Detection of Emerging Environmental Pollutants using LC-MS/MS

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Outline

- Using SCIEX LC-MS/MS for:
- EPA 537 (PFAA)
- Investigation of PFAA from packaging into food
- EPA 539 (hormones in drinking water)
- Marine and freshwater toxins

EPA 537 – Analysis of Perfluoroalkyl Acids (PFAA) Specified Under the UCMR3

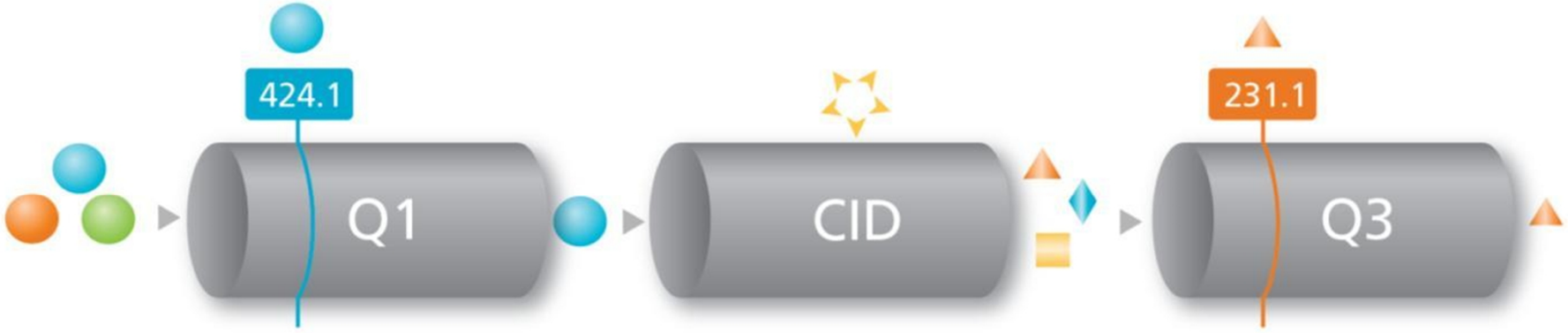
* The authors would likes to thank Lily Sanchez, Lee Yoo , and Mike Wehner of Orange County Water District (Fountain Valley, California,).

Experimental

- Solid Phase Extraction (SPE) following the EPA 537 method section 10, 11, and 12
  - All required quality control parameters (section 9.3) were met or exceeded
- Agilent 1260 with Eksigent ULC 100 HTC-xt autosamples
  - Atlantis T3 analytical column (150 x 2.1 mm, 5 µm)
  - Gradient with water + 20mM ammonium acetate and methanol
  - 5 µL injection (vs. 10 µL in EPA 537)
  - Atlantis T3 column (50 x 2.1mm, 5 µm) was also used as a delay column (installed between pump and autosampler)
- SCIEX QTRAP® 6500 system with IonDrive® source (400ºC)
  - Negative polarity Electrospray Ionization (ESI)
  - Multiple Reaction Monitoring (MRM)

Triple Quadrupole – Multiple Reaction Monitoring (MRM)

Highest Selectivity and Sensitivity for Screening and Quantitation



Filter Fragment Filter

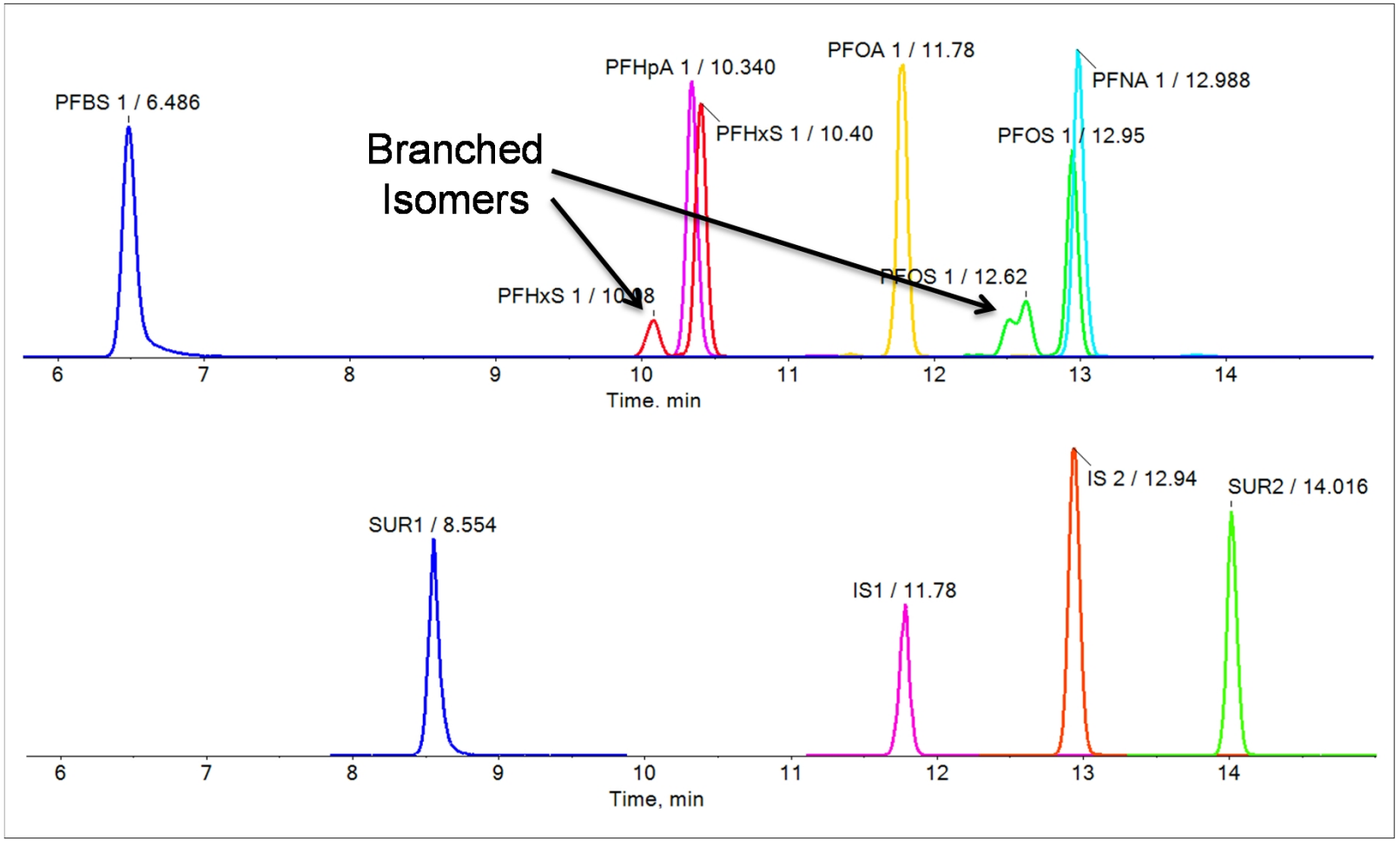
MRM Transitions for PFAAs, ISTDs and Surrogates

2 MRM Transitions Were Monitored for Each Target PFAA

Compound	Q1	Q3	RT	DP (V)	CE (V)
PFBS 1	298.8	79.8	6.8	-60	-68
PFBS 2	298.8	98.9	6.8	-60	-36
PFHpA 1	362.8	318.8	10.7	-5	-12
PFHpA 2	362.8	168.8	10.7	-5	-22
PFHxS 1	398.9	79.7	10.7	-70	-86
PFHxS 2	398.9	98.7	10.7	-70	-74
PFOA 1	412.8	368.9	12.1	-5	-14
PFOA 2	412.8	168.7	12.1	-5	-24
PFOS 1	498.9	79.8	13.2	-60	-122
PFOS 2	498.8	98.9	13.2	-60	-98
PFNA 1	462.9	418.9	13.3	-30	-14
PFNA 2	462.9	218.9	13.3	-30	-24
*$^{13}C_2$-PFOA**	414.9	369.8	12.1	-20	-14
*$^{13}C_4$-PFOS**	502.9	79.8	13.3	-10	-102
*$^{13}C_2$-PFHxA^*	314.8	269.8	8.9	-15	-12
*$^{13}C_2$-PFDA^*	514.9	469.9	14.3	-25	-16

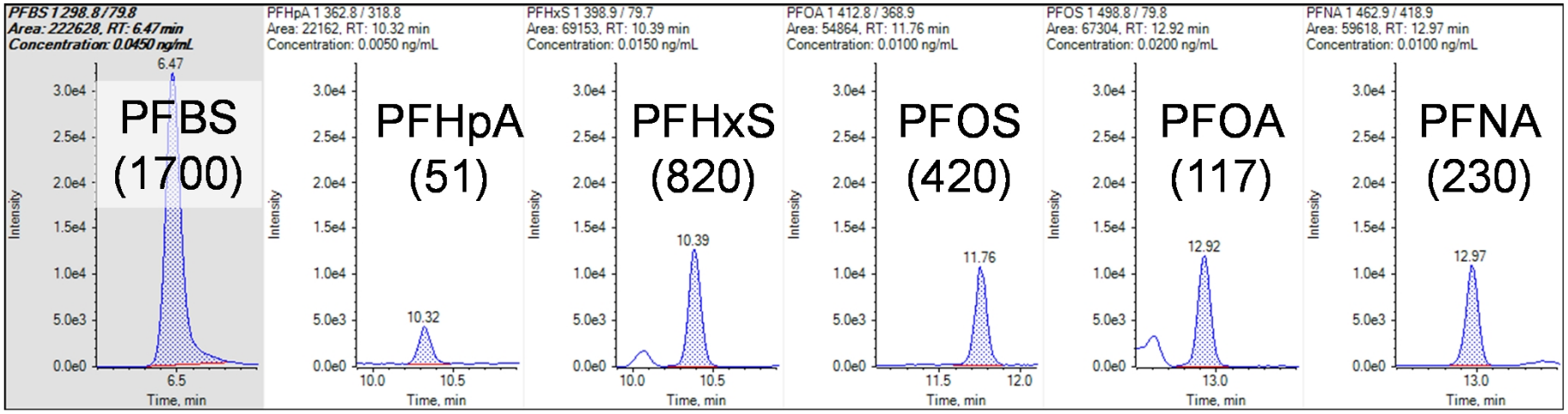
LC Separation of PFAA

Target PFAA (Top) and ISTDs and Surrogates (Bottom)



S/N of Target PFAA

Low Calibrator at ½ of UCMR Reporting Limits

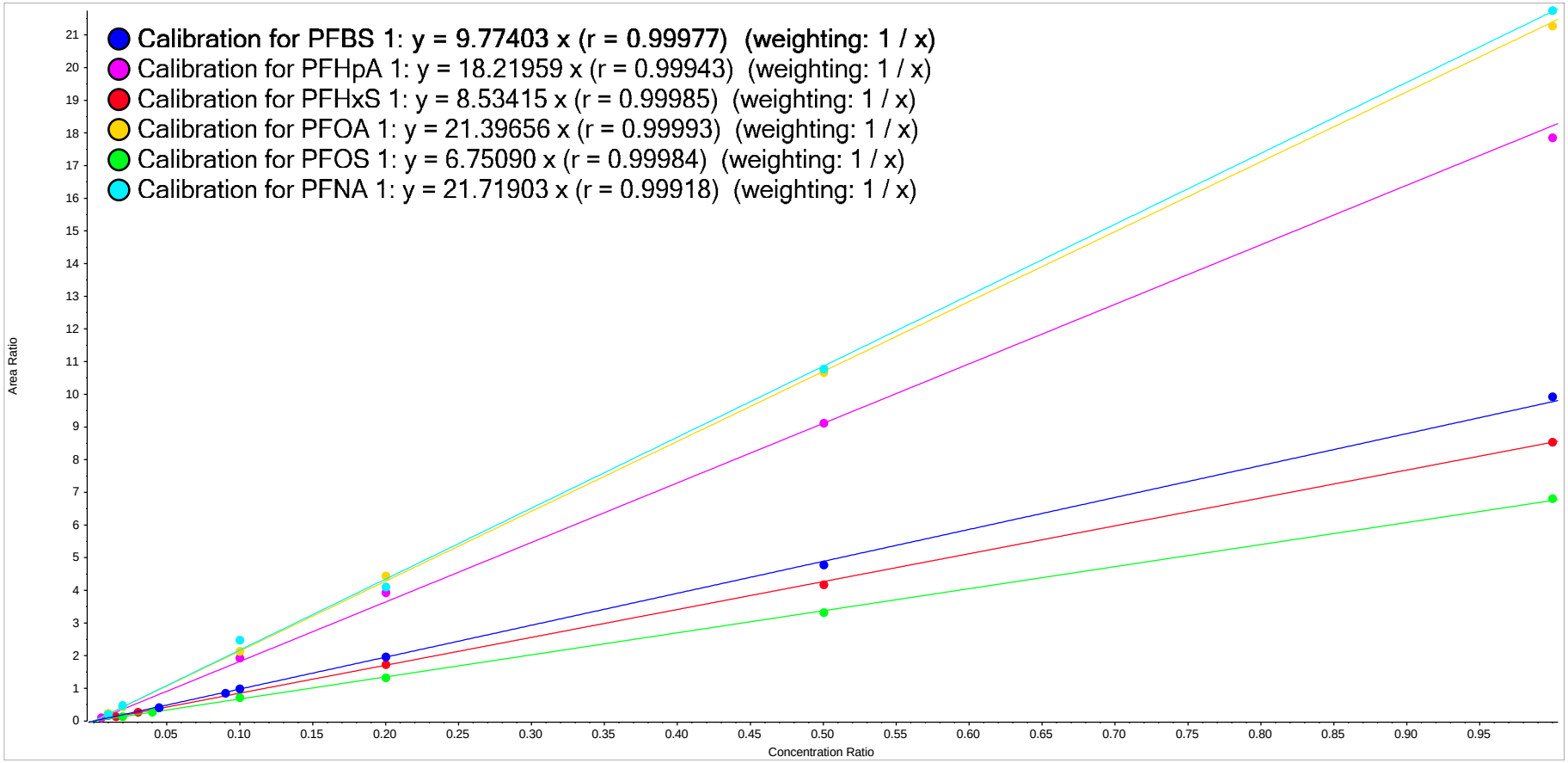


45 ng/mL | 5 ng/mL | 15 ng/mL | 20 ng/mL | 10 ng/mL | 10 ng/mL

Low level ng/L levels were easily obtained for all PFAA.

Calibration Lines for all Six PFAA

R > 0.999 for all Target PFAA



Accuracy QCS with < ± 30% and precision of 3.1 to 9.8%

MRL and MDL and Statistical Verification

PIR must be within 50 and 150% to be a validated MRL

Compound	Fortification Level (ng/L)	Lower PIR (%)	Upper PIR (%)	MDL (ng/L)
PFBS	90	81	99	8.3
PFHpA	10	75	114	1.4
PFHxS	30	86	99	1.6
PFOA	20	77	109	3.1
PFOS	40	56	144	35.9
PFNA	20	75	98	7.0

$$HR_{PIR} = 3.963s$$

$$\frac{Mean + HR_{PIR}}{Fortified\ Concentration} \times 100\%$$

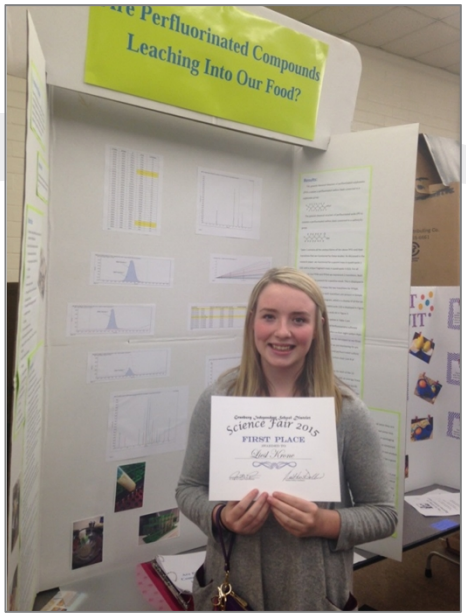
HR_{PIR} = Half Range for the prediction interval of results

s = the standard deviation of replicate analyses

3.963 = a constant value for seven replicates

Using the MRL extracts, the calculated MDLs ranged from 1.4 to 35.9 ng/L.

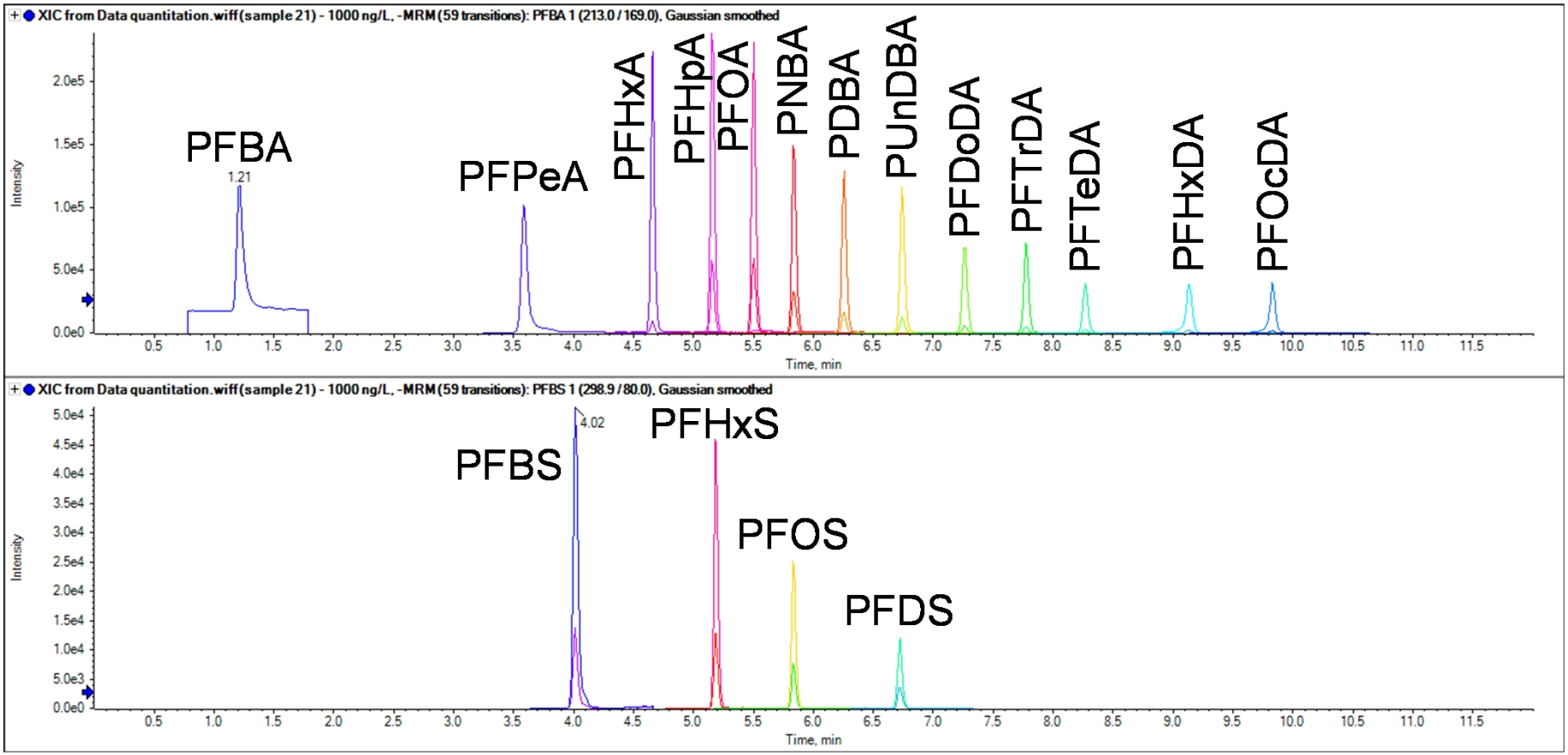
Liesl Krone (15): “Are perfluorinated compounds leaching into your food?”



* The authors would like to thank Nicole Riddell (Wellington Laboratories) for providing standards and Jack Cochran (Restek) for supplying LC columns.

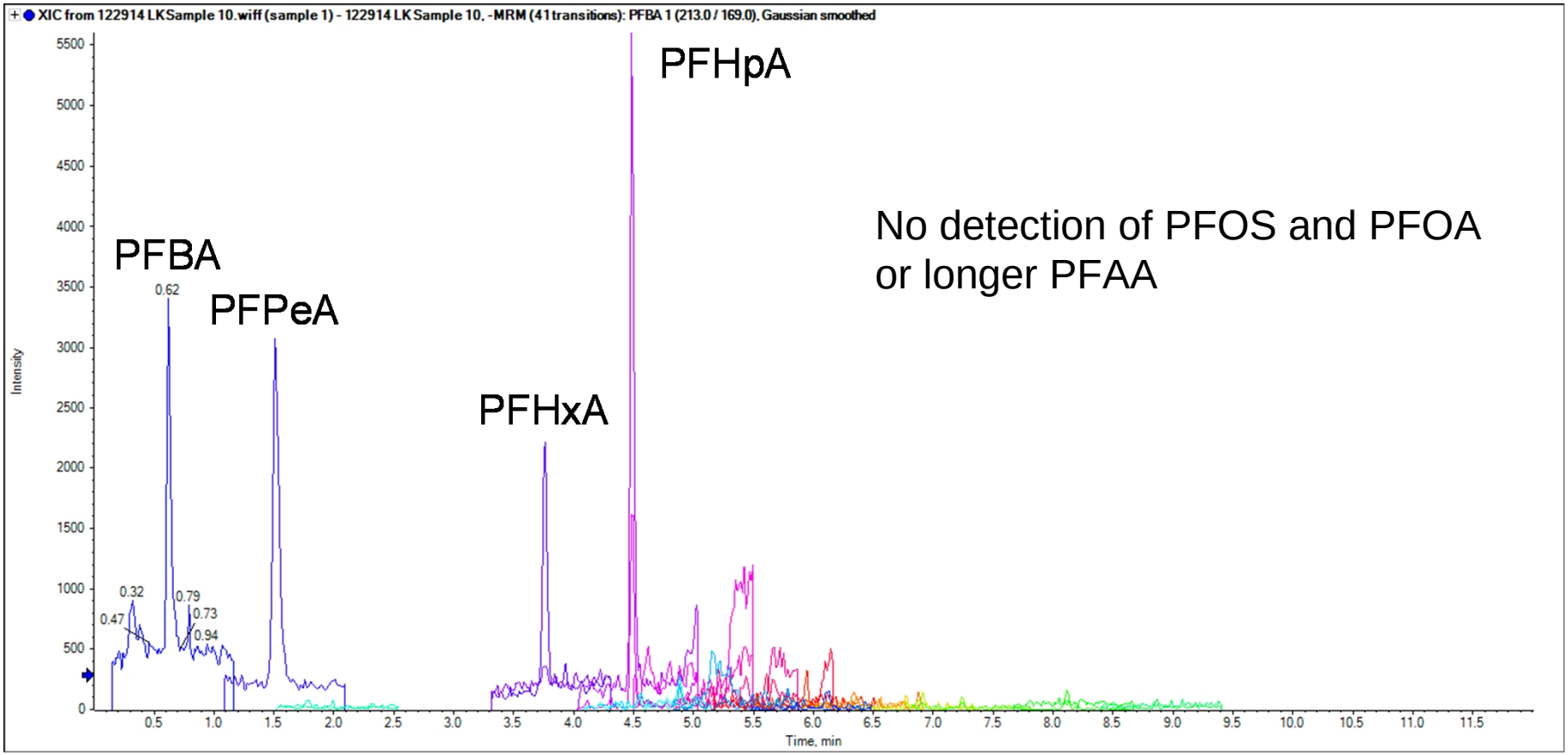
We also would like to thank Jeffrey McDonald from University of Texas Southwestern Medical Center Dallas, TX for assistance in sample analysis.

Extended Panel to Screen and Quantify PFAA



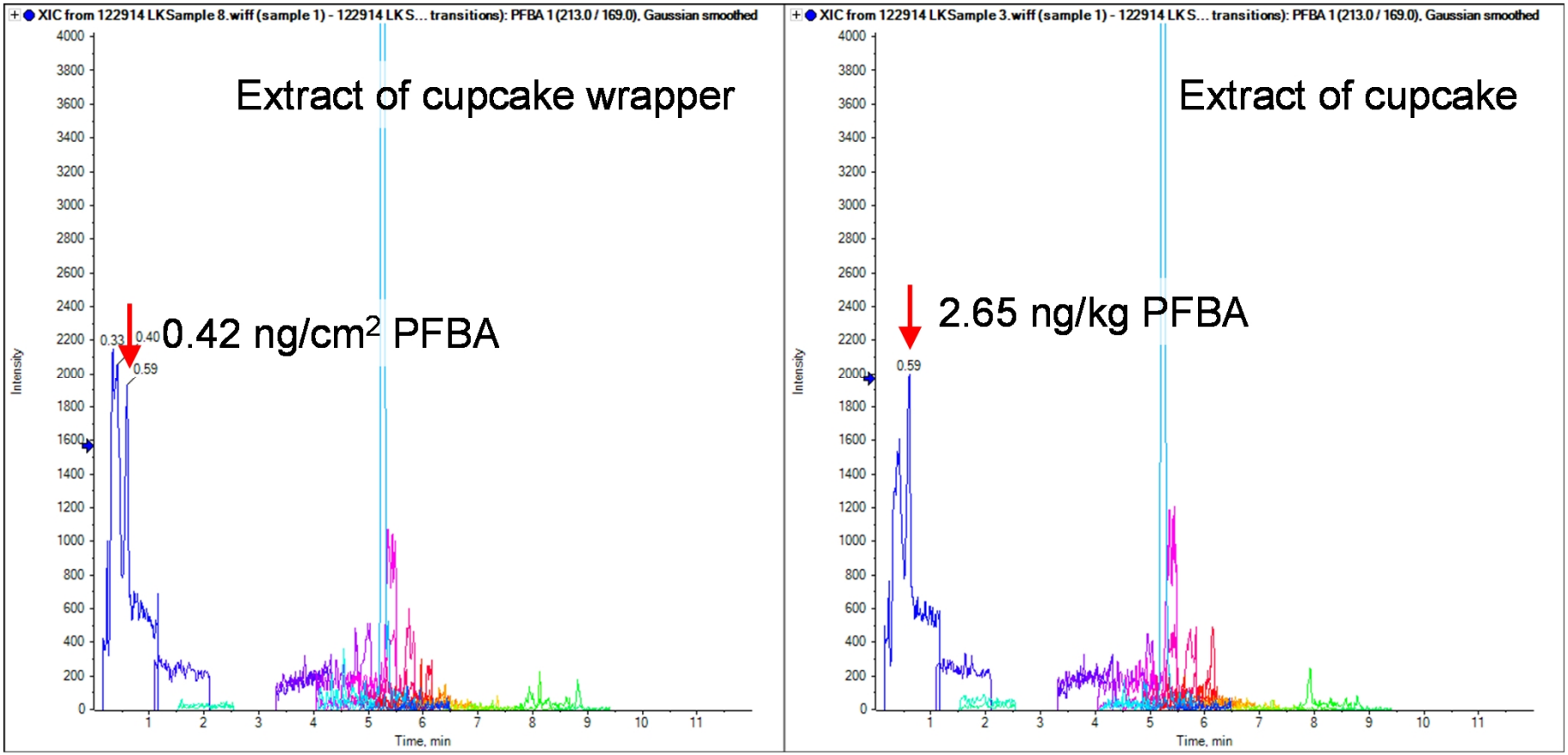
Restek Raptor C18 (50 x 2 mm, 2.7 µm) column and gradient of water/methanol with 5 mM ammonium formate

Detection of Short-Chain PFAA in Corn Chip Bag



Concentrations: 0.30 to 0.79 ng/cm^2

Migration of PFBA from Cupcake Wrapper into Cupcake



EPA 539 – Analysis of Hormones in Drinking Water

* The authors would like to thank Lily Sanchez and Lee Yoo of Orange County Water District (Fountain Valley, California,).

Experimental

- Solid Phase Extraction (SPE) following the EPA 539 method section 10, 11, and 12
  - All required quality control parameters (section 9.3) were met or exceeded
- Agilent 1260 with Eksigent ULC 100 HTC-xt autosamples
  - Phenomenex Kinetex C18 column (100 x 2.1 mm, 5 µm)
  - Gradient with 0.02% NH4OH and 0.02% NH4OH in methanol
  - 10 µL injection (vs. 50 µL in EPA 539)
- SCIEX QTRAP® 6500 system with IonDrive® source (600ºC)
  - Electrospray Ionization (ESI)
  - 3 separate experiments to facilitate polarity switching (25 msec)
  - Multiple Reaction Monitoring (MRM)

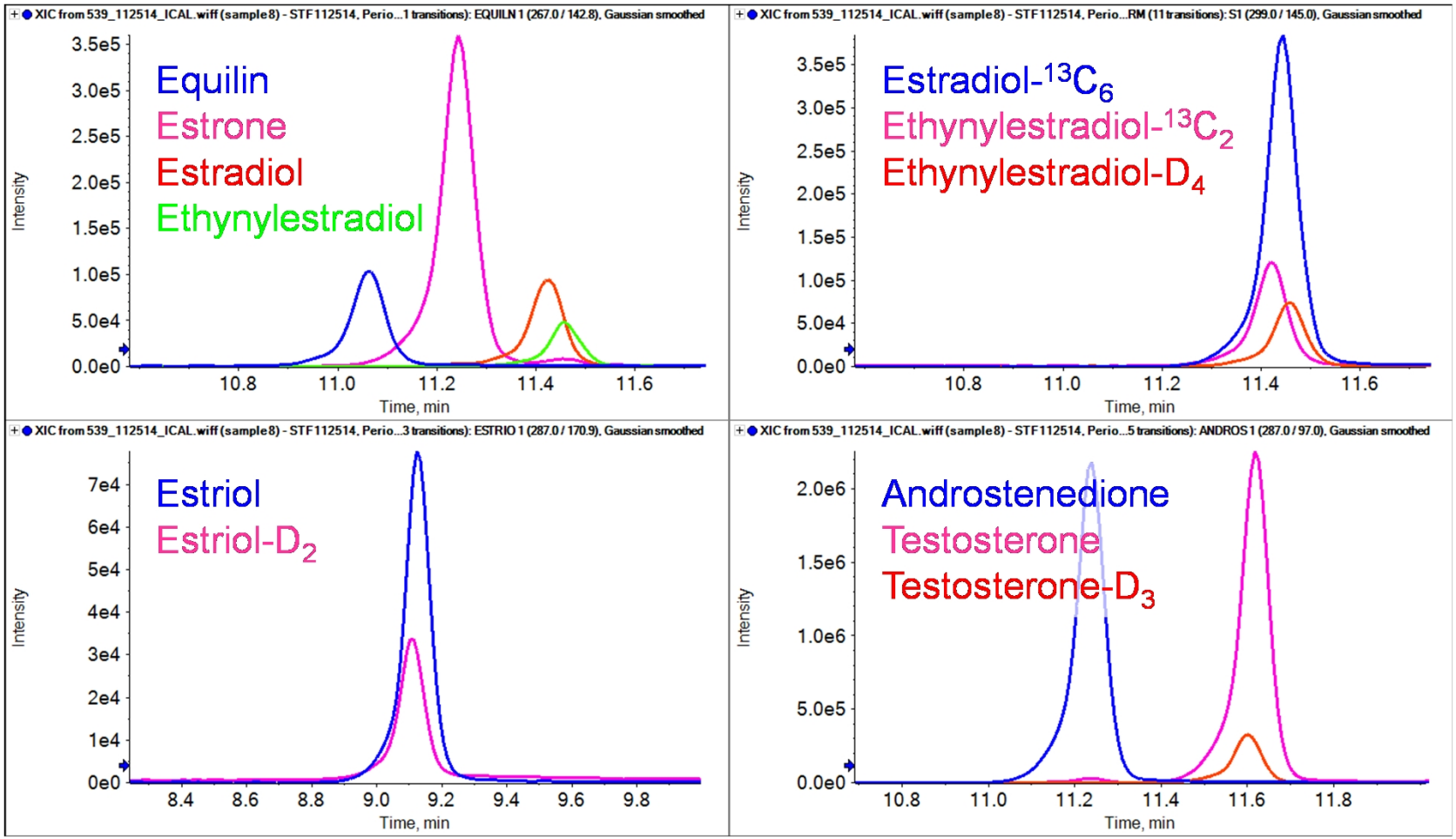
MRM Transitions for Hormones, ISTDs and Surrogate

2 MRM Transitions Were Monitored for Each Target Compound

Compound	Experiment (Polarity)	Q1	Q3	Dwell (ms)	DP (V)	CE (V)
Estriol 1	1 (-)	287.0	170.9	200	-115	-46
Estriol 2	1 (-)	287.0	144.9	200	-115	-50
Equilin 1	2 (-)	267.0	142.8	20	-100	-42
Equilin 2	2 (-)	267.0	223.0	20	-100	-44
Estrone 1	2 (-)	269.0	145.0	20	-130	-48
Estrone 2	2 (-)	269.0	143.0	20	-130	-64
Estradiol 1	2 (-)	271.0	145.1	20	-140	-50
Estradiol 2	2 (-)	271.0	143.0	20	-140	-66
Ethynylestradiol 1	2 (-)	295.0	143.0	20	-130	-70
Ethynnylestradiol 2	2 (-)	295.0	159.0	20	-130	-44
Androstenedione 1	3 (+)	287.0	97.0	35	116	27
Androstenedione 2	3 (+)	287.0	109.0	35	116	29
Testosterone 1	3 (+)	289.0	97.0	40	96	27
Testosterone 2	3 (+)	289.0	108.9	40	96	31
*Estriol-D_2**	1 (-)	289.0	146.9	200	-165	-54
*Estradiol-$^{13}C_6$**	1 (-)	277.0	144.9	20	-145	-50
*Ethynylestradiol-$^{13}C_2$**	1 (-)	297.0	145.0	20	-155	-54
*Testosterone-D_3**	3 (+)	292.0	97.1	35	106	27
*Ethynylestradiol-D_4^*	2 (-)	299.0	145.0	20	-145	-72

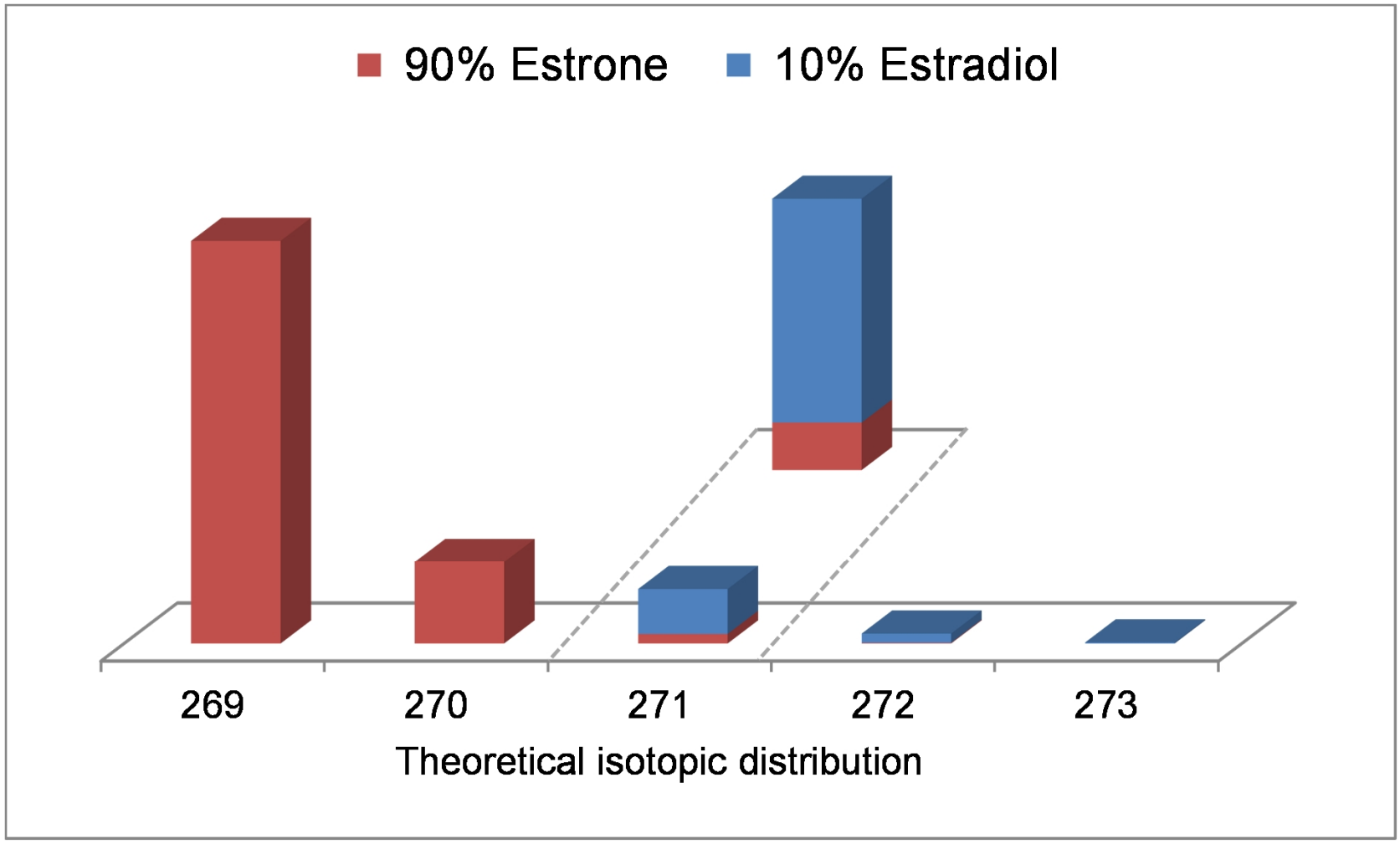
LC Separation of Hormones

Target Hormones, ISTDs and Surrogate (EE2-D4)



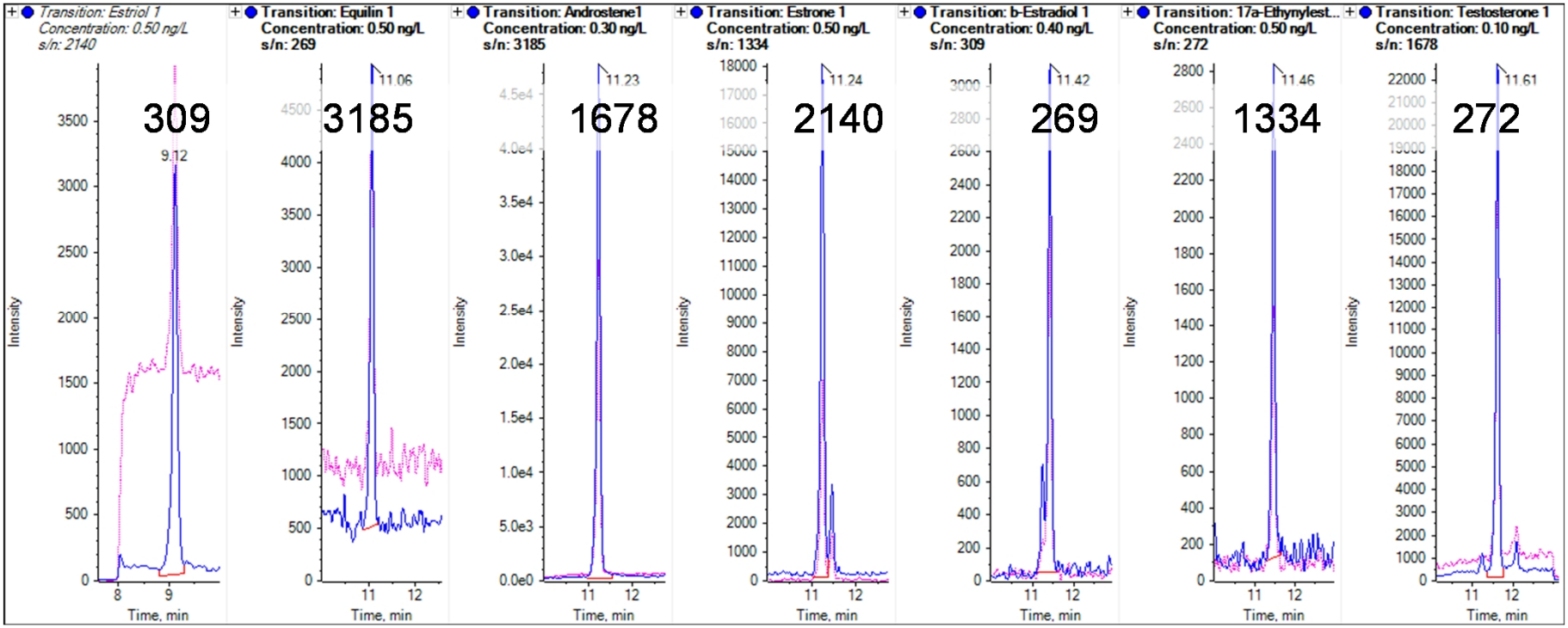
Three Critical Pairs of Isotopes Require LC Separation

Estrone/Estradiol, Equilin/Estrone, and Testosterone/Androstenedione



S/N of Target Hormones

Low Level Calibrator Corresponding at UCMR3 Reporting Limits

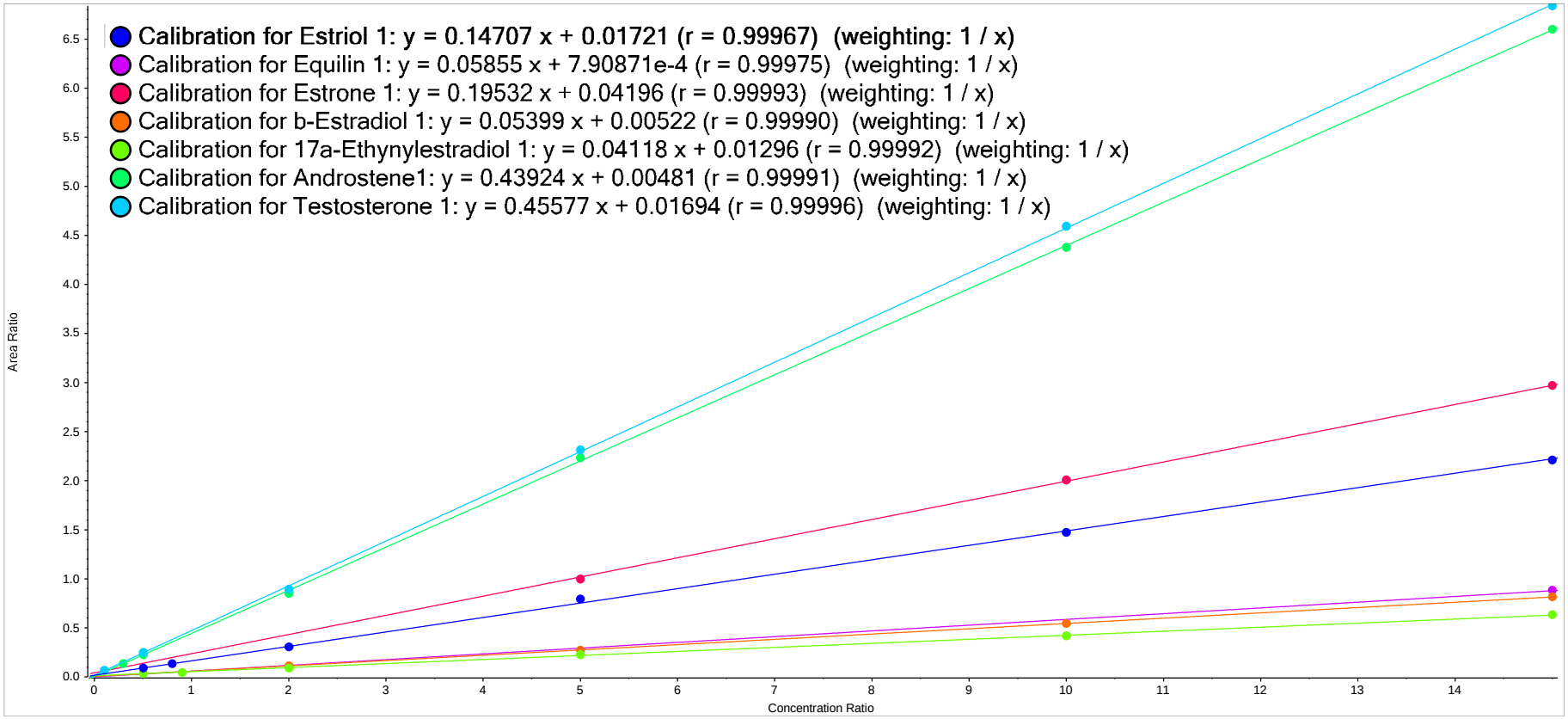


E3	Equilin	Androstenedione	E1	E2	EE2	Testosterone
@0.5	@0.5	@0.3	@0.5	@0.4	@0.5	@0.1
0.8	4	0.3	2	0.4	0.9	0.1

UCMR3 Reporting Limits (ng/L)

Calibration Lines for all Hormones

R > 0.999 for all Target Hormones



Accuracy QCS with < ± 30% and precision of 3.1 to 9.8%

MRL and MDL and Statistical Verification

PIR must be within 50 and 150% to be a validated MRL

Compound	Fortification Level (ng/L)	Lower PIR (%)	Upper PIR (%)	MDL (ng/L)
Estriol	0.8	77%	98%	0.068
Equilin	4.0	61%	93%	0.50
Androstenedione	0.3	82%	101%	0.023
Estrone	2.0	79%	100%	0.17
Estradiol	0.4	81%	126%	0.071
Testosterone	0.1	68%	104%	0.014
Ethynylestradiol	0.9	62%	112%	0.18

$$HR_{PIR} = 3.963s$$

$$\frac{Mean + HR_{PIR}}{Fortified\ Concentration} \times 100\%$$

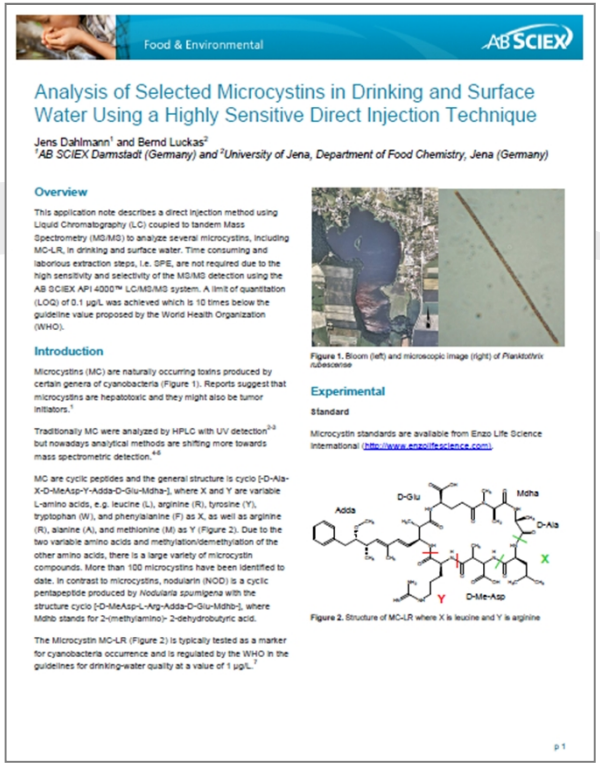
HR_{PIR} = Half Range for the prediction interval of results

s = the standard deviation of replicate analyses

3.963 = a constant value for seven replicates

Using the MRL extracts, the calculated MDLs ranged from 0.014 to 0.50 ng/L.

EPA 539 – Freshwater and Shellfish Toxins



Food & Environmental

AB SCIEX

Analysis of Selected Microcystins in Drinking and Surface Water Using a Highly Sensitive Direct Injection Technique

Jens Dahlmann[1] and Bernd Luckas[2]
[1]AB SCIEX Darmstadt (Germany) and [2]University of Jena, Department of Food Chemistry, Jena (Germany)

Overview

This application note describes a direct injection method using Liquid Chromatography (LC) coupled to tandem Mass Spectrometry (MS/MS) to analyze several microcystins, including MC-LR, in drinking and surface water. Time consuming and laborious extraction steps, i.e. SPE, are not required due to the high sensitivity and selectivity of the MS/MS detection using the AB SCIEX API 4000™ LC/MS/MS system. A limit of quantitation (LOQ) of 0.1 µg/L was achieved which is 10 times below the guideline value proposed by the World Health Organization (WHO).

Introduction

Microcystins (MC) are naturally occurring toxins produced by certain genera of cyanobacteria (Figure 1). Reports suggest that microcystins are hepatotoxic and they might also be tumor initiators.[1]

Traditionally MC were analyzed by HPLC with UV detection[2,3] but nowadays analytical methods are shifting more towards mass spectrometric detection.[4-6]

MC are cyclic peptides and the general structure is cyclo [-D-Ala-X-D-MeAsp-Y-Adda-D-Glu-Mdha-], where X and Y are variable L-amino acids, e.g. leucine (L), arginine (R), tyrosine (Y), tryptophan (W), and phenylalanine (F) as X, as well as arginine (R), alanine (A), and methionine (M) as Y (Figure 2). Due to the two variable amino acids and methylation/demethylation of the other amino acids, there is a large variety of microcystin compounds. More than 100 microcystins have been identified to date. In contrast to microcystins, nodularin (NOD) is a cyclic pentapeptide produced by *Nodularia spumigena* with the structure cyclo [-D-MeAsp-L-Arg-Adda-D-Glu-Mdhb-], where Mdhb stands for 2-(methylamino)- 2-dehydrobutyric acid.

The Microcystin MC-LR (Figure 2) is typically tested as a marker for cyanobacteria occurrence and is regulated by the WHO in the guidelines for drinking-water quality at a value of 1 µg/L.[7]

Figure 1. Bloom (left) and microscopic image (right) of *Planktothrix rubescense*

Experimental

Standard

Microcystin standards are available from Enzo Life Science International (http://www.enzolifescience.com).

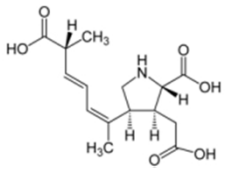
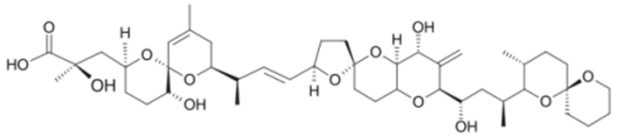
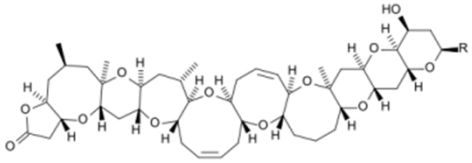
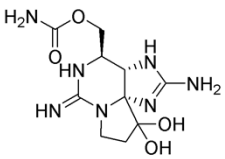
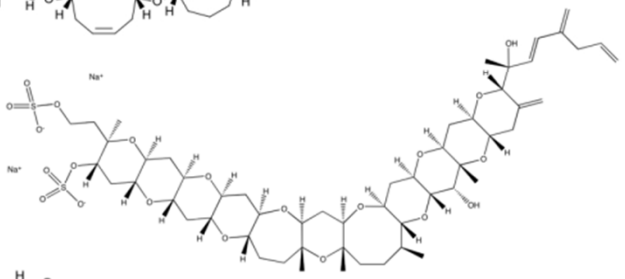
Figure 2. Structure of MC-LR where X is leucine and Y is arginine

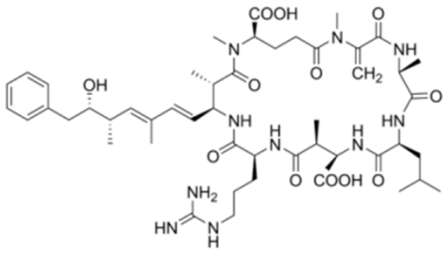
p 1

* The authors would like to thank Pearse McCarron and Michael Quilliam of National Research Council of Canada, Halifax, NS (Canada); Philipp Hess of Ifremer, Nantes (France); Bernd Krock of Alfred Wegener Institute, Bremerhafen (Germany)

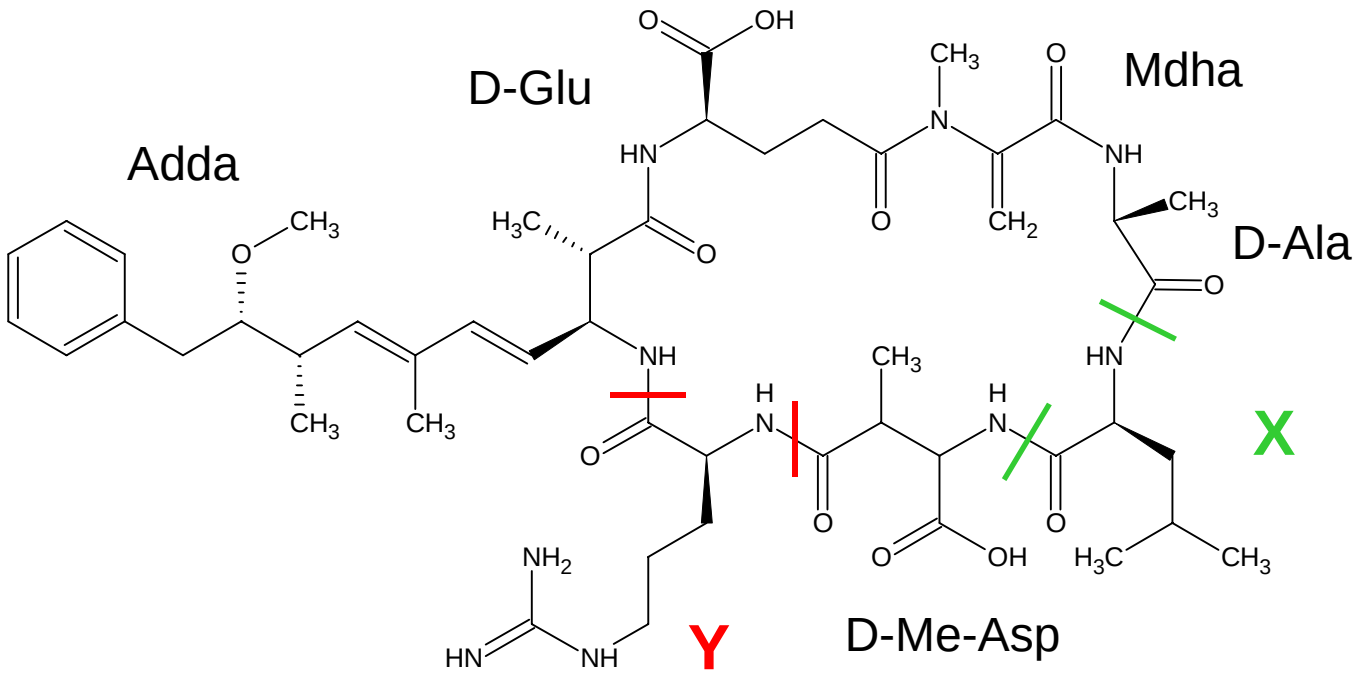
Toxins Overview

- Amnesic shellfish poisoning (ASP)
  - Amino acids: Domoic acid
- Diarrhetic shellfish poisoning (DSP)
  - Polyethers: Okadaic acid and derivatives
- Neurotoxic shellfish poisoning (NSP)
  - Cyclic polyether: Brevetoxin, Yessotoxin
- Paralytic shellfish poisoning (PSP)
  - Alkaloids: Anatoxin, Saxitoxin
- Cyanotoxins
  - Cyclic peptides: Microcystins (MC), Nodularin
- ***Analytical challenges***
  - Both hydrophilic and lipophilic compounds
  - ionic and non-ionic substances
  - Some compounds are not detectable without derivatization (UV, FLD),
  - Different matrices (algae, cyanobacteria, mussel, fish, drinking water etc.)



Structure of Microcystin-LR (MC-LR) Regulated by WHO



Why Use LC/MS/MS for marine biotoxin analysis?

- Chromatography with spectrometric detection
  - LC with UV or FLD, GC-MS, HPTLC
  - Non- specific, low sensitivity
  - Rigorous sample preparation and derivatization often required
  - Difficulty to analyze multiple analytes in a single run
- Immunoassay (i.e. ELISA) and Bioassays
  - Not specific, problems with cross contamination, false positive
  - Difficulty to distinguish between multiple analytes
  - Mouse or rat bioassay – ethical issues
- LC-MS/MS
  - Highly selective, sensitive and accurate quantitation
  - Reduced sample preparation
  - No issue with false positives
  - Provides both qualitative and quantitative analysis in a single run
  - Meet all requirements for modern residue analysis

Regulation

- Old official method for detection of biotoxins
  - Mouse bioassay, rat bioassay
- Commission regulation EU No. 5/2011
  - “these bioassays have shortcomings and are not considered an appropriate tool for control purposes because of the high variability in results, the insufficient detection capability and the limited specificity”
  - “A liquid chromatography-mass spectrometry (LC-MS/MS) method was validated under the coordination of the European Union Reference Laboratory on marine biotoxins (EU-RL)”
- EU-harmonized SOP using LC-MS/MS for lipophilic marine biotoxins:
  - Okadaic acid (OA), Pectenotoxin (PTX), Azaspiracid (AZA), Yessotoxin (YTX) group toxins

11.1.2011 EN Official Journal of the European Union L 6/3

COMMISSION REGULATION (EU) No 15/2011

of 10 January 2011

amending Regulation (EC) No 2074/2005 as regards recognised testing methods for detecting marine biotoxins in live bivalve molluscs

(Text with EEA relevance)

THE EUROPEAN COMMISSION,

Having regard to the Treaty on the Functioning of the European Union,

Having regard to Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin (1), and in particular Article 11(4) thereof,

Having regard to Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption (2), and in particular Article 18(1)(a) thereof,

Whereas:

(1) Regulation (EC) No 854/2004 lays down specific rules for the organisation of official controls on products of animal origin and Regulation (EC) No 853/2004 lays down specific requirements concerning hygiene rules for food of animal origin. Implementing measures for those Regulations as regards recognised testing methods for marine biotoxins are set out in Commission Regulation (EC) No 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004

Chair of EFSA noted that these bioassays have shortcomings and are not considered an appropriate tool for control purposes because of the high variability in results, the insufficient detection capability and the limited specificity.

(4) Recently developed alternatives to the biological methods for the determination of the marine biotoxins with lower limits of detection (LOD) have successfully been tested in prevalidation studies.

(5) A liquid chromatography-mass spectrometry (LC-MS/MS) method was validated under the coordination of the European Union Reference Laboratory on marine biotoxins (EU-RL) in an inter-laboratory validation study carried out by the Member States. This method is publicly available for consultation in the web page of the EU-RL (http://www.aesan.msps.es/en/CRLMB/web/home.shtml). This validated technique of liquid chromatography (LC) mass spectrometry (MS) should be applied as the reference method for the detection of lipophilic toxins and used as matter of routine, both for the purposes of official controls at any stage of the food chain and own-checks by food business operators.

(6) Any other recognised method, different from the liquid chromatography (LC) mass spectrometry (MS), could be applied for the detection of lipophilic toxins provided

MINISTERIO DE SANIDAD Y POLITICA SOCIAL

agencia española de seguridad alimentaria y nutrición

EU-RL-MB

EUROPEAN UNION REFERENCE LABORATORY FOR MARINE BIOTOXINS

EU-Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS

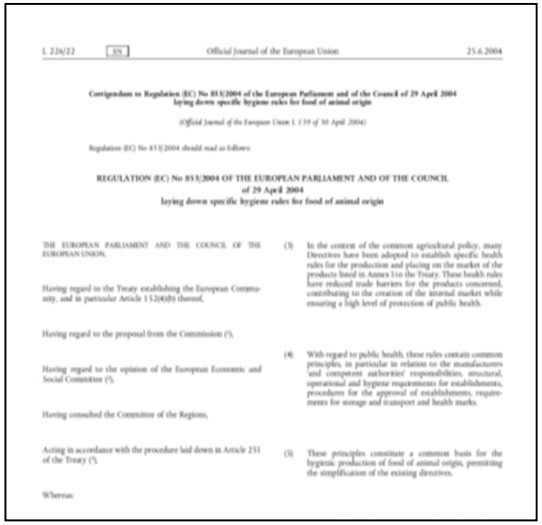
Version 2, July 2010

Coordination:

European Union Reference Laboratory for Marine Biotoxins (EU-RL-MB)
Agencia Española de Seguridad Alimentaria y Nutrición (AESAN)
Estación Marítima S/N
36200 Vigo, Spain
E-mail: eurlmb@msps.es
http://www.aesan.msps.es/en/CRLMB/web/home.shtml

Regulated Residue Levels

- Regulation (EC) No. 853/2004
- Food business operators must ensure that live bivalve molluscs placed on the market for human consumption… must not contain marine biotoxins in total quantities.. that exceed the following limits:
  - 800 µg/kg of PSP toxins
  - 20 mg/kg of domoic acid for ASP
  - 160 µg/kg of okadaic acid equivalents
  - 1 mg/kg of yessotoxin equivalents
  - 160 µg/kg of azaspiracid equivalents
- ***Cyanotoxins***
  - WHO guidelines for drinking water quality 1 µg/L
  - Many countries, especially with geographies in warmer climate zones detect at 0.1 µg/L or lower



L 226/22 EN Official Journal of the European Union 25.6.2004

Corrigendum to Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin

(Official Journal of the European Union L 139 of 30 April 2004)

Regulation (EC) No 853/2004 should read as follows:

REGULATION (EC) No 853/2004 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 29 April 2004 laying down specific hygiene rules for food of animal origin

THE EUROPEAN PARLIAMENT AND THE COUNCIL OF THE EUROPEAN UNION,

Having regard to the Treaty establishing the European Community, and in particular Article 152(4)(b) thereof,

Having regard to the proposal from the Commission (1),

Having regard to the opinion of the European Economic and Social Committee (2),

Having consulted the Committee of the Regions,

Acting in accordance with the procedure laid down in Article 251 of the Treaty (3),

Whereas:

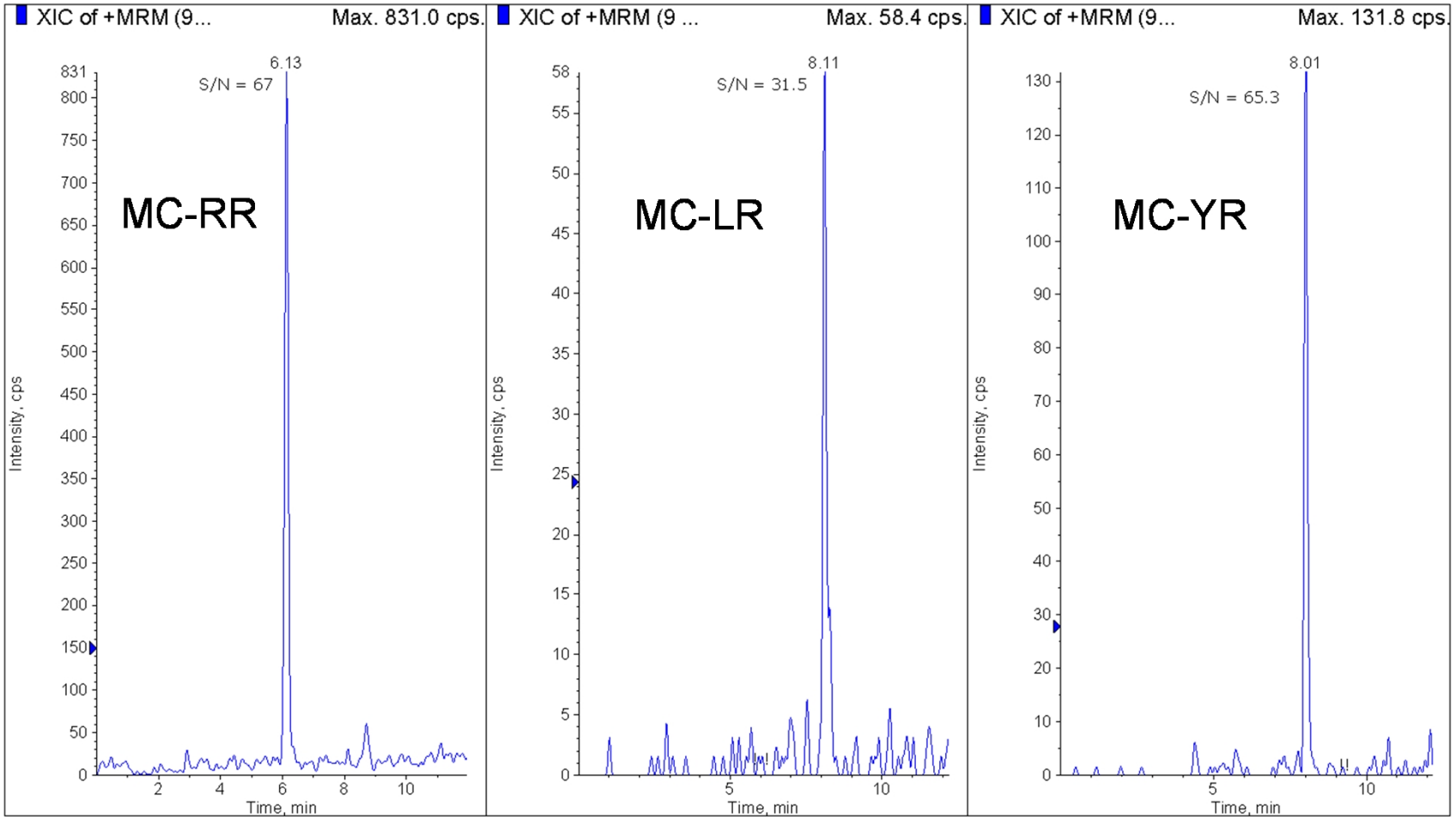
(1) In the context of the common agricultural policy, many Directives have been adopted to establish specific health rules for the production and placing on the market of the products listed in Annex I to the Treaty. These health rules have reduced trade barriers for the products concerned, contributing to the creation of the internal market while ensuring a high level of protection of public health.

(4) With regard to public health, these rules contain common principles, in particular in relation to the manufacturers and competent authorities' responsibilities, structural, operational and hygiene requirements for establishments, procedures for the approval of establishments, requirements for storage and transport and health marks.

(5) These principles constitute a common basis for the hygienic production of food of animal origin, permitting the simplification of the existing directives.

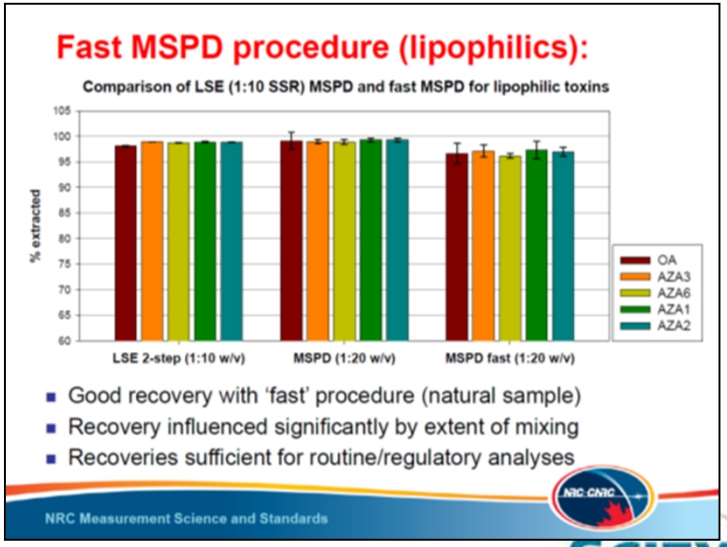
Detection of 0.1 µg/L of Microcystins in Drinking Water

25 µL injected into an API 4000™ System (over 10 years ago)



Standards and Sample Preparation

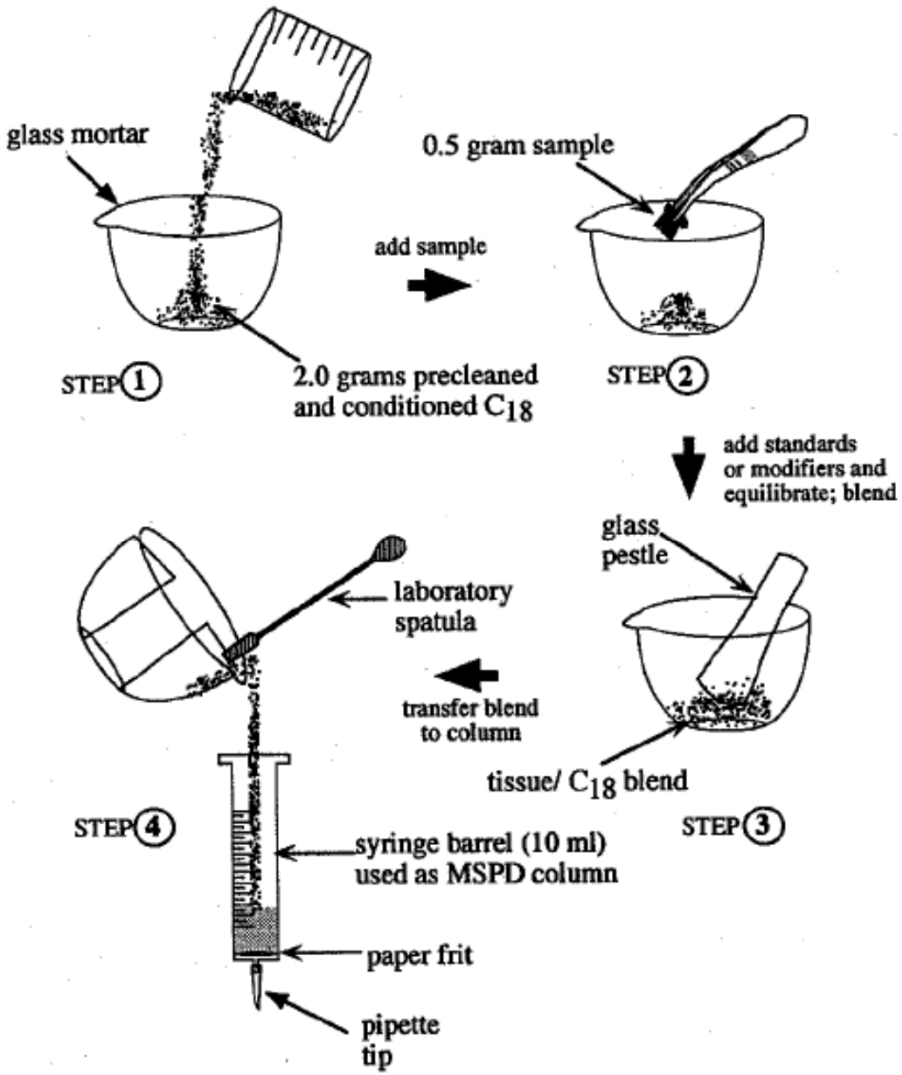
- Standards and CRM from National Research Council of Canada, NRC (Halifax, Canada)
- EU-harmonized SOP
  - Methanol extraction from homogenized tissue
  - Alkaline hydrolysis to determine total content of OA group toxins
- New developments by M. Quilliam and P. McCarron at NRC
  - Matrix Solid Phase Dispersion (MSPD)
  - Fast MSPD procedure: mixing, elution
  - and in-line filtration of extracts
  - Presented at AOAC Annual Meeting (2011) and AOAC Annual Meeting of Pacific NW Section (2012) on 'Marine and Freshwater Toxins Analysis'



Basic MSPD Procedure

- Mix sample and extraction material (bonded silica)
  - Typical ratio 0.5 g sample : 2.0 g dispersant
- Prepare column
- Extraction by eluting column with appropriate solvent(s)
  - Analyte dependent

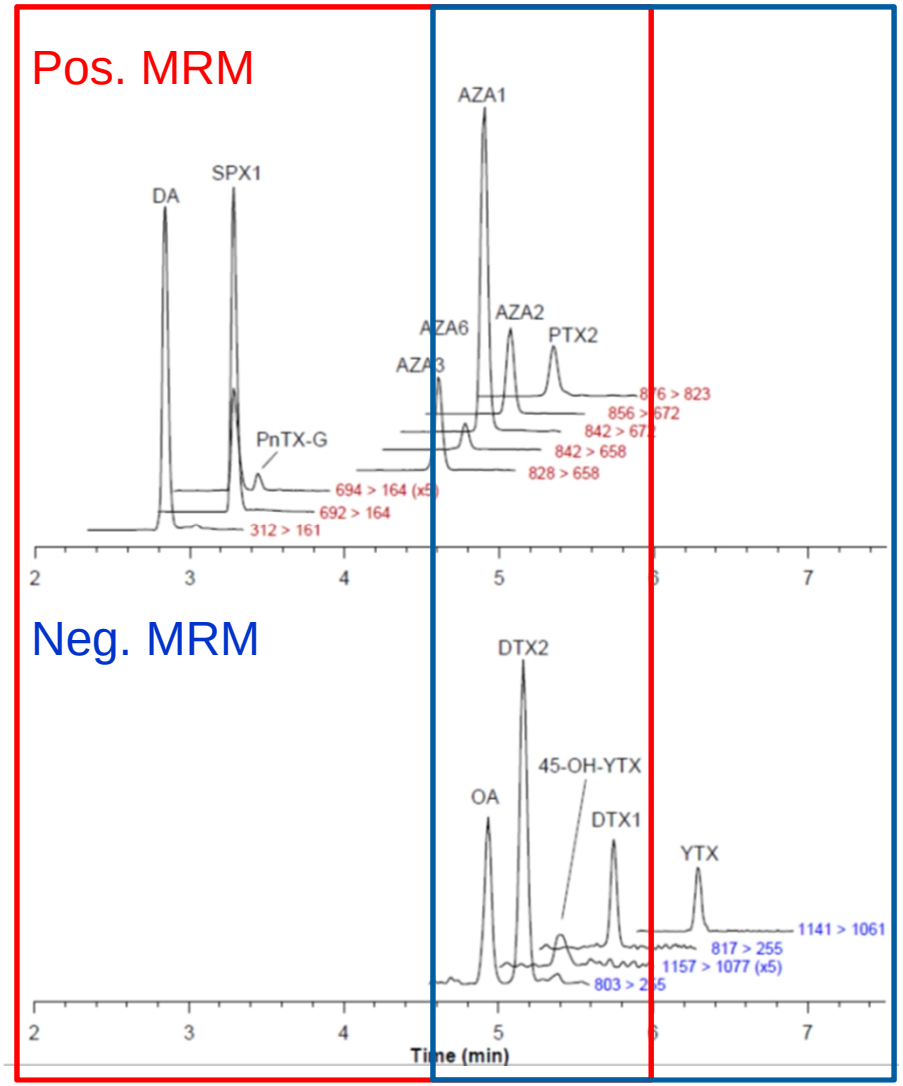
S. A. Barker, J. Chromatogr. A 885 (2000) 115-127



Liquid Chromatography

- EU-harmonized SOP
  - Reversed phase (RP column)
  - Acidic conditions (water, acetonitrile, formic acid)
  - Basic conditions (water, acetonitrile, pH=11 with ammonia or bicarbonate
- New developments by M. Quilliam and P. McCarr
  - Neutral conditions (water, acetonitrile, ammonium acetate)
- HILIC conditions for PSP toxins
- Faster analysis using UHPLC conditions
  - Small particle size columns, 2.5 or 3 µm
  - Core-shell particle columns, i.e. Kinetex, UHPLC speed and separation efficiency at reduced pressure

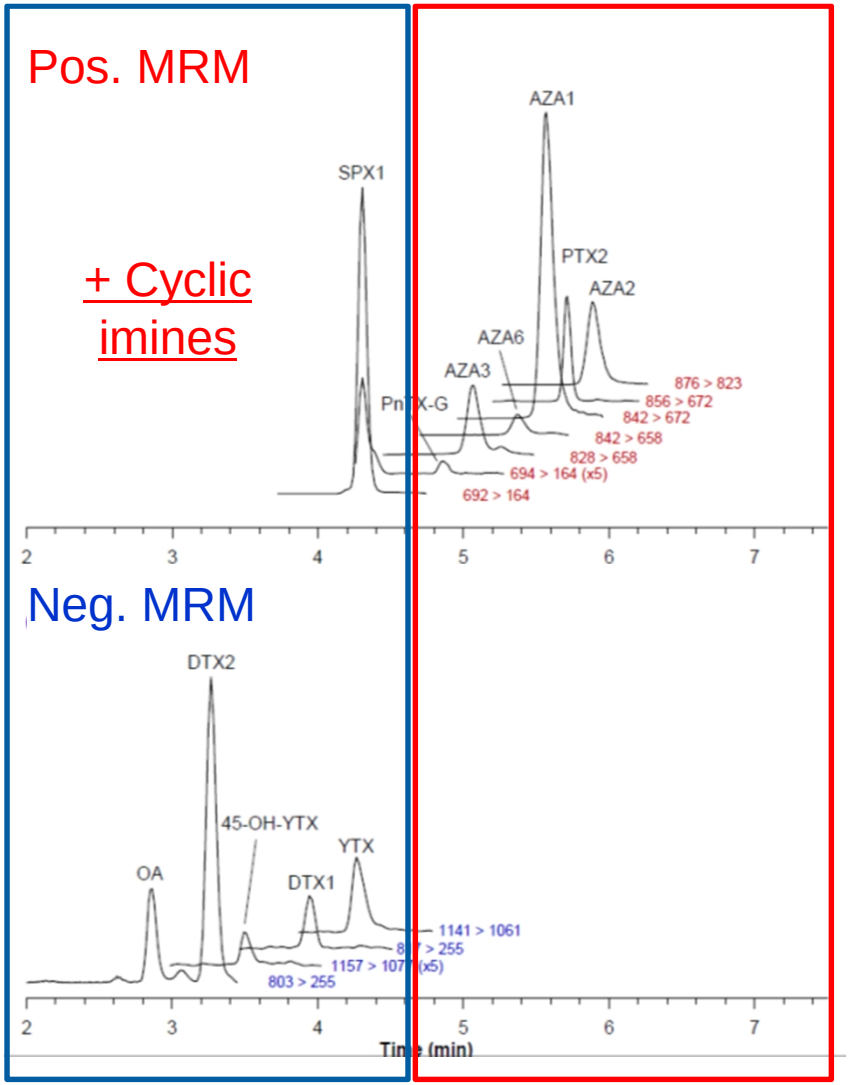
Acidic LC Conditions



CRM-FDMT1

- Phenomenex LUNA C18(2) 2.5 µm (50 x 2 mm) with gradient of water / acetonitrile + 50 mM formic acid and 2 mM ammonium formate
- Good peak shape and resolution
- Suited to MS/MS instruments capable of rapid polarity switching

Neutral LC Conditions



CRM-FDMT1

- Phenomenex Gemini-NX C18 3 µm (50 x 2 mm) with gradient of water / acetonitrile + 5 mM ammonium aceate
- Good peak shape and resolution
- Separation of regulated toxins into periods according to best ionization conditions
- Not useful for domoic acid (analyzed by LC-FLD at the NRC)

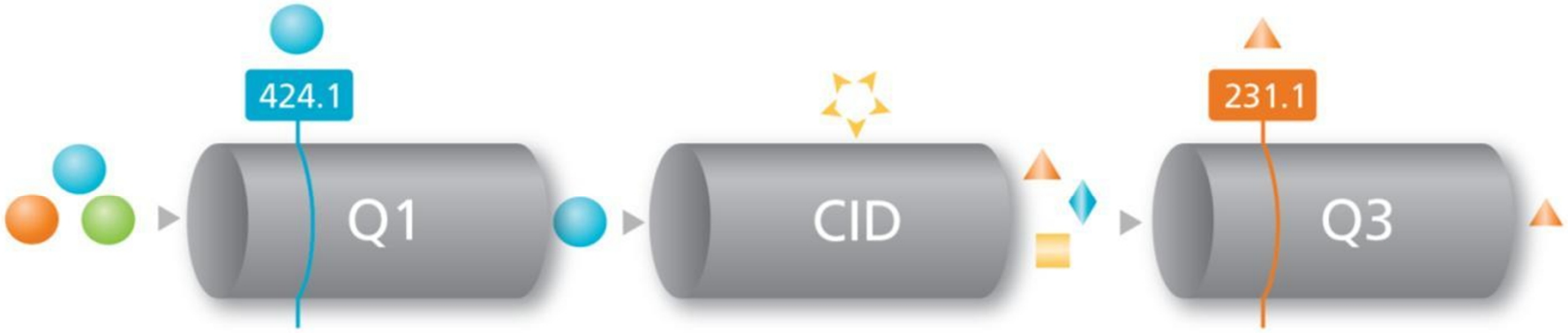
MS/MS Detection

SCIEX QTRAP® 4500 system

- EU-harmonized SOP
  - AB SCIEX 3200 QTRAP® system
  - Electrospray Ionization (ESI)
  - Multiple Reaction Monitoring (MRM)
  - 2 Transitions per compound (ratio for identification)
- Newer triple quadrupole LC-MS/MS instruments offer
  - Higher sensitivity
    - Reduced matrix effects and interferences after dilution of extract
    - Detection at lower levels allows prediction of upcoming problems when analyzing phytoplancton and water samples
  - Fast polarity switching
- QTRAP® and TripleTOF® technology offers
  - Unique features for quantitation and identification (EPI, MRM^3) and unknown screening and identification of new toxins (TOF-MS and MS/MS)

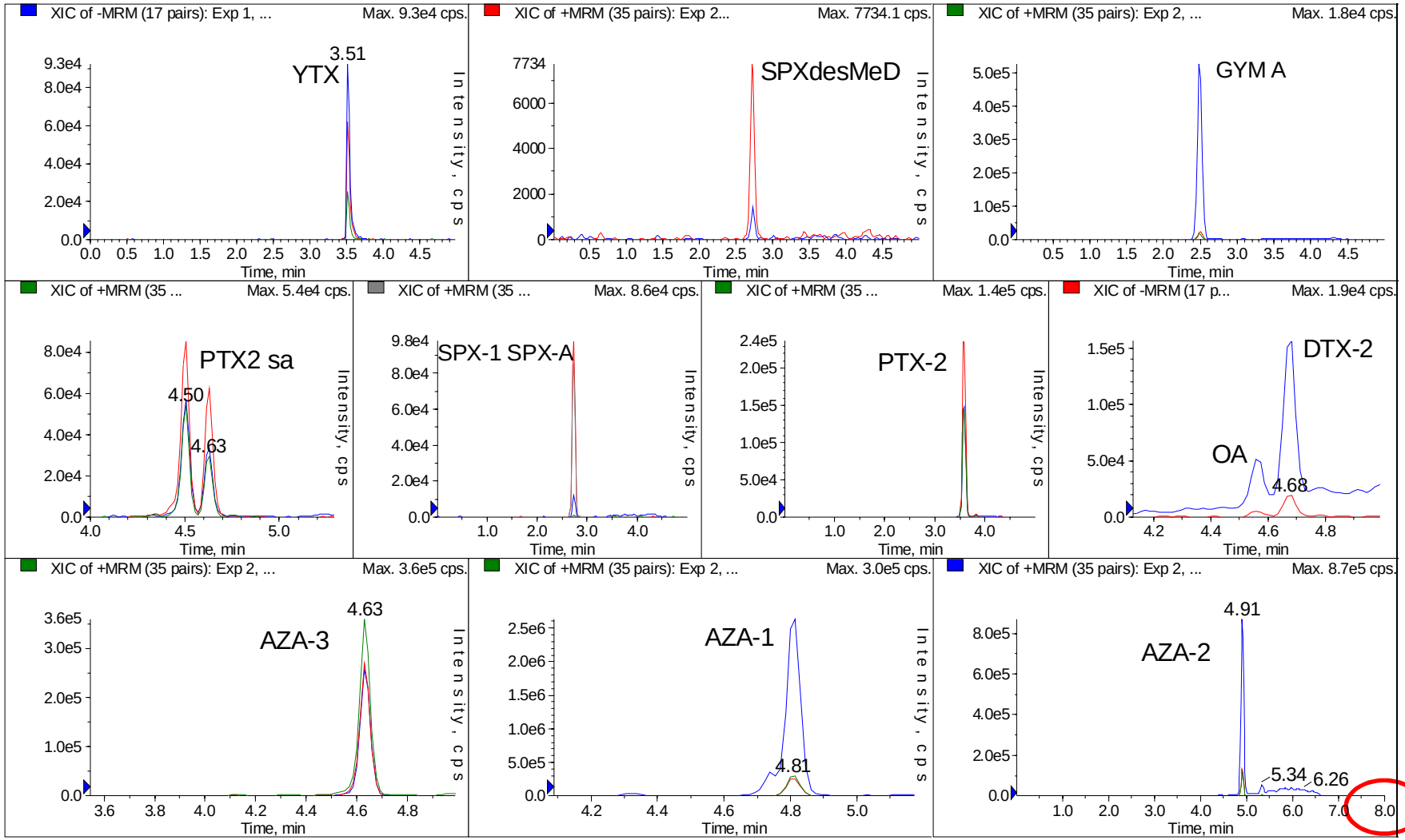
Triple Quadrupole – Multiple Reaction Monitoring (MRM)

Highest Selectivity and Sensitivity for Screening and Quantitation



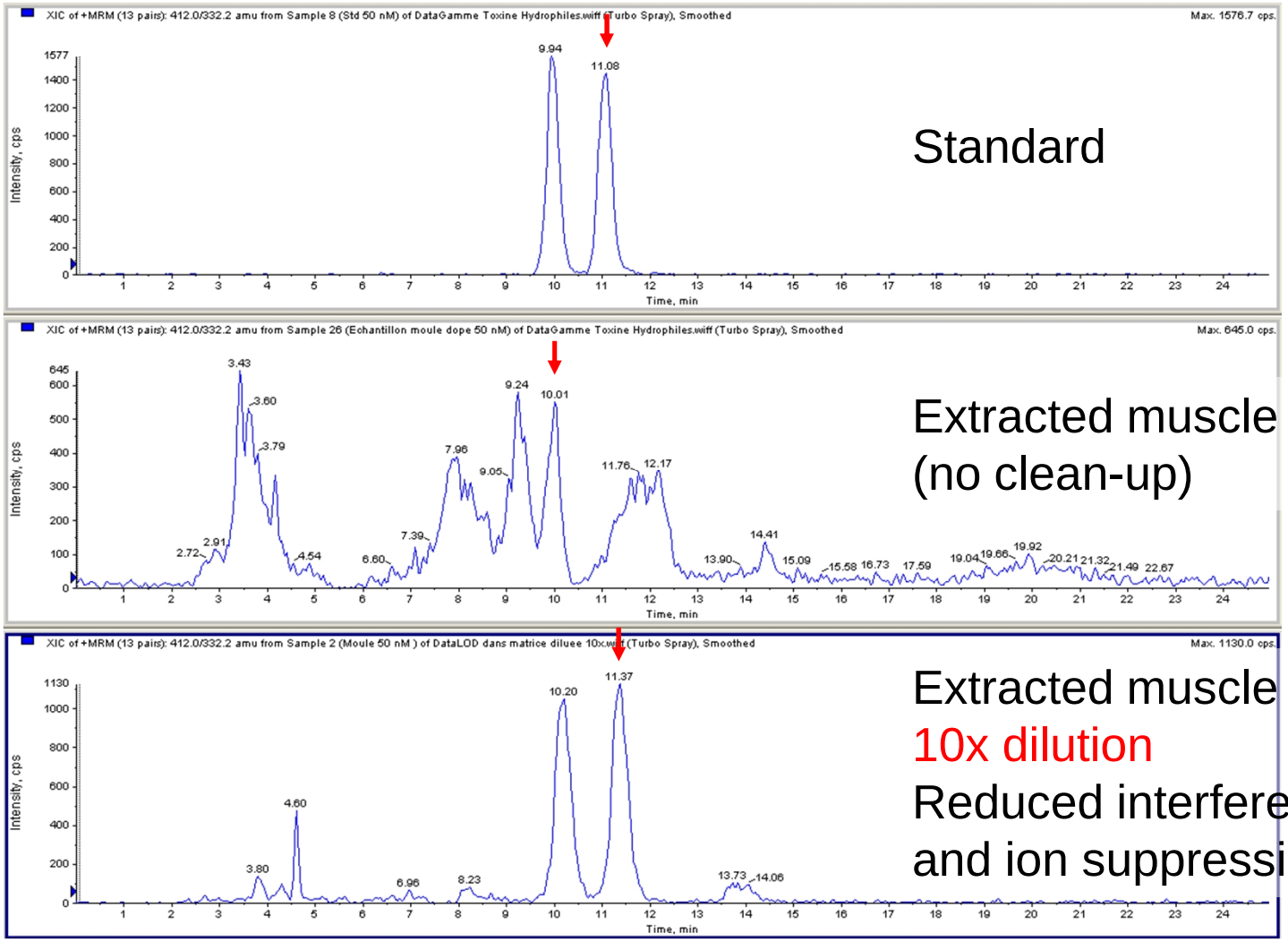
Filter Fragment Filter

QTRAP® 5500 System with Polarity Switching



Fast acidic LC using Phenomenex Kinetex C18 2.7µm (50x2mm) column

QTRAP® 5500 System – Reduced Matrix Effect



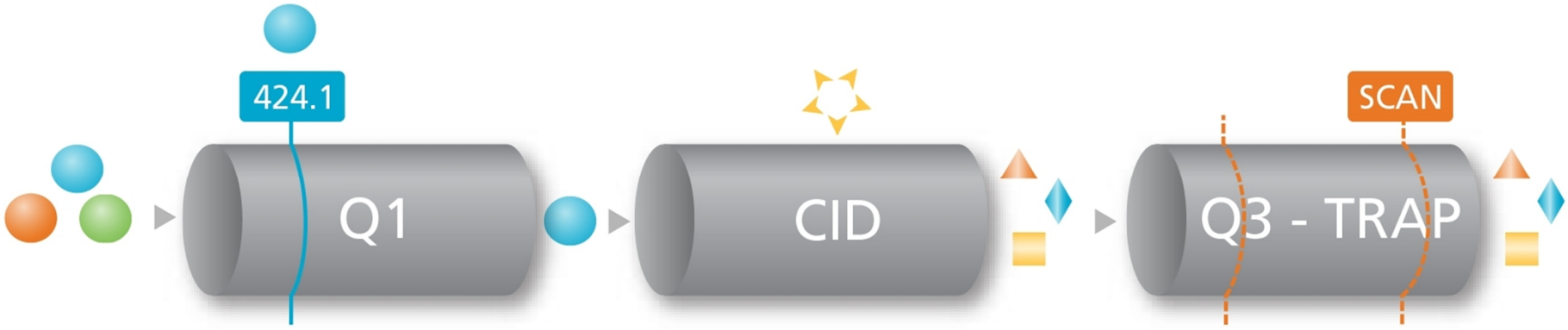
Standard

Extracted muscle sample (no clean-up)

Extracted muscle sample
10x dilution
Reduced interferences and ion suppression

QTRAP® System – MS/MS Full Scan and Library Searching

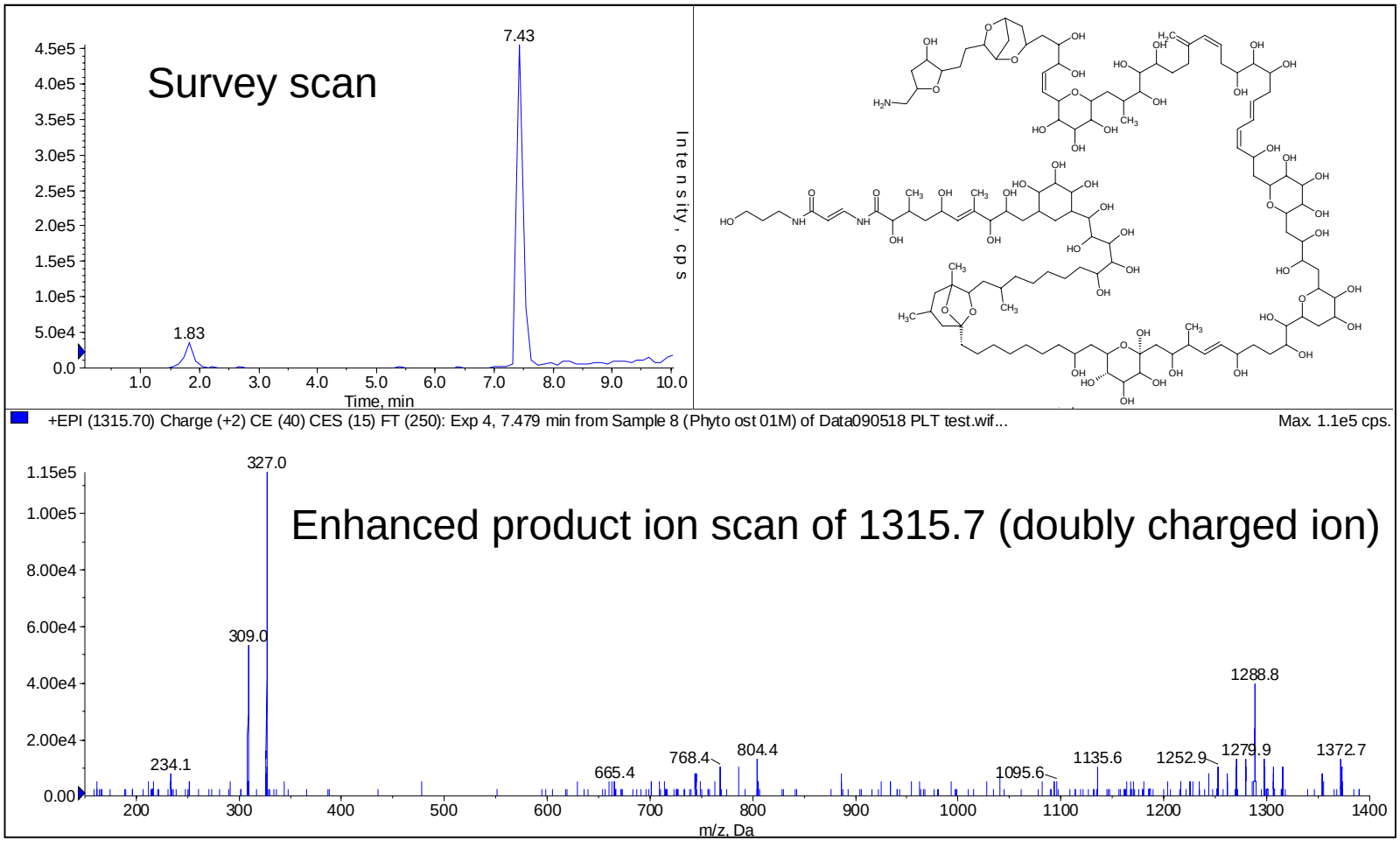
High Selectivity and Fast MS/MS Scan to Increase Confidence



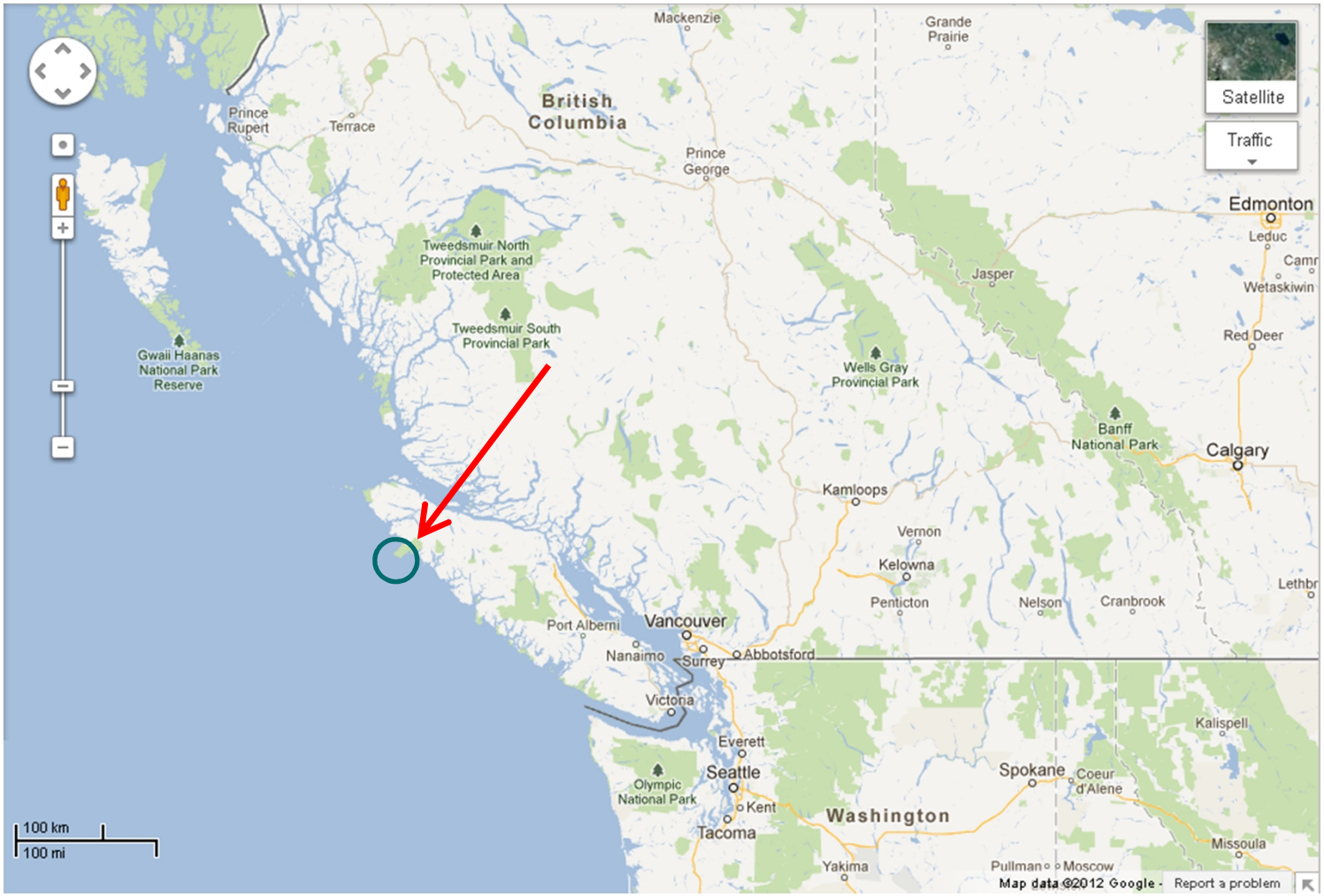
Filter Fragment Trap and Scan

MRM Quantitation and QTRAP® Full Scan MS/MS can be combined in Information Dependent Acquisition (IDA) methods for simultaneous quantitation and Identification

QTRAP® 4500 System – MS/MS Identification

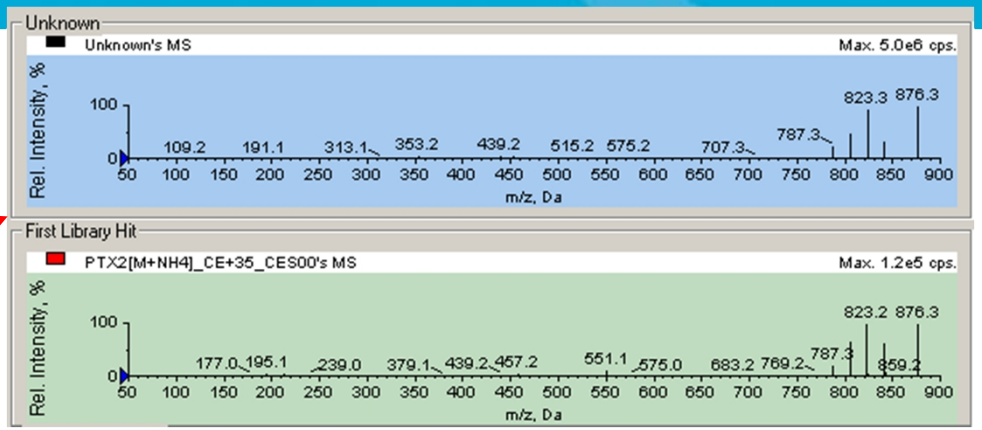


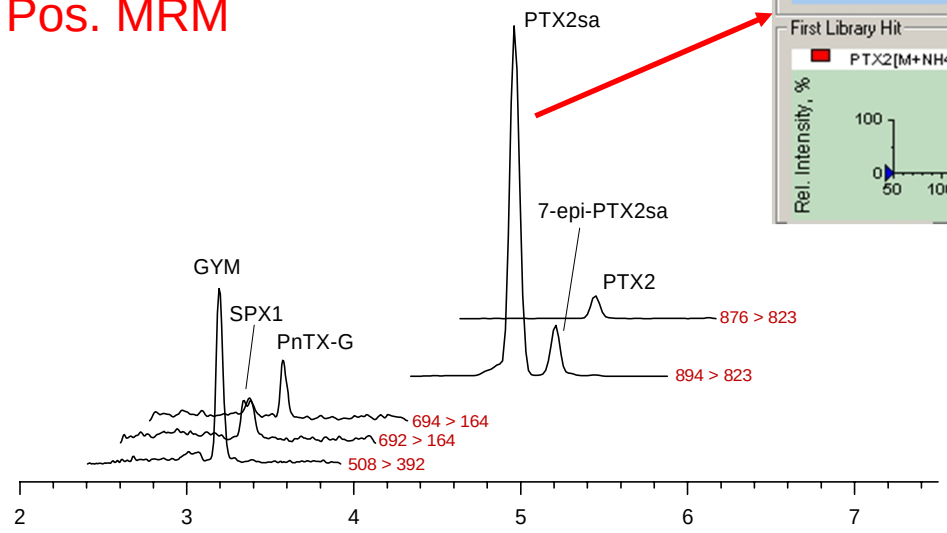
2011 DSP outbreak: British Columbia

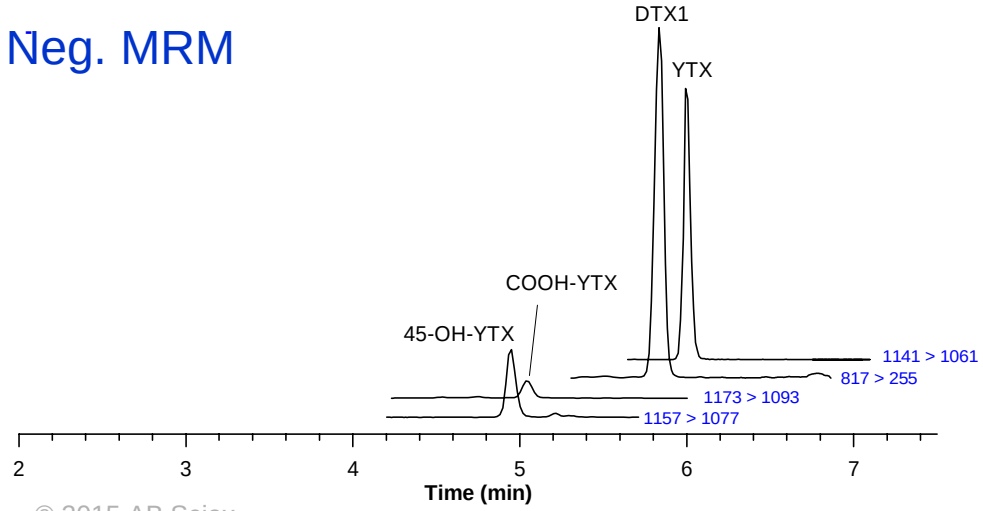


Application of methods to Gorge Harbour sample (July 2011)

Toxin Profile

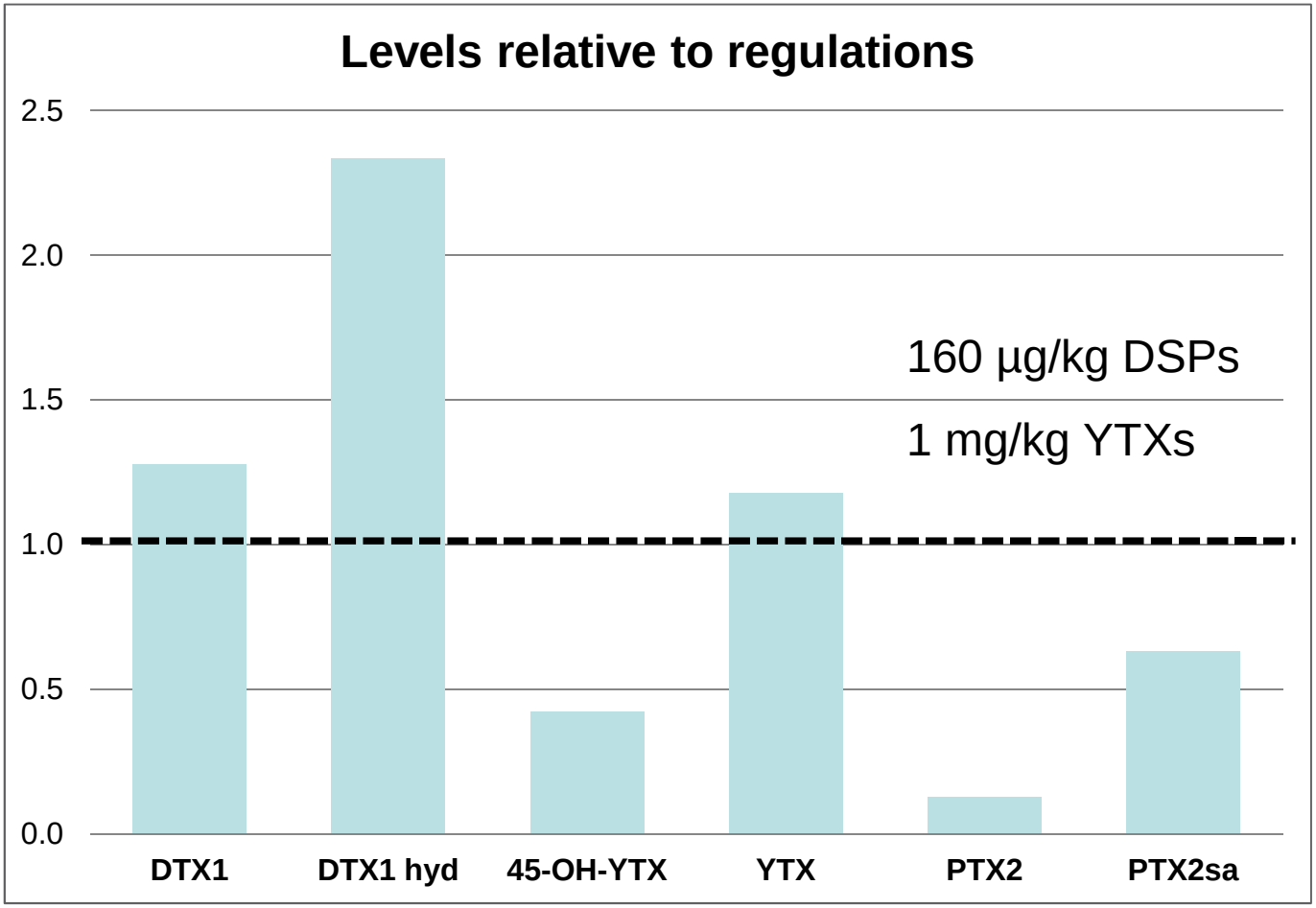






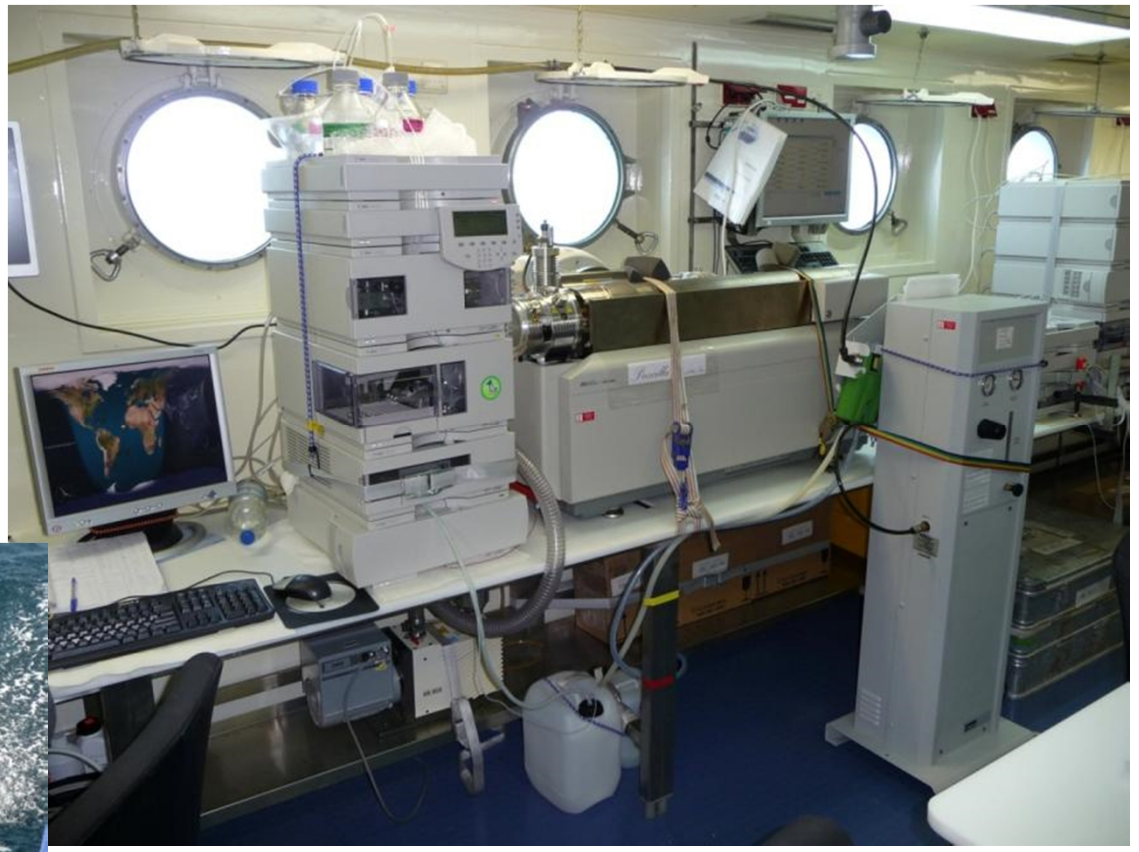
- ~~AZAs~~
- DTX1 (esters)
- PTXs (esters)
- YTXs
- Cyclic imines
  - GYM
  - SPX
  - PnTX-G

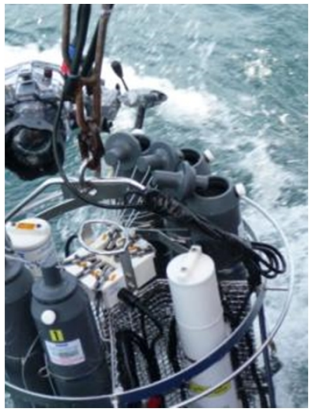
Concentrations in BC sample (Gorge Harbour)



Low levels of cyclic imine toxins (GYM, SPX, PnTX-G)

4000 QTRAP® System on Board a Research Vessel



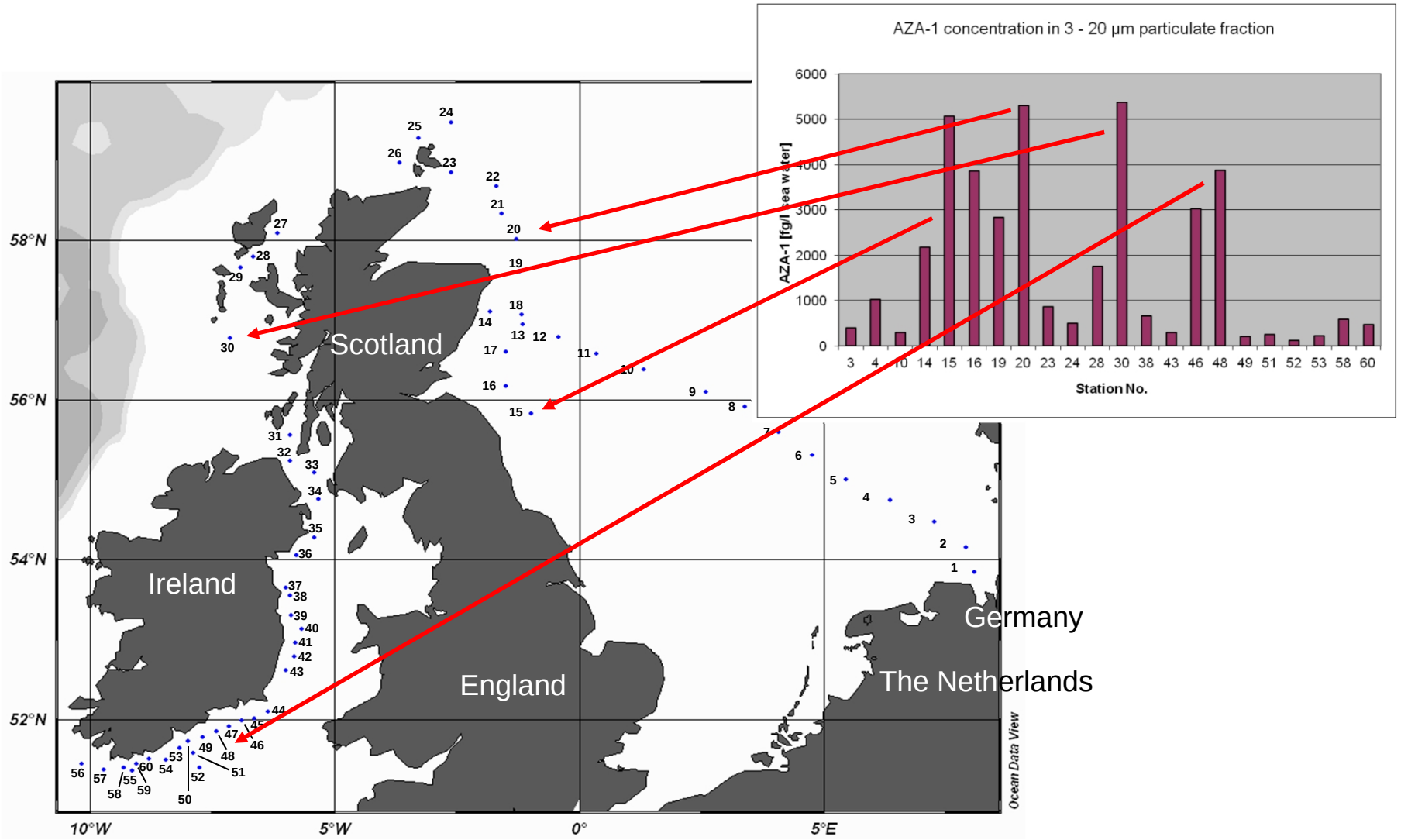


Rosette sampler and phytoplankton net

Live data acquisition and processing

(ruggedness !!!)

Sampling (15. April to 05. May)



Summary

- LC-MS/MS strategies for monitoring of emerging environmental pollutants
  - EPA 537
  - Migration of PFAA from packaging into food
  - EPA 539
  - Marine and freshwater toxins

Trademarks/Licensing

For Research Use Only. Not for use in diagnostic procedures.

Thank you for your attention!

Holy Ghost and surrounding rocks in Meteora (Greece).