Use of chemical detection methods for determination of marine biotoxins in shellfish

Cefas INTERNATIONAL CENTRES OF EXCELLENCE

SEAFOOD SAFETY

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Natural Aquatic Toxins Team

- Official control testing of molluscs in Great Britain
- Research activities in toxins field
- Collaborative science with other countries
- Active publishers of new toxin science
- Reference materials, ring trials
- Method validation activities
- Marine and freshwater
- Invertebrates, fish, water and algae/bacteria
- “One Health” approach

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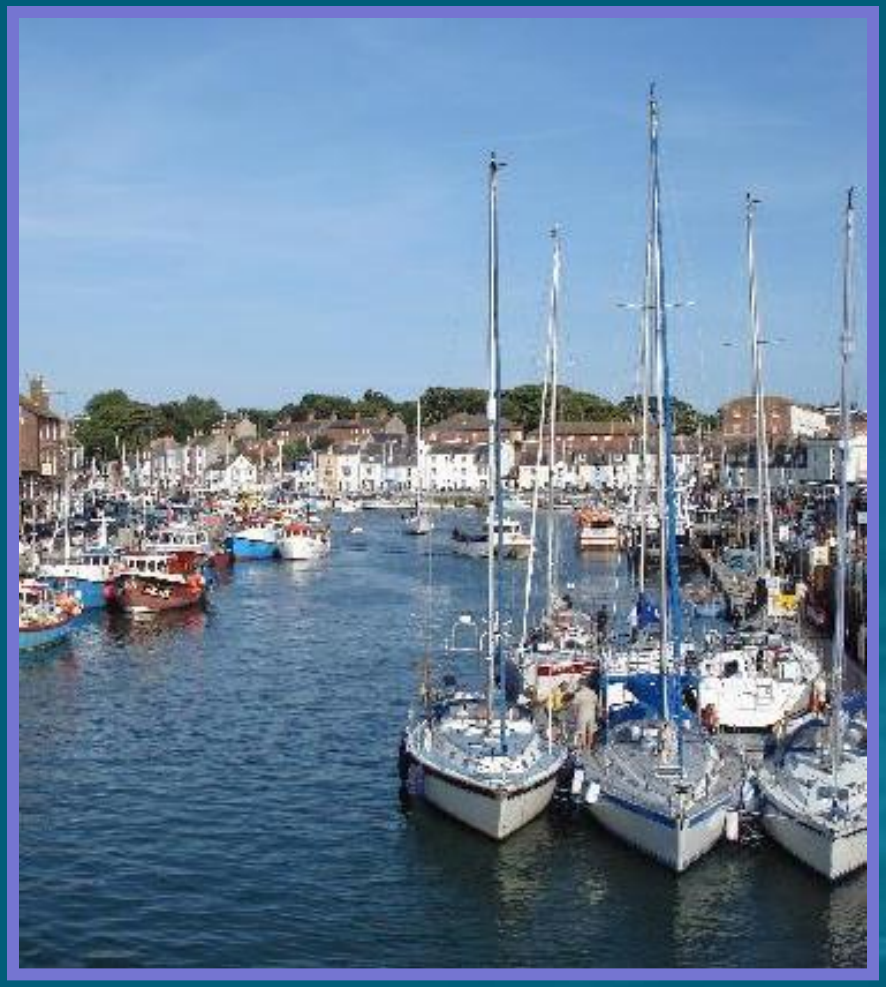
Overview



- Shellfish toxin background
- Laboratory processes
- Current testing methods
- Validation / implementation
- Method developments
- Emerging toxin threats

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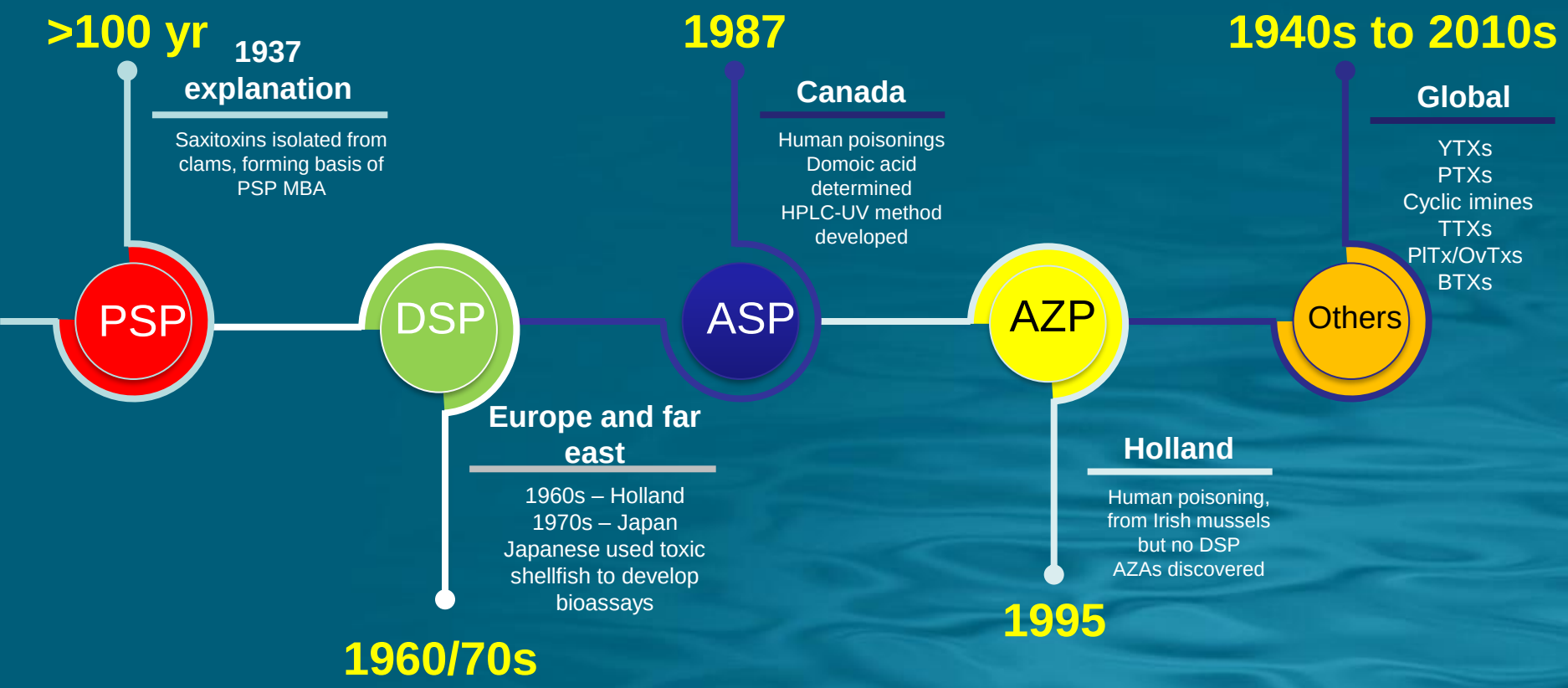
Background

A brief history of shellfish toxin discovery



1947 – Brevetoxins (Gulf of Mexico); 1986 – Yessotoxins (Japan); 1989 – Pectenotoxins (Japan); 1980s – Palytoxin/ovatoxins (Hawaii, Japan, Mediterranean); 1990s – Cyclic imines (Canada, NZ); 2000s – Tetrodotoxins (Japan, NZ, UK);

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Regulated toxins and microalgal producers:

ASP

(Domoic/epi-domoic acid)

- Nausea
- Diarrhoea
- Vomiting
- Confusion
- Memory loss
- Can be fatal

20 mg/kg

LT

(Lipophilic toxins include:

OA, DTX. YTX, PTX and AZA groups)

- Nausea
- Abdominal pains
- Vomiting
- Diarrhoea
- May be tumourigenic

160 µg/kg
3.75 mg/kg

PSP

(Saxitoxins)

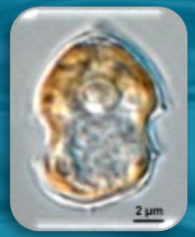
- Numbness/tingling
- Headaches
- Nausea, Vomiting
- Respiratory distress
- Paralysis
- Can be fatal

800 µg STX eq./kg

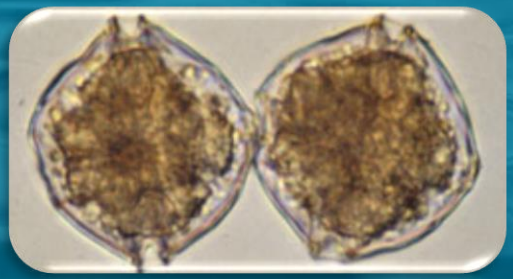
Pseudo-nitzschia spp.

Prorocentrum lima

Dinophysis spp.



Azadinium spinosum



Alexandrium spp.

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Toxin testing methods – history

- ASP – HPLC-UV (Reference Method)
- PSP – until 2006 – all using PSP MBA[a]
  - Pressure to replace use of animals
  - New methods required
- DSP - until 2011 – all using DSP MBA[b]
  - Similar pressure to replace animals
  - Methods required detection of all other lipophilic toxins (LT) – DSP, PTXs, AZAs, YTXs,

[a]AOAC 959.08; [b]based on Yasumoto et al., 1978

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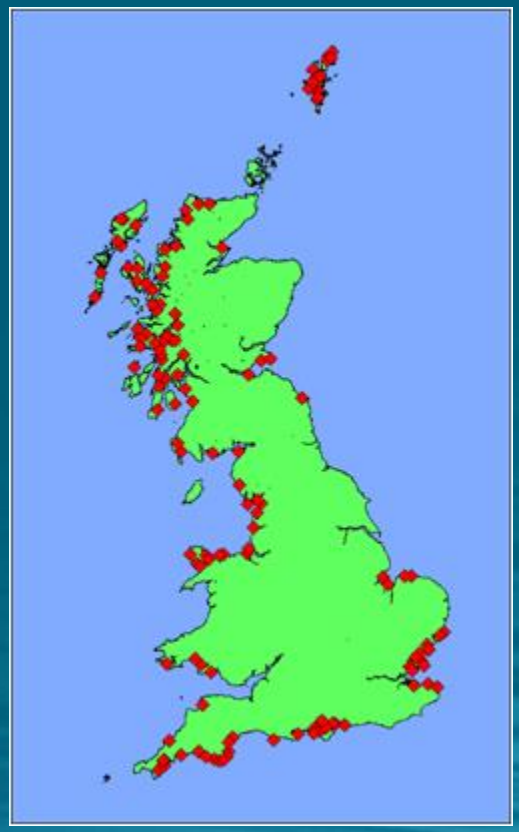
Typical monitoring e.g. UK

Flesh monitoring programme

- Samples of shellfish are collected from pre-determined monitoring points
- Results used to inform opening of harvesting areas

Phytoplankton monitoring programme

- Water samples are also collected from pre-determined sites
- Presence of microalgal species/genus of concern above thresholds results in increased shellfish testing



•>200 sites

•>3,000 samples per year

•Covering all of GB

•5-55 per day

Shellfish testing process

- Samples received - daily
- Shellfish shucked, >50g tissue homogenised
  - Min 10 organisms per sample
- Sub-samples for each of three testing methods
- Extraction, clean-up
- Analysis overnight
- Results reported next day (customer requirement)
  - Results >MPL = shellfish beds closed for harvest
  - Two consecutive <MPL to re-open

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Testing methods

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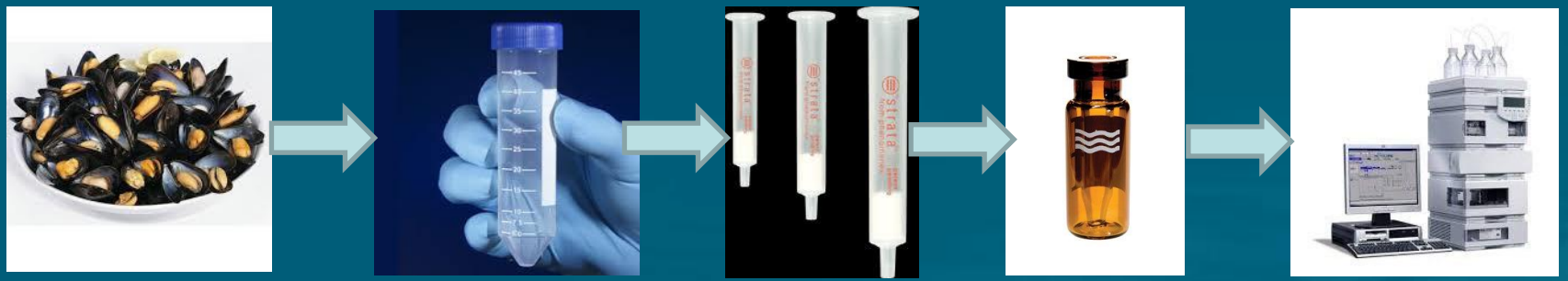
Biotoxin testing methods

All involve:



- Homogenisation (blending)
- Solvent extraction (to remove toxins from shellfish)
- Clean-up (chemical and/or physical)
- Analysis
  - Separation
  - Detection

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Homogenisation step

Shellfish

Homogenisation step

- Sample representative of sampling area
- Homogenisation (blending) – Critical step

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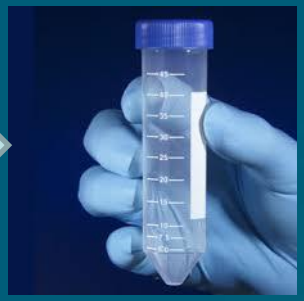
Extraction step

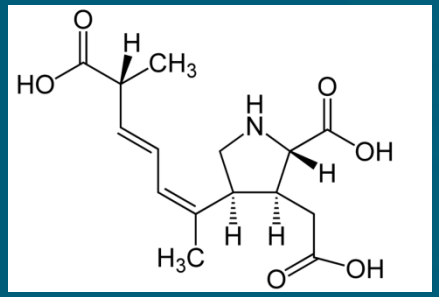
Shellfish



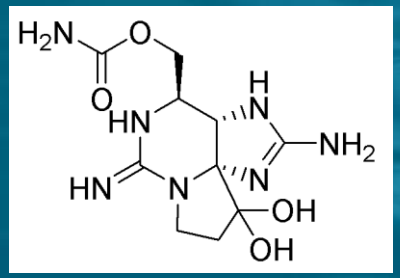
Extract

Biotoxin extractions

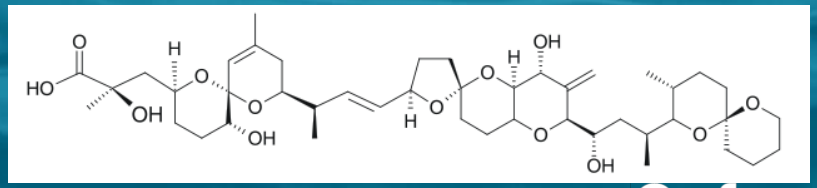
ASP	PSP	DSP
Hydrophilic	Hydrophilic (ionic)	Lipophilic
•Contains many hydroxyl groups •Forms many H-bonds with water		•Long chain carbon ring structure (non-polar) •Few H-bond forming substituents
•Methanol/water	•Weak acetic acid	•Methanol
•Will extract mainly polar compounds	•Extract hydrophilic and ionic compounds	•Methanol will solubilise lipophilics, but less tendency to extract very hydrophobic compounds



Domoic acid (ASP)



Saxitoxins (PSP)



Lipophilic toxins (*e.g.* DSP)

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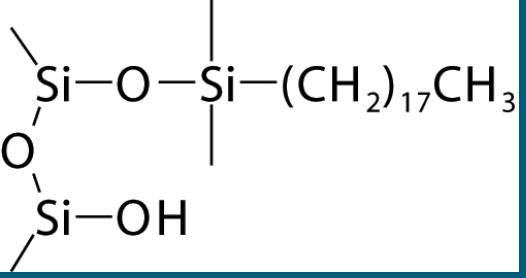
Clean-up step

Shellfish

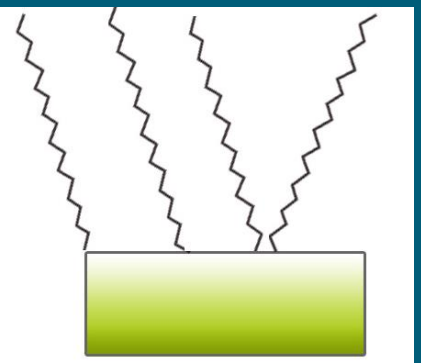
Extract

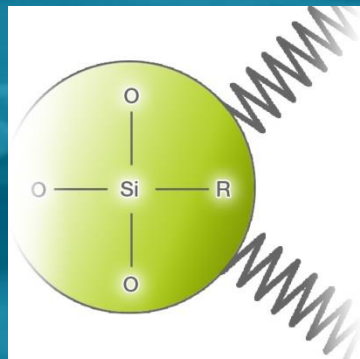
Clean-up



SPE

- PSTs (LC-FLD) – non-polar materials to remove interferences
- LT – can also use non-polar SPE
- Domoic acid – can use ion exchange SPE
- PST (LC-MS/MS) – carbon for salt removal



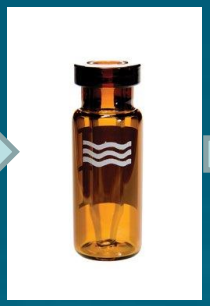


Analysis - separation

Shellfish

Extract

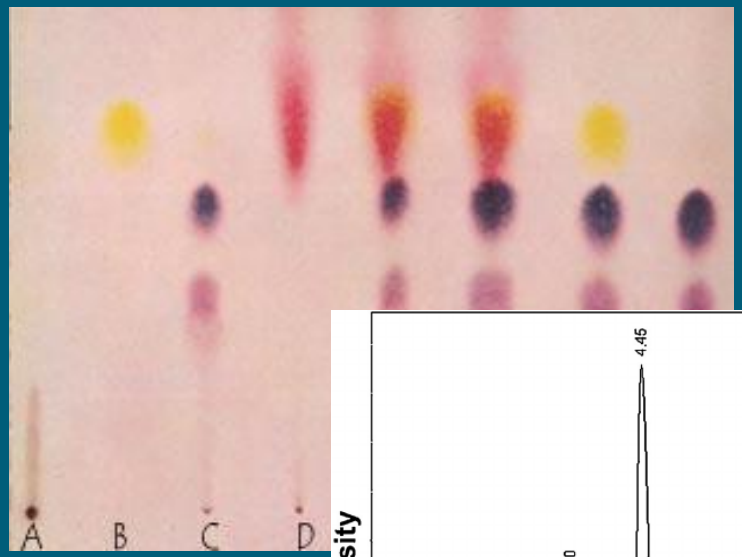
Clean-up

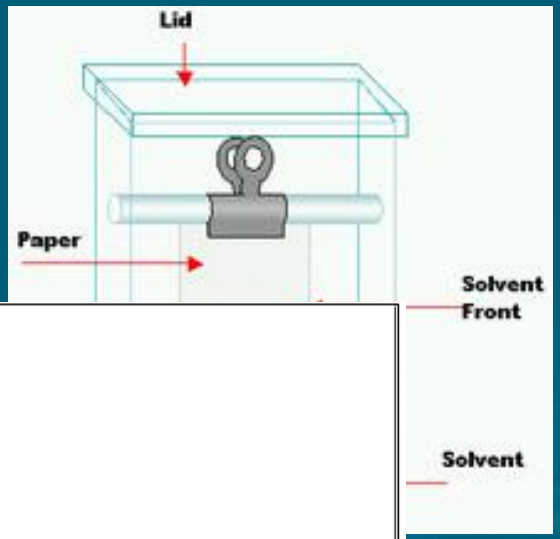


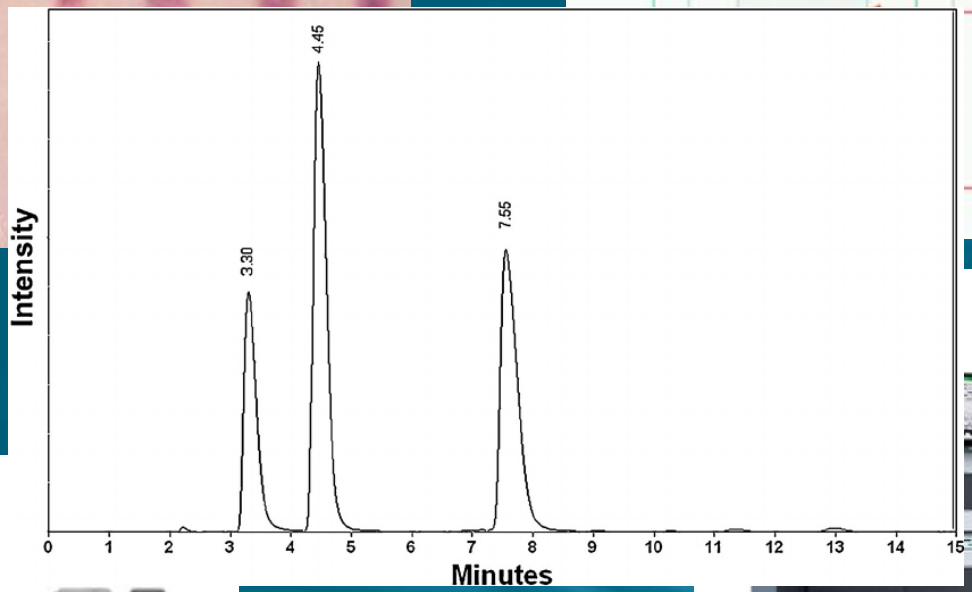
Final sample

Analysis

Separation







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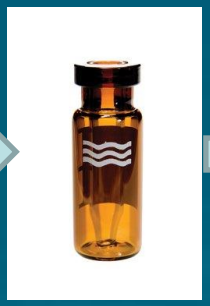
Analysis - detection

Shellfish

Extract

Clean-up



Final sample

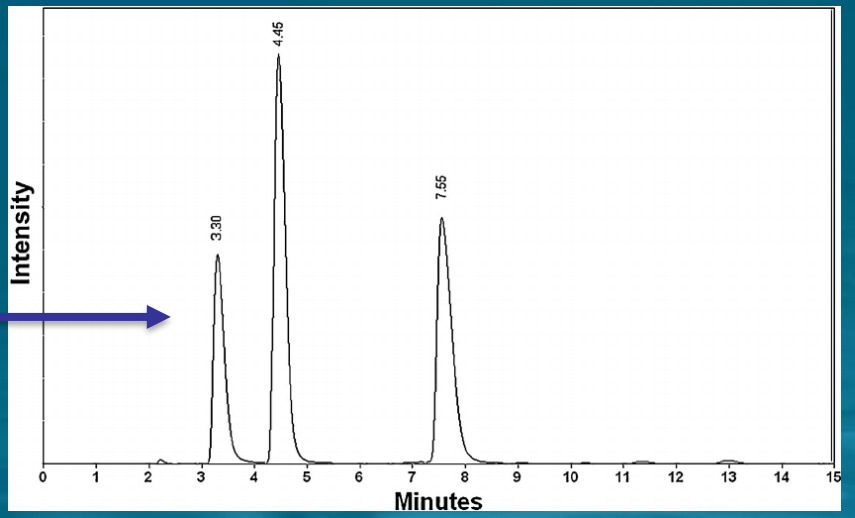
Analysis

Detection

Ultraviolet (UV)

Fluorescence (FLD)

Tandem mass spectrometry (MS/MS)



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Instrumentation - HPLC

Bottles containing mobile phases

Pump to deliver the mobile phase to the column

Autosampler – automatically injects sample extracts into mobile phase

Column compartment where LC column is held

Detector – detects the compounds when they elute from the end of the column

Computer to run the system and display results

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Instrumentation

Currently:

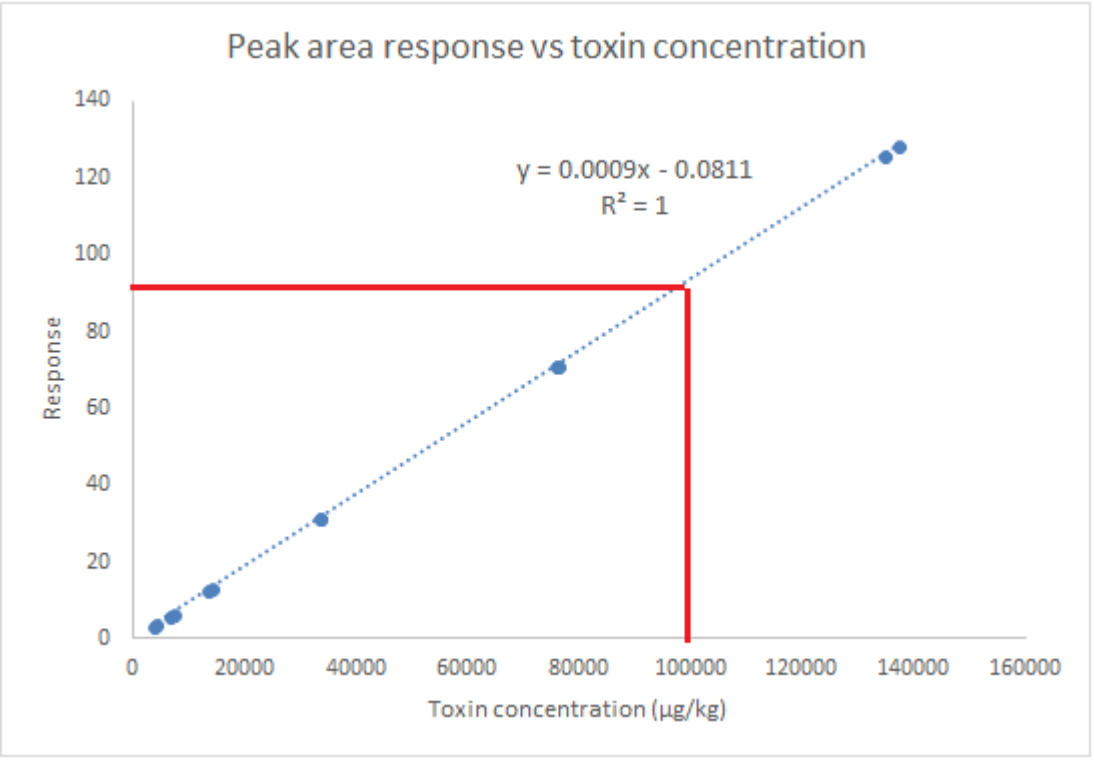
- ASP – LC + UV
- PSP – LC + FLD
- LTs – LC + MS/MS

Methods written in European regulations

Analysis of known toxins

Analysis involves:

- Use of certified reference standards (CRM)
  - Generate external calibrations
- Quantitation against known concentrations



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Testing methods for regulated toxins

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ASP

Domoic/epi-domoic acid

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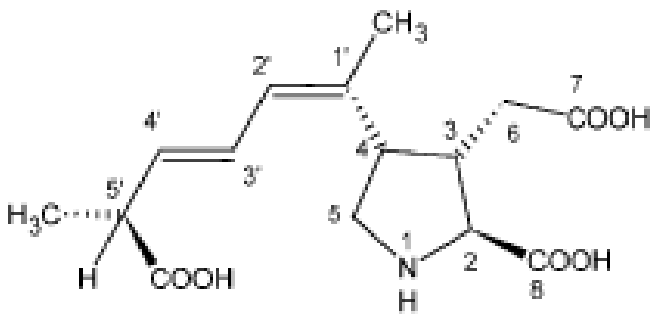
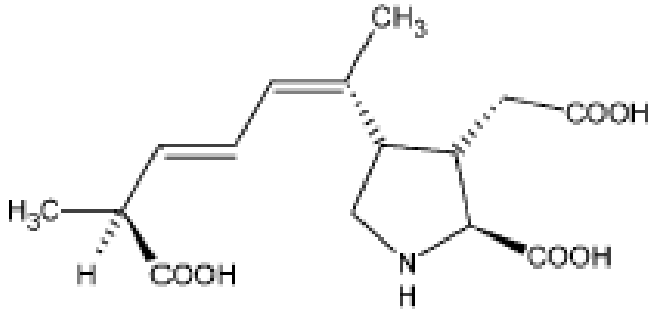
ASP

- Domoic acid & epi-domoic acid – total content of whole shellfish or edible part alone



Domoic acid



Epi-domoic acid (Domoic acid C5'-diastereomer)

- <u>EU reference method: HPLC-UV</u>
- Shellfish + 50% Methanol extraction
- With or without SPE clean-up
- Very simple, reproducible – no major issues

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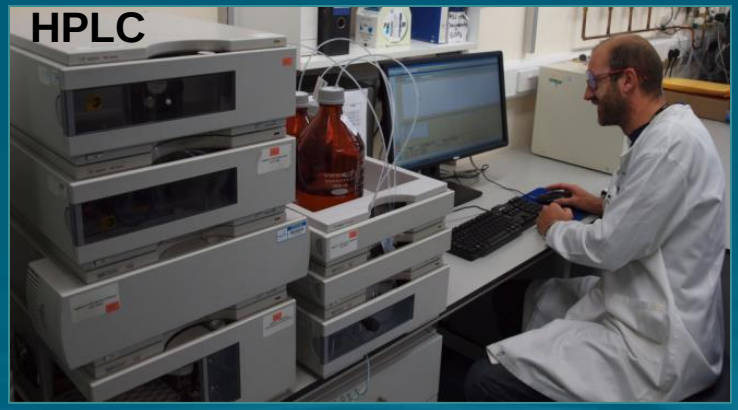
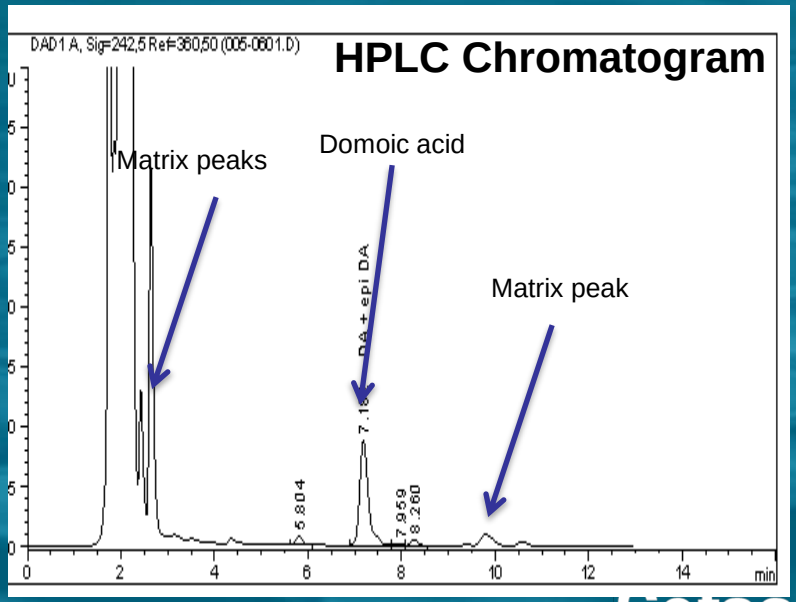
HPLC-UV

- EU reference method: HPLC-UV
- Shellfish + 50% Methanol extraction
- Without SPE clean-up
- Very simple, reproducible – no major issues





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LTs

OA, DTXs, YTXs, AZAs, PTXs

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LC-MS/MS for Lipophilic Toxins

From 1st July 2011

- EU Reference Method
- EURL SOP specifies:
  - Aims and scope
  - Extraction and general conditions
  - Performance characteristics

OA-Group
- *OA, DTX1, DTX2*
- *Esters of OA-group (DTX3)*
- *PTXs (PTX2, 1, 11)*

AZA-Group
- *AZA1, AZA2, AZA3*

YTX-Group
- *YTX*
- *Homo-YTX*
- *45 OH YTX*
- *45 OH homo YTX*

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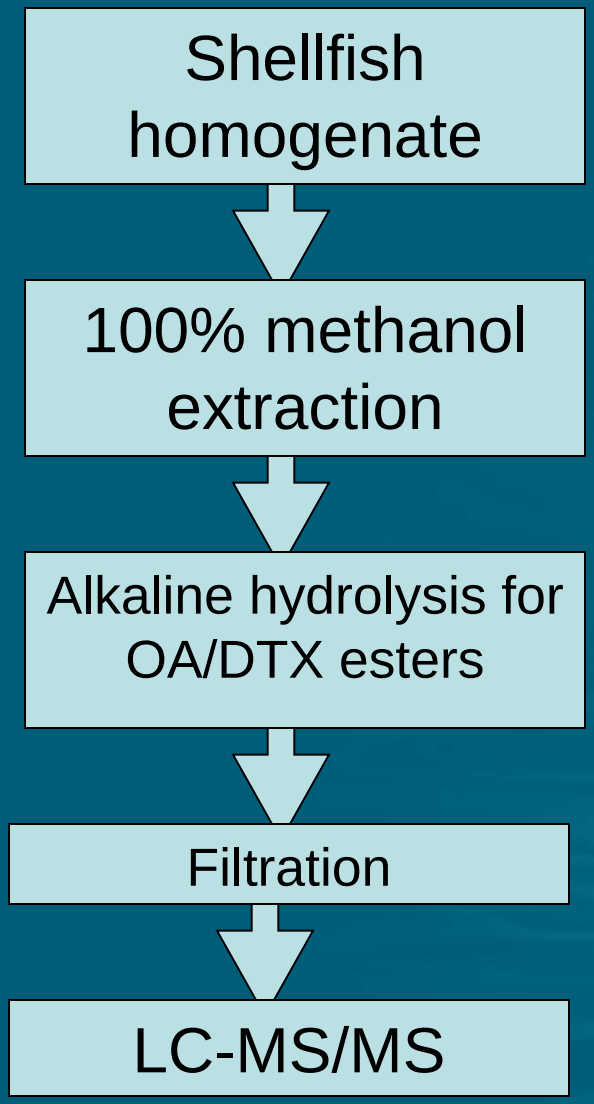
LT method overview



- Results report as:
  - Total OA-group
  - Total AZAs
  - Total YTXs
- Direct determination of toxins available as reference standards
  - Indirect determination of other toxins
- High pH mobile phase (pH 11)
  - Ammonium hydroxide
  - Low pH methods can also be used

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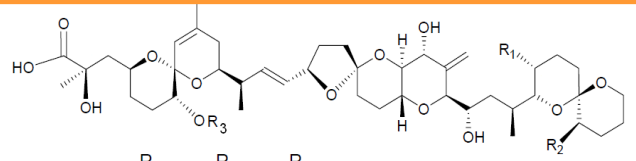
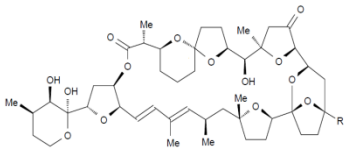
LT LC-MS/MS

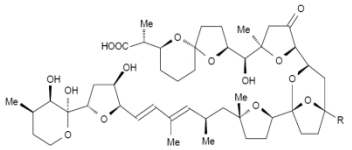
- High proportion of OA/DTXs present as acyl-esters
  - Alkaline hydrolysis to liberate
- +/- switching to encompass all groups
- Now implemented throughout EU



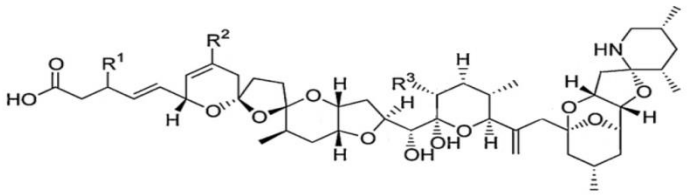
R_1	R_2	R_3	
CH_3	H	H	Okadaic acid (OA)
CH_3	CH_3	H	Dinophysistoxin-1 (DTX1)
H	CH_3	H	Dinophysistoxin-2 (DTX2)
H	CH_3	acyl	Dinophysistoxin-3 (DTX3)



R	
CH_3	Pectenotoxin-2 (PTX2)
CH_2OH	Pectenotoxin-1 (PTX1)
CHO	Pectenotoxin-3 (PTX3)
COOH	Pectenotoxin-6 (PTX6)

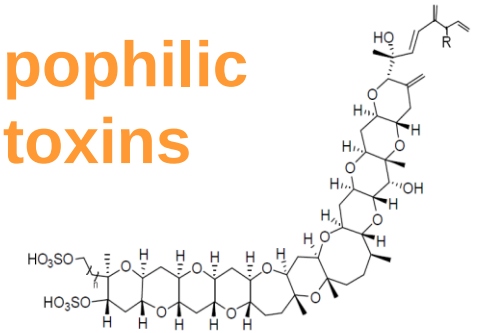


PTX2 seco acid (PTX-2-SA)



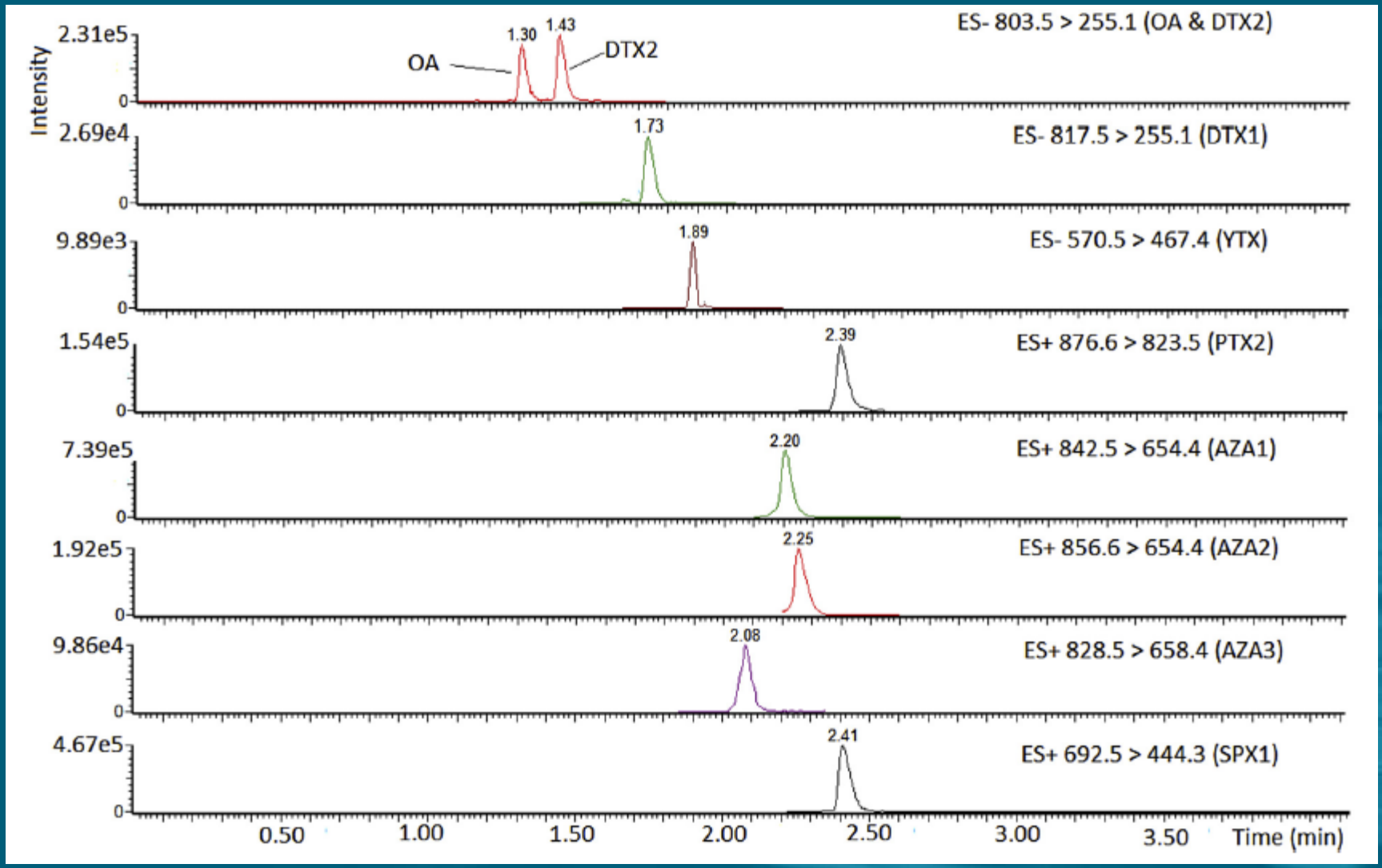
R_1	R_2	R_3	
H	H	CH_3	Azaspiracid-1 (AZA1)
H	CH_3	CH_3	Azaspiracid-2 (AZA2)

Lipophilic toxins



R	n	
H	1	Yessotoxin (YTX)
OH	1	45-Hydroxy-YTX (45-OH-YTX)
H	2	1a-homo-yessotoxin (homo-YTX)
OH	2	45-Hydroxy-homo-YTX (45-OH-homo-YTX)

LT LC-MS/MS



- Now implemented in throughout EU

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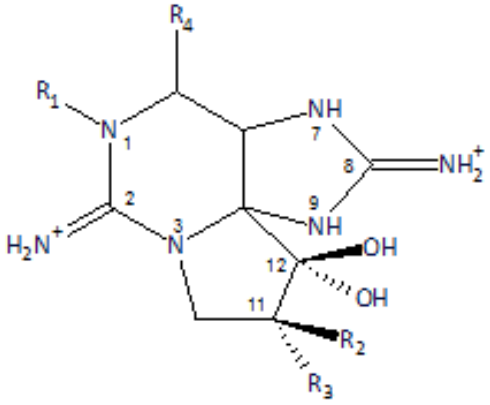
PSTs

Saxitoxins

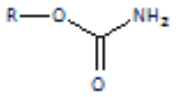
Cefas

PSP toxins

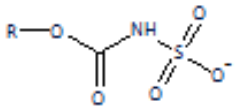
Saxitoxin derivatives



OCONH$_2$:



OCONHSO$_3^-$:



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	$OCONHSO_3^-$
STXs (+2)	dcSTX	H	H	H	H
	dcSTX	H	H	H	OH
	dcNEO	OH	H	H	OH
	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	$OCONH_2$

- N-hydroxyl
  - Carbamate NEO, GTX1&4
  - Decarbamoyl dcNEO, dcGTX1&4
  - N-sulfocarbamate GTX6, C3&4
- Non N-hydroxyl
  - STX, GTX2&3, dcSTX, dcGTX2&3, GTX5, C1&2
- Others
  - M toxins, GC toxins and more…
- All have different toxicities; TEF of some still unknown

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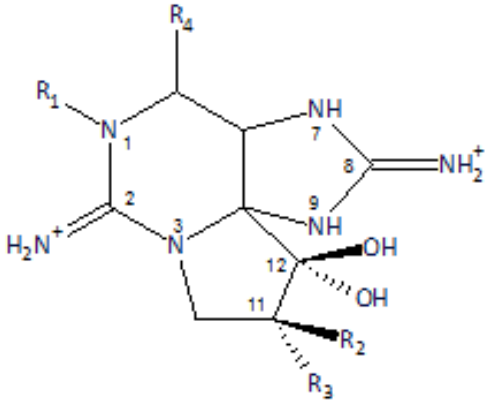
PSP toxins

Saxitoxin derivatives



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	$OCONHSO_3^-$
STXs (+2)	doSTX	H	H	H	H
				H	OH
				H	OH
				H	$OCONH_2$
				H	$OCONH_2$
				OH	$OCONH_2$
				H	$OCONH_2$
				OH	$OCONH_2$

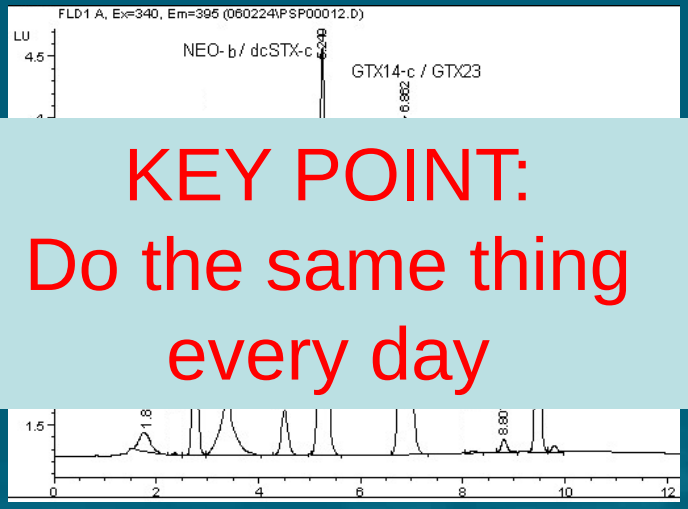
Thankfully: PSTs commonly occurring in naturally contaminated shellfish are available as standards **and** most have fairly well described TEFs

- N-hydr
  - Carb
  - Deca
  - N-sulfocarbamate GTX6, C3&4
- Non N-hydroxyl
  - STX, GTX2&3, dcSTX, dcGTX2&3, GTX5, C1&2
- Others
  - M toxins, GC toxins and more…
- All have different toxicities; TEF of some still unknown

Cefas

PSP LC-FLD

(AOAC 2005.06 OM)



KEY POINT:
Do the same thing every day

- Shellfish homogenate
  - Extraction (1% Acetic acid)
    - C18 SPE clean-up / pH adj
      - Periodate ox (screen)
        - HPLC-FLD
          - Peroxide oxidation
            - HPLC-FLD
              - STX, dcSTX, GTX2/3, GTX5, C1/2, dcGTX2,3
          - Ion exchange SPE (fractionation)
            - Fraction#1 (C toxins)
              - Perox. → C1/2
              - Period → C3/4*
            - Fraction#2 (GTX1/4, GTX2/3, GTX5, GTX6*)
              - Perox. → GTX2/3, GTX5
              - Period → HPLC-FLD → GTX1/4 GTX6*
            - Fraction#3 (STX, NEO, dcNEO, dcSTX)
              - Perox. → STX, dcSTX
              - Period → HPLC-FLD → NEO, dcNEO
          - Unoxidised Non-toxic co-extractives
            - HPLC-FLD

** No certified standards currently available*

Cefas

Current approach for PSP

- Qualitative screen of every sample
- Semi-quantitative “toxicity” reported
- Only samples >400 µg STX eq/kg are subjected to full clean up and quantitation
- All others reported as either:
  - Not detected
  - Detected (< 400)
- Reduces requirement for quantitation significantly

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Validation and Implementation

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Validation of Methods

Not an easy, quick or cheap process:

- Initial testing of method
- Assessment of issues
- Resolve practical issues and pitfalls
- In-house validation to define performance
- Comparison with other methods
- Define implementation approaches
- Implement

Validation

Selectivity
LOD/LOQ (screen & quant)
Linearity and range
Accuracy (CRM)
Toxin recovery
Precision (short, medium, long term)
Ruggedness
Uncertainty of measurement

To be done for each species

IUPAC
International Union of Pure and Applied Chemistry

Implementation of “new” methods

In EU: Process is time-consuming:

- Method developed and single-lab validated:
  - Must follow full EC / IUPAC guidelines
  - Demonstrate “equivalence” with current ref method
- Formal multi-lab collaborative study
  - Following specific guidelines (*e.g*. AOAC)
- Publication as Official Method (*e.g*. AOAC, CEN)
- Method acceptable within EU legislation
- Approval by Competent Authority and COT
- Accreditation to ISO17025

Implementation now may be possible

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Practical Application of Methods

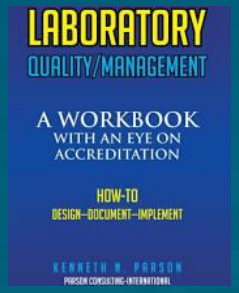
Key Points

- ISO 17025
- Highly trained analysts
- Robust instrumentation
- Automated processes
- Risk awareness, mitigation and contingency
- Availability of reference materials

- Internal Quality Control
- Positive controls
- Blanks
- Calibrations
- Calibration checks
- Trend analysis

- External Quality Assurance
- Proficiency testing schemes
- Ring trials
- External materials

Starting from scratch?



Requirements

- Lab space + facilities (temp control)
- Instruments for each method
- Other associated instruments (centrifuges, water baths, pipettes – long list)
- Chemicals, reagents and standards
- Trained personnel
- Quality management programme
- Workbooks and systems for sample logging and tracking
- Results reporting framework
- Contingency for everything!

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Ongoing method developments

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1. Fast Chromatography

- UHPLC hardware:
  - Sub 2µm columns; high pressure; quick analysis
  - Expensive!
- “Fused core”, “Superficially Porous” HPLC:
  - 2.6µm – 5µm pore; use with normal HPLC
  - Much cheaper!

Approaches taken

- Lipophilic toxins:
  - UHPLC with MS/MS – essential for throughput
  - 5.5 min method; 3.5 min for DTX3s
- ASP and PSP:
  - Test & validated fused core HPLC

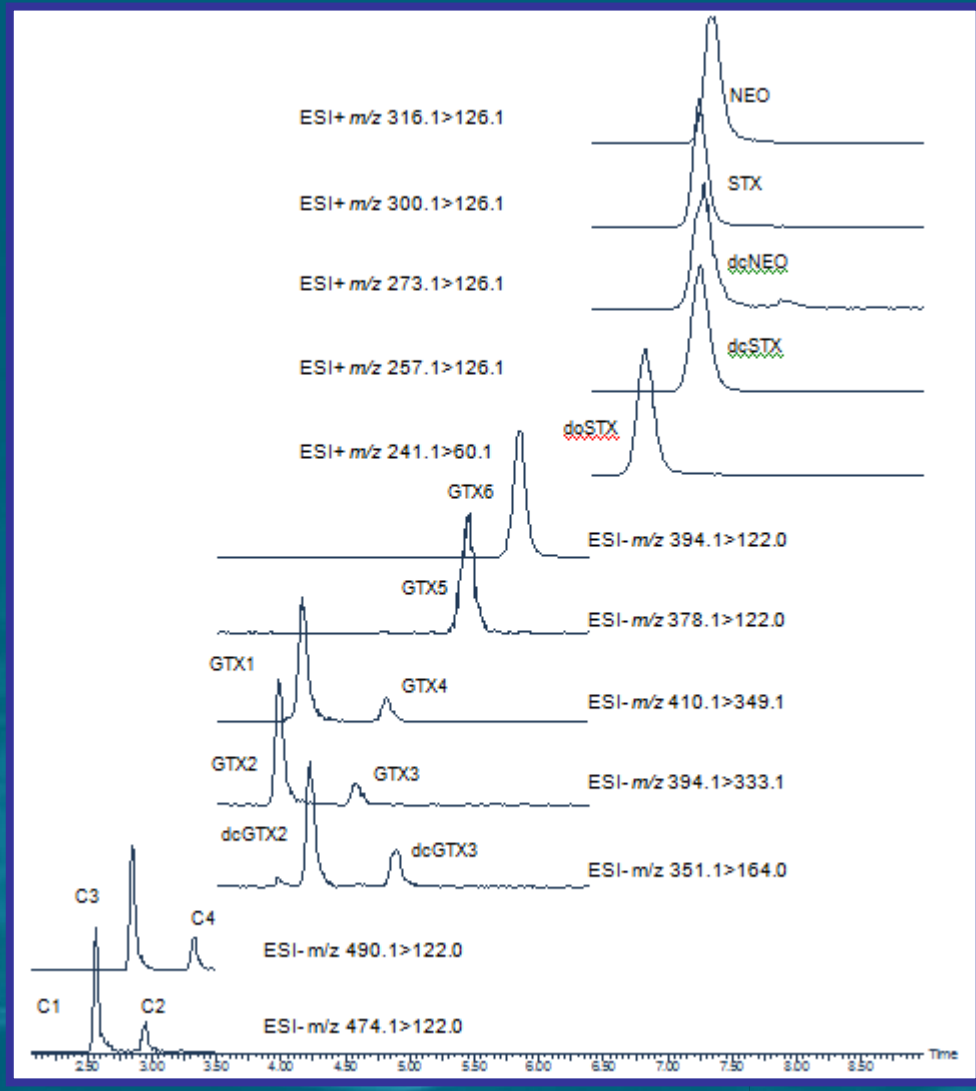
2. Other PSP methods

- AOAC 2011.02 – PCOX LC-FLD:
  - US/Canada
  - Requires at least 2 columns/systems to run each sample
- AOAC 2011.27 - Receptor binding assay (RBA)
  - US States

Cefas

HILIC-MS/MS

- Fast single step extraction
- One SPE clean-up
- One analysis per sample for rapid results
- Full separation of critical pairs, including epimers
- Total cycle time of 11.5 min for all PSTs
- Fully validated and collaborative study complete
- Compares well with LC-FLD



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Opportunities

- Use of chemical detection methods gives you LOTS of opportunity for research:
  - Rapid screening of toxicity (spatial/temporal change) – risk management
  - Toxin profiles – links to microalgal source
  - Discovery of new toxin threats to food safety and animal health
  - Valuable tools suitable for assessment of toxins in food webs
  - Collaboration with other organisations
  - Greater quality assurance of monitoring programmes

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marine drugs

MDPI

Article

Application of Six Detection Methods for Analysis of Paralytic Shellfish Toxins in Shellfish from Four Regions within Latin America

Andrew D. Turner [1,*], Sophie Tarnovius [1,2], Robert G. Hatfield [1], Mickael Teixeira Alves [1], Maggie Broadwater [3], Frances Van Dolah [3], Ernesto Garcia-Mendoza [4], Dinorah Medina [5], Maria Salhi [5], Alejandra B. Goya [6], Fernanda Barrera [7], Daniel Carrasco [7], Ignacio Rubilar [7] and Benjamin A. Suarez-Isla [7]

Harmful Algae 99 (2020) 101910

Contents lists available at ScienceDirect

Harmful Algae

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

ELSEVIER

Paralytic shellfish toxins and associated toxin profiles in bivalve mollusc shellfish from Argentina

Alejandra B. Goya[a], Sophie Tarnovius[b,c], Robert G. Hatfield[c], Lewis Coates[c], Adam M. Lewis[c], Andrew D. Turner[c,*]

[a] Marine Biotoxin Department, Mar del Plata Regional Laboratory, Agri-food Health and Quality National Service (SENASA)
[b] Technische Universität München, Walther-Meißner-Straße 3, 85748 Garching, German
[c] Centre for Environment, Fisheries and Aquaculture Science (Cefas), Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB, United Kingdom

applied sciences

MDPI

Article

Presence of Cyanotoxins in a Mexican Subtropical Monomictic Crater Lake

José Jesús Bustillos-Guzmán [1,*], Andrew Turner [2], Oscar Ubisha Hernández-Almeida [3], Christine Johanna Band-Schmidt [4,*], Carlos Alberto Romero-Bañuelos [3], Francisco Eduardo Hernández-Sandoval [1], Erick Julián Núñez-Vázquez [1] and Yolotzin Apatzingan Palomino-Hermosillo [3]

marine drugs

MDPI

Article

Multiple New Paralytic Shellfish Toxin Vectors in Offshore North Sea Benthos, a Deep Secret Exposed

Karl J. Dean [1,*], Robert G. Hatfield [1], Vanessa Lee [1,2], Ryan P. Alexander [1], Adam M. Lewis [1], Benjamin H. Maskrey [1], Mickael Teixeira Alves [1], Benjamin Hatton [3], Lewis N. Coates [1], Elisa Capuzzo [1], Jim R. Ellis [3] and Andrew D. Turner [1]

Marine Biology (2019) 166:82
https://doi.org/10.1007/s00227-019-3529-x

ORIGINAL PAPER

The invasive sea slug *Pleurobranchaea maculata* is a vector of two potent neurotoxins in coasts of Argentina

Nahuel E. Farías[1,2] · Alejandra B. Goya[3] · Evangelina Schwindt[4] · Sandra Obenat[1,2] · Monika Dhanji-Rapkova[5] · Andrew D. Turner[5]

Toxicon 140 (2017) 147–156

Contents lists available at ScienceDirect

Toxicon

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

ELSEVIER

Assessing the presence of marine toxins in bivalve molluscs from southwest India

Andrew D. Turner [a, *], Monika Dhanji-Rapkova [a], Stephanie Rowland-Pilgrim [a], Lucy M. Turner [b, c], Ashwin Rai [d], Moleyur N. Venugopal [d], Indrani Karunasagar [e], Anna Godhe [b]

TURNER ET AL.: JOURNAL OF AOAC INTERNATIONAL VOL. 103, NO. ■, 2020 1

FOOD BIOLOGICAL CONTAMINANTS

Ultrahigh-Performance Hydrophilic Interaction Liquid Chromatography with Tandem Mass Spectrometry Method for the Determination of Paralytic Shellfish Toxins and Tetrodotoxin in Mussels, Oysters, Clams, Cockles, and Scallops: Collaborative Study

ANDREW D. TURNER, MONIKA DHANJI-RAPKOVA, and SUM Y.T. FONG
Centre for Environment, Fisheries and Aquaculture Science, Barrack Rd, The Nothe, Weymouth, Dorset DT4 8UB, United Kingdom

frontiers in Physiology

ORIGINAL RESEARCH
published: 04 April 2019
doi: 10.3389/fphys.2019.00373

Toxic Algae Silence Physiological Responses to Multiple Climate Drivers in a Tropical Marine Food Chain

Lucy M. Turner[1,2], *Jonathan N. Havenhand[1]*, *Christian Alsterberg[3]*, *Andrew D. Turner[4]*, *Girisha S. K[5]*, *Ashwin Rai[5]*, *M. N. Venugopal[5]*, *Indrani Karunasagar[5] and Anna Godhe[1]*

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Emerging toxin threats

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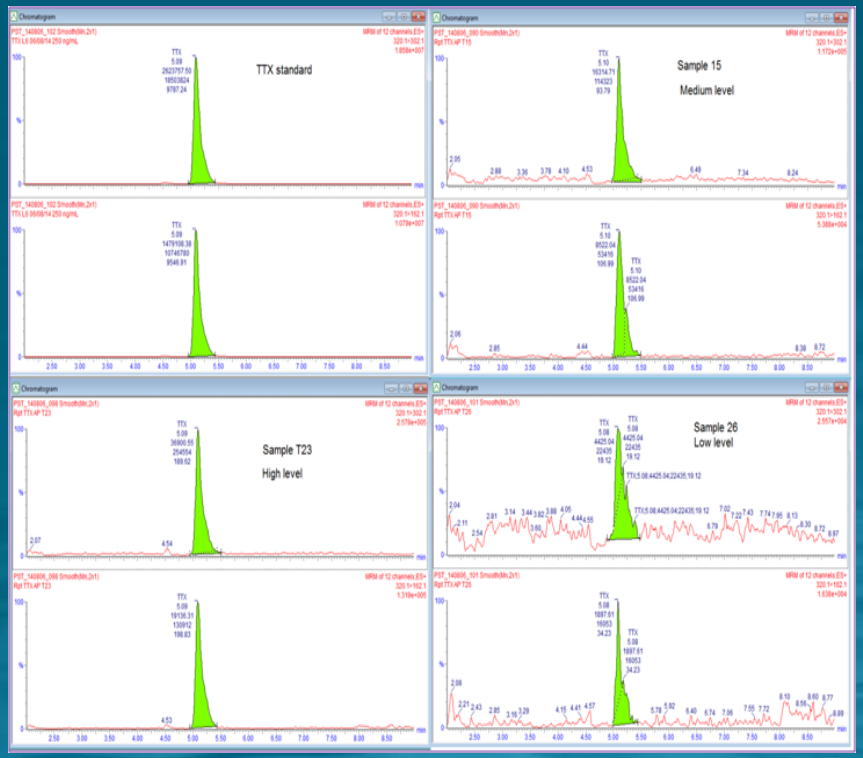
Tetrodotoxins (TTXs)

LC-MS/MS method includes TTX

- Found in UK molluscs + other parts Europe, NZ
- Potential bacterial source e.g. *Vibrio* sp.
- *Vibrio*-positive oysters & mussels from south coast found contain TTX
- Also detected in *Vibrio* cultures



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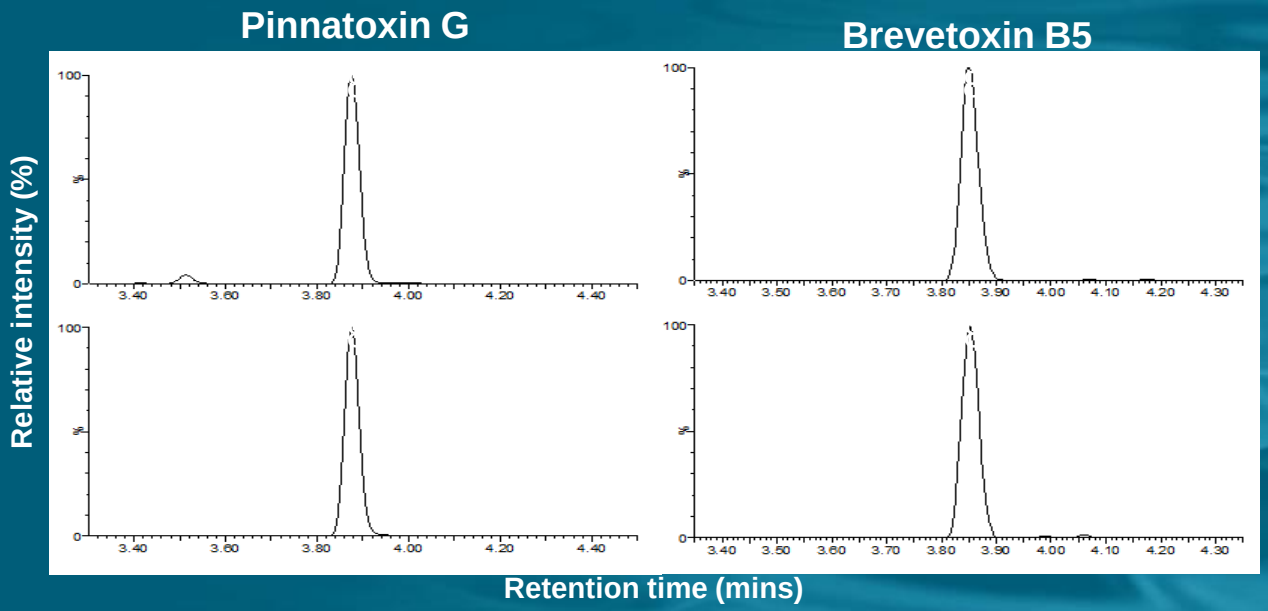
Pinnatoxins & Brevetoxins

- LC-MS/MS method for LTs extended
- Includes PnTx E, F, G
- Brevetoxins (BTX B2, B4, B5; PbTx2, PbTx3, S desoxy BTX B2,)
- Evidence for PnTx G noted in N. Europe



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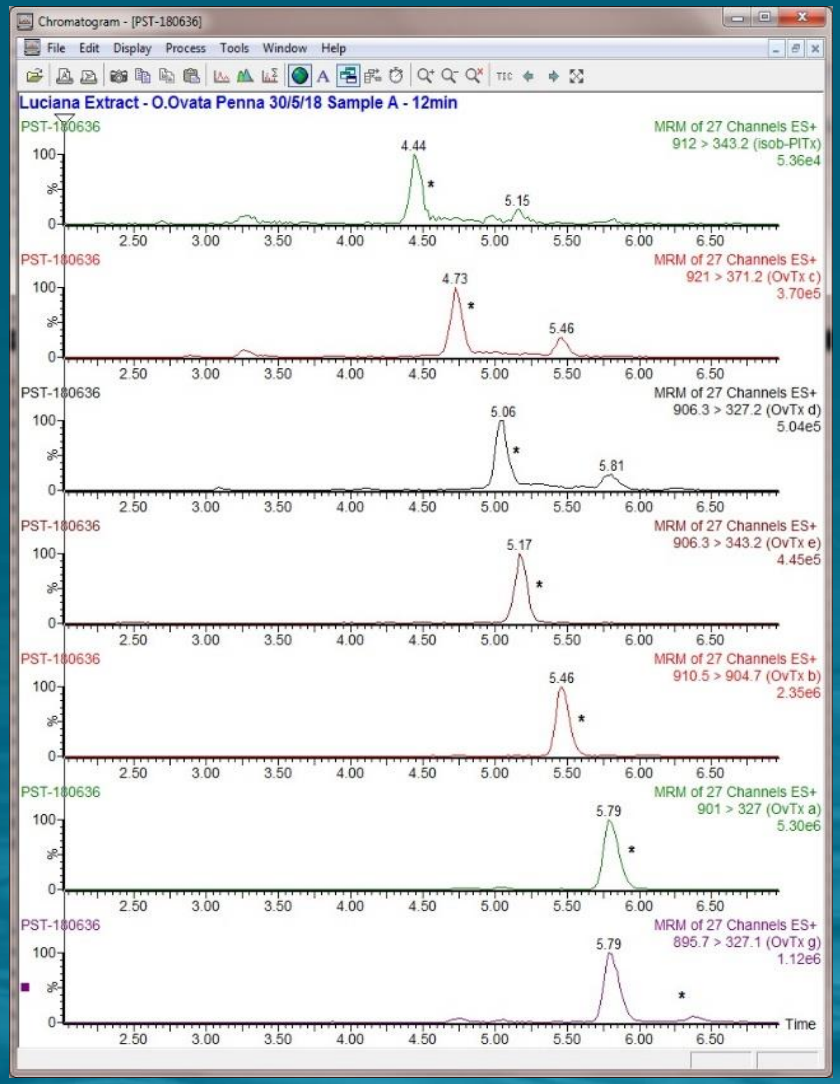
Palytoxins/Ovatoxins

- Issues in Mediterranean Sea + other regions
- LC with high resolution MS reported from Italy
- LC-MS/MS also useful



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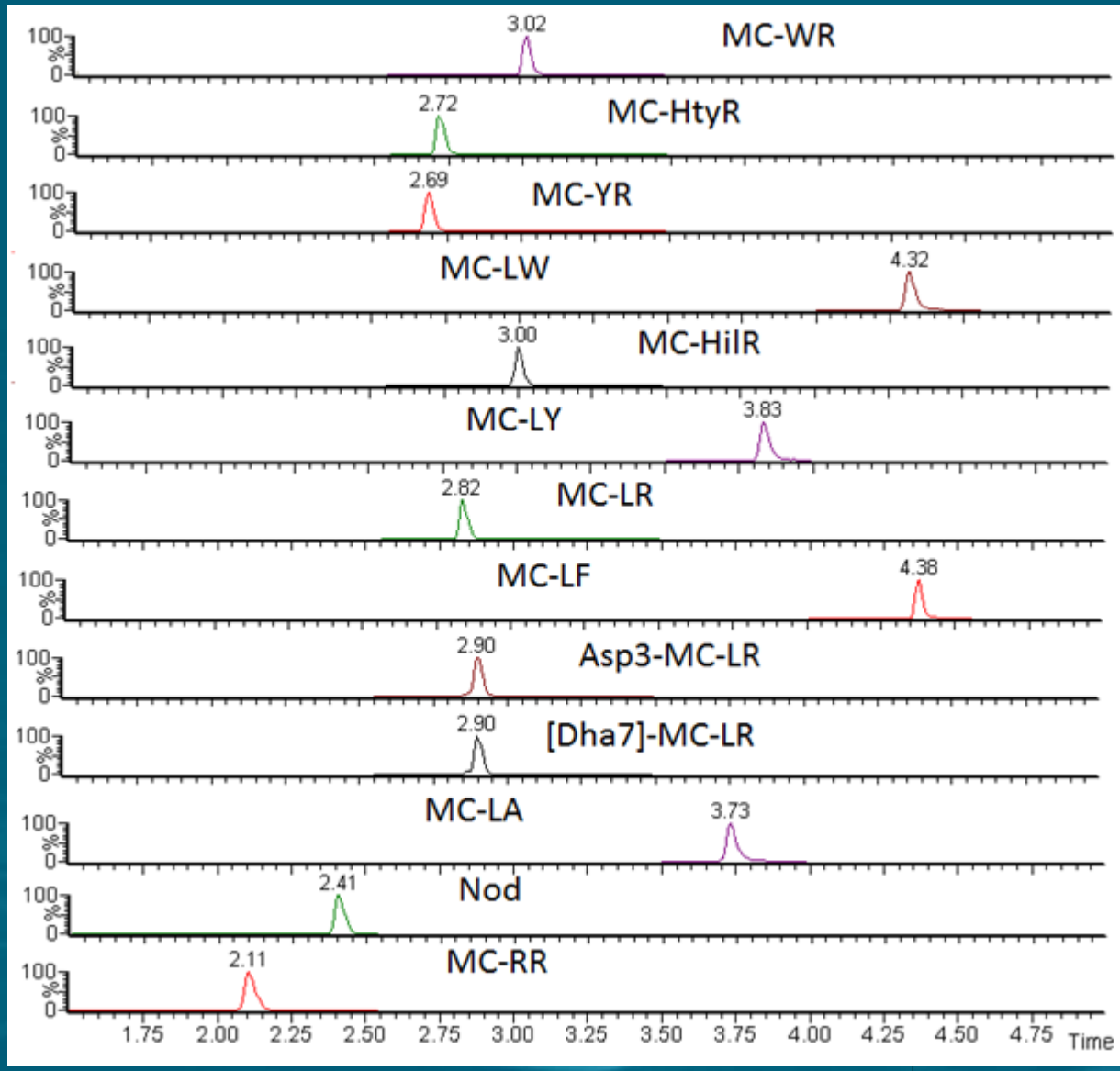
Microcystins

- LC-MS/MS

- Water
- Algae
- Shellfish
- Powders

- 5.5 min method



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Overall

- Chemical detection methods provide powerful tools for the protection of shellfish consumers from contaminated shellfish products

- Methods need to be tested and validated in each lab for the species of relevance

- Labs must participate in IQC and EQA procedures routinely

- Ideally, new biological assays to complement chemical detection tools

- Need to be aware of the potential for “new” or “emerging” toxin threats, now and in the future

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How can we help?

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