



Beyond the mice test: mass spectrometry analysis of marine biotoxins

Ana Gago-Martínez

**European Reference Laboratory for Marine
Biotoxins and University of Vigo, Spain**

Rome, October 2012



OUTLINE

- **Overview on Marine Biotoxins**
- **EU Legislation**
- **LC-MS/MS an alternative to MBA**
- **Concerns and Needs**
- **Update on the EURLMB efforts for LC-MS/MS implementation**
- **Update on the LC-MS/MS development for emerging toxins**
- **Conclusions**



Marine Toxic Syndromes

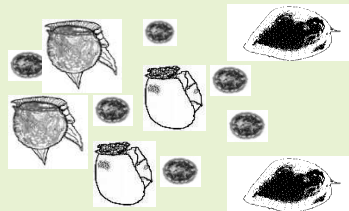
- **Bivalve molluscs filter large volumes of water**

Mussels, clams, scallops, oysters affected

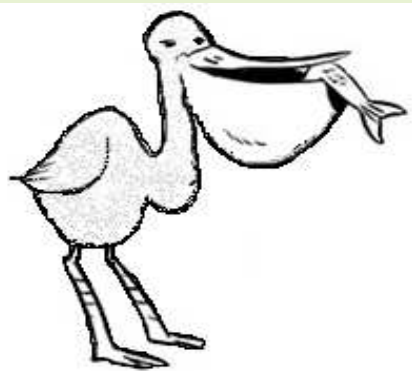
Five major toxic syndromes:

- **Diarrhetic Shellfish Poisoning (DSP)**
- **Azaspiracid Shellfish Poisoning (AZP)**
- **Neurotoxin Shellfish Poisoning (NSP)**
- **Amnesic Shellfish Poisoning (ASP)**
- **Paralytic Shellfish Poisoning (PSP)**

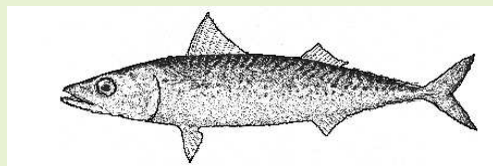




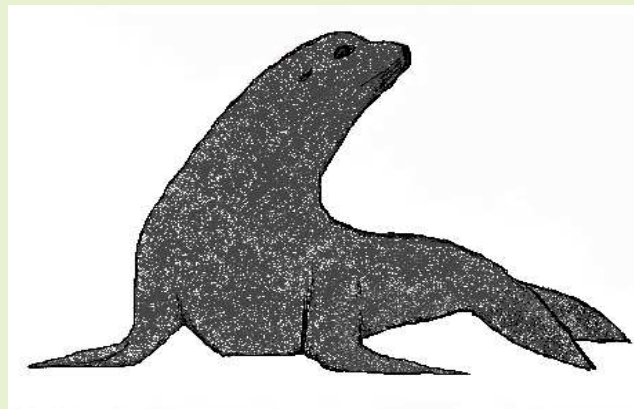
Phytoplankton & Bacteria



Birds



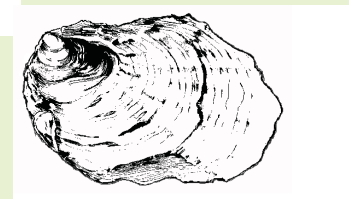
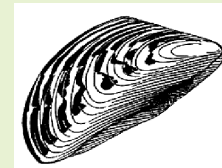
Finfish



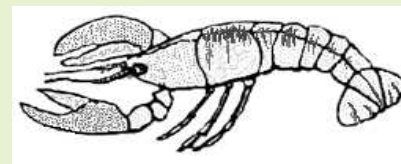
Marine Mammals



Human

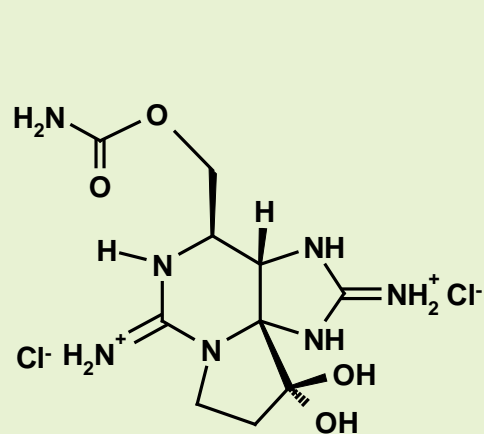


Bivalves

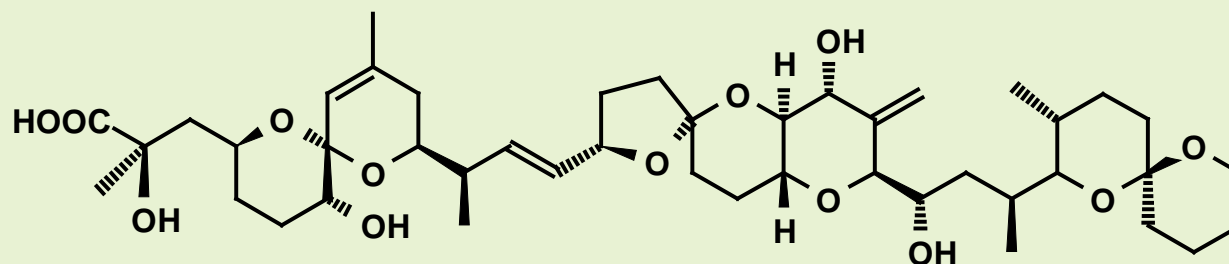


Crustaceans

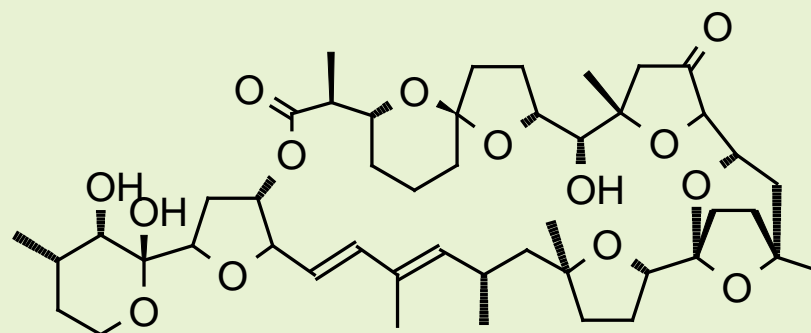




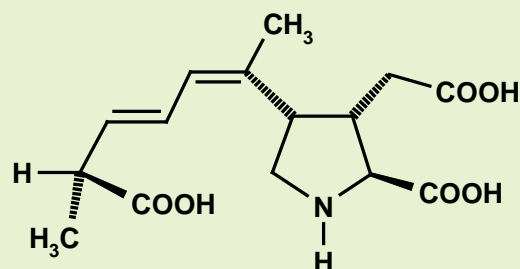
Saxitoxin



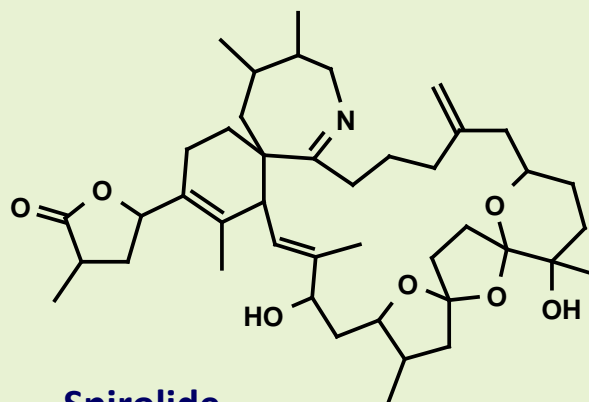
Okadaic Acid



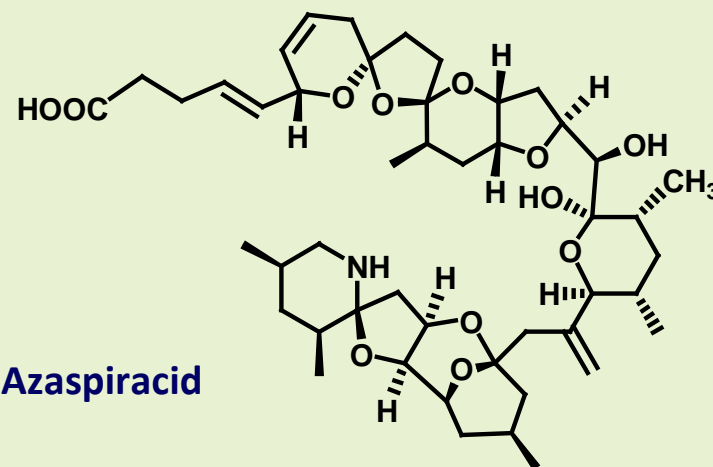
Pectenotoxin



Domoic Acid



Spirolide



Azaspiracid

MBA: the beginning of the Story!!



Specifically, animal assays have been used to monitor two main toxin groups:

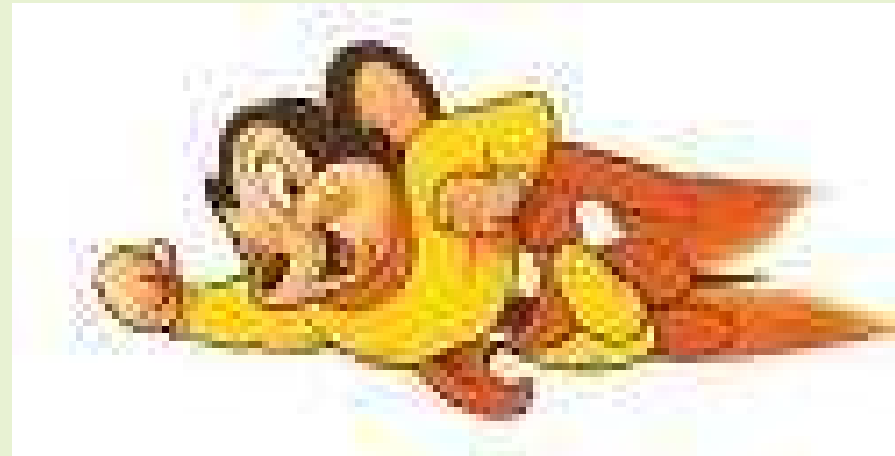


1. Paralytic shellfish poisoning (PSP) toxins.

2. Lipophilic (eg. Okadaic acid group) toxins.

The Mouse Bioassay

the long-standing reference method for shellfish toxins



Disadvantages

Poor precision ($\pm 20-30\%$)

Poor accuracy, especially near regulatory levels

Subject to false positives

Subject to false negatives due to either poor
detection limit or unsuitability for some toxins

Animal rights concerns

Analytical reasons to move away from MBA

AOAC-MBA

Never validated for accuracy; prone to false positives/negatives

Lipophilic toxin MBA

Never interlab validated. No single protocol for analytical testing

Difficulties in implementing alternative non-animal methods:

- 1. Long time success of animal methods.**
- 2. Ease of use of animals- no need for highly trained staff.**
- 3. Chemical/ instrumental methods can be expensive especially to set up.**



SCIENTIFIC OPINION

Marine biotoxins in shellfish – Summary on regulated marine biotoxins

Scientific Opinion of the Panel on Contaminants in the Food Chain

(Question No EFSA-Q-2009-00685)

Adopted on 13 August 2009

The mouse bioassay (MBA) is the official reference method for lipophilic biotoxins. The Panel on Contaminants in the Food Chain (CONTAM Panel) noted that this bioassay has shortcomings and is not considered an appropriate tool for control purposes because of the **high variability in results, the insufficient detection capability and the limited specificity.**

ALTERNATIVE METHODS

In vitro Assays

Functional assays (based on the toxicological mode of action)

(OA group toxins, YTXs, PTXs)

PP2A (OA group)

Citotoxicity (OA and PTXs, YTX, AZAs)

Biochemical Methods

ELISA (OA group , YTXs)

Inmunosensors (OA group)

Chemical Methods

HPLC/FLD (OA group, AZAs, YTXs and PTXs)

LC/ MS-MS (OA group, PTXs, YTXs)

AN ALTERNATIVE BY LC-MS/MS

- Alternate methods to the mouse bioassay are required
- Many alternate assay and analysis methods are now available
- For many labs, multi-toxin chemical analysis methods are most desirable in order to avoid establishing a wide range of individual toxin assays
- **LC and LC-MS methods are rapidly becoming the method of choice**
- **But why has it taken so long?**



- **Were methods not available until recently?**
 - No, LC-MS methods started being published in 1989
 - But instruments have become more advanced and easier to use
- **Are methods not comprehensive enough compared to MBA?**
 - New toxin groups are not detectable, but ...
 - LC-MS/MS scan techniques are getting better; LC-MS has in fact led to the discovery of many new structural analogues!
 - MBA does not really cover all toxin groups or analogues!
- **Are chemical analytical methods too expensive?**
 - No, studies have shown that multi-toxin methods can be less costly
- **The lack of accurate calibration standards and reference materials has been the biggest hold-up in the development, validation and routine implementation of analytical methods for seafood toxins.**



EU EFFORTS ON THE ANALYSIS OF LIPOPHILIC TOXINS

Interlaboratory Validation study of the “EU-harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS

- **COORDINATION: EUROPEAN UNION REFERENCE LABORATORY FOR MARINE BIOTOXINS**
- **JUNE-SEPTEMBER 2010**

ANNEX

In Annex III to Regulation (EC) No 2074/2005, Chapter III is **replaced by the following:**

CHAPTER III

LIPOPHILIC TOXIN DETECTION METHODS

A. Chemical methodology

(1) The EU-RL LC-MS/MS method shall be the reference method for the detection of marine toxins as referred to in Chapter V(2)(c), (d) and (e) of Section VII of Annex III, to Regulation (EC) No 853/2004. This method shall determine at least the following compounds:

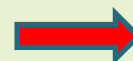
- **okadaic acid group toxins: OA, DTX1, DTX2, DTX3 including their esters**
- **pectenotoxins group toxins: PTX1 and PTX2**
- **yessotoxins group toxins: YTX, 45 OH YTX, homo YTX, and 45 OH homo YTX**
- **azaspiracids group toxins: AZA1, AZA2 and AZA3**

SCOPE OF THE LC-MS/MS METHOD

Applicable to the determination of the lipophilic marine biotoxins in molluscan shellfish in the live, frozen or processed state and in different shellfish matrices such as mussels, clams, scallops and oysters spiked and/or naturally contaminated

Lipophilic toxins extraction (SOP EURLMB)

- 2g of tissue homogenate
- Add 9 mL of 100% MeOH
- Vortex mix (3 min)
- Centrifuge at 2000g (10 min)
- Re-extract residual pellet (9 mL 100% MeOH).
- Homogenate (1 min) and centrifuge at 2000g (10 min)
- Combine supernatants (to 20 mL 100% MeOH)

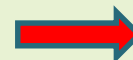


**Determination of free
OA, PTX, AZA and YTX
group toxins**



HYDROLYSIS

- 2.5 mL of methanolic extract
- Add 313µL of NaOH 2.5M
- Heat at 76 °C for 40 min
- Neutralise with 313µL of HCl 2.5M



**Determination of
total OA group**



- **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,

- **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 of the European Parliament and of the Council and Regulation (EC) No 852/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004 (3). It is necessary to modify those implementing measures in the light of new scientific evidence.
- (2) In July 2006 the Commission requested the European Food Safety Authority (EFSA) to provide a scientific opinion to assess the current limits and methods of analysis with regard to human health for various marine biotoxins as established in the Community legislation, including new emerging toxins. The last of a series of opinions was published on 24 July 2009.
- (3) The mouse bioassay (MBA) and the rat bioassay (RBA) are the official methods for the detection of lipophilic biotoxins. The Panel on Contaminants in the Food Chain 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.aesam.sps.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 that they fulfil the method performance criteria stipulated by the EU-RL. Such methods should be intra-laboratory validated and successfully tested under a recognised proficiency test scheme. If the results are challenged, the reference method shall be the EU-RL LC-MS/MS method.
- (7) To allow Member States to adapt their methods to the chemical method, the biological methods should continue to be used for a limited period of time. After this period, the biological methods should be used not as a matter of routine and only during the periodic monitoring of production areas for detecting new or unknown marine toxins.
- (8) Therefore, Regulation (EC) No 2074/2005 should be amended accordingly.
- (9) The measures provided for in this Regulation are in accordance with the opinion of the Standing Committee on the Food Chain and Animal Health, EN 11.1.2011 Official Journal of the European Union L 6/3
- (9) The measures provided for in this Regulation are in accordance with the opinion of the Standing Committee on the Food Chain and Animal Health

- **HAS ADOPTED THIS REGULATION:**

- *Article 1*
- Annex III to Regulation (EC) No 2074/2005 is amended in accordance with the Annex to this Regulation.
- *Article 2*
- This Regulation shall enter into force on the 20th day following its publication in the *Official Journal of the European Union*.
- It shall apply from 1 July 2011.
- This Regulation shall be binding in its entirety and directly applicable in all Member States.
- Done at Brussels, 10 January 2011.

- *For the Commission*
- *The President*
- José Manuel BARROSO EN L 6/4 Official Journal of the European Union 11.1.2011



- **Amending Regulation (EC) No 2074/2005 as regards recognized testing methods for detecting marine biotoxins in live bivalve molluscs**

- 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 interlaboratory validation study carried out by the Member States. This method is publicly available for consultation in the web page of the EURL (<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

A series of mouse bioassay procedures, may be still used until 31 December 2014 for detecting marine toxins.

After that period, the mouse bioassay shall be used only during the periodic monitoring of production areas and relaying areas for detecting new or unknown marine toxins on the basis of the national control programmes elaborated by the Member States.



MBA/LC-MS/MS

Routine labs specific concerns

- Are LC-MS/MS methods fast enough? How the change of methodology is going to affect the shellfish industry (producers)???
- Lack of standards?? Health protection?
- LC-MS/MS very expensive/ MBA???

Their main conclusions:

Economic losses and

Lack of health protection



LOOKING FOR ANSWERS:

Time and Efficiency: Some Considerations

Are sample preparation protocols taken into account?

MBA	HPLC –MS/MS
<ul style="list-style-type: none">- Hepatopancreas extraction- Toxin Extraction-Homogenate (2 min)-Centrifuge (10 min)-Evaporation-Dissolve (10 mL deionized water) + 50 mL Diethyl ether-Vortex-Liquid – Liquid extraction 3 times-Take organic phase (3 extracts) (twice) +20 mL deionised water , mix softly , phase separation, discard aqueous phase (twice)-Evaporate organic solvent (dryness)-Residue + Tween 60 1%-Injection (ip) in mice (1 mL) <p>Tedious and time consuming , prompted to errors</p>	<ul style="list-style-type: none">--Whole tissue from shells-Toxin extraction-Homogenate (MeOH) (3 min)-Centrifuge 10 min-Transfer supernatant (volumetric flask)- Repeat (residual tissue pellet) extraction (MeOH) Homogenate (1 min)- Centrifuge (10 min)- Combine supernatants (to 20 mL MeOH 100%) <p>Simpler and Faster, Considerable reduction of organic solvents</p>

LOOKING FOR ANSWERS: LACK OF STANDARDS!!

Big efforts are being made for developing Standards and Reference materials

NRC CRM-FDMT-1



A new CRM for multiple lipophilic toxins



Thanks to Mike Quilliam (NRC) for his permanent support and collaboration!!



EURLMB PRESENT EFFORTS ON THE ANALYSIS OF LIPOPHILIC TOXINS

- **Implementation of the existing LC-MS/MS method for Lipophilic Toxins**
 - **Collaboration with the Analytical Chemistry and Food Dept. University of Vigo**
 - **Collaboration with Agilent Tech.**



**UNIVERSIDAD
DE VIGO**



Aim of the implementation

- **Faster Analysis**
- **Sensitive Analysis**
- **Confirmation**

How to approach?

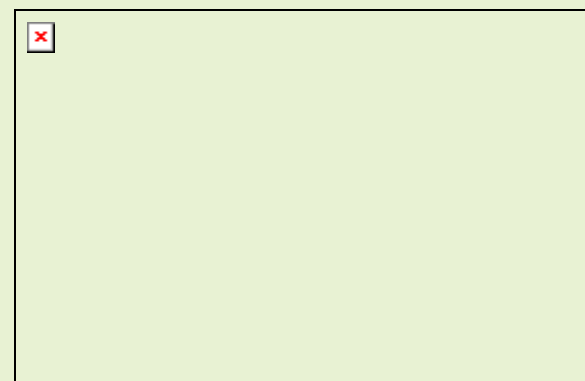
- **UHPLC – MS/MS**
- **Replacing multiple reaction monitoring (MRM) acquisition mode by Dynamic mode (DMRM) (fast polarity switching allowing single injection) (lower LODs)**
- **Triggered MRM Acquisition (Confirmation even up to 8 additional transitions)**

An implemented method has been developed and SLV was carried out

LC-MS/MS Method

Acidic method (based on McNabb et al., 2005)

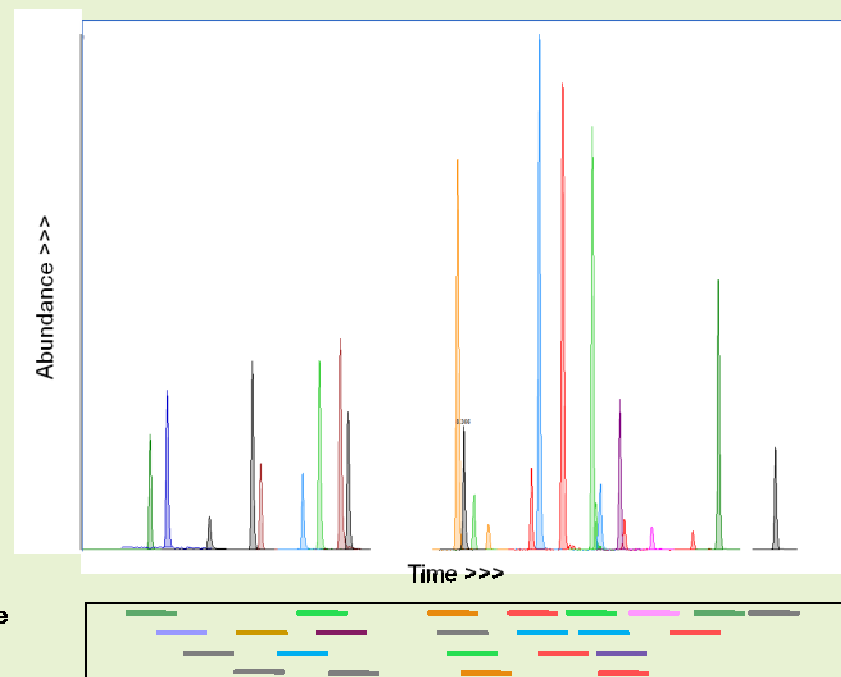
- Reverse phase conditions (Zorbax SB C8, 50mm x 2.1 mm, 1.8 μm)
- Acidic mobile phases (A: 50mM Formic Acid, 2 mM Ammonium Formiate, 100% Water; B: 50mM Formic Acid, 2 mM Ammonium Formiate 5% Water, 95% Acetonitrile)
- Electrospray ionization with polarity switching
- Dynamic Multiple reaction monitoring
- Triggered MRM for confirmation



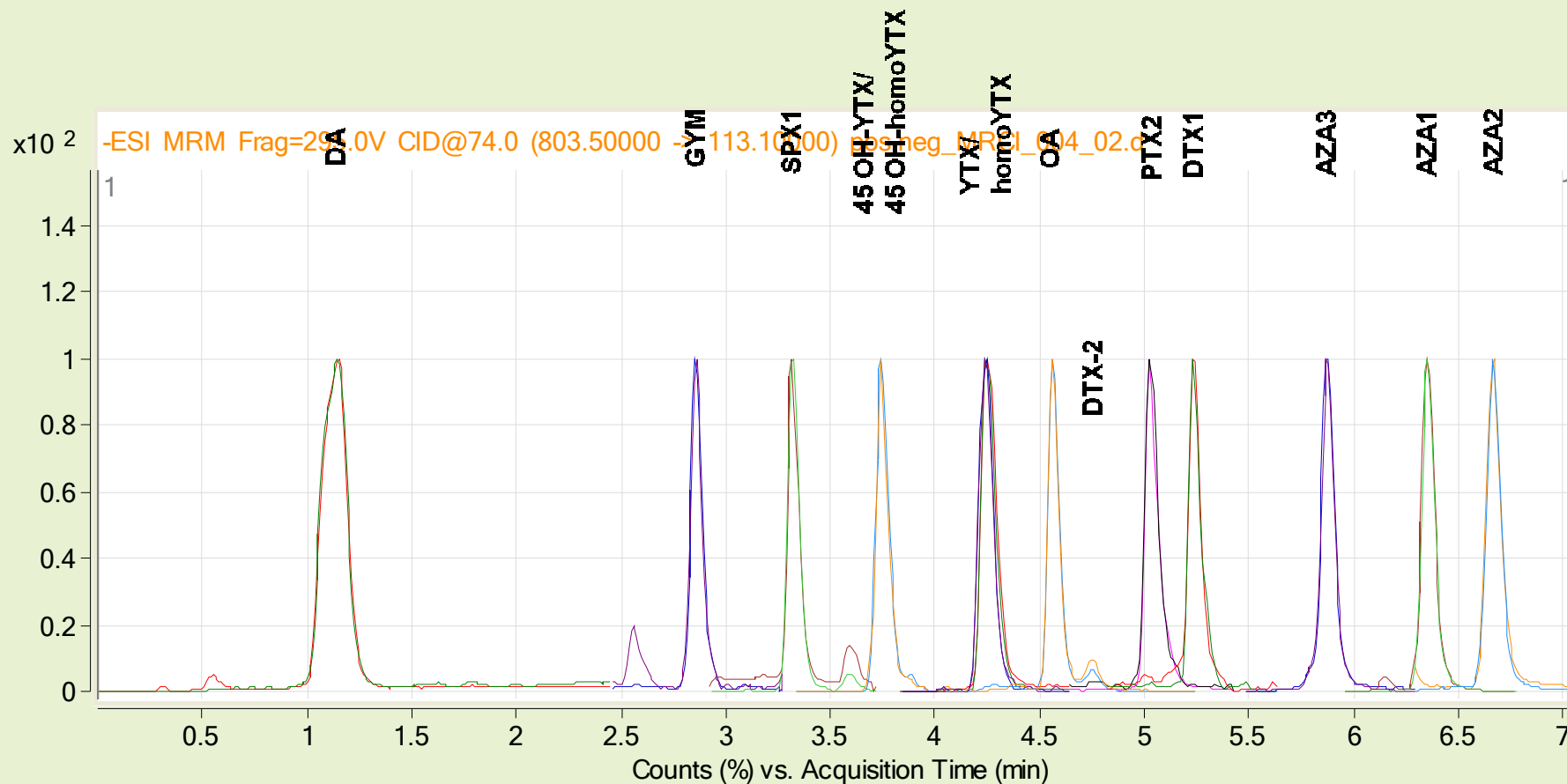
Agilent G6460A

Dynamic MRM

- Multiple Reaction Monitoring with Retention Time Windows
- Maximize MS Duty Cycle
- Fewer Concurrent MRMs
- Longer Dwell Times
 - Lower LODs
 - Lower RSDs
- Constant Cycle Time Assures Accurate Quantitation



UHPLC-MS/MS of mussel extract



* Mussel extract, responses normalized to 100%

Introduction

- CFP the most common type of marine biotoxin food poisoning
- As a result of biotransformation of precursor gambiertoxins produced by benthic dinoflagellate *Gambierdiscus toxicus*
- Primarily associate with the consumption of large predator fish
- Wide variety of signs such as gastrointestinal, neurological and cardiovascular effects
- Fatalities can occur due to cardio-respiratory failure

Chemical Structures CIGUATOXINS

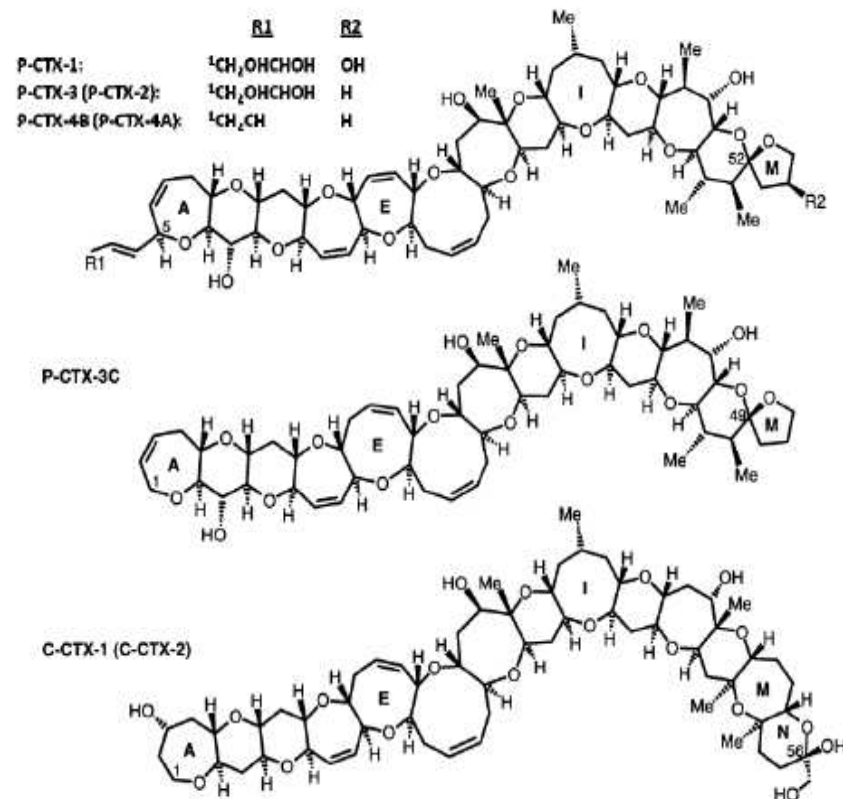
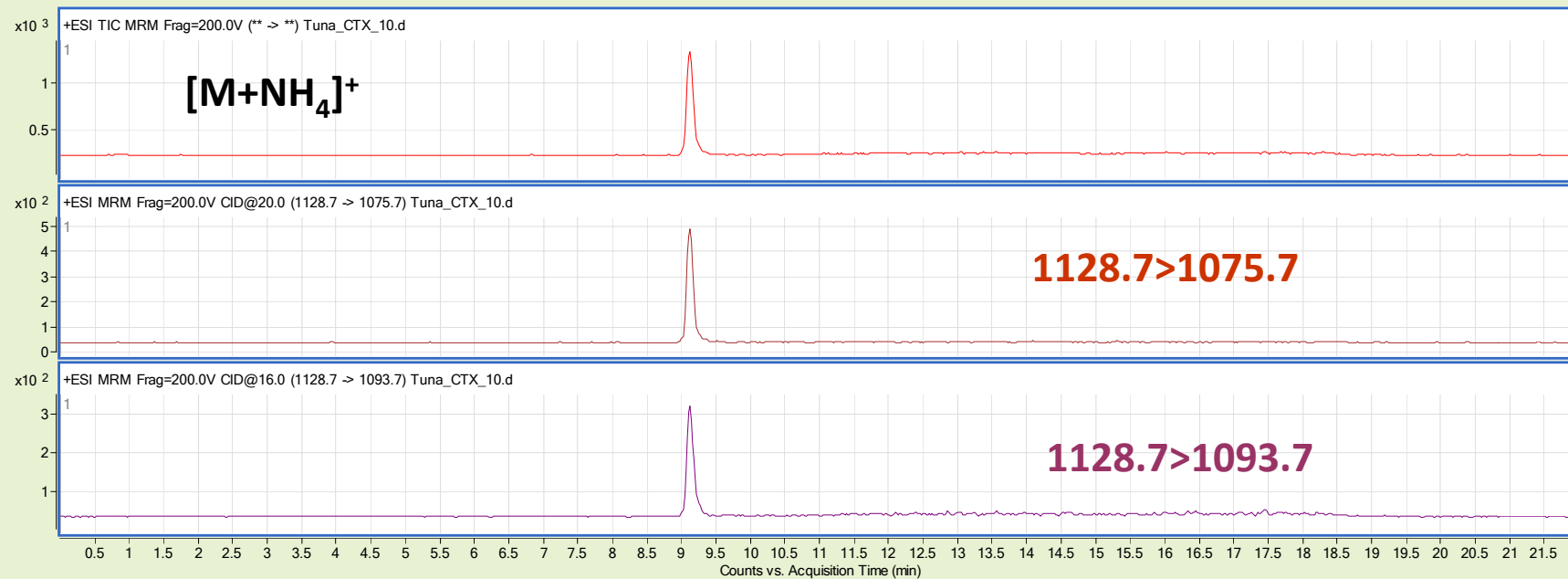


Figure 1: Structures of Pacific (P) and Caribbean (C) CTX-group toxins (modified from Lewis, 2001). The energetically less favoured epimers of P-CTX-3, P-CTX-4B and C-CTX-1 are stereoisomers at C52, C52 and C56, respectively (in brackets). Structures of Indian Ocean CTX-group toxins have not been reported.

HPLC Conditions:

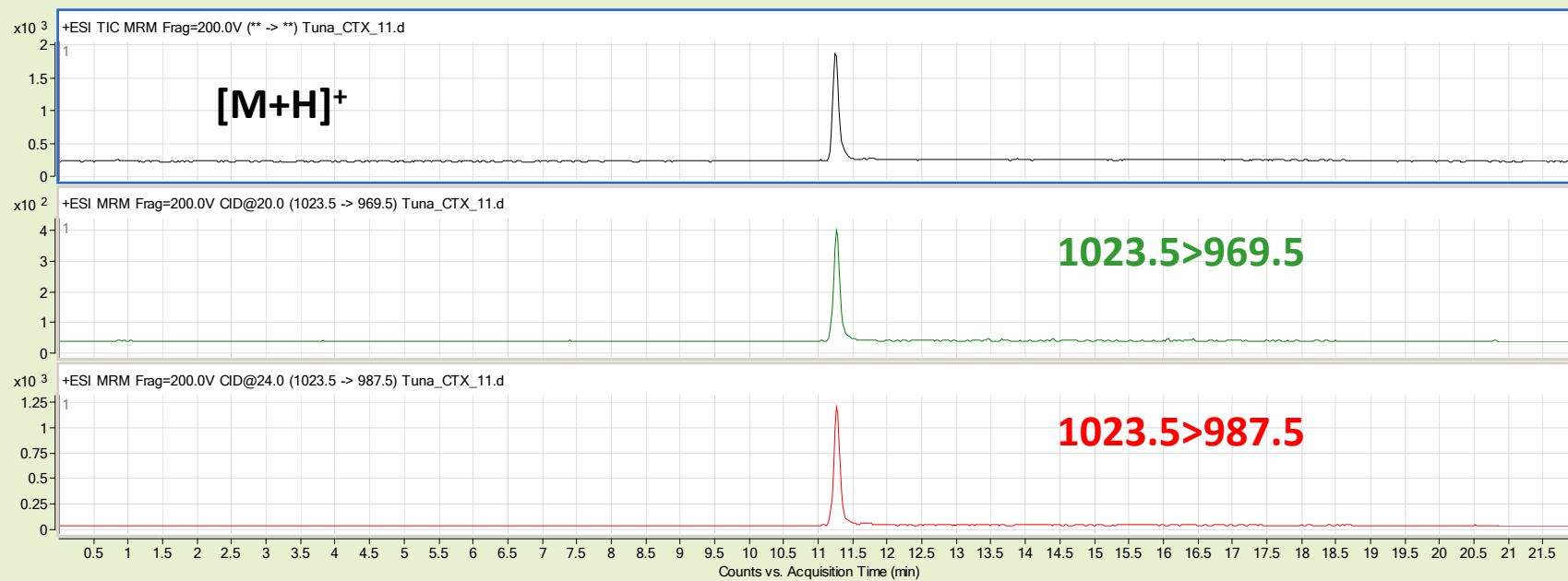
Column:	C18, Luna 3 μ m, 50 x 2 mm (Phenomenex)		
Mobile Phase A:	Methanol:Water (50:50) with 3.3 mM ammonium formate/50mM Formic acid		
Mobile Phase B:	Methanol:Water (95:5) with 3.3 mM ammonium formate/50mM Formic acid		
Flow	0.2mL/min		
Gradient	min	A (%)	B(%)
	0	100	0
	10	0	100
	17	0	100
	22	100	0
Injection Volume:	10 μ L		
Ionization Mode	Positive		

LC-MS/MS P-CTX1B



PCT1-B kindly provided by Prof. Yasumoto

LC-MS/MS P-CTX3C



PCT3-C kindly provided by Prof. Yasumoto

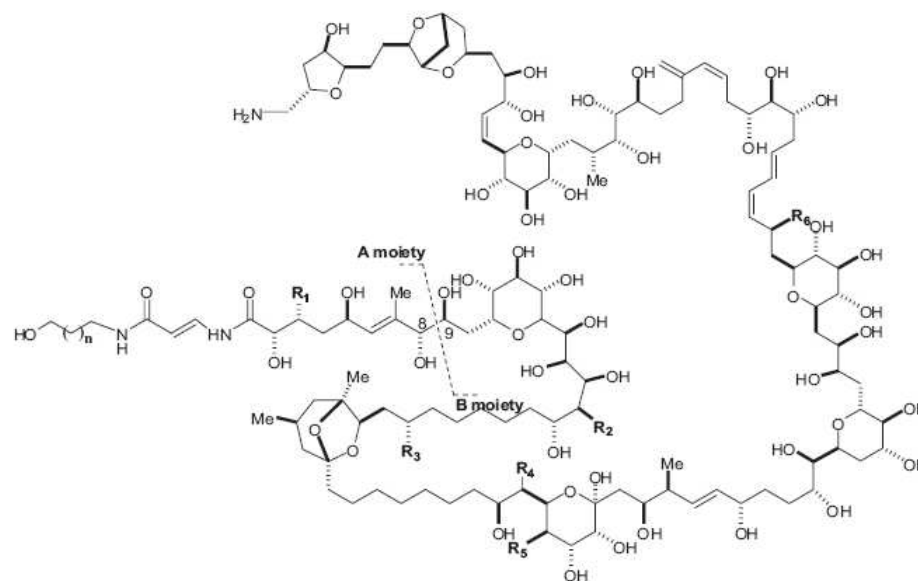
Introduction

- PltX-group have been mainly detected marine zoanthids (soft corals) *Palythoa* y benthic dinoflagelates *Ostreopsis*
- First reported in 1981 (Japan y Hawaii)
- Blooms of *ostreopsis spp.* have been also reported in European countries (Italy, Greece, France y Spain)
- Signs and symptoms are not well-defined including myalgia, weakness, fever, nausea, vomiting
- Fatalities are rare but after about 15h
- No under EU legislation yet

Chemical structure of Palytoxin

P. Ciminiello et al. / *Toxicon* 57 (2011) 376–389

377



	n	R1	R2	R3	R4	R5	R6
Palytoxin	1	Me	OH	Me	H	OH	OH
Ostreodol-D	1	H	H	H	OH	H	OH
42-Hydroxy-palytoxin	1	Me	OH	Me	OH	OH	OH

Fig. 1. Structure of palytoxin, ostreodol-D and 42-hydroxy-palytoxin. In MS spectra, cleavage between carbons 8 and 9 is highly favoured and divides the molecule in two moieties, A and B. Basing only on MS evidence, ovatoxin-a and mactenotoxin-A and -B were suggested to have the same A moiety as palytoxin, at least for elemental composition.

HPLC Conditions:

Column:	Extended C18 , Strata 3.5μm, 150 x 3 mm (Agilent)		
Mobile Phase A:	Water 50mM AcH		
Mobile Phase B:	Acetonitrile 50mM AcH		
Flow	0.2 mL/min		
Gradient	min	A (%)	B(%)
	0	95	5
	10	0	100
	14	0	100
	15	95	5
Injection Volume:	5 μL		
Ionization Mode	Positive		

LC-MS/MS Method

MS Conditions:

a) Jet Stream Settings

Gas Temp.: 200 °C

Flow Gas: 8 L/min

Nebulizer Pressure: 25 Psi

Sheath Gas Temp.: 250°C

Sheath Gas Flow: 9 L/min

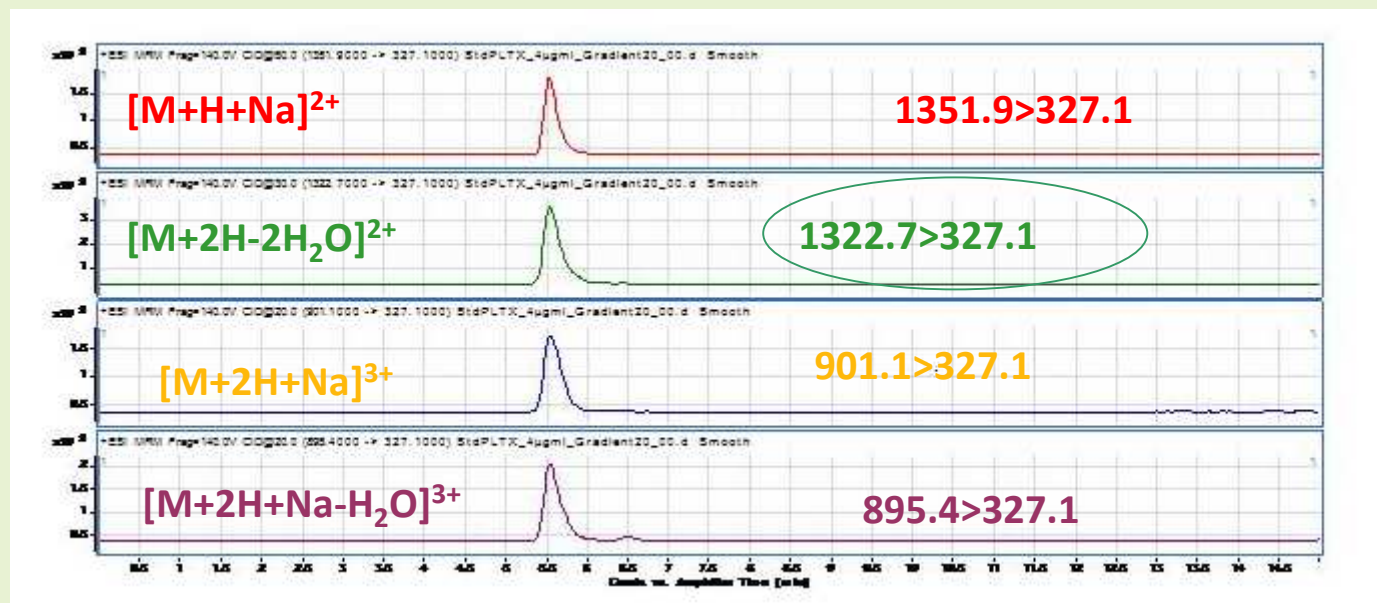
Capillary Voltage: 3000V

Nozzle Voltage: 400 V

b) Mass Spectrometry

PITX , MRM (m/z = 1351.9-1322.7-901.1-895.4) → 327.1

UHPLC-MS/MS PITX



Std PLTx (Wako) 4 μ g/mL in methanol:water (1:1)

Same molecular ions from bibliography Ciminiello *et al* 2008



CONCLUSIONS

MBA has been successfully replaced by LC-MS/MS for the analysis of Lipophilic toxins

MASS SPECTROMETRY became the most powerful technique for the analysis of these toxins

Improved MS methods are being developed which demonstrate the potential of this technique for this particular application

Mass Spectrometry plays also an important role in the identification of New and Emerging toxins

ACKNOWLEDGEMENTS

- EURLMB STAFF
- DG SANCO (EU COMMISSION)
- EU NRLs
- UVIGO STAFF
- AESAN (Spanish Food Safety and Nutrition Agency)
- ALL OUR COLLABORATORS IN THE FIELD OF MARINE TOXINS