HILIC-MS/MS method for determination of PSP and TTX toxins in shellfish using the Agilent 1290 UHPLC and 6495B tandem mass spectrometer

Dr Andrew Turner CChem CSci

Principal Chemist

Food Safety Group

Cefas, Weymouth, UK

Agilent Webinar

Dec 2019

Agilent Technologies

Cefas

Centre for Environment Fisheries and Aquaculture Science (Cefas)

- UK Government agency
- Two specialist laboratories
- 500 staff
- Aquatic science, research and advice

Food Safety Group

- Water quality
- Primary focus on shellfish (bivalve molluscs)
- Risks from bioaccumulation of pathogens and chemicals
- Official control testing for government
- Research programmes
- Focus on **applied science**

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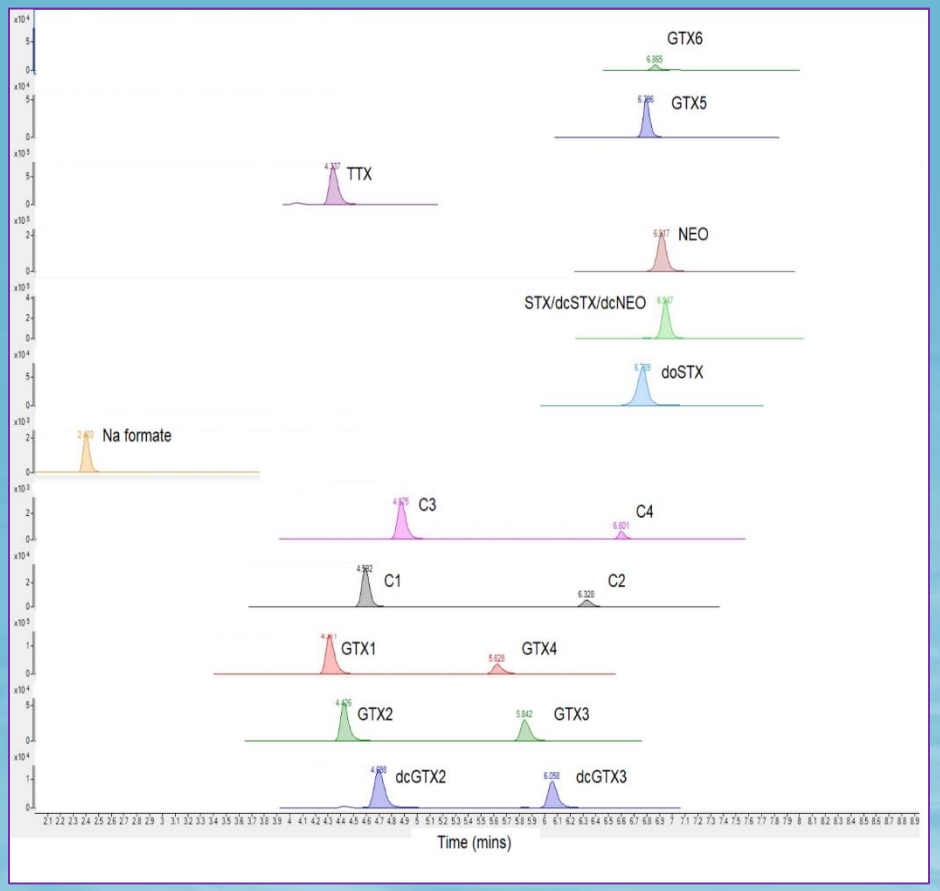
Presentation overview

1. Background and testing methods
2. Use of Agilent 1290-6495B for PST/TTX analysis
3. Multi-laboratory validation
4. Column options
5. Overall conclusions



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Background and testing methods

Agilent 6495B Triple Quadrupole LC/MS

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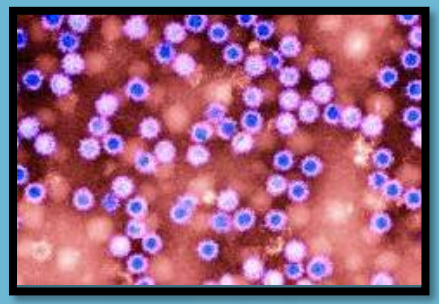
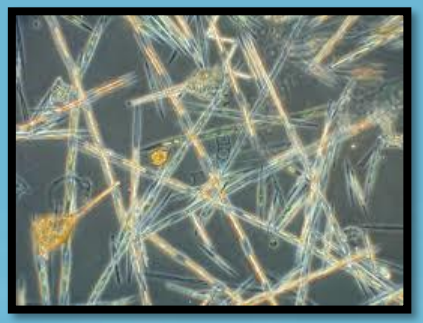
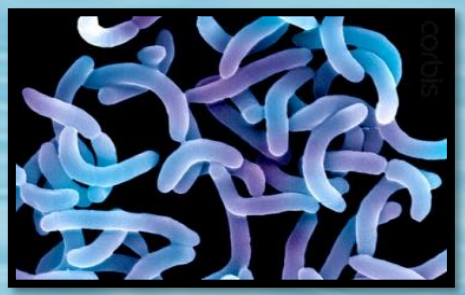
From seawater to shellfish, why it's the little things that matter:

Bivalve shellfish (mussels, oysters, cockles, etc) filter feed and concentrate whatever is in the seawater they grow in:

- Bacteria
- Viruses
- Heavy metals
- Radiological wastes
- Chemical wastes
- Pharmaceuticals
- Microplastics
- **Toxic algae**







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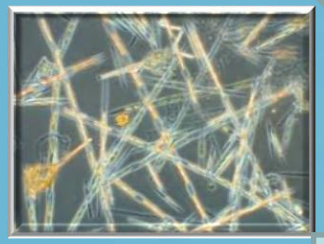
Marine Biotoxins



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EU-regulated Biotoxins in Shellfish

ASP

(Domoic/epi-domoic acid)

- Nausea
- Diarrhoea
- Vomiting
- Confusion
- Memory loss (may be permanent)
- Can be fatal

Lipophilic toxins

(OA, DTX. YTX, PTX and AZA groups)

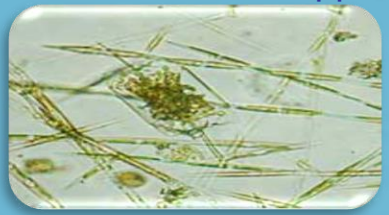
- Nausea
- Abdominal pains
- Vomiting
- Diarrhoea
- Tumourigenic

PSP

(Saxitoxins)

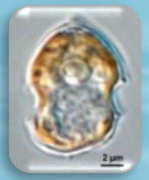
- Numbness/tingling
- Headaches
- Nausea, Vomiting
- Respiratory distress
- Paralysis
- Can be fatal

Pseudo-nitzschia spp.



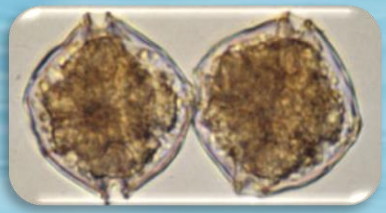
Prorocentrum lima

Dinophysis spp.



Azadinium spp.

Alexandrium spp.



OVERALL ~20 algal species &>30 toxins all present in EU waters and requiring monitoring

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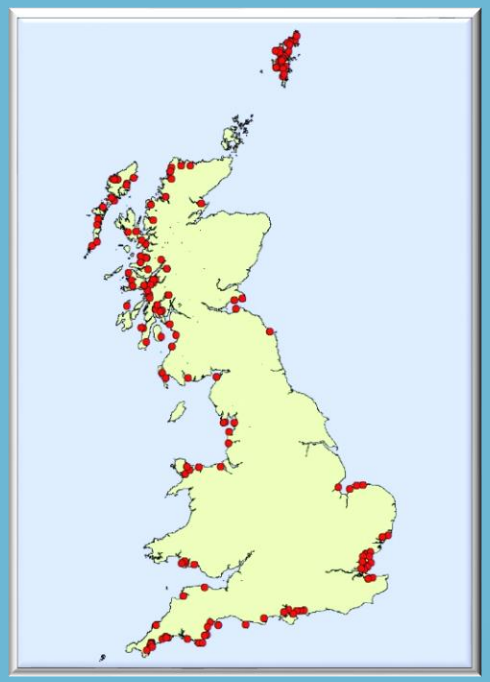
GB Monitoring Programme



- Cefas conduct official control monitoring of bivalve molluscs conducted for FSA/FSS
- >100 monitoring sites round GB
- >3000 samples/year
- Samples arrive 7.30am each morning
- Results reported next day (24 hr turnaround)

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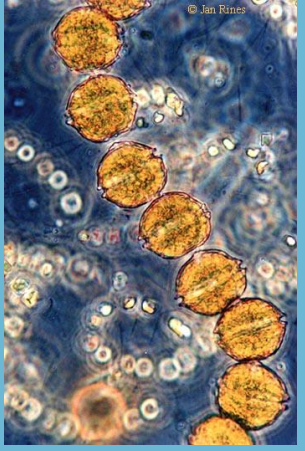
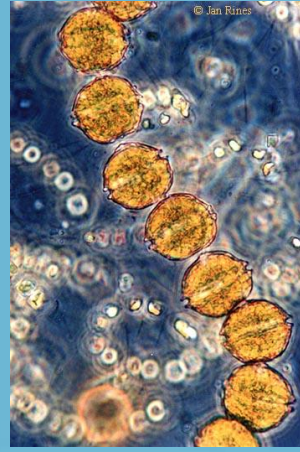


PSP Toxins



- Saxitoxins (>55 analogues)
- Highly potent neurotoxins
- Variable toxicities
- Paralysis, death by asphyxiation
- No antidote

- Max permitted limit = 800 µg STX eq/kg shellfish
- Max UK concentration >27,000 µg STX eq/kg (x34 MPL)
- Globally >1,000,000 (1g/kg) (x1,250 MPL)

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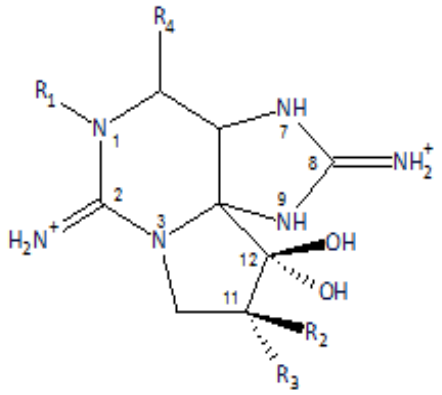
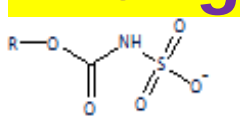
PSP Toxins



Group (Charge state)	Analogue	R1	R2	R3	R4
C toxins (0)	C1	H	H	OSO_3^-	$OCONHSO_3^-$
	C2	H	OSO_3^-	H	$OCONHSO_3^-$
	C3	OH	H	OSO_3^-	$OCONHSO_3^-$
	C4	OH	OSO_3^-	H	$OCONHSO_3^-$
GTXs (+1)	dcGTX2	H	H	OSO_3^-	OH
	dcGTX2	H	OSO_3^-	H	OH
	dcGTX1	OH	H	OSO_3^-	OH
	dcGTX4	OH	OSO_3^-	H	OH
	GTX2	H	H	OSO_3^-	$OCONH_2$
	GTX3	H	OSO_3^-	H	$OCONH_2$
	GTX1	OH	H	OSO_3^-	$OCONH_2$
	GTX4	OH	OSO_3^-	H	$OCONH_2$
	GTX5 (B1)	H	H	H	$OCONHSO_3^-$
	GTX6 (B2)	OH	H	H	$OCONHSO_3^-$
	M1α	H	H	OH	$OCONHSO_3^-$
	M1β	H	OH	H	$OCONHSO_3^-$
	M3	H	OH	OH	$OCONHSO_3^-$
	STX	H	H	H	$OCONH_2$
	NEO	OH	H	H	$OCONH_2$
	M2α	H	H	OH	$OCONH_2$
	M2β	H	OH	H	$OCONH_2$
	M4	H	OH	OH	$OCONH_2$

$OCONH_2$:



$OCONHSO_3^-$:

R4 =
- N-sulfocarbamoyl
- Carbamoyl
- Decarbamoyl

A range of methods available for testing

- All congeners contain two $=NH_2^+$ substituents
- R_4 substituents vary:
  - N-sulfocarbamoyl – impart 1 –ve charge
  - Others (carbamoyl, OH and H) are neutral
- R_2/R_3 C11 substituents, either H, OH or hydroxysulphate OSO_3^-
- Three charge groups result: Neutral, +1 or +2

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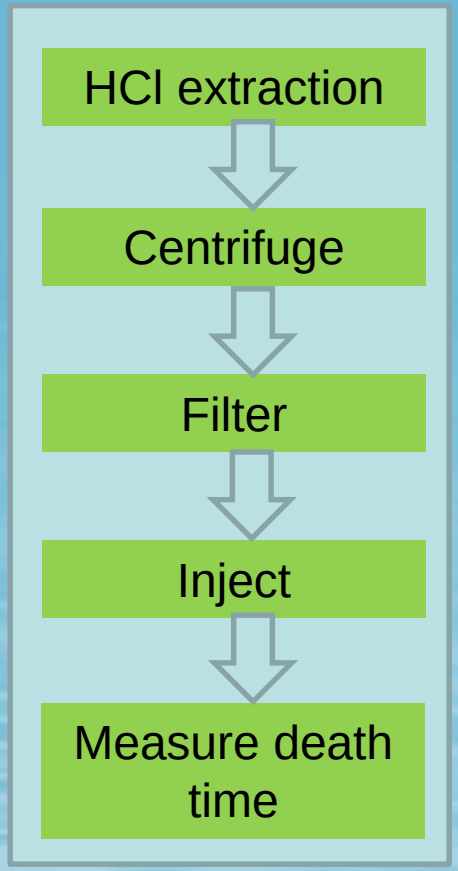
a) PSP Mouse bioassay

- EU reference method until Jan 2019
- Shellfish homogenised & extracted (HCl)
- IP injection into replicate mice
- Death time gives PSP toxicity
- Simple, BUT, lots of issues:
  - Matrix effects
  - Reproducibility
  - Sensitivity
  - Ethics
- Until Oct 2006 – all PSP testing conducted by MBA



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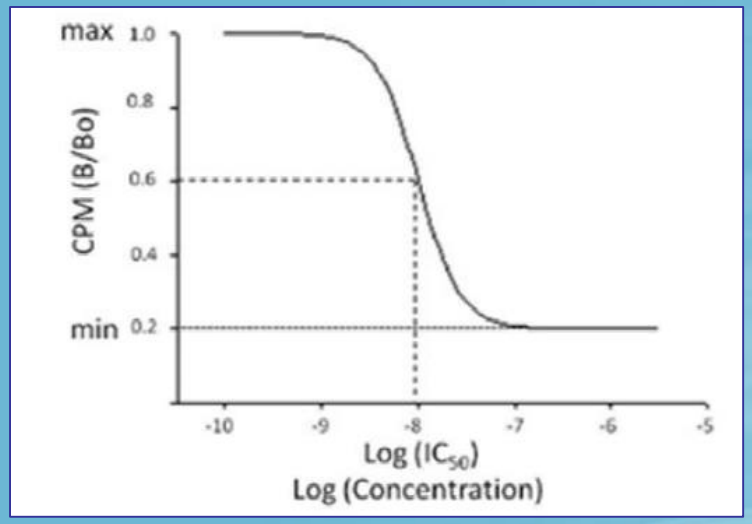
b) PSP by Receptor Binding Assay (RBA)

- Competitive binding assay:
  - [^{3}H]STX competes with unlabelled STX in samples
  - Receptor sites in rat brain membrane prep
- AOAC 2011.27 OMA
- ISSC approved (Mussels and clams)



HCl extraction

Centrifuge

Add reagents to microplate

Incubate

Filter plate

Scintillation cocktail add

Scintillation counting

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c) PSP by post-column LC-FLD (PCOX)

- AOAC 2011.02 OMA
- ISSC approved
- Routine use in Canada & parts of USA, Norway

HCl extraction

↓

Centrifuge

↓

25 µL 30% TCA

↓

35 µL NaOH

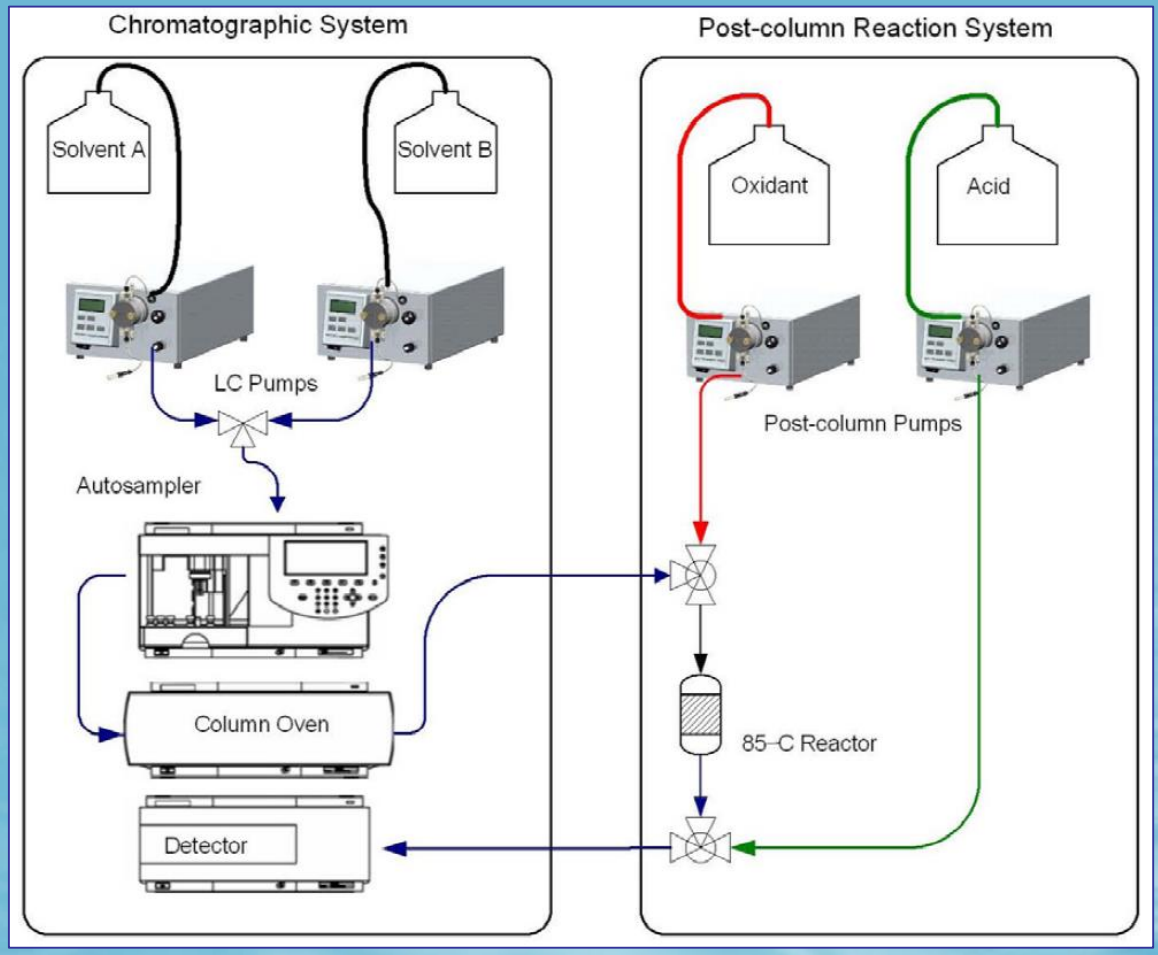
↓

Filter

↓

GTXs STXs

C toxins



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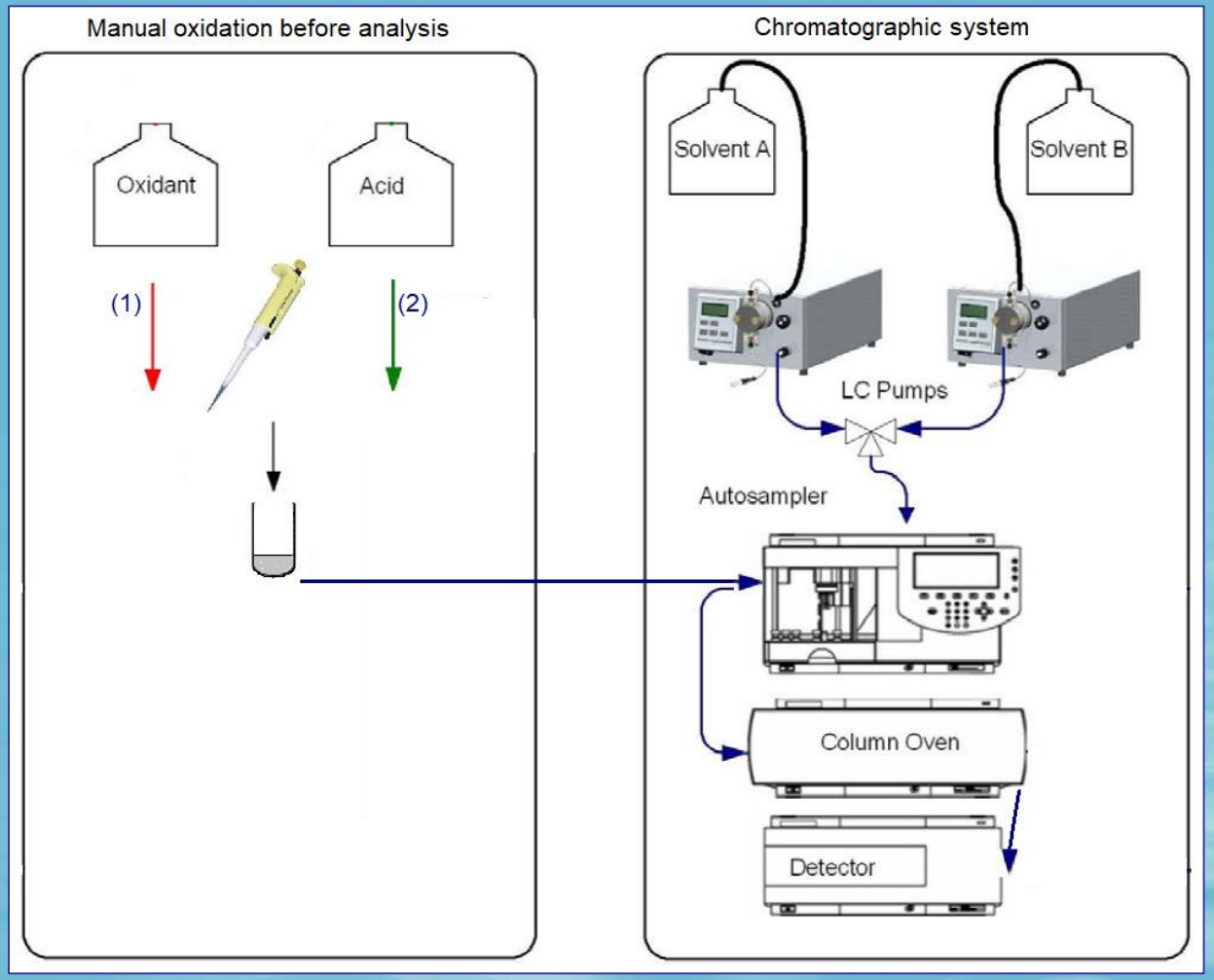
d) PSP by pre-column LC-FLD

- AOAC 2005.06
- EU Ref Method
- Routine in UK, Ire, Aus, NZ, Portugal – more and more



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e) PSP by pre-column LC-FLD

- Method performance well validated
  - Some recovery issues (SPE related)
- Use for routine monitoring > 12 years in UK
  - No contingency MBA required
- Frustrations are voiced:
  - Significant analyst time needed
  - ≥ 4 analytical runs for each quantitative sample
  - Full quantitation requires 2 days
  - A known over-estimation in total PST for some analogues
  - Accurate quant not easy for some analogues
  - Not validated for all analogues (GTX6, C3&4, dcGTX1&4)
- A method which is simple, fast, accurate and applicable to all known PSTs desirable

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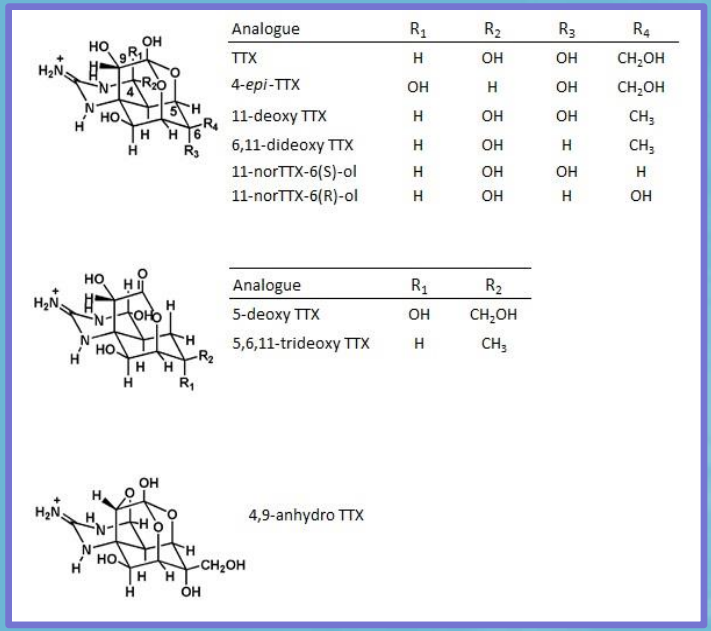
e) PSP by pre-column LC-FLD

- One other (more recent) issue…

Tetrodotoxins (TTXs)

- TTXs also found in UK and EU molluscs
- Also highly toxic and heat-stable
- Multiple TTX analogues identified
- TTX not incorporated into either LC-FLD method



Analogue	R_1	R_2	R_3	R_4
TTX	H	OH	OH	CH_2OH
4-*epi*-TTX	OH	H	OH	CH_2OH
11-deoxy TTX	H	OH	OH	CH_3
6,11-dideoxy TTX	H	OH	H	CH_3
11-norTTX-6(S)-ol	H	OH	OH	H
11-norTTX-6(R)-ol	H	OH	H	OH

Analogue	R_1	R_2
5-deoxy TTX	OH	CH_2OH
5,6,11-trideoxy TTX	H	CH_3

4,9-anhydro TTX

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TTX prevalence

- Japan
- New Zealand

- England
- Greece
- Netherlands
- Italy
- Spain
- France
- Portugal

Linked primarily to marine bacteria *e.g. Vibrio* spp.

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f) PSP/TTX by LC-MS/MS – an opportunity

- Dell'Aversano 2005 – use of HILIC chromatography for PST
- TSK-gel Amide-80 (250mm) column
  - ✓ More sensitive than LC-FLD
  - ✗ Matrix ion-suppression
  - ✗ Chromatographic variability

- Subsequent reports:
  - Sensitivity issues
  - Toxin recovery
  - Long run times (> 60 mins)
  - Inadequate peak resolution

- Issues needed resolving


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JOURNAL OF CHROMATOGRAPHY A

Journal of Chromatography A, 1081 (2005) 190–201

www.elsevier.com/locate/chroma

Hydrophilic interaction liquid chromatography–mass spectrometry for the analysis of paralytic shellfish poisoning (PSP) toxins

Carmela Dell'Aversano [a,b], Philipp Hess [a,c,1], Michael A. Quilliam [a,*]

[a] Institute for Marine Biosciences, National Research Council of Canada, 1411 Oxford Street, Halifax, Nova Scotia, Canada B3H 3Z1
[b] Dipartimento di Chimica delle Sostanze Naturali, Università degli Studi di Napoli "Federico II", Via D. Montesano 49, Napoli 80131, Italy
[c] FRS Marine Laboratory Aberdeen, Victoria Road, P.O. Box 101, Aberdeen AB11 9DB, Scotland, UK

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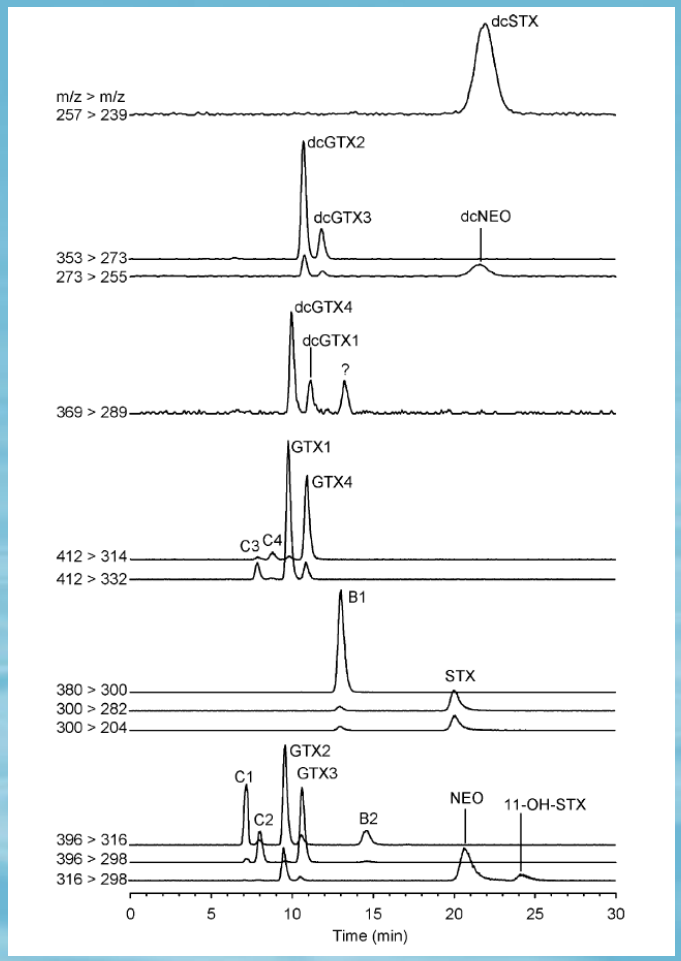
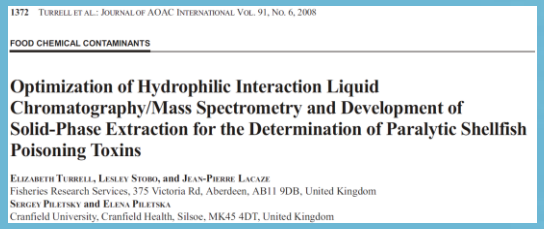
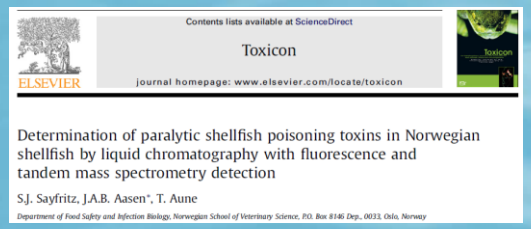




FOOD CHEMICAL CONTAMINANTS

Optimization of Hydrophilic Interaction Liquid Chromatography/Mass Spectrometry and Development of Solid-Phase Extraction for the Determination of Paralytic Shellfish Poisoning Toxins

ELIZABETH TURRELL, LESLEY STOBO, and JEAN-PIERRE LACAZE
Fisheries Research Services, 375 Victoria Rd, Aberdeen, AB11 9DB, United Kingdom
SERGEY PILETSKY and ELENA PILETSKA
Cranfield University, Cranfield Health, Silsoe, MK45 4DT, United Kingdom


Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

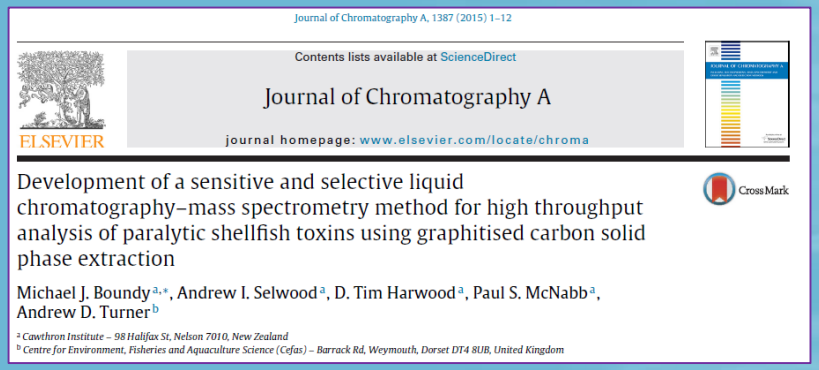
Determination of paralytic shellfish poisoning toxins in Norwegian shellfish by liquid chromatography with fluorescence and tandem mass spectrometry detection

S.J. Sayfritz, J.A.B. Aasen*, T. Aune

Department of Food Safety and Infection Biology, Norwegian School of Veterinary Science, P.O. Box 8146 Dep., 0033, Oslo, Norway

Method goals

- Rapid, simple extraction (>80 per day)
- One simple clean-up
- No derivatisation steps
- 1 column / system required
- 1 run for full quant of all analytes
- All within 1 day
- Able to use multiple columns and consumables



Journal of Chromatography A, 1387 (2015) 1–12

Contents lists available at ScienceDirect

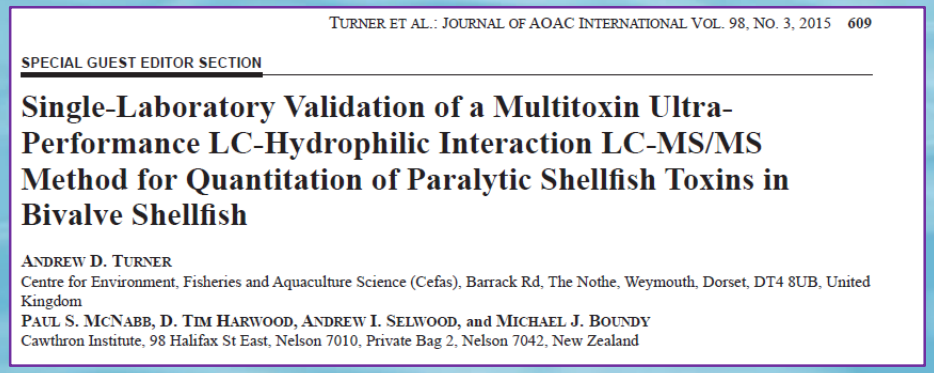
Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Development of a sensitive and selective liquid chromatography–mass spectrometry method for high throughput analysis of paralytic shellfish toxins using graphitised carbon solid phase extraction

Michael J. Boundy[a,*], Andrew I. Selwood[a], D. Tim Harwood[a], Paul S. McNabb[a], Andrew D. Turner[b]

[a] Cawthron Institute – 98 Halifax St, Nelson 7010, New Zealand
[b] Centre for Environment, Fisheries and Aquaculture Science (Cefas) – Barrack Rd, Weymouth, Dorset DT4 8UB, United Kingdom



SPECIAL GUEST EDITOR SECTION

Single-Laboratory Validation of a Multitoxin Ultra-Performance LC-Hydrophilic Interaction LC-MS/MS Method for Quantitation of Paralytic Shellfish Toxins in Bivalve Shellfish

ANDREW D. TURNER
Centre for Environment, Fisheries and Aquaculture Science (Cefas), Barrack Rd, The Nothe, Weymouth, Dorset, DT4 8UB, United Kingdom
PAUL S. MCNABB, D. TIM HARWOOD, ANDREW I. SELWOOD, and MICHAEL J. BOUNDY
Cawthron Institute, 98 Halifax St East, Nelson 7010, Private Bag 2, Nelson 7042, New Zealand

Use of Agilent 1290 UHPLC and 6495B MS/MS for PST/TTX analysis

Agilent 6495B Triple Quadrupole LC/MS

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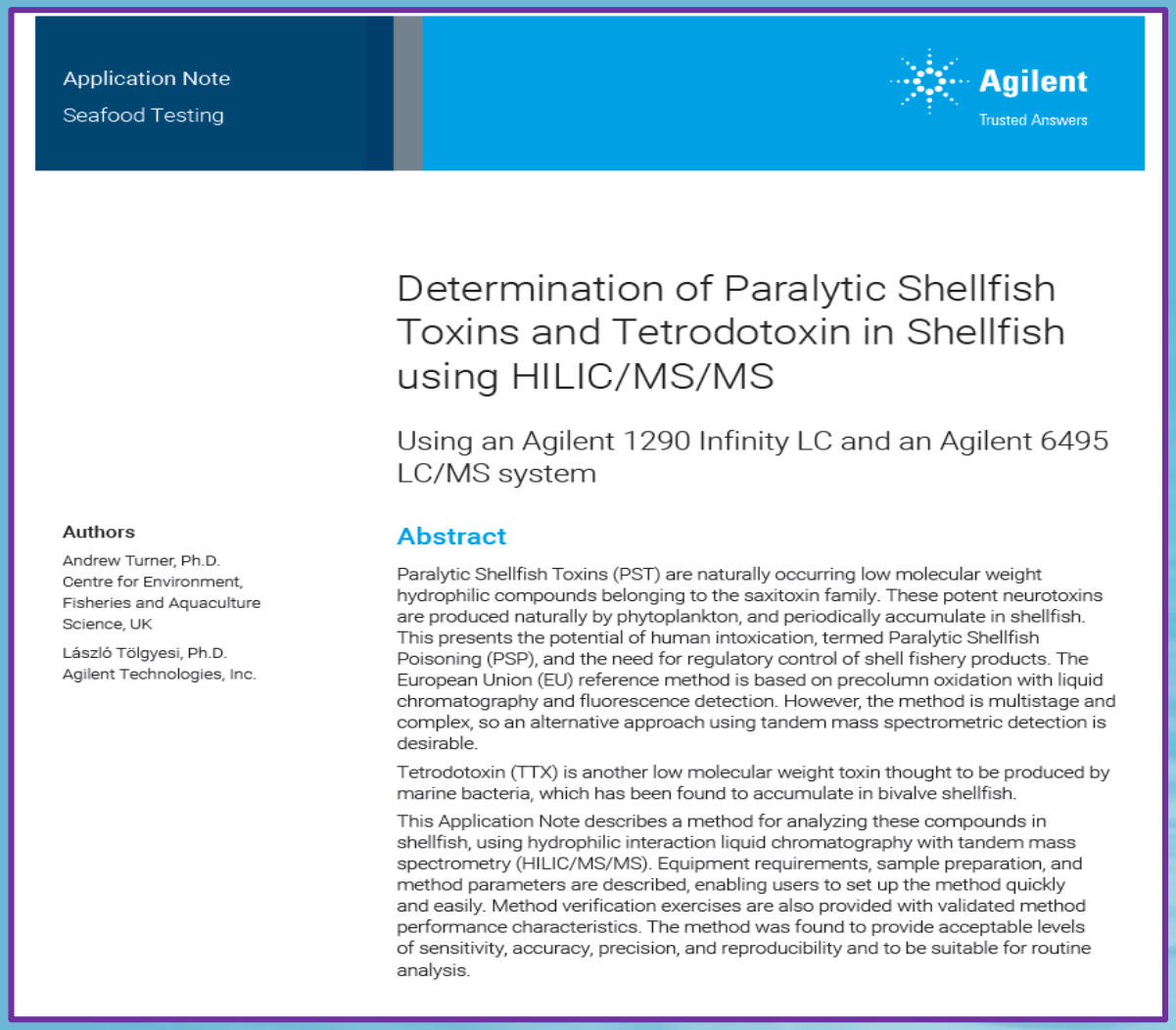
Method



Application Note

Seafood Testing

Agilent

Trusted Answers

Determination of Paralytic Shellfish Toxins and Tetrodotoxin in Shellfish using HILIC/MS/MS

Using an Agilent 1290 Infinity LC and an Agilent 6495 LC/MS system

Authors

Andrew Turner, Ph.D.
Centre for Environment, Fisheries and Aquaculture Science, UK

László Tölgyesi, Ph.D.
Agilent Technologies, Inc.

Abstract

Paralytic Shellfish Toxins (PST) are naturally occurring low molecular weight hydrophilic compounds belonging to the saxitoxin family. These potent neurotoxins are produced naturally by phytoplankton, and periodically accumulate in shellfish. This presents the potential of human intoxication, termed Paralytic Shellfish Poisoning (PSP), and the need for regulatory control of shell fishery products. The European Union (EU) reference method is based on precolumn oxidation with liquid chromatography and fluorescence detection. However, the method is multistage and complex, so an alternative approach using tandem mass spectrometric detection is desirable.

Tetrodotoxin (TTX) is another low molecular weight toxin thought to be produced by marine bacteria, which has been found to accumulate in bivalve shellfish.

This Application Note describes a method for analyzing these compounds in shellfish, using hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC/MS/MS). Equipment requirements, sample preparation, and method parameters are described, enabling users to set up the method quickly and easily. Method verification exercises are also provided with validated method performance characteristics. The method was found to provide acceptable levels of sensitivity, accuracy, precision, and reproducibility and to be suitable for routine analysis.

https://www.agilent.com/cs/library/applications/application-pst-tetrodotoxin-shellfish-lcmsms-5994-0967en-agilent.pdf

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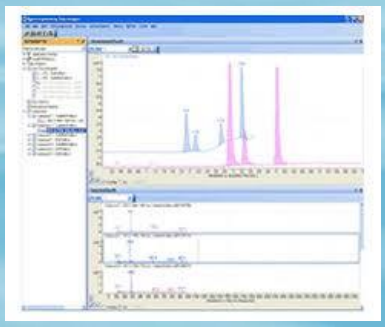
Methodology: Instrumentation

- Chromatography
  - Agilent 1290 Infinity II LC with 20 µL injection loop
- Mass spectrometer
  - Agilent 6495B triple quadrupole LC/MS with iFunnel and Jet Stream technology
- Analysis
  - Fast polarity switching mode with electrospray ionisation
- Data acquisition
  - Agilent MassHunter Acquisition software (Version B.08.00)
- Data processing
  - Agilent MassHunter Quantitative and Qualitative Analysis software (Version 10.0)

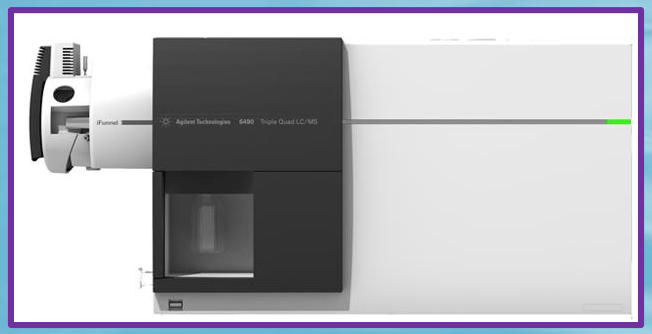




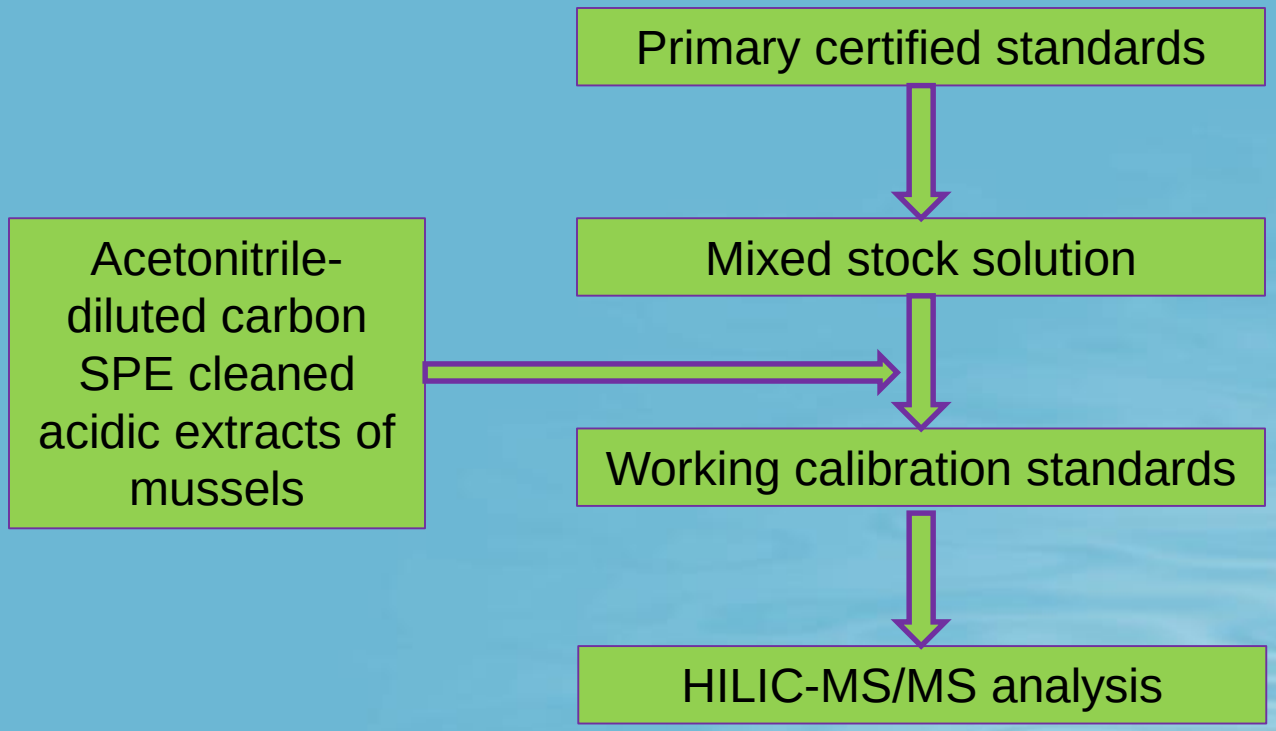


Methodology: other equipment needed

- Boiling water bath
- Centrifuge, operating at 4,500 × g
- Vortex mixer
- Vacuum Manifold for manual SPE (Solid Phase Extraction), if required
- Automated SPE liquid handler for SPE clean-up (optional for high throughput)
- Autopipettes
- Duran bottles
- Volumetric flasks for standards and samples
- 50 mL polypropylene centrifuge tubes
- 15 mL polypropylene centrifuge test tubes
- Polypropylene 700 µL autosampler vials
- Suitable analytical HILIC column (optional guard cartridge)
- Supelco Supelclean ENVI-Carb 250 mg/3 mL or equivalent carbon SPE column
- Use of glassware should be avoided



Methodology: toxin standards



Stable for 1 week (ALS)

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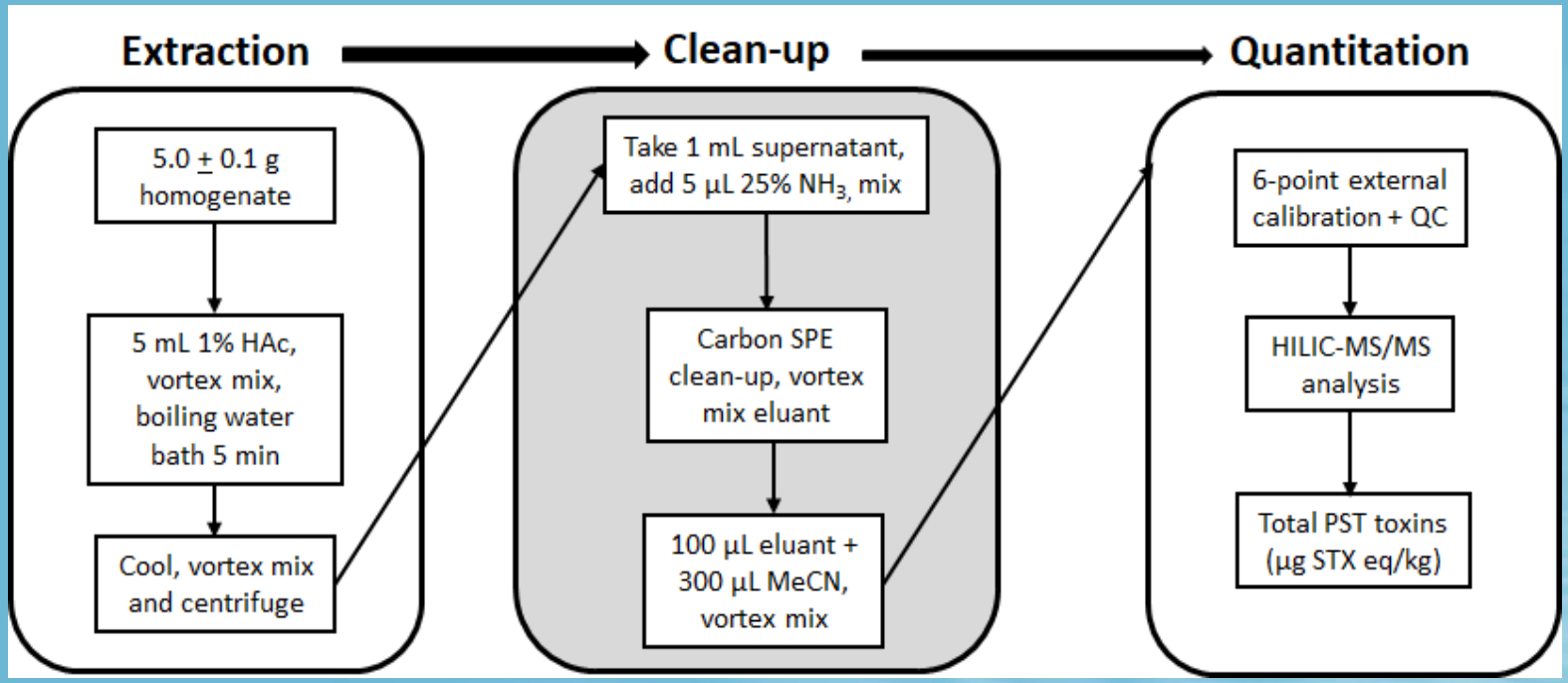
Methodology: protocol overview



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Methodology: Analytes

- Saxitoxin congeners

Group (Charge state)	Analog	R1	R2	R3	R4	Chemical Name	TEF	Calibrant
C toxins (0)	C1	H	H	OSO_3^-	$OCONHSO_3^-$	N21-sulfocarbamoyl-11α-hydroxysulfate-saxitoxin	0.01	C1
	C2	H	OSO_3^-	H	$OCONHSO_3^-$	N21-sulfocarbamoyl-11 β -hydroxysulfate-saxitoxin	0.1	C2
	C3	OH	H	OSO_3^-	$OCONHSO_3^-$	N21-sulfocarbamoyl-11α-hydroxysulfate-neosaxitoxin	0.02	C1
	C4	OH	OSO_3^-	H	OSO_3^-	N21-sulfocarbamoyl-11β-hydroxysulfate-neosaxitoxin	0.1	C2
GTXs (1)	dcGTX3	H	H	OSO_3^-	OH	Decarbamoyl-11α-hydroxysulfate-saxitoxin	0.2	dcGTX3
	dcGTX2	H	OSO_3^-	H	OH	Decarbamoyl-11β-hydroxysulfate-saxitoxin	0.4	dcGTX2
	dcGTX1	OH	H	OSO_3^-	OH	Decarbamoyl-11α-hydroxysulfate-neosaxitoxin	0.5[a]	dcGTX2
	dcGTX4	OH	OSO_3^-	H	OH	Decarbamoyl-11β-hydroxysulfate-neosaxitoxin	0.5[a]	dcGTX3
	GTX2	H	H	OSO_3^-	$OCONH_2$	11α-hydroxysulfate-saxitoxin	0.4	GTX2
	GTX3	H	OSO_3^-	H	$OCONH_2$	11β-hydroxysulfate-saxitoxin	0.6	GTX3
	GTX1	OH	H	OSO_3^-	$OCONH_2$	11α-hydroxysulfate-neosaxitoxin	1	GTX1
	GTX4	OH	OSO_3^-	H	$OCONH_2$	11β-hydroxysulfate-neosaxitoxin	0.7	GTX4
	GTX5	H	H	H	$OCONHSO_3^-$	N-sulfocarbamoyl-saxitoxin	0.1	GTX5
	GTX6	OH	H	H	$OCONHSO_3^-$	N-sulfocarbamoyl-neosaxitoxin	0.1	GTX6
STXs (2)	doSTX	H	H	H	H	Deoxydecarbamoyl-saxitoxin	0.05[b]	doSTX
	dcSTX	H	H	H	OH	Decarbamoyl-saxitoxin	1	dcSTX
	dcNEO	OH	H	H	OH	Decarbamoyl-neosaxitoxin	0.4	dcNEO
	STX	H	H	H	$OCONH_2$	Saxitoxin	1	STX
	NEO	OH	H	H	$OCONH_2$	N1-hydroxy-saxitoxin (Neosaxitoxin)	1	NEO

- Tetrodotoxin (TTX)

Based on commercially available toxin CRMs

Cefas

Note on quantitation from calibrants

- Quantitation for all PST analytes available as CRMs

$$\text{Conc}_{\mu g\ STX.2HCl\ eq/kg} = \frac{\text{Conc}_{nmol/L} \times M_{STX} \times DF \times TEF}{1000}$$

Equation 1.

Where:

$\text{Conc}_{nmol/L}$	Measured concentration of sample extract in nmol/L
$\text{Conc}_{\mu g\ STX.2HCl\ eq/kg}$	Saxitoxin dihydrochloride equivalents in sample homogenate in µg STX.2HCl eq/kg
M_{STX}	Molecular weight of saxitoxin dihydrochloride salt (372.4)
DF	Dilution factor of shellfish homogenate to diluted SPE eluent for injection (40)
TEF	Molar toxicity equivalency factor

- For other PSTs quantify against calibration generated from most appropriate analyte
- TEFs based on EFSA, 2009

Cefas

Methodology: HILIC Mobile phases

- Mobile Phase A1:
  500 mL water + 75 µL formic acid + 300 µL ammonium hydroxide.

- Mobile Phase B1:
  700 mL acetonitrile + 300 mL water + 100 µL formic acid.

- Mobile Phase A2:
  200 mL water + 1 mL formic acid.

- Mobile Phase B2:
  Methanol.

Cefas

Methodology: HILIC conditions

- Column: Acquity UPLC BEH Amide, 130 Å, 1.7 µm, 2.1 × 150 mm
- Guard: Acquity UPLC BEH Amide pre-column, 130 Å, 1.7 µm, 2.1 mm × 5 mm

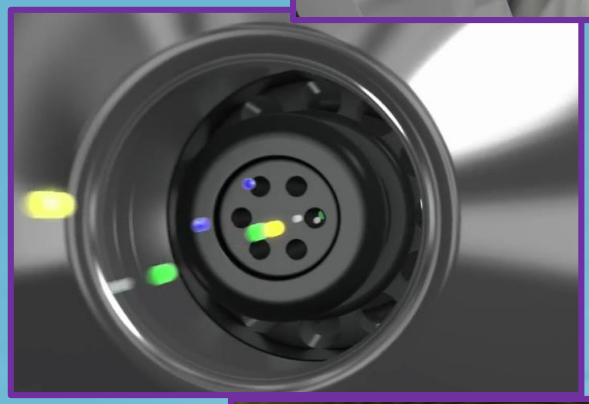
Time	Flow Rate (mL/min)	% A1	% B1
Initial	0.400	2.0	98.0
5.00	0.400	2.0	98.0
7.50	0.400	50.0	50.0
9.00	0.500	50.0	50.0
9.50	0.500	2.0	98.0
10.0	0.800	2.0	98.0
10.60	0.800	2.0	98.0
10.61	0.400	2.0	98.0
11.00	0.400	2.0	98.0

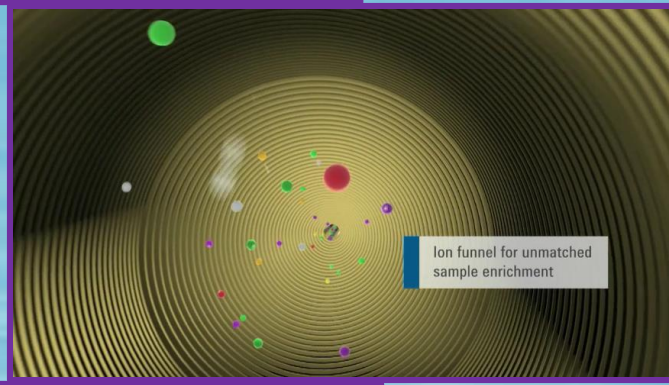
- 60 °C column temp
- 2 µL injection volume
- Columns to be conditioned, with start-up procedure

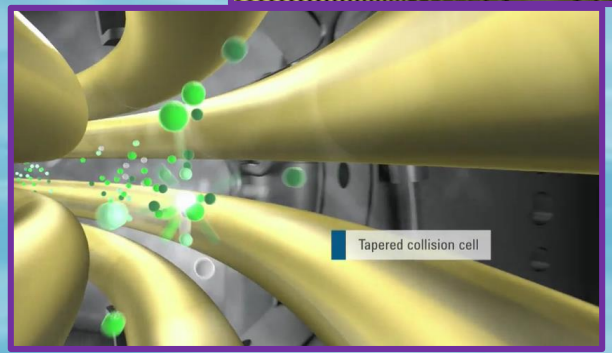
Cefas

Methodology: Agilent 6495B

- Gate valve to enable capillary to be changed without venting
- Jet stream and ion funnel technology – high sensitivity
- Hexabore capillary for signal intensity increase
- Enhanced Q1 ion options
- Curved collision cell – pull out more neutrals
- Detector with high energy conversion dynode



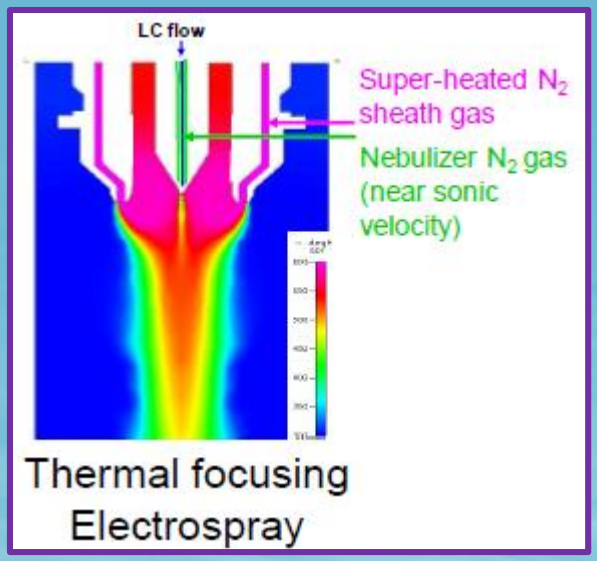


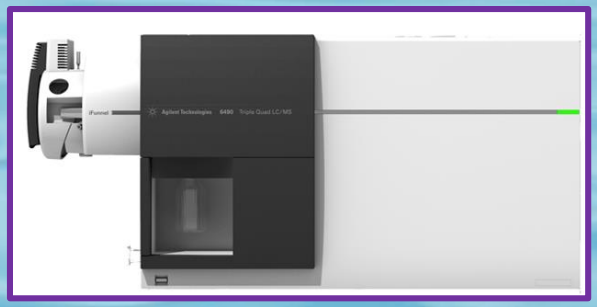


Cefas

Methodology: Agilent Jet Stream conditions

Source settings	
Drying gas temperature	150 °C
Drying gas flow	15 L/min
Nebulizer pressure	50 psi
Sheath gas temperature	400 °C
Sheath gas flow	12 L/min
Capillary voltage	2,500 V (positive) | 2,500 V (negative)
Nozzle voltage	0 V (positive) | 0 V (negative)
High/low Pressure funnel RF (V)	150/80 V (positive) | 150/80 V (negative)
Acquisition settings	
Acquisition mode	Dynamic MRM
Cycle time	400 ms
Time filter	0.04 minutes
ΔEMV	0 V (positive) | 0 V (negative)

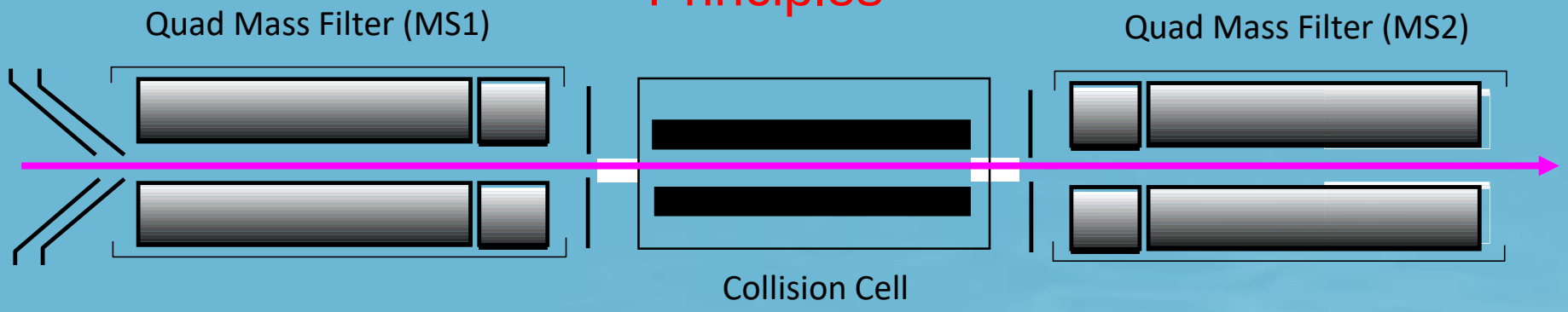




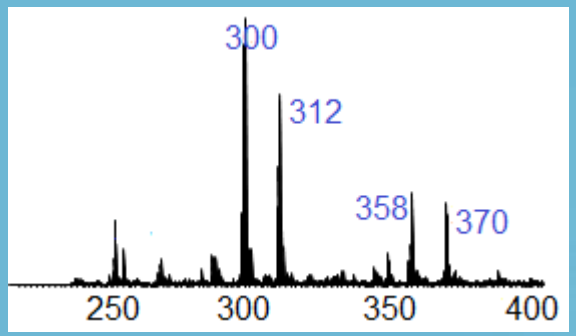
Cefas

Multiple Reaction Monitoring

Principles

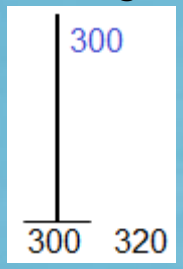


Spectrum with background ions

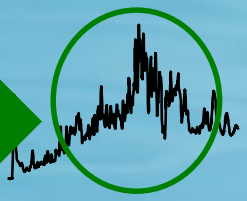


Chromatogram

Q1 lets target ion 300 pass through



High background

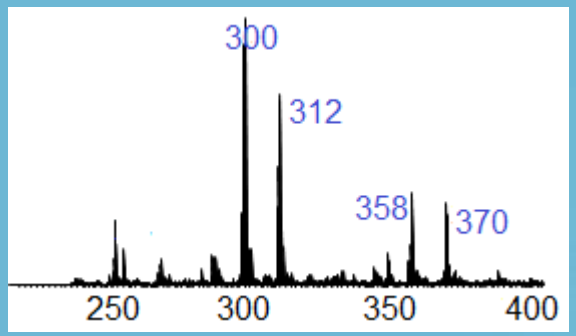


Cefas

Multiple Reaction Monitoring

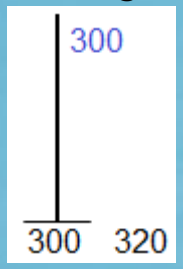
Principles

Spectrum with background ions

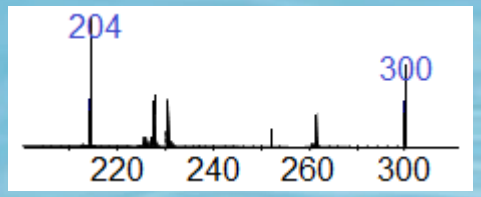


Chromatogram

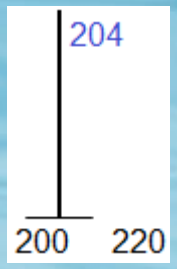
Q1 lets target ion 300 pass through



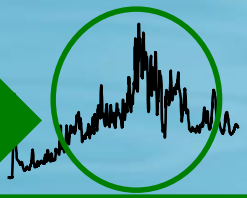
Collision cell breaks ion 300 apart



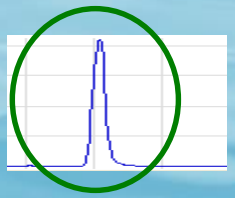
Q3 monitors 204 fragment from 300 ion



High background



Low background



Cefas

Methodology: MRM transition parameters

- MS1 Res = Unit, MS2 Res = Unit, cycle time = 400 ms, retention time (RT) window = 1 min, cell accelerator voltage = 5 V
- Tested on 3 systems – transferable to other systems

Analog	ESI+ Transition	CE +ve	RT (min)	ESI- Transition	CE -ve
STX	300.1>204.1, 138.0	31, 24	7.76		
NEO	316.1>126.1, 298.1, 220.1	30, 20	7.82		
dcSTX	257.1>126.1, 222.0	20, 20	7.73		
dcNEO	273.1>126.1 ,225.1	24, 18	7.73		
doSTX	241.1>60.0, 206.1	25, 20	7.50		
TTX	320.1>302.1, 162.1	28, 44	6.55		
GTX2			5.10	394.1>351.1, 333.1	20, 18
GTX3	396.1>298.1	16–20	5.90	394.1>333.1	22
GTX1			5.36	410.1>367.1, 349.1	15, 20
GTX4	412.1>314.1	18	6.36	410.1>367.1	15
GTX5	380.1> 300.1	15	6.98	378.1>122.0	22
GTX6	396.1> 316.1	12	7.26	394.1>122.0	24
dcGTX2			5.44	351.1>164.0, 333.1	22, 12
dcGTX3	353.1>255.1	15	6.43	351.1>333.1	18
dcGTX1			5.55	367.1>274.1, 349.1	20, 17
dcGTX4	369.1>271.1	20	6.60	367.1>349.1	16
C1			3.15	474.1>122.0, 351.1	38, 30
C2	396.1>298.1	15	3.71	474.1>122.0	38
C3	412.1>332.1	12	3.56	490.1>410.1	16
C4	412.1>314.1		4.28	490.1>392.1	20
Sodium			2.89	452.7>133.0, 588.6>316.8	26

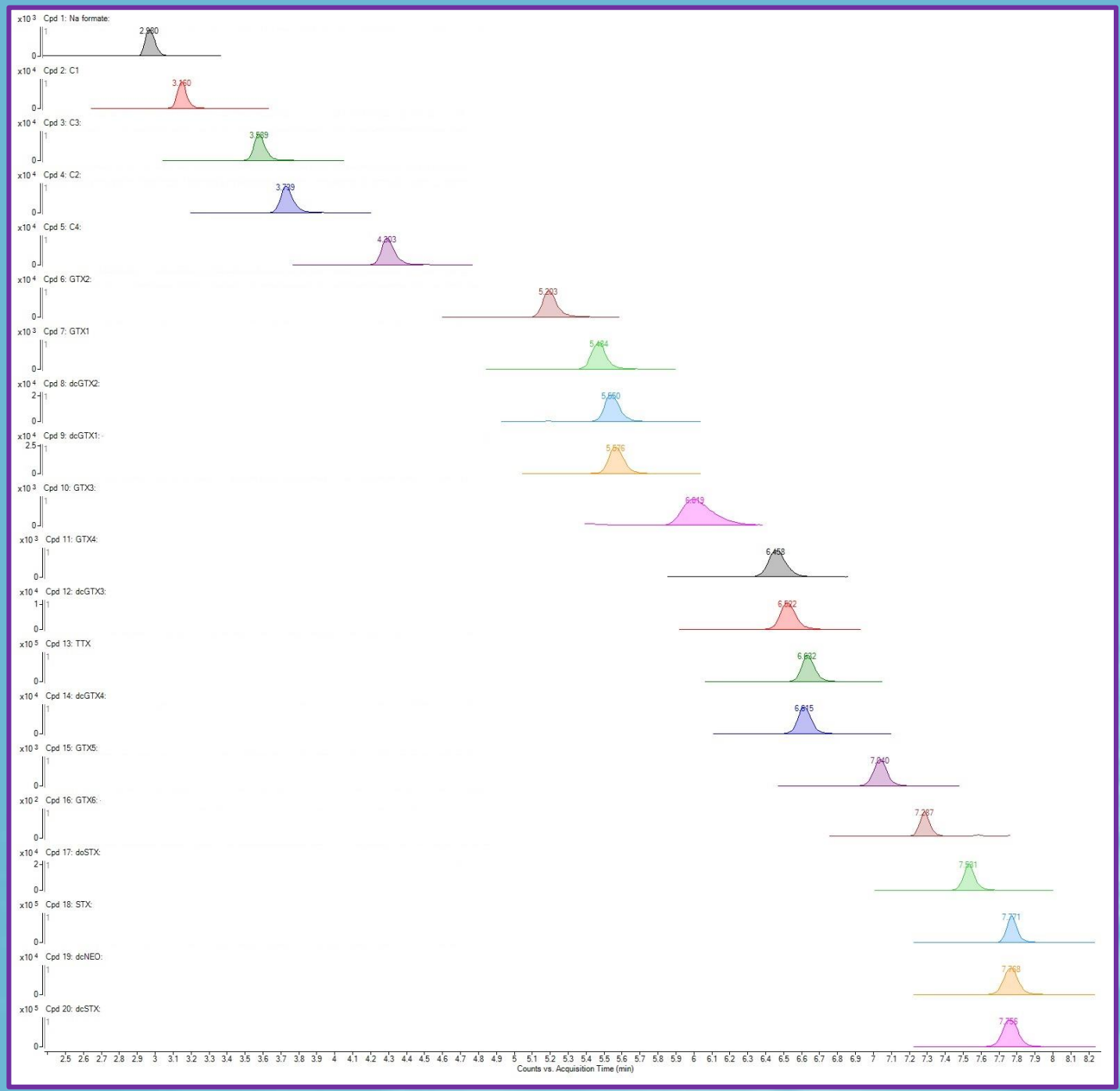
Preparing for analysis

- Scouting run to establish retention times
- Acquisition windows: 1 to 9 mins

Analogs	ESI+ Transitions	CE		ESI- Transition	CE
STX, dcSTX, dcNEO	300.1>204.1	31			
NEO	316.1>126.1	30			
C1&2				474.1>122.0	38
C3&4				490.1>410.1	16
doSTX	241.1>60.0, 206.1	20–25, 20			
TTX	320.1>302.1	28			
GTX2&3				394.1>333.1	20
GTX1&4				410.1>367.1	15
dcGTX2&3				351.1>164.1	15
GTX5	380.1>300.1	15			
GTX6	396.1>316.1	12			
Sodium				588.6>316.8	26

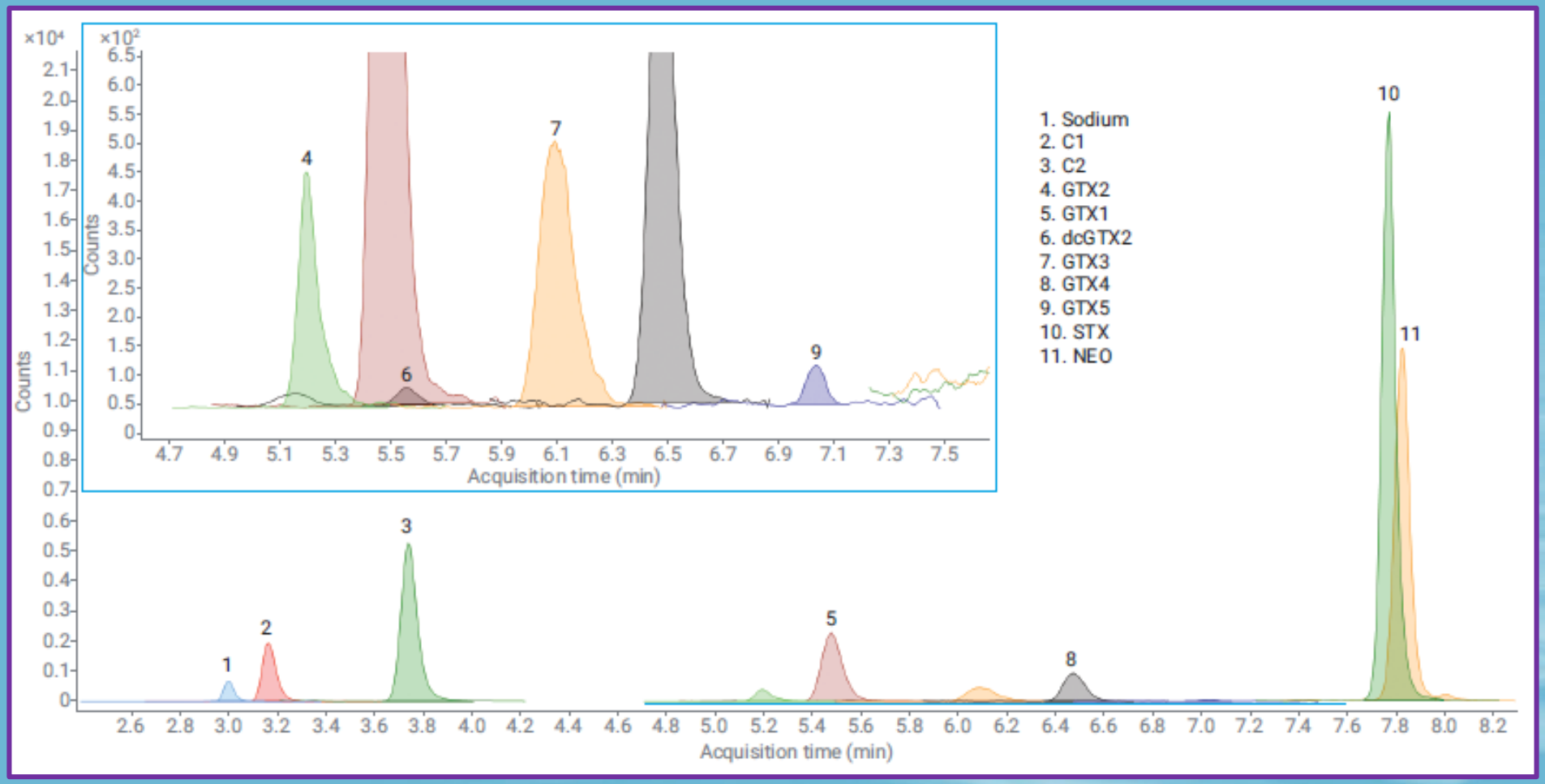
Cefas

- QC Mix
- dMRMs


x10 3 Cpd 1: Na formate:
2.980
x10 4 Cpd 2: C1
3.160
x10 4 Cpd 3: C3:
3.589
x10 4 Cpd 4: C2:
3.739
x10 4 Cpd 5: C4:
4.203
x10 4 Cpd 6: GTX2:
5.203
x10 3 Cpd 7: GTX1
5.484
x10 4 Cpd 8: dcGTX2:
5.660
x10 4 Cpd 9: dcGTX1:
5.876
x10 3 Cpd 10: GTX3:
6.613
x10 3 Cpd 11: GTX4:
6.458
x10 4 Cpd 12: dcGTX3:
6.622
x10 5 Cpd 13: TTX
6.832
x10 4 Cpd 14: dcGTX4:
6.815
x10 3 Cpd 15: GTX5:
7.040
x10 2 Cpd 16: GTX6:
7.287
x10 4 Cpd 17: dcSTX:
7.501
x10 5 Cpd 18: STX:
7.771
x10 4 Cpd 19: dcNEO:
7.768
x10 5 Cpd 20: dcSTX:
7.756
Counts vs. Acquisition Time (min)

Chromatograms for oyster matrix CRM

- Cefas POCRM used for trueness QC assessment



Chromatograms – separation requirements

1) In-source fragmentation

- MRMs are shared between some analytes
- MRMs are shared with other components e.g. M toxins

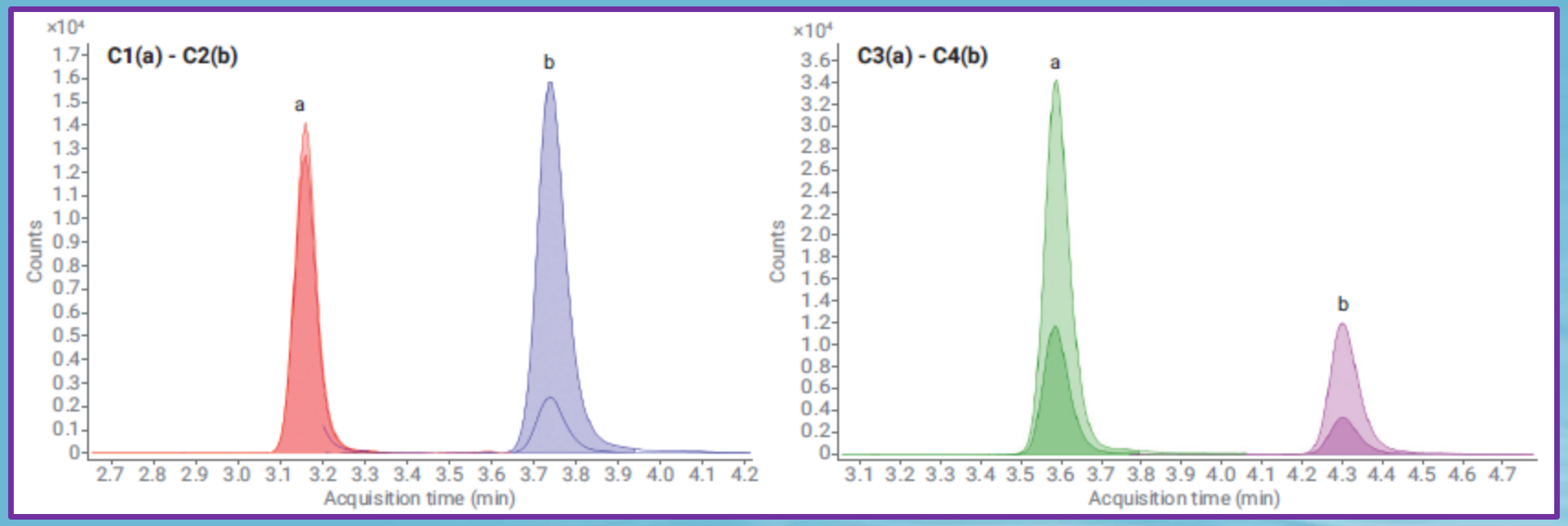
2) Epimeric pairs

- Identical masses + different toxicities = separation needed

Cefas

Chromatograms – separation of epimeric pairs

- C1 & C2
- C3 & C4



Cefas

Method verification on 1290-6495B

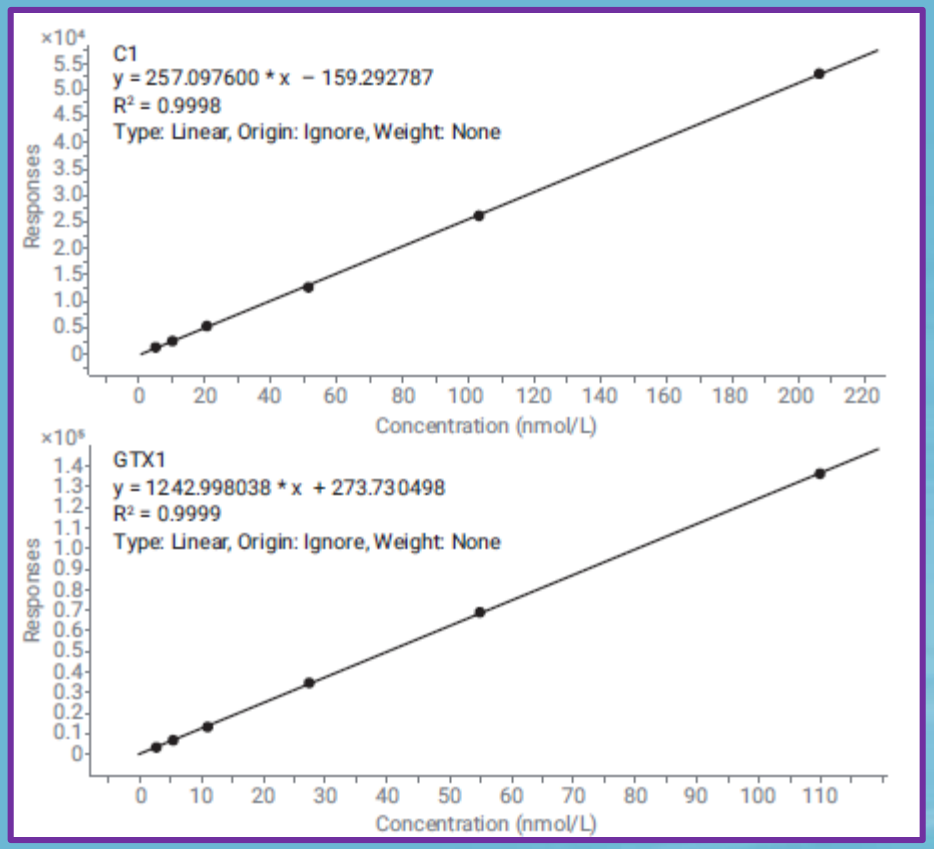
Agilent 6495B Triple Quadrupole LC/MS

EXPERIENCE THE HIGHEST LEVEL OF CONFIDENCE

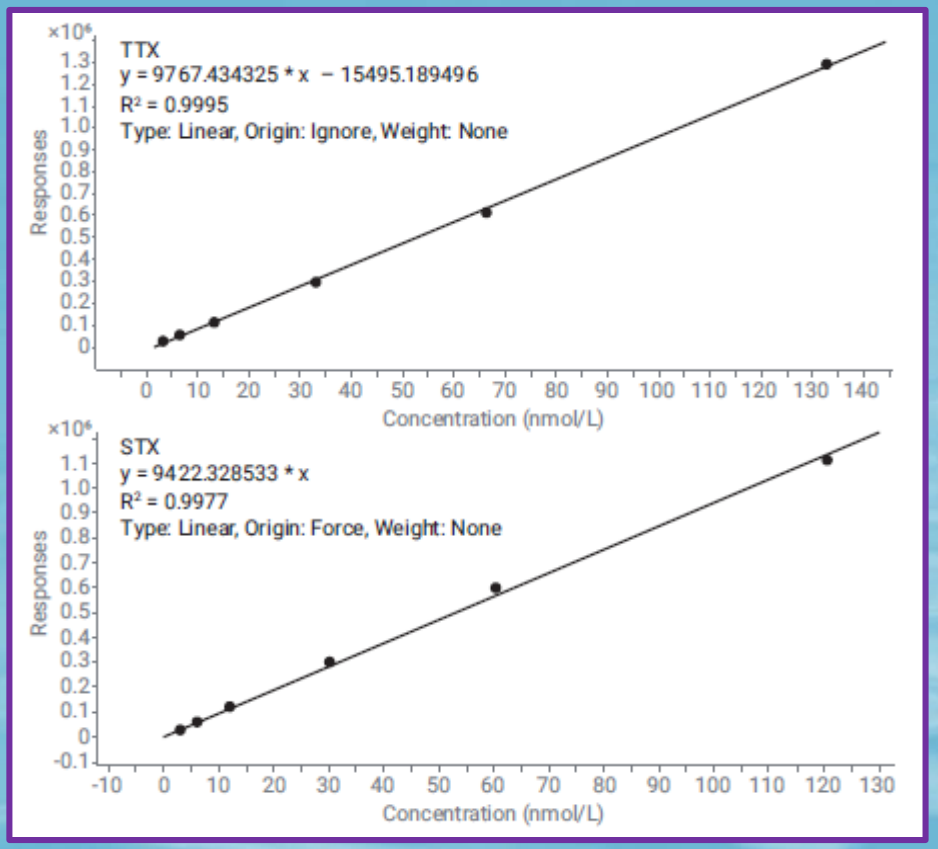
Cefas

Calibrations

- Matrix-modified calibrations
- Linearity acceptable over full calibration range
- Excellent dynamic range for quantitation



Negative mode



Positive mode

Cefas

Method verification assessment

- LOD = conc (nmol/L) with MRM S/N ratio = 3.0
- LOQ = conc (nmol/L) with MRM S/N ratio = 10.0
- Trueness = Repeat (n=33) analysis of matrix CRM over 1 year, comparing accuracy against certified values
- Precision (within batch) = repeat analysis of QC (n=42)
- Repeatability (between batch) = repeat analysis of QC (n=20) over one week
- Reproducibility (over one year) = repeat CRM (n=31)

Cefas

Verification results

Analyte	LOD	LOQ	Accuracy % (n=33)	Precision (1 batch; n=42)	Repeatability (1 week; n=20)	Reproducibility (1 yr; n=31)
C1	0.07	0.24	116%	0.06	0.10	0.24
C2	0.05	0.17	86%	0.06	0.11	0.19
C3	0.28	0.92	-	0.07	0.09	-
C4	0.33	1.09	-	0.09	0.11	-
dcGTX1	0.08	0.27	-	0.08	0.12	-
dcGTX2	0.24	0.78	-	0.08	0.12	-
dcGTX3	0.04	0.14	-	0.09	0.09	-
dcGTX4	0.05	0.15	-	0.17	0.18	-
dcNEO	0.18	0.59	-	0.14	0.08	-
dcSTX	0.15	0.49	-	0.15	0.12	-
doSTX	0.05	0.16	-	0.12	0.11	-
GTX1	0.02	0.08	96%	0.06	0.10	0.22
GTX2	0.13	0.43	107%	0.05	0.12	0.23
GTX3	0.30	0.99	112%	0.08	0.16	0.24
GTX4	0.29	0.72	89%	0.04	0.14	0.21
GTX5	0.37	1.22	96%	0.07	0.23	0.27
GTX6	0.15	0.50	-	0.15	0.20	-
NEO	0.06	0.19	96%	0.07	0.10	0.16
STX	0.02	0.08	115%	0.07	0.12	0.17
TTX	0.04	0.15	-	0.11	0.15	-

LOD/Q concentrations in nmol/L

Cefas

Verification results

Analyte	LOD	LOQ	Accuracy % (n=33)	Precision (1 batch; n=42)	Repeatability (1 week; n=20)	Reproducibility (1 yr; n=31)
C1	0.07	0.24	116%	0.06	0.10	0.24
C2	0.05	0.17	86%	0.06	0.11	0.19
C3						
C4						
dcGTX1						
dcGTX2						
dcGTX3						
dcGTX4						
dcNEO						
dcSTX						
doSTX						
GTX1						22
GTX2						23
GTX3						24
GTX4						21
GTX5						27
GTX6						
NEO						16
STX	0.02	0.08	115%	0.07	0.12	0.17
TTX	0.04	0.15	-	0.11	0.15	-

Note on sensitivity (LOD & LOQ)

When calculated in terms of µg STX eq/kg:

- LOD: 0.005 to 3.4 µg STX eq/kg (mean 0.85)
- LOQ of 0.02 to 11.4 µg STX eq/kg (mean = 2.85)

In comparison to MPL of 800 µg STX eq/kg, this represents excellent level of sensitivity

Provides excellent advance warning of developing toxicity event

LOD/Q concentrations in nmol/L

Cefas

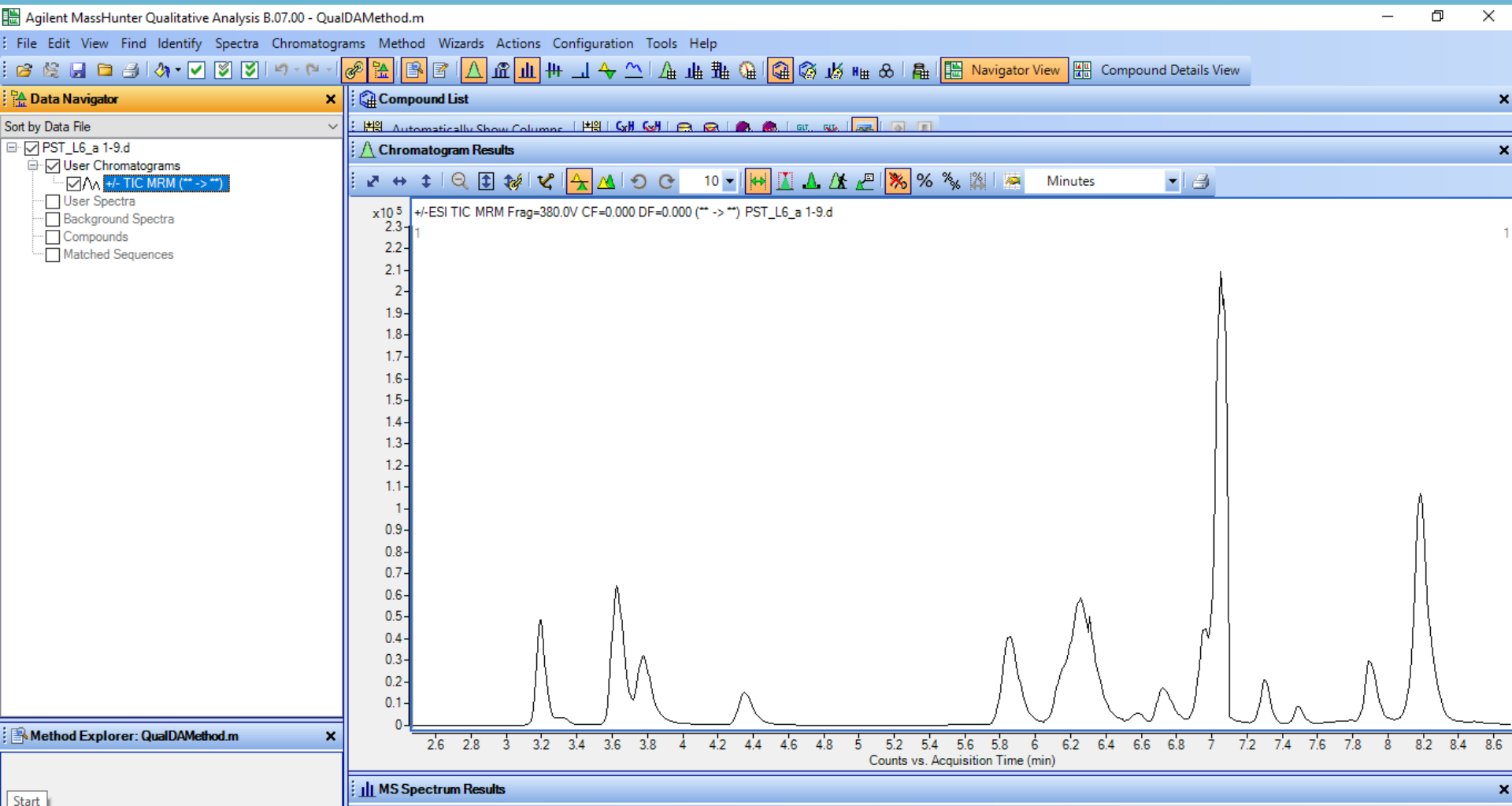
Quality control

- Keep sequence running continually with BEH Amide
- Correct column conditioning and shutdown/start-up cycles
- Use of “scouting run” to update dMRM acquisition method
- Resolution of all critical pairs
- Retention time drift for HILIC ≤ 5%
- Acquisition of sodium formate cluster for C toxin checks
- Linearity of calibrations – use of bracketed calibrations
- Correlation for both calibrations ≥ 0.98 across the batch
- Continuing calibration checks for extra long sequences
- Sensitivity check on low standards (both MRMs)
- Use of CRM + positive and negative control samples
- No carryover in procedural blanks

Cefas

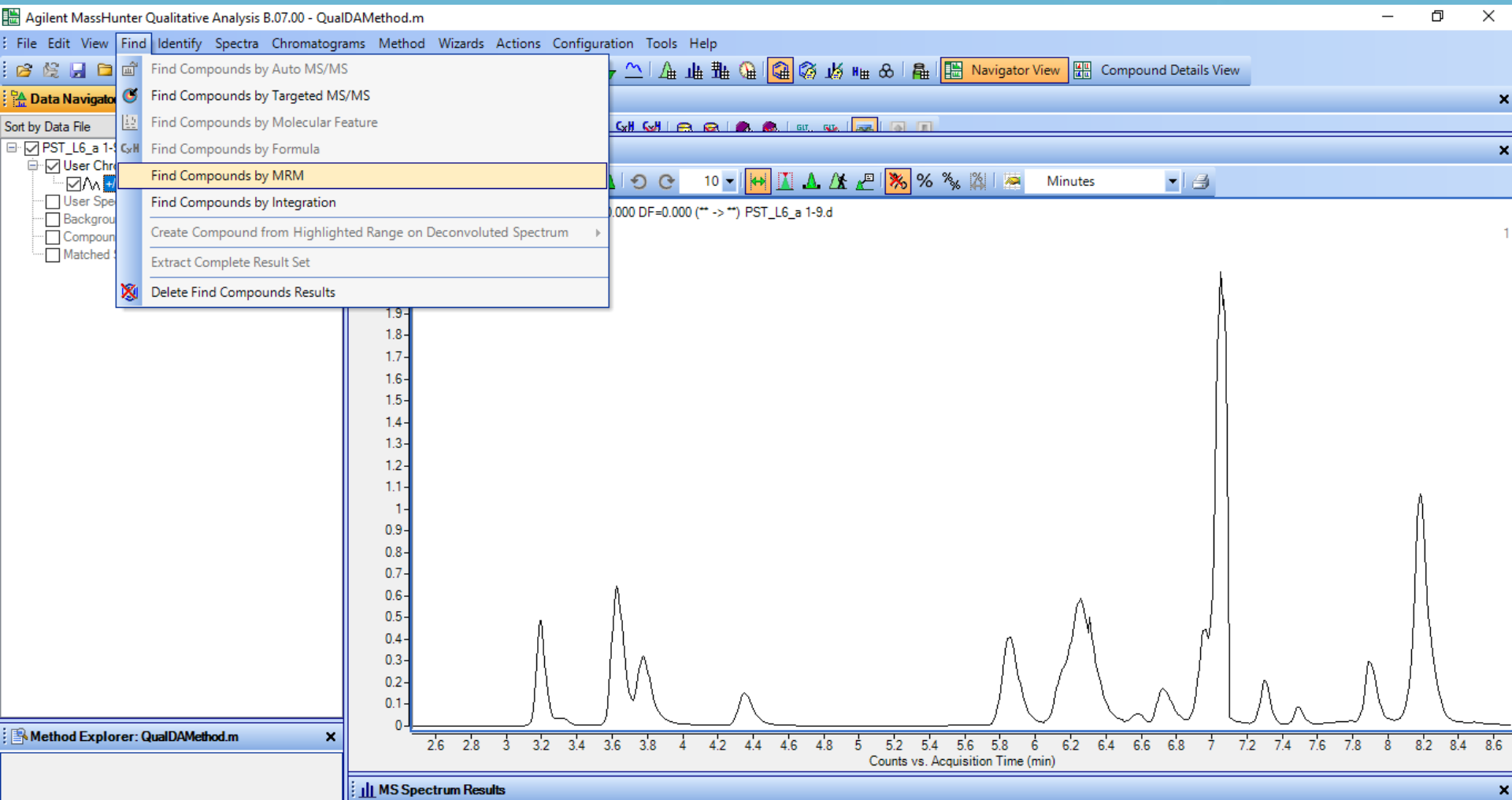
Qualitative analysis of data

- Using MassHunter Qualitative Analysis
- Provides useful system suitability check
- Load samples – check TIC



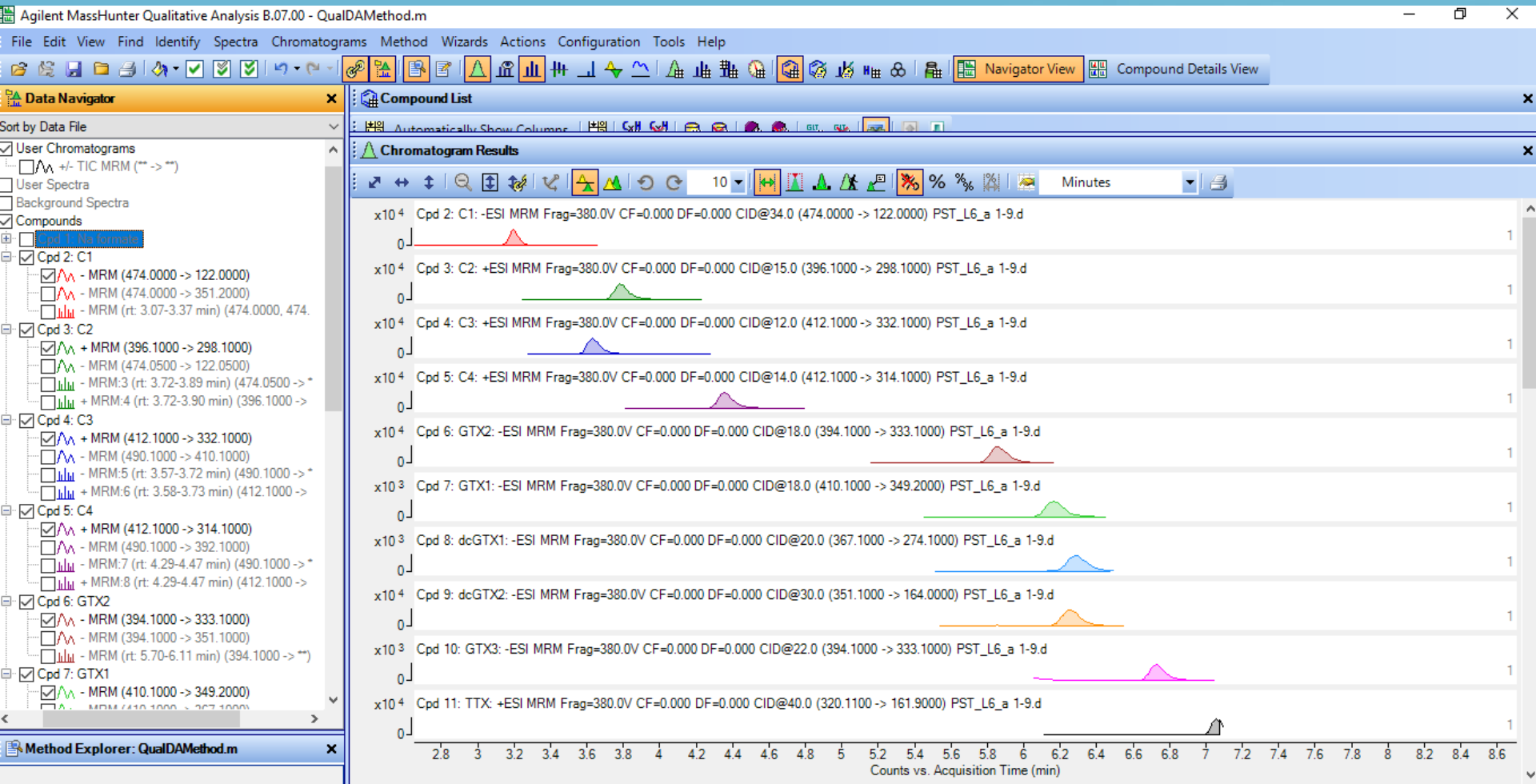
Qualitative analysis of data

- Using MassHunter Qualitative Analysis
- Click “Find compounds by MRM” to check all analytes:



Qualitative analysis of data

- Using MassHunter Qualitative Analysis
- Find compounds by MRM:

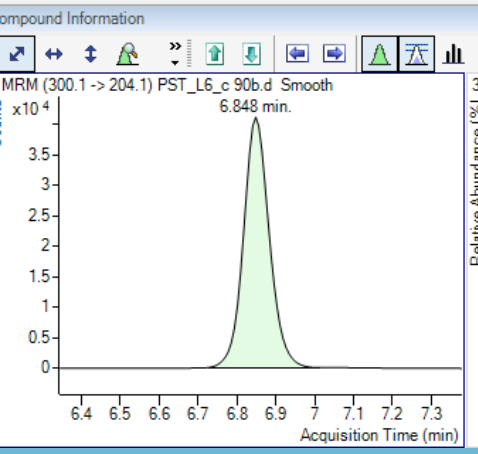


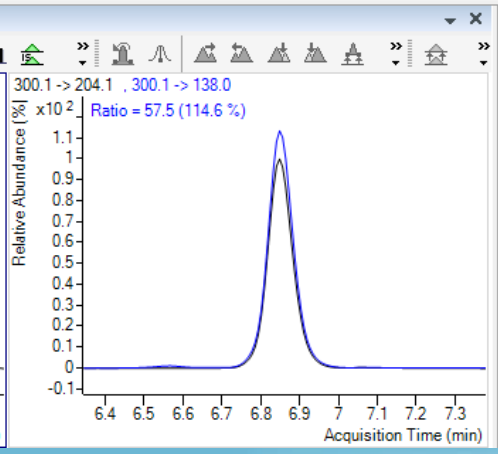
Identification of analytes in batch review

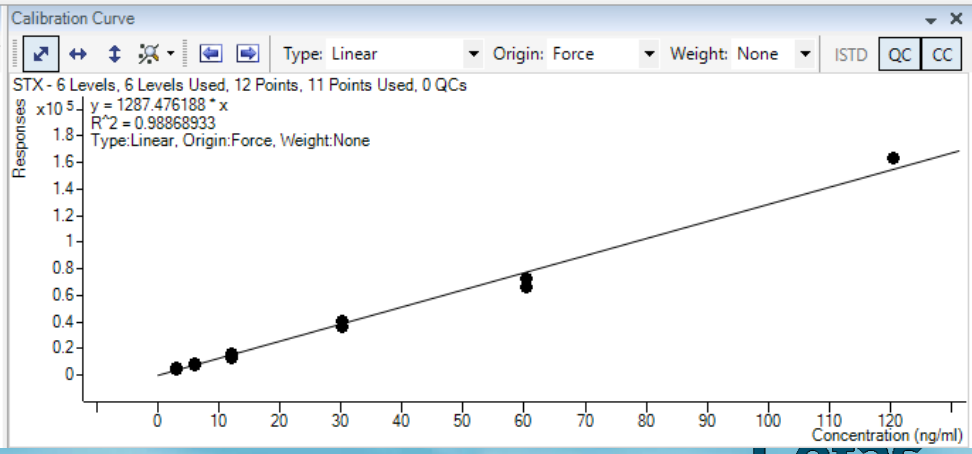
- Using MassHunter Quantitative Analysis
- Easy assessment of peak integration, ion ratios and calibration characteristics
- Clear flags – enables issue assessment

Sample: PST_L6_c 90b | Sample Type: <All> | Compound: STX | ISTD:

Sample						C4 Results		GTX5 Results			NEO Results			dcNEO Results			STX Results		
	Name	Data File	Type	Level	Acq. Date-Time	Final Conc.	Accuracy	RT	Final Conc.	Accuracy	RT	Final Conc.	Accuracy	RT	Final Conc.	Accuracy	RT	Final Conc.	Accuracy
	PST_L6_c 90b	PST_L6_c 90b.d	Cal	6	05/10/2019 01:35	68.1021	110.5	6.792	113.8418	107.1	6.832	166.7183	139.7	6.833	57.9442	108.4	6.848	147.8037	122.7
	PST_L5_c 90b	PST_L5_c 90b.d	Cal	5	05/10/2019 01:47	29.0487	94.3	6.792	51.0947	96.1	6.832	65.4471	109.7	6.833	25.8299	96.6	6.848	56.1932	93.2
	PST_L4_c 90b	PST_L4_c 90b.d	Cal	4	05/10/2019 01:59	15.2841	99.2	6.792	28.1105	105.7	6.824	32.4915	109.0	6.833	13.2342	99.1	6.848	31.3625	104.1
	PST_L3_c 90b	PST_L3_c 90b.d	Cal	3	05/10/2019 02:11	7.5477	122.4	6.792	12.5619	118.2	6.832	15.5368	99.1	6.833	6.6211	101.0	6.848	12.9773	107.7
	PST_L2_c 90b	PST_L2_c 90b.d	Cal	2	05/10/2019 02:23	3.2973	107.0	6.792	6.1811	105.7	6.824	6.0213	101.0	6.833	3.9344	112.4	6.848	6.8835	114.2
	PST_L1_c 90b	PST_L1_c 90b.d	Cal	1	05/10/2019 02:34	0.0000	0.0	6.792	4.2665	118.2	6.840	3.3512	112.4	6.818	3.4481	105.7	6.848	4.1744	138.5
	EMFF_Routine_ LRM 164	EMFF_Routine_ LRM 164.d	Sample		05/10/2019 02:47	0.0000	0.0	6.792	3.8305	116.3	6.832	13.4934		6.896	2.5175	118.2	6.848	19.1600	
	EMFF_Routine_ PB	EMFF_Routine_ PB.d	Sample		05/10/2019 02:58	0.0000	0.0							6.911	0.3250		6.754	0.0000	0.0
	EMFF_Routine_ EC81 w	EMFF_Routine_ EC81 w.d	Sample		05/10/2019 03:10	0.0000	0.0	7.009	0.0000	0.0									
	EMFF_Routine_ EC 81 b	EMFF_Routine_ EC 81 b.d	Sample		05/10/2019 03:22	0.0000	0.0												
	EMFF_Routine_ EC 82 w	EMFF_Routine_ EC 82 w.d	Sample		05/10/2019 03:34	0.0000	0.0	7.071	0.0000	0.0	6.871	0.0000	0.0	6.659	0.0000	0.0			
	EMFF_Routine_ EC 82 b	EMFF_Routine_ EC 82 b.d	Sample		05/10/2019 03:46	0.0000	0.0							6.754	0.0000	0.0			

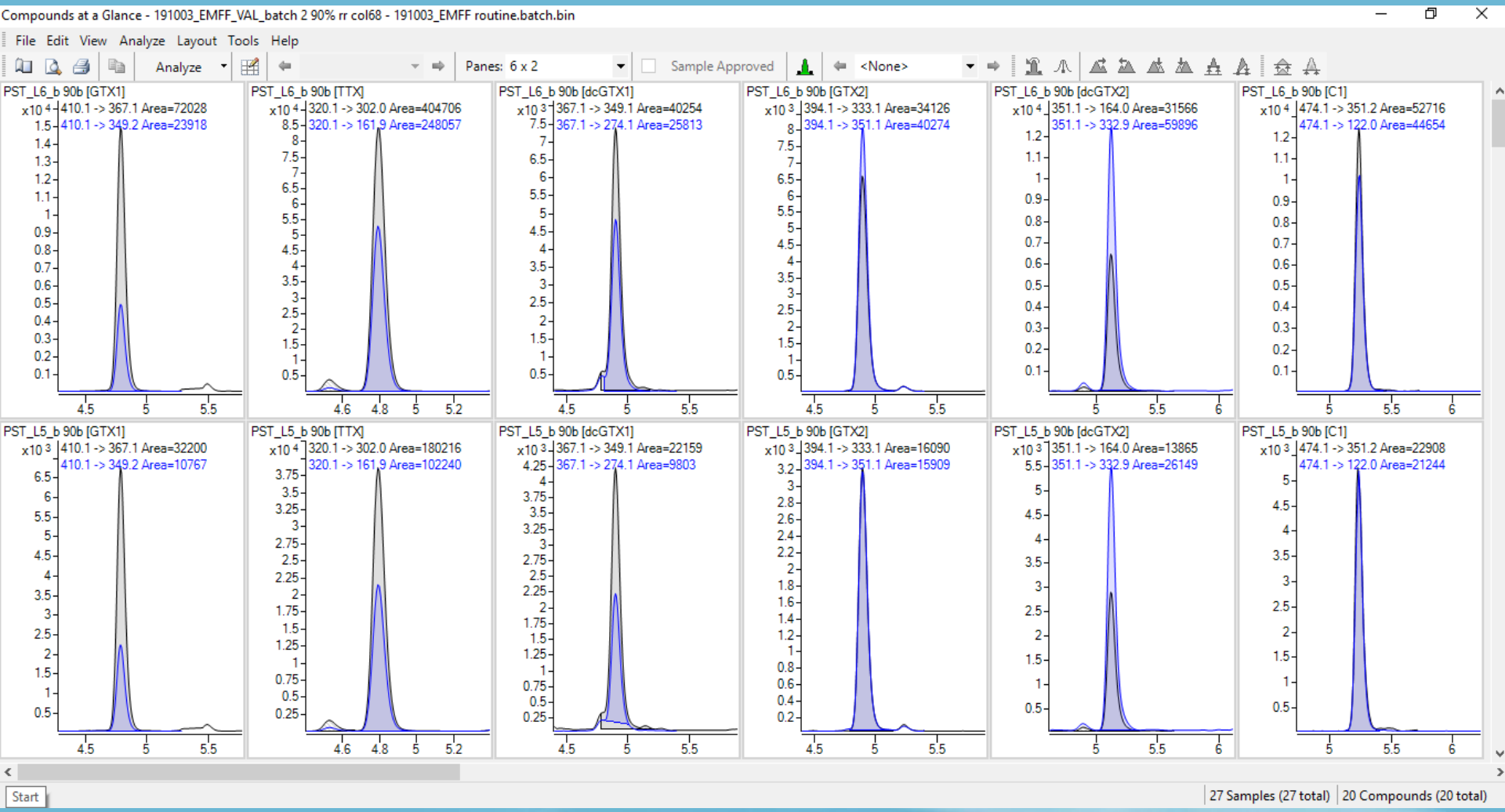






“Compounds at a glance”

- Using MassHunter Quantitative Analysis
- Easy integration and mass “noise peak” removal – speeding up data assessment hugely



International collaborative validation study

Agilent 6495B Triple Quadrupole LC/MS

EXPERIENCE THE HIGHEST LEVEL OF CONFIDENCE

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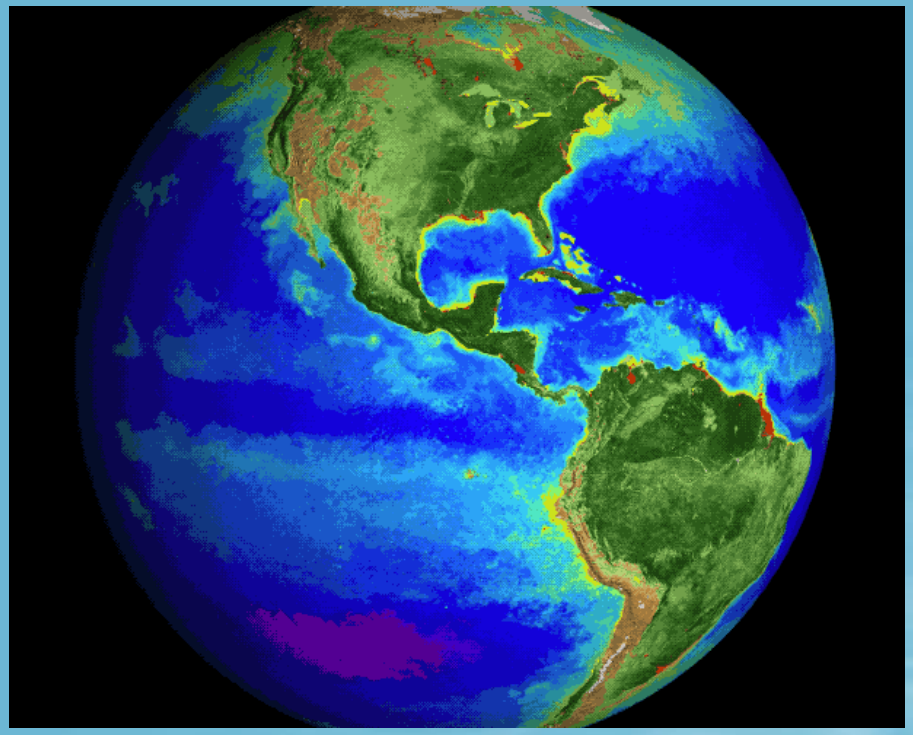
Collaborative Study



Global study with globally-sourced materials for a global end user group

- In order to use method for official control, must be “Internationally Accepted”
- Following AOAC guidance

Main Study

- 24 participating laboratories
- 5 continents

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Materials – raw tissues

- 94 separate shellfish tissues provided for study
- 6 continents

15 Shellfish species:

- **Mussels** (*Mytilus edulis, Perna canaliculus, Mytilus galloprovincialis, Mytilus chilensis*)
- **Clams** (*Spisula solida, Venerupis pullastra, Mesodesma donacium, Mercenaria mercenaria, Paphies australis*)
- **Oysters** (*Magallana gigas*)
- **Cockles** (*Cerastoderma edule, Acantocardia tuberculata*)
- **Scallops** (*Pecten maximus*)
- **Razorshells** (*Ensis* sp.)
- **Geoduck** (*Panopea generosa*)

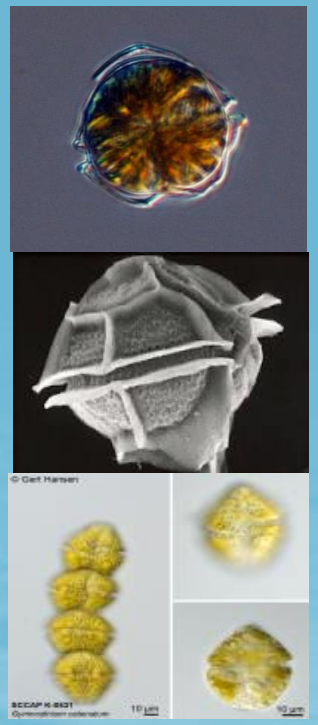






Materials – raw tissues

- Source algae
  - *Alexandrium* (*catenella*, *(fundyense), minutum*, *pacificum*)
  - *Pyrodinium bahamense*
  - *Gymnodinium catenatum*
- Toxicity & toxin profiles
  - Highly variable
- Analogues
  - 20 included (all except M toxins)
- TTX
  - Naturally present in 2 samples
  - Spiked into 1 other sample



Sensitivity & Linearity

Sensitivity dependent on system used

Highest sensitivity:

<1 µg STX eq/kg quantifiable

Lowest sensitivity:

Mean lowest calibration std conc = 15 µg STX eq/kg

Linearity

- Mean r^2 = 0.995
- Range 0.989-1.000
- Across all systems
- Not system dependent

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Performance summary

- Trueness – mean results vs expected
  - 85 characterised values for assessment in 21 labs
  - Total PST trueness = 1.02 ± 0.09
  - Mean PST analogue trueness = 1.03 ± 0.19

- Repeatability (RSD_r) – duplicate variability
  - Acceptable for majority of duplicates

- Reproducibility (RSD_R) – variability between labs
  - Total PST: RSD_R = 17% across 21 labs

- HorRat – acceptability of RSD_R (<2.0)
  - 1 out of 157 results >2.0 (>99% acceptable)

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Comparison with other methods

	RBA	Pre-COX	PCOX	LC-MS/MS
Analogues	Toxicity	16	15	25
M toxins	?	n	n	Y
TTX	(Y)	n	n	Y
Trueness	89%	73%	104%	102%
RSD_r %	nd	33%	21%	17%
RSD_R %	33%	29%	27%	26%
HorRat	2.0	1.5	1.2	1.6
% HorRat >2.0	-	10.6%	12.2%	3.3%
Species	9	4	4	15

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HILIC column options

Agilent 6495B Triple Quadrupole LC/MS

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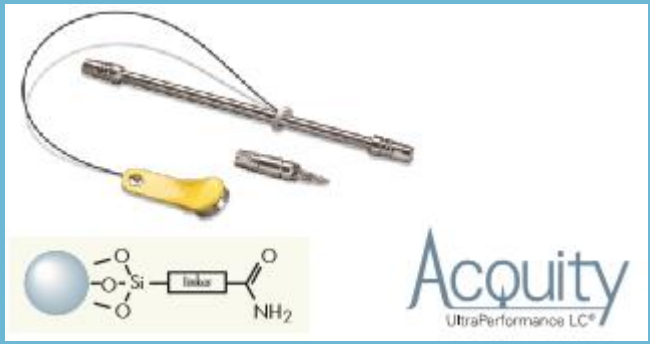
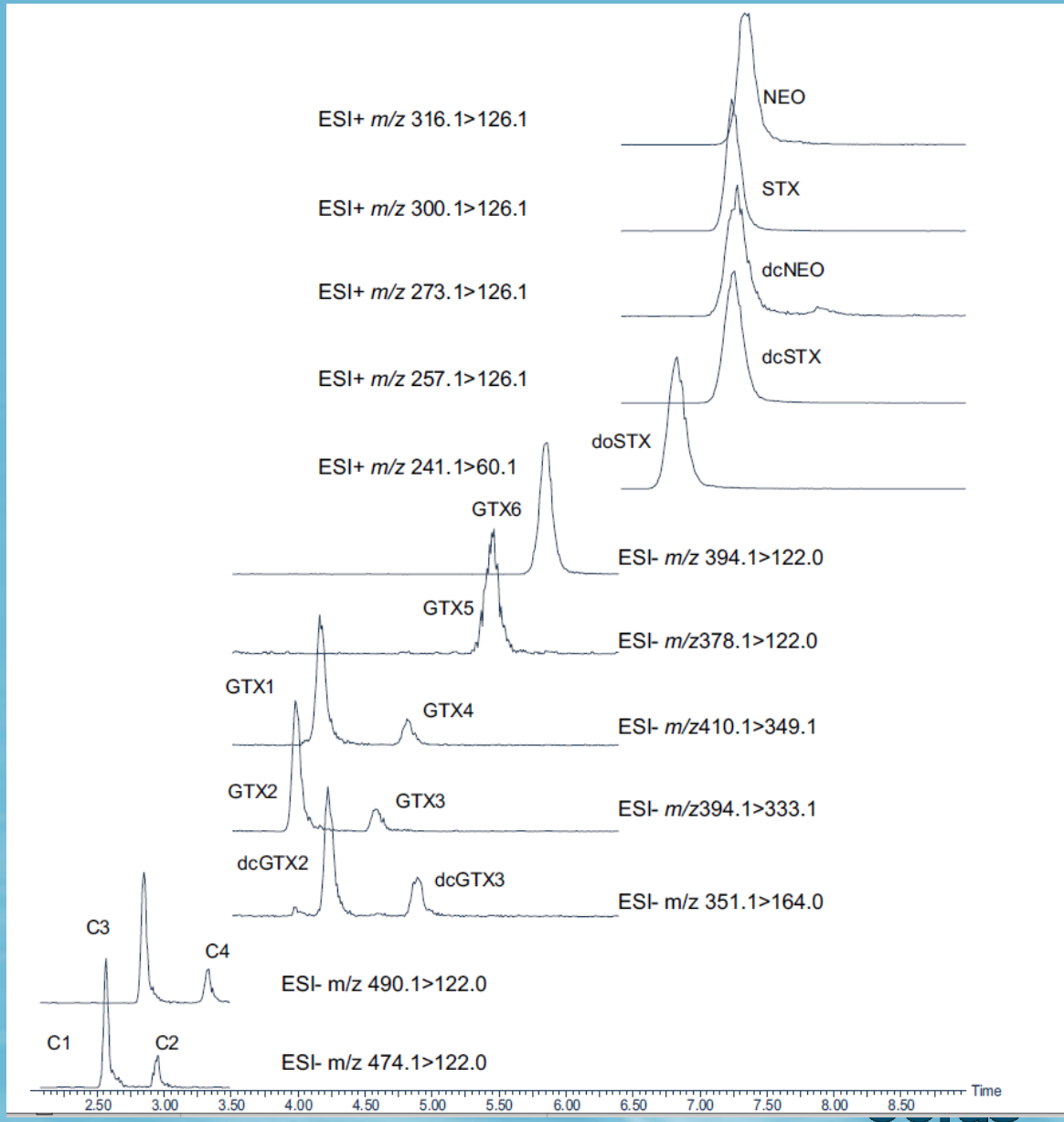
UHPLC column options

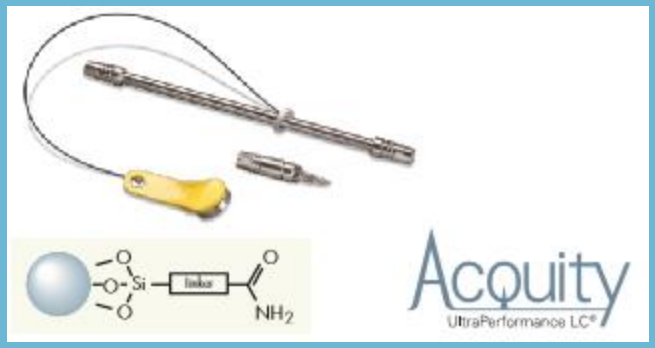
BEH Amide HILIC



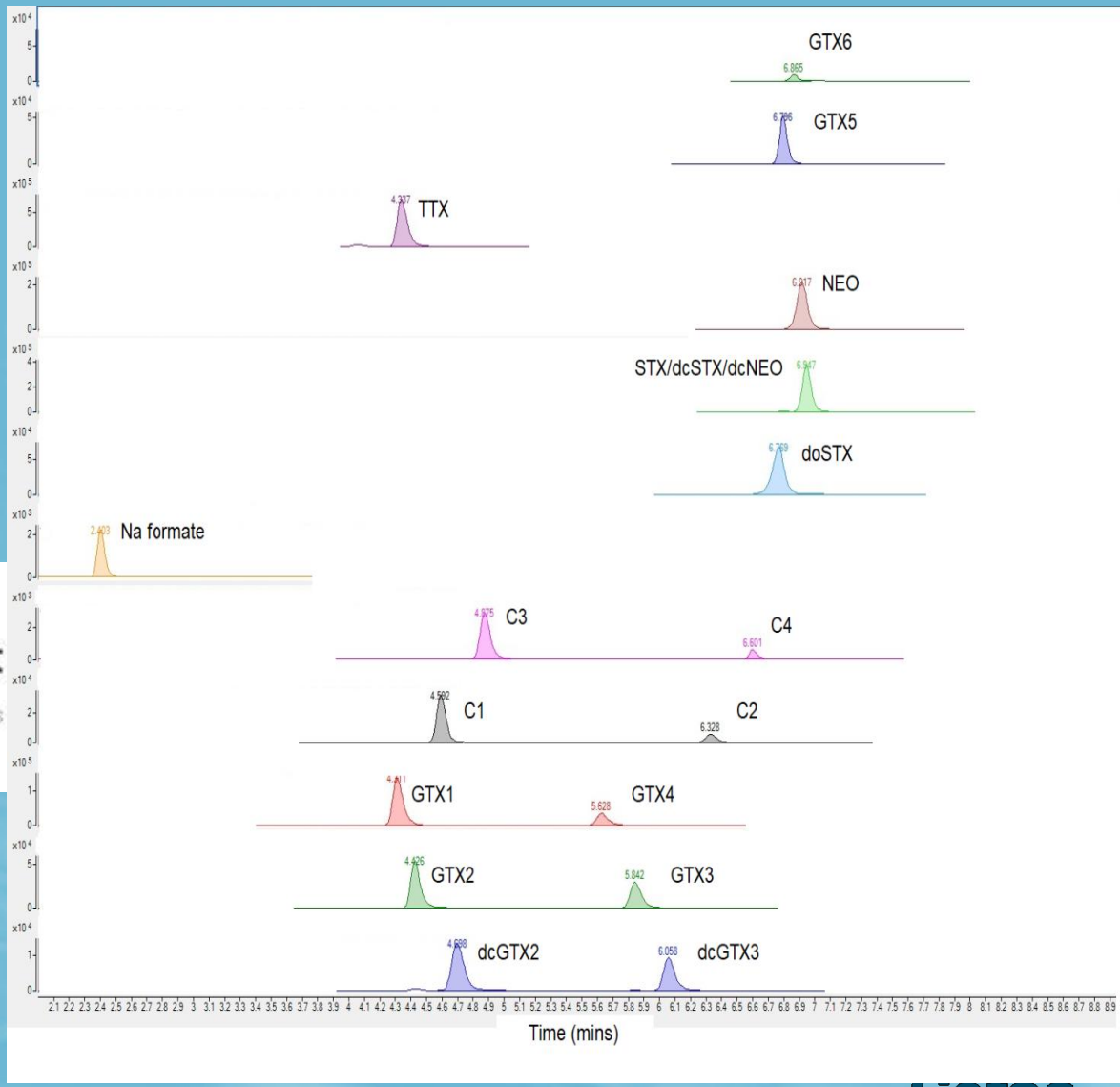


UHPLC column options

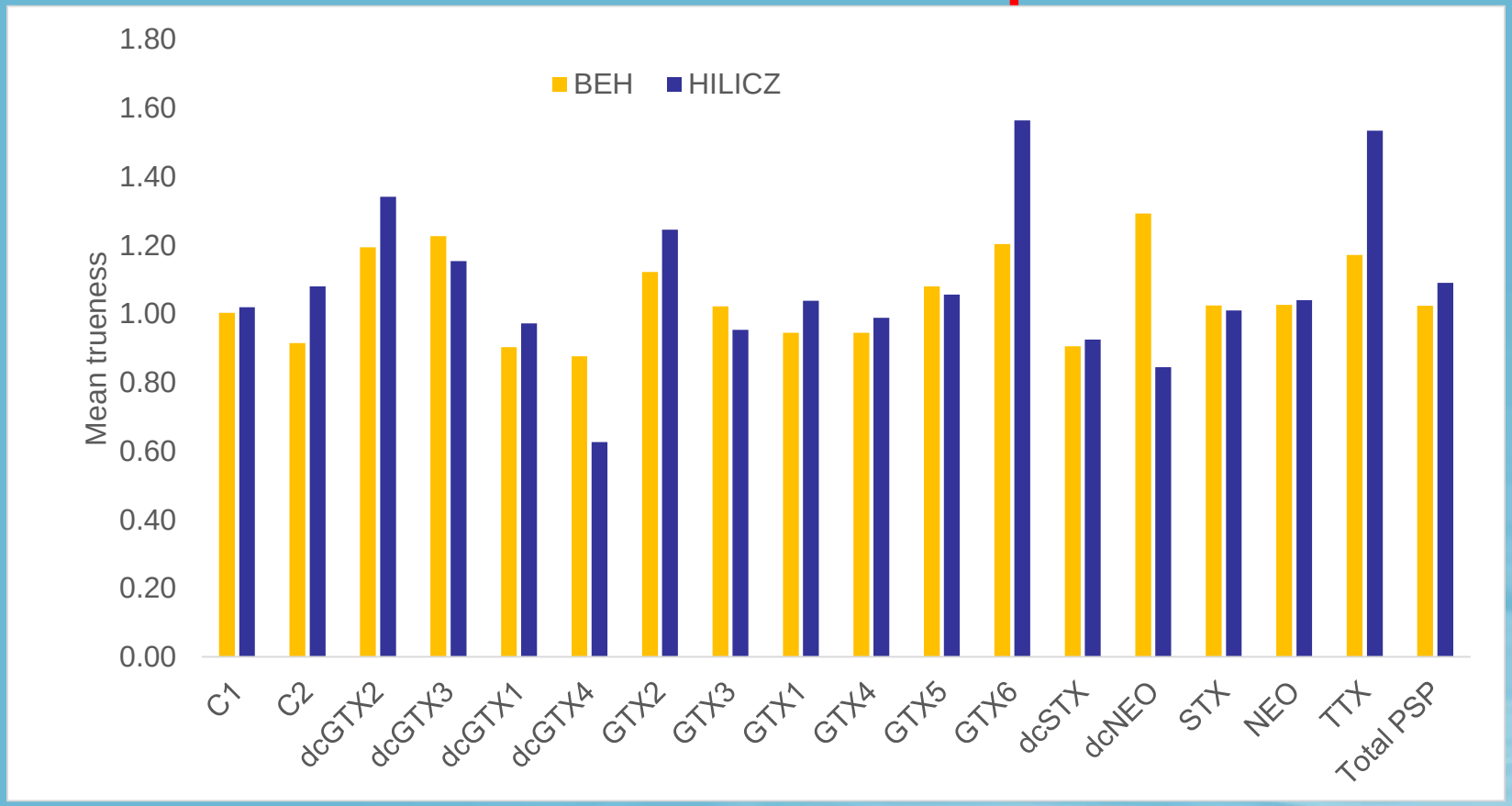
Waters BEH Amide HILIC



Agilent Poroshell HILIC-Z



UHPLC column options



Mean trueness

Agilent Poroshell HILIC-Z
1.06

Waters BEH Amide HILIC
1.04

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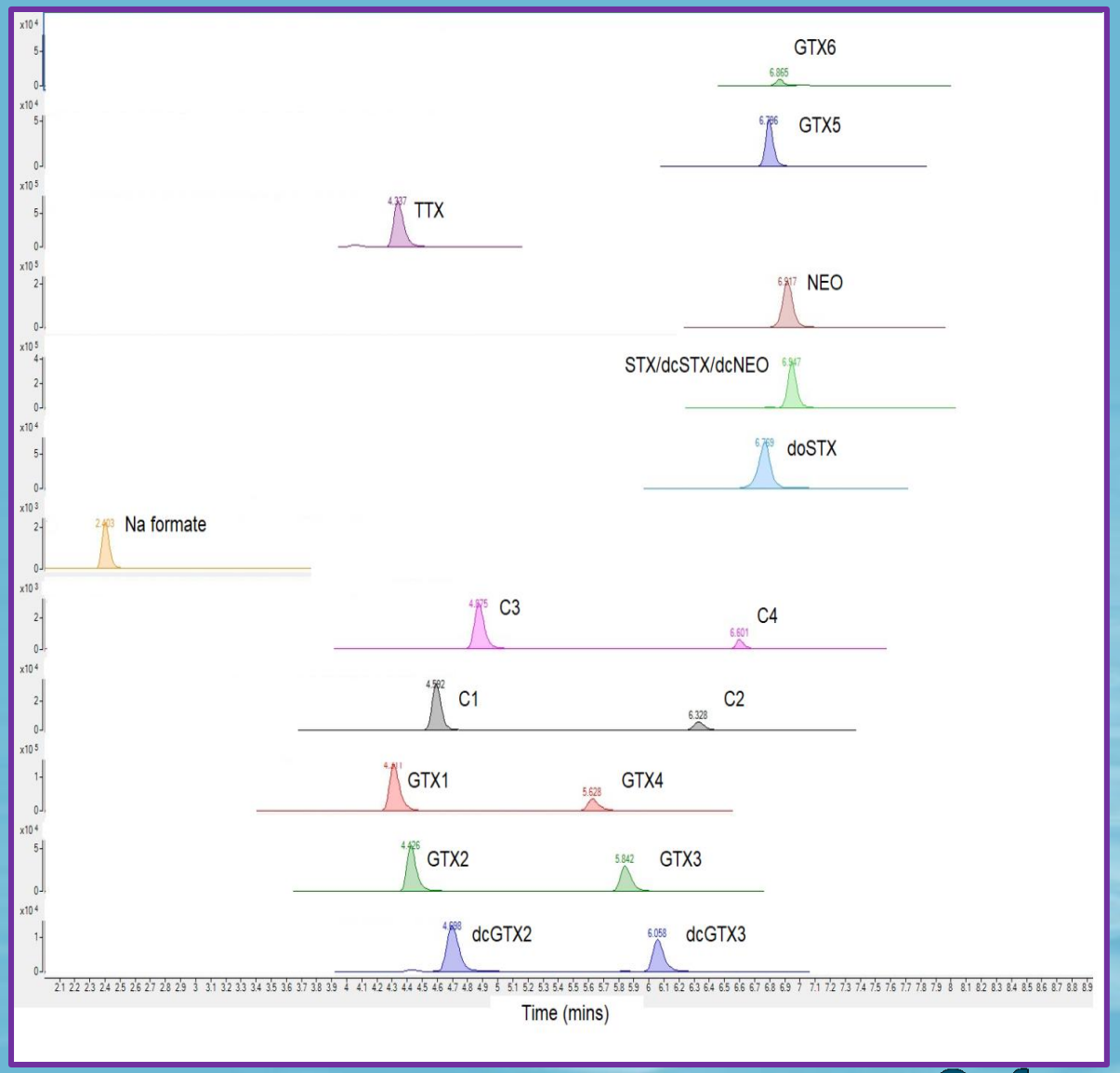
Agilent HILIC-Z column

Agilent Poroshell HILIC-Z

- No complex conditioning and start-up/shutdown cycles required
- Retention times more stable
- Greater separation from sodium

- Less separation between some analogues

- Work ongoing assessing multiple batches of columns



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Overall conclusions

Agilent 6495B Triple Quadrupole LC/MS

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Use of Agilent 1290-6495B

- Application note - PSTs & TTX in shellfish in 11 mins
- Two interference-free MRM transitions for each compound
- LOQs ≤16 µg STX eq/kg
- Robust, repeatable and reproducible.
- Matrix-modified calibration curves with excellent linearity
- Simple, rapid, and cost-effective in terms of analyst time, consumables and the absence of expensive internal standards

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Agilent Technologies

Interreg Atlantic Area European Regional Development Fund

ALERTOXNET ATLANTIC AREA NETWORK FOR INNOVATIVE TOXICITY ALERT SYSTEMS FOR SAFER SEAFOOD PRODUCTS

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Collaborative study participants

Names	Laboratory	Country
Ana Gago-Martinez; Jose Manuel Leao	EURLMB	Spain
Eva Cagide; Mercedes Alvarez; Alvaro Queijo; Carmen Alfonso	CIFGA	Spain
Fabiola Arévalo; Angeles Morono; Covadonga Salgado	Intecmar	Spain
Veronique Savar; Damien Reveillon; Zouher Amzil	Ifremer	France
Marina Nicolas; Vincent Hort	ANSES	France
Anna Milandri; Marinella Pompei; Monica Cangini; Stefani Milandri; Sonia Dall'Ara	CRM	Italy
John Bunaes; Stine Therese Aanrud	NMBU	Norway
Bob Hatfield	CTL	Great Britain
Ben Maskrey	Cefas	Great Britain
Dermot Faulkner	AFBI	Northern Ireland
Sara McGrath	USFDA	USA
Krista Thomas; Pearse McCarron	NRC	Canada
Eric Braekevelt	Health Canada	Canada
Wade Rourke; Ryan Gibbs; Melanie Casey	CFIA	Canada
Maria Fernanda Barrera; Daniel Carrasco; Benjamin Suarez-Isla	University of Chile	Chile
Tim Jordan; Joe Hutchinson	AST	Australia
Bryan Stokes	Cawthron Institute	New Zealand
Colin Hayman; Sam Murray	CNC	New Zealand
Pui-Kwan Chan	Government Laboratory	Hong Kong
Toshi Suzuki; Oikawa Horoshi; Ryuichi Watanabe	Fisheries Research Agency	Japan

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Thank you for listening

Questions about application note please contact:

László Tölgyesi (Agilent) laszlo_toelgyesi@agilent.com

Andrew Turner (Cefas) andrew.turner@cefas.co.uk

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