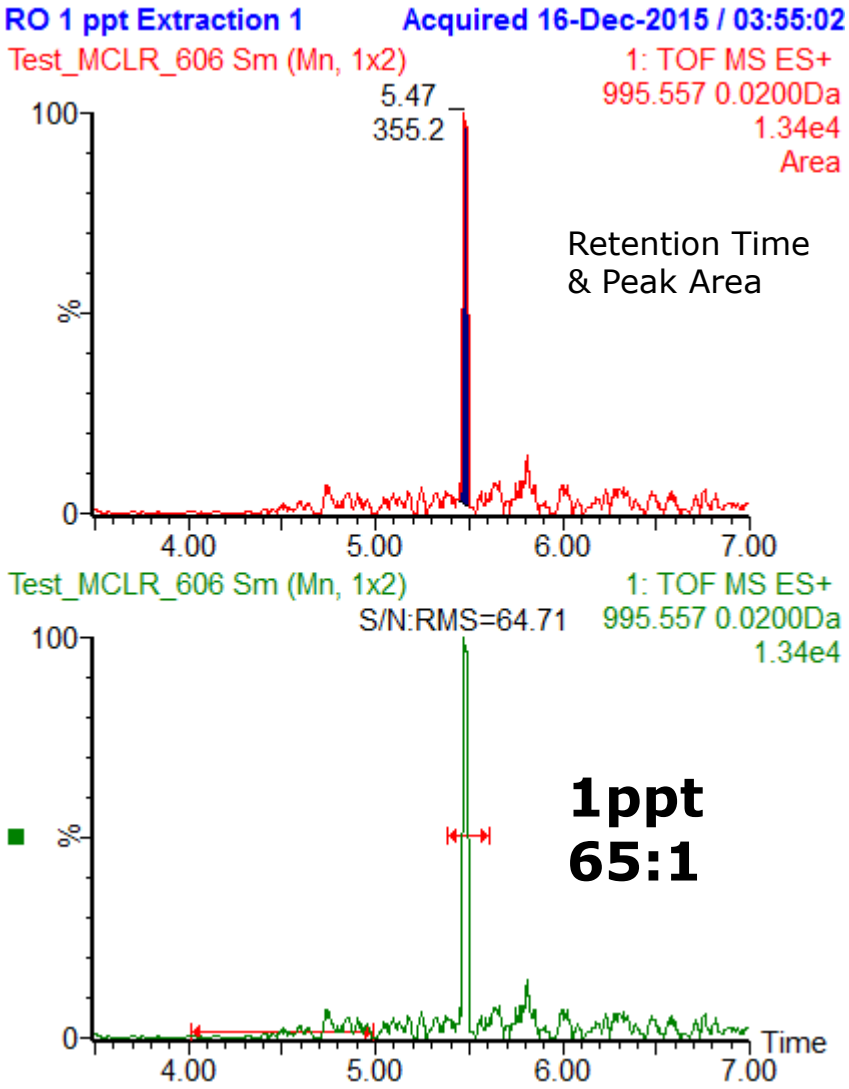

RO Water Injections 2 & 3

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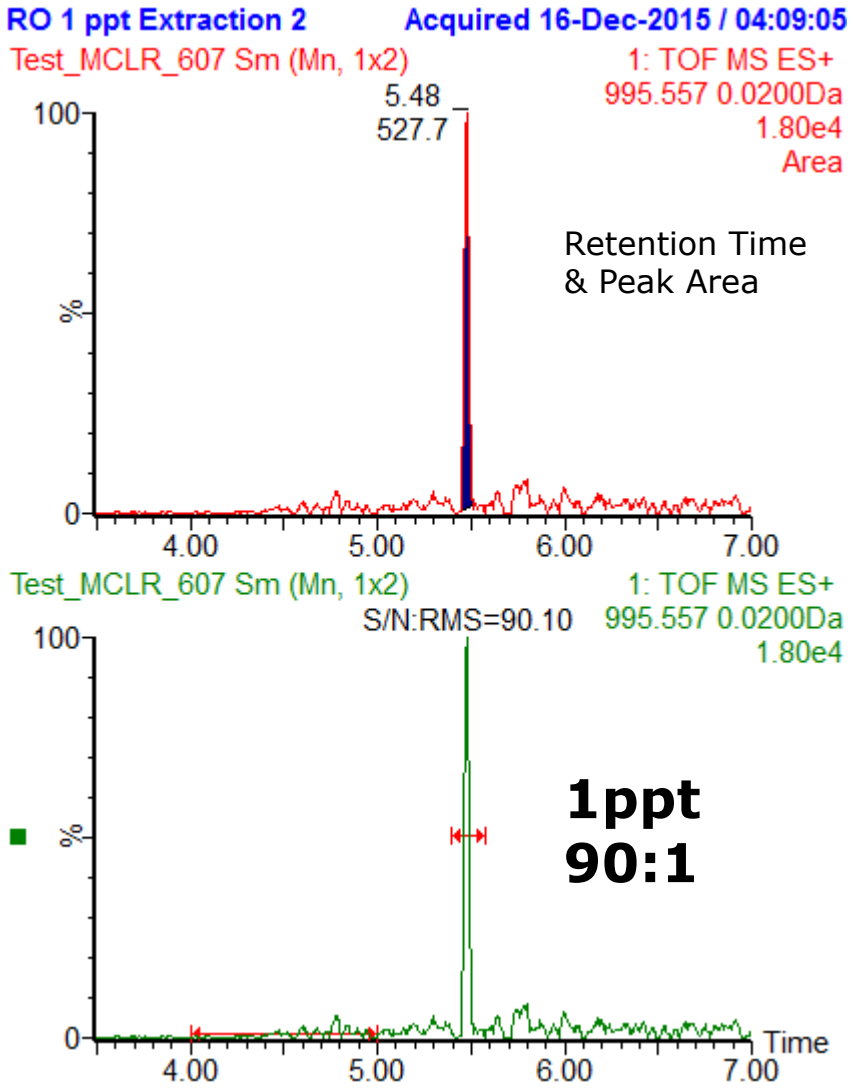

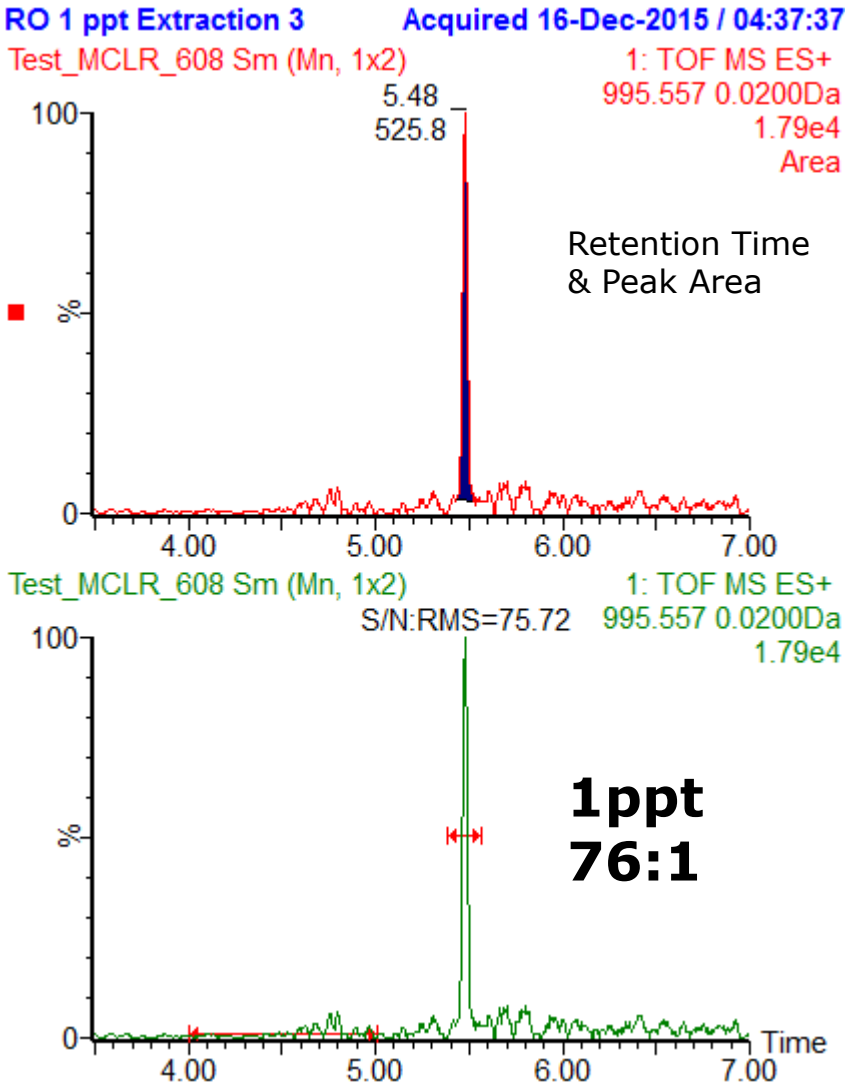

RO Water Injections 3 & 4

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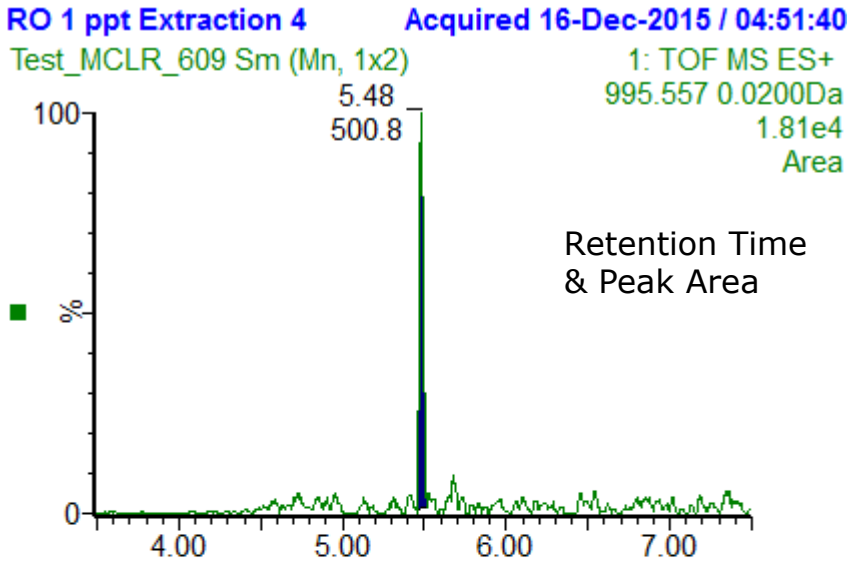

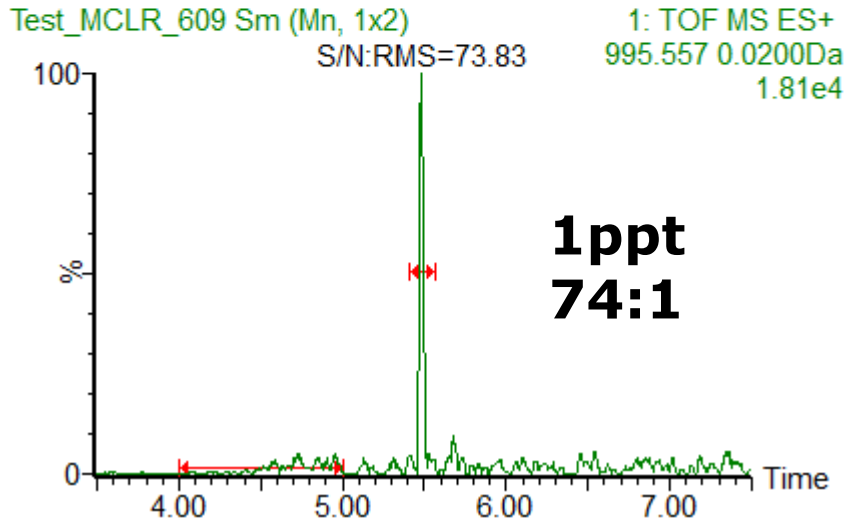

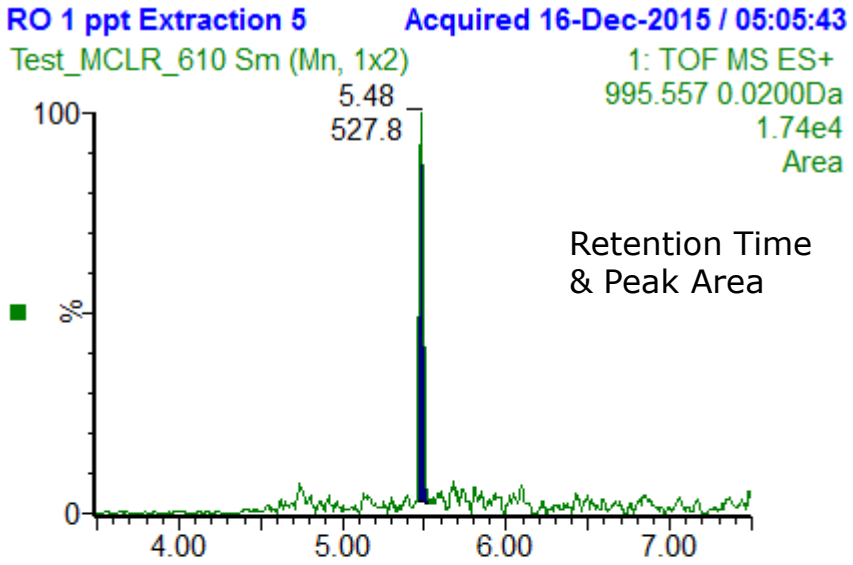

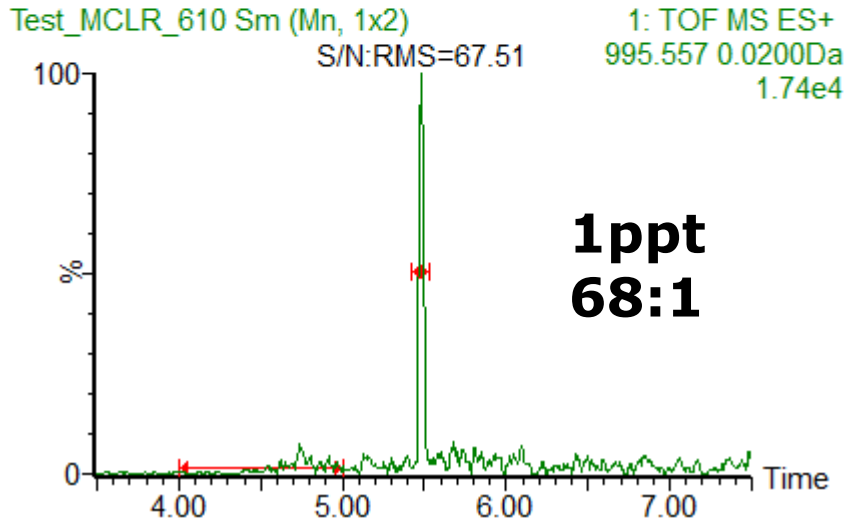

Instrument Detection Limits

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Five Analyses of RO Water Samples Spiked at 1 ppt

Injection	RT	Area	RMS S:N
1	5.48	355.2	64.71
2	5.48	527.7	90.10
3	5.48	525.8	75.72
4	5.48	500.8	73.83
5	5.48	527.8	67.51
	Average =	487.5	**74.37**
	Std Dev =	74.8	9.87
	RSD =	15.3%	**13.3%**

Translates to LOD of approximately <100ppq

Spectrum for 1 ppt of Microcystin LR

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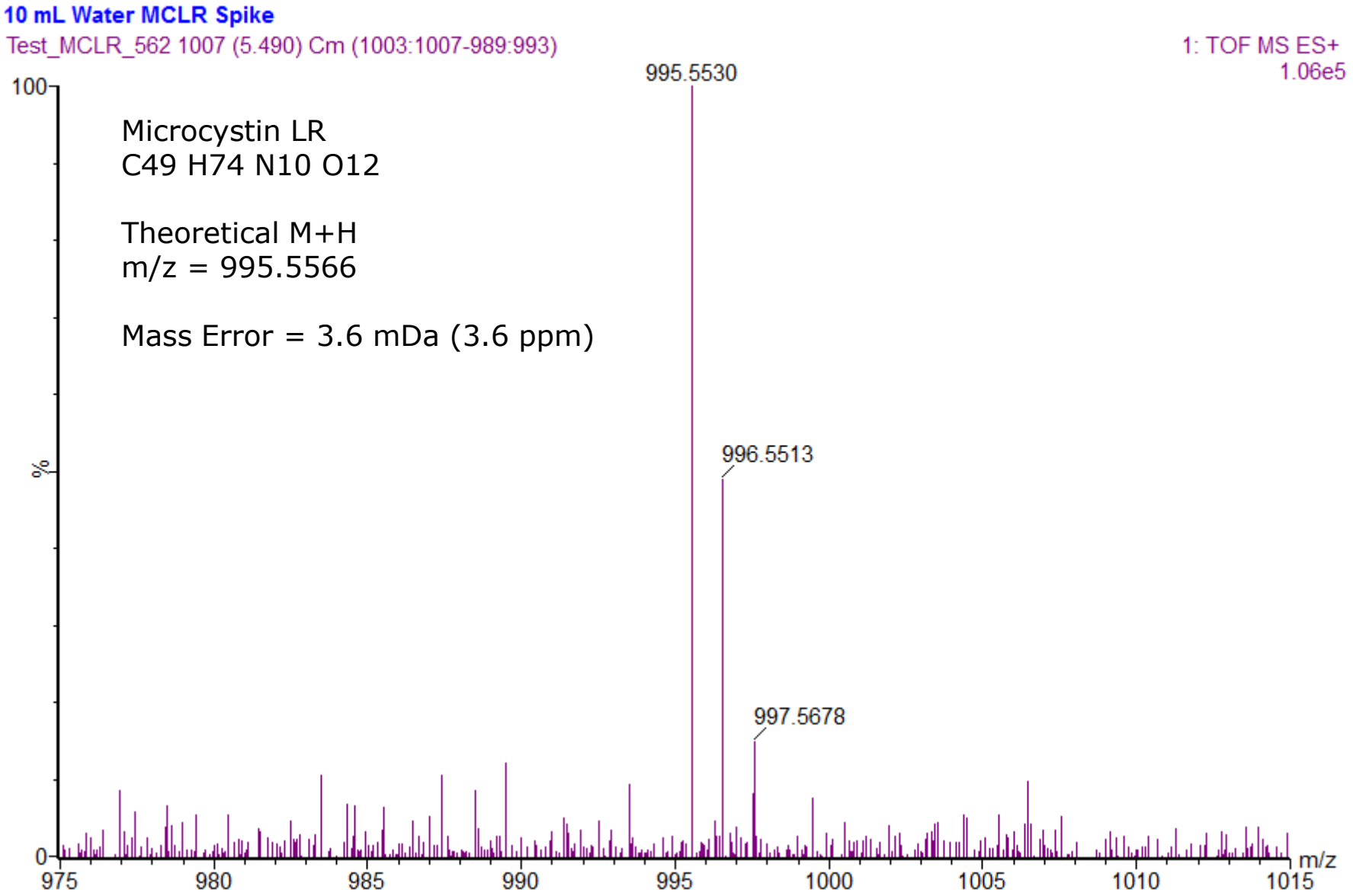

Why Use a QTof?

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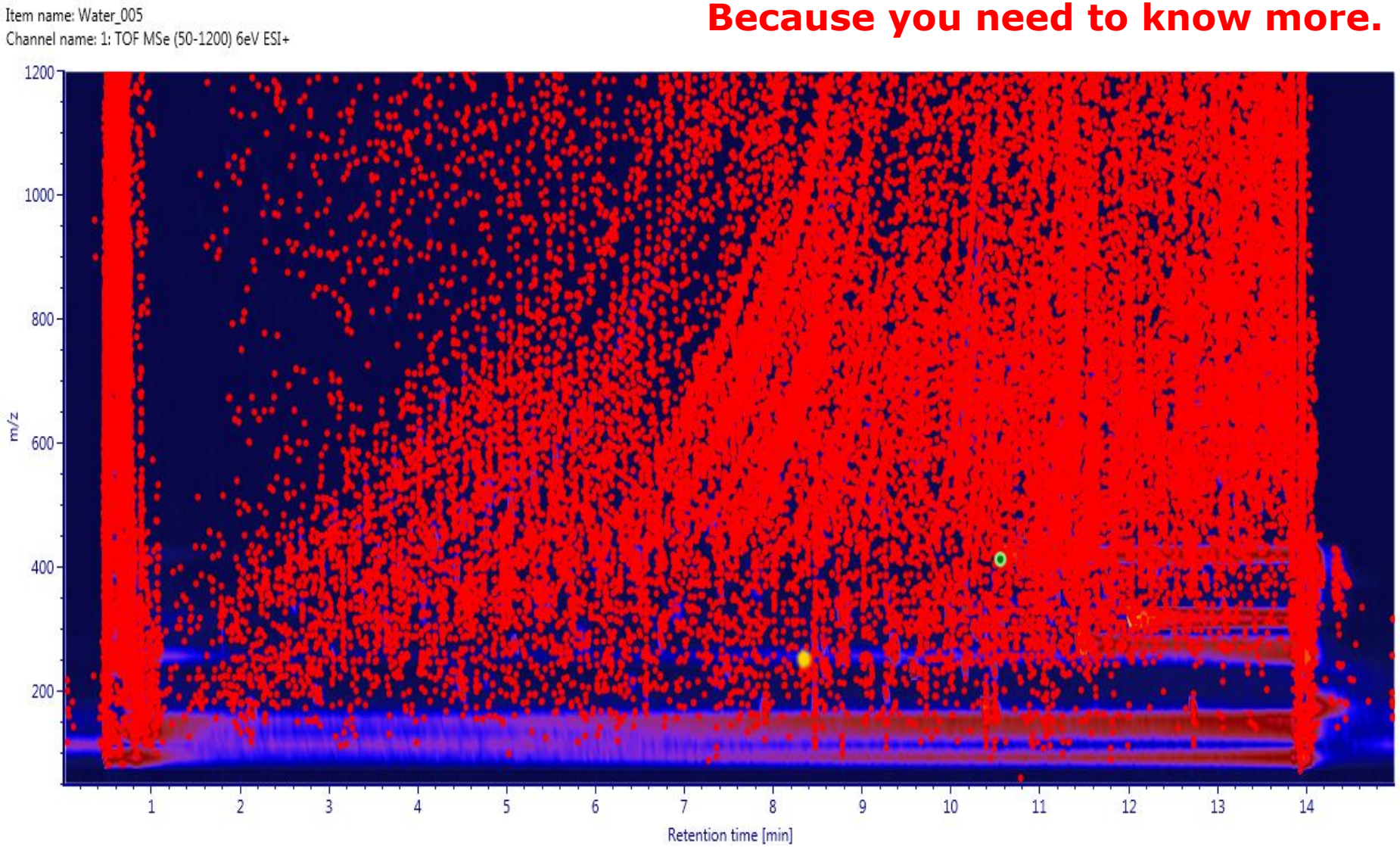
Because you need to know more.



Linearity for Microcystin LR

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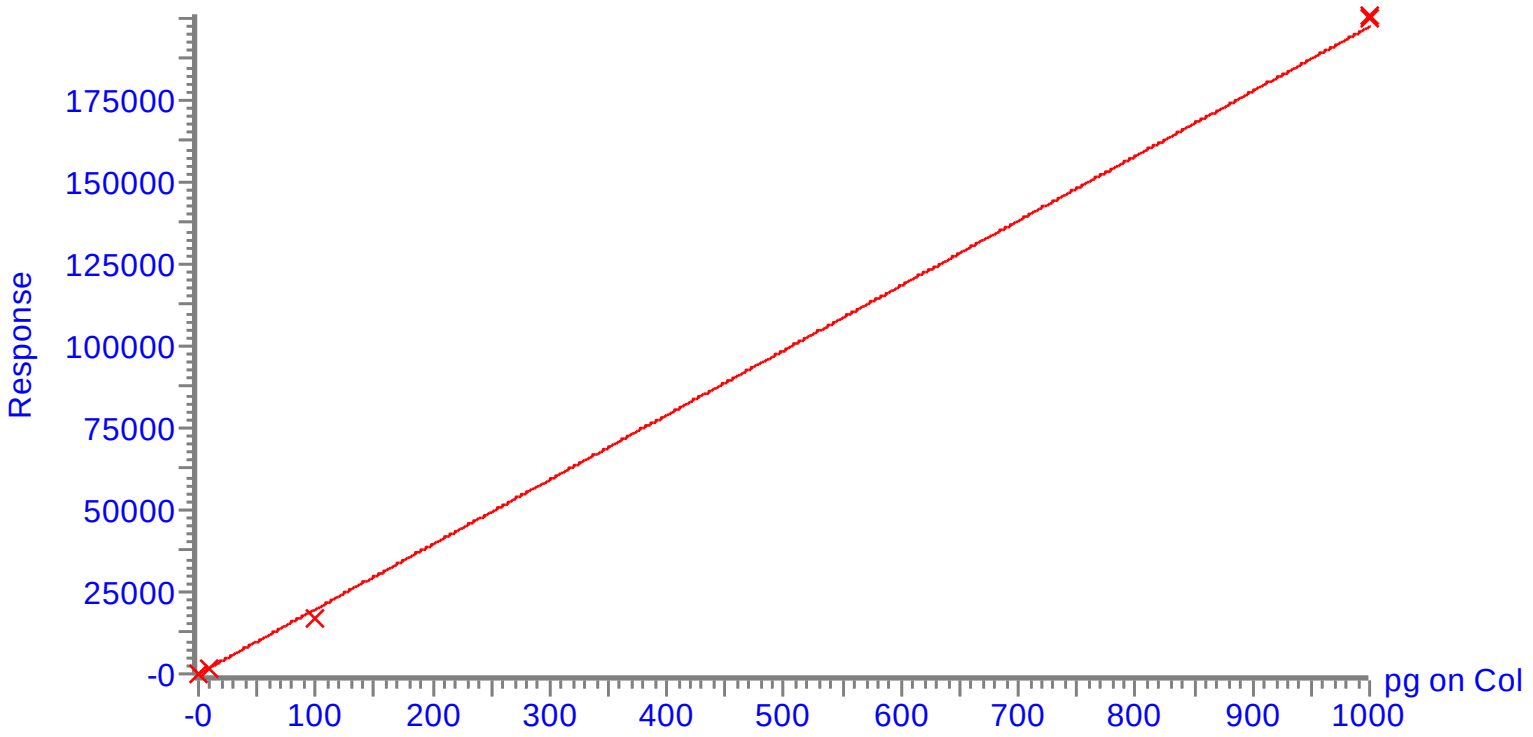
Compound name: Microcystin LR
Correlation coefficient: r = 0.999008, r^2 = 0.998017
Calibration curve: 197.411 * x + -5.88617
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

Calibration Curve for Microcystin LR 0.010ppb to 100ppb



Summary of 2D LC QTof Method

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- 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