Catalogue of Methods and Procedures on Toxin Detection.

Contents.

ALERTOX-NET project aims to facilitate market delivery of safer seafood products by putting at disposal of affected industries a new toxicity alert system for prevention strategies/processes across the value chain.

This document is a catalogue of toxin detection methods and procedures focused on emerging toxins detection and it was written by the consortia of the ALERTOX-NET project. The catalogue is a contribution of the available methods and procedures for toxicity detection in food products.

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Catalogue of methods and procedures:

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2. Universidade de Porto (U.Porto), Portugal.

3. Fundación Azti (AZTI), Spain.

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10. Commisariat à lÉnergie Atomique (CEA), France.

11. Centre for Environment Fisheries and Aquaculture Science (CEFAS), UK.

1. Universidad de Santiago de Compostela (USC).

1.1. Receptor-based methods

1.1.1. Surface plasmon resonance biosensor method for palytoxin detection based on Na+,K+-ATPase affinity.

This method represents a useful approach to easily detect the presence of PLTX-like compounds in marine products using the mechanism of action of these toxins. The detection method for PLTX is based on its binding to the Na+,K+-ATPase. The method uses the phenomenon of surface plasmon resonance (SPR) to monitor biomolecular reactions. This technique does not require any labeling of components. The interaction of PLTX over immobilized Na+,K+-ATPase is quantified by injecting different concentrations of toxin in the biosensor and checking the binding rate constant. In this way the PLTX-Na+,K+-ATPase association is used as a suitable method for determination of the toxin concentration in a sample. The limit of detection (LOD) for the method is 3.73 pg PLTX and the limit of quantification (LOQ) is 11.2 pg PLTX (*A. Alfonso, et al.* Toxins 2013; 6 (1): 96-107).

1.1.2. Detection of spirolides by fluorescence polarization using Torpedo electrocyte membranes based on the competition of cyclic imine marine biotoxins with a-bungarotoxin for binding to nicotinic acetylcholine receptor.

This assay can be used to detect the spirolides such as 13-desmethyl spirolide C and 13,19-didesmethyl spirolide C in shellfish as a screening assay. Toxins (13-desmethyl C spirolide and 13,19-didesmethyl C spirolide) are detected in mussel matrix after an acetone: chloroform extraction with an average recovery rate of 77.7 ± 1.9%. The quantification range for toxins in mussel is 40–200 µg/kg of shellfish meat (*E. Fonfría, et al*. Anal Biochem 2010; 403: 102-7).

1.1.3. Detection and quantification of spirolides in mussel samples using a direct fluoresce polarization assay with nicotinic acetylcholine receptor from *Torpedo marmorata* membranes.

This assay is a fluorescence polarization (FP) method that detects and quantifies spirolides in mussel samples. Nicotinic acetylcholine receptor from *Torpedo marmorata* membranes labelled with a derivative of fluorescein is used. After 10 incubation minutes, there is a decrease in FP when toxin concentration is increased. Thus, a direct relationship between the FP units and the spirolides amount present in a sample is obtained. This assay is a reproducible, simple and very sensitive direct method with a detection limit about 25 nM for 13 desmethyl spirolide C and 150 nM for 13,19-didesmethyl spirolide C. The procedure is used to measure spirolides in mussel samples using an extraction and clean up protocol. This method is useful to quantify 13-desmethyl spirolide C in the range of 50–350 µg/kg meat (*P. Otero, et al.* Anal Chim Acta 2011;701(2):200-8).

1.1.4. High-throughput receptor-based assay for the detection of spirolides by chemiluminescence.

This method is based on the competition between 13-desmethyl spirolide C and biotin-α-bungarotoxin immobilized on a streptavidin-coated surface, for binding to nicotinic acetylcholine receptors. In this inhibition assay the amount of nAChR bound to the well surface is quantified using a specific antibody, followed by a second anti-mouse IgG antibody labelled with horseradish peroxidase (HRP). The sensitivity of the assay for 13-desmethyl spirolide C ranged from 5 to 150 ng/mL. The performance of

the assay in scallop extracts is adequate, with an estimated detection limit for 13-desmethyl spirolide C of 50 μg/kg of shellfish meat. The recovery rate of 13-desmethyl spirolide C is 80% and the inter-assay coefficient of variation is 8%. This chemiluminescence method can be used as a high-throughput screening assay to detect 13-desmethyl spirolide C in shellfish meat to reduce the number of samples to be processed through bioassays or analytical methods (*L. Rodriguez, et al.* Toxicon. 2013;75:35-43).

1.1.5. Solid-Phase Receptor-Based Assay for the detection of cyclic imines using a microsphere-flow cytometry system.

The method consists in a receptor-based detection method for spirolides using a microsphere/flow cytometry Luminex system. For the assay, two alternatives were considered as binding proteins, the *Torpedo marmorata* nicotinic acetylcholine receptor (nAChR) and the *Lymnaea stagnalis* acetylcholine binding protein (Ls-AChBP). A receptor-based inhibition assay was developed using the immobilization of nAChR or Ls-AChBP on the surface of carboxylated microspheres and the competition of cyclic imines with biotin-α-bungarotoxin (α-BTX) for binding to these proteins. The method allows the detection of the 13-desmethyl spirolide C in three different shellfish extracts, mussels, clams and scallops, in the range of 10–6000 μg/kg of shellfish meat and a LOD of 3 μg/kg. This microsphere-based assay provides a rapid, sensitive, and easily performed screening method that could be multiplexed for the simultaneous detection of several marine toxins (*L. Rodriguez, et al.* Anal Chem 2011; 83(15):5857-63).

1.2. Cell-based methods

1.2.1. Fluorimetric method based on neurblastoma changes in membrane potential for screening TTXs , paralytic shellfish toxins, ciguatoxins and brevetoxins in shellfish.

This method screen toxins based on its functional activity on neuroblastoma, the toxins bind to the voltage-gated Na+ channels and block their activity. The method is a fluorimetric assay that allows quantification of the toxins by detecting changes in the membrane potential of human excitable cells such as PSP or TTX. The detection limits for PSP are 1 ng STX equivalents/mL. This assay gives an estimate of toxicity, since each toxin present in the sample binds to sodium channels with an affinity which is proportional to its intrinsic toxic potency (*MC Louzao, et al*, Analytical Biochemistry 2001; 289: 246-250).

1.2.2. Detection of palytoxins by *in vitro* microplate assay with human neuroblastoma cells.

In this method, rat-derived hepatocytes (Clone 9) and BE (2)-M17 human neuroblastoma cells are used to test palytoxin or palytoxin-like compounds by measuring the cell metabolic rate with Alamar Blue. This is a functional, dynamic and simple test for palytoxins with high sensitivity (0.2 ng/mL). This method is suitable for the routine determination and monitoring of palytoxins and palytoxin-like compounds in *Ostreopsis* extracts and naturally contaminated mussel sample (*B Espiña, et al.* Biosci Rep. 2009; 29(1):13-23.

1.2.3. Palytoxin detection and quantification by fluorescence polarization technique.

The detection method is based on the interaction between the Na,K-ATPase and the palytoxin. The Na,K-ATPase is labeled with a reactive succinimidyl ester of carboxyfluorescein, and the fluorescence polarization of protein-dye conjugate is measured when the amount of palytoxin in the medium is

modified. The method is suitable to detect palytoxin in mussel samples and dinoflagellates cultures. The LOQ is 10nM and the LOD is 2 nM (*A Alfonso et al*. 2012;424(1):64-70).

1.2.4. Detection of palytoxin-like compounds by a flow cytometry-based immunoassay assay.

This is an immuno-detection method for PLTX-like molecules based on the use of microspheres coupled to flow-cytometry detection. This single detection method is useful for the screening of PLTX-like molecules in different matrixes. The assay consists of the competition between free PLTX-like compounds in solution and PLTX immobilized on the surface of microspheres for binding to a specific monoclonal anti-PLTX antibody. This method displays an IC_{50} of 1.83 ± 0.21 nM and a dynamic range of 0.47-6.54 nM for PLTX. This method is suitable for mussel samples providing a recovery rate of 104 ± 8% and a range of detection from 374 ± 81 to 4430 ± 150 µg/kg. Extracts of *Ostreopsis cf. siamensis* and *Palythoa tuberculosa* were tested and yielded positive results for PLTX-like molecules (*M Fraga, et al.* Anal Chem. 2017; 89(14):7438-7446).

1.3. Analytical methods

1.3.1. Detection of TTX and analogues by ultra-Hight-performance liquid chromatography system coupled to triple quadrupole mass spectrometer (UPLC-MS/MS).

Chromatographic separation is performed using ACQUITY UPLC BEH Amide column at 35°C and a gradient system of mobile phases. Mobile phase A comprises 100% water with 10mM formic acid and 10mM ammonium formate and mobile phase B is acetonitrile: water (95:5), containing 5mM formic acid and 2 mM ammonium formate. MS detection is performed using a triple quadrupole mass spectrometer equipped with a Jet Stream ESI source (*I Rodriguez, et al.* Sci Rep. 2017;7:40880. doi: 10.1038/srep40880).

1.3.2. UPLC-MS/MS method for identification and quantification of hydrophilic (PSPs, TTX and DA) marine toxins.

Chromatographic separation is performed using ACQUITY UPLC BEH Amide column (2.1 x 100 mm, 1.7µm) at 35°C and a gradient system of mobile phases. Mobile phase A: 100% water with 0.1% formic acid and 10mM ammonium formate. Mobile phase B is 98 % acetonitrile with 0.1% formic acid and 2% 100 mM ammonium formate. Gradient from 95% to 5% B. The injection volume is 5 µL and the mobile phase flow rate is 0.3 mL/min for a total run time of 15 min. MS detection is performed using a triple quadrupole mass spectrometer equipped with a Jet Stream ESI source. (*I Rodriguez*, et al. Talanta. 2018; 189:622-628).

1.3.3. Detection of TTX and analogues by ultra-Hight-performance liquid chromatography system coupled to an ion trap with time-of-flight mass spectrometer (UPLC-IT-TOF-MS).

As before, chromatographic separation is performed using ACQUITY UPLC BEH Amide column at 35°C and a gradient system of mobile phases. Mobile phase A comprises 100% water with 10mM formic acid 10mM ammonium formate and mobile phase B is acetonitrile: water (95:5), containing 5mM formic acid and 2 mM ammonium formate. Gradient from 95% to 5% B. The injection volume is 5 µL and the mobile phase flow rate is 0.4 mL/min for a total run time of 14 min. MS detection is performed in an IT-TOF-MS system with an electrospray ionization (ESI) interface. TTXs are analyzed using MS^{1}, MS^{2} and MS^{3} data by $[M+H]^{+}$, several water loses and 178 and 162 adducts. (*I Rodriguez, et al.* Sci Rep. 2017;7:40880. doi: 10.1038/srep40880).

1.3.4. Detection of emerging mycotoxins by UPLC-IT-TOF-MS.

The LC separation of fungi metabolites is performed by a Waters ACQUITY HSS T3 column at 40 °C. The separation is performed by an elution gradient of 13 min using a mobile phase composed by (A) water containing 0.1% formic acid and 5mM ammonium formate and (B) methanol. The MS method is operated in positive and negative full scan MS mode (MS^1 data) within the mass range 150-850 and toxins are identified by $[M+H]^+$, $[M+K]^+$, $[M+Na]^+$, $[M+NH_4]^+$ ions and several loses *(JM González-Jartín, et al.* Talanta 2018;178:37-42. doi: 10.1016/j.talanta.2017.09.009).

1.3.5. Detection and quantification of mycotoxins by UPLC-MS/MS.

As before, the LC separation of fungi metabolites is performed by a Waters ACQUITY HSS T3 column at 40 °C. The separation is performed by an elution gradient of 13 min using a mobile phase composed by (A) water containing 0.1% formic acid and 5mM ammonium formate and (B) methanol. The MS detection is performed by multiple reaction monitoring (MRM) selecting two transitions per toxin. The limit of detection is comprised between 0.75-3.25 ng/mL depending on the compound analysed (JM González-Jartín, et al. J Agric Food Chem. 2017;65(23):4843-4852. doi: 10.1021/acs.jafc.7b01845).

1.3.6. Detection and quantification of Lipophilic toxins by UPLC-MS/MS (EU reference method for lipophilic toxins).

The toxins are separated using a column AQUITY UPLC BEH C18 (2.1 x 100 mm, 1.7μm) at 40°C. Mobile phase A was 100% water and B acetonitrile-water (95:5), both containing 50 mM formic acid and 2 mM ammonium formate. The gradient program with a flow rate of 0.4 mL/min was started with 30% B and then a linear gradient to 70% B in 3 min. After an isocratic hold time linear of 1.5 min at 70% B and return to the starting conditions of 30% B in 0.1 min. Finally, 30% B was kept for 1.99 min before the next injection. MS detection is performed in MRM mode selecting two transitions per toxin *(I Rodriguez, et al.* Toxins 2016, 8(6), 175; https://doi.org/10.3390/toxins8060175).

1.3.7. Detection of azaspiracids analogs by UPLC-MS/MS

Toxins are separated using a column AQUITY UPLC BEH C18 (2.1 x 100 mm, 1.7μm) at 40°C. Mobile phase A was 100% water and B acetonitrile-water (95:5), both containing 50 mM formic acid and 2 mM ammonium formate. The gradient program with a flow rate of 0.4 mL/min was started with 30% B and then a linear gradient to 70% B in 20 min. After an isocratic hold time linear of 2.5 min at 70% B and return to the starting conditions of 30% B in 0.5 min. Finally, 30% B was kept for 2 min before the next injection. MS detection is performed in MRM mode selecting two transitions per toxin.

1.3.8. UPLC-MS/MS method for identification and quantification of hydrophilic and lipophilic marine toxins.

Chromatographic separation is performed using ACQUITY UPLC BEH Amide column (2.1 x 100 mm, 1.7μm) at 35°C and a gradient system of 3 mobile phases: mobile phase A: 100% water with 0.1% formic acid and 10mM ammonium formate; mobile phase B is 98 % acetonitrile with 0.1% formic acid and 2% 100 mM ammonium formate; mobile phase C is 100% acetonitrile. The injection volume is 5 μL and the mobile phase flow rate is 0.3 mL/min for a total run time of 19 min. MS detection is

performed using a triple quadrupole mass spectrometer equipped with a Jet Stream ESI source in MRM mode selecting two transitions per toxin (*I Rodriguez*, et al. Talanta. 2018; 189:622-628).

1.3.9.   Identification and characterization of palytoxins-like molecules by UPLC-IT-TOF-MS.

UPLC separation is carried out with an HSS T3 column coupled to an in-line filter kit. Mobile phases A and B are water and acetonitrile, respectively, acidified with 30 mM formic acid. The flow rate is 0.4 mL/min, and temperature is maintained at 35 °C. PLTXs are analyzed using MS^1 and MS^2 data. MS^1 spectra, with tri-, bi-, and monocharged ions, and the fragmentation pattern in MS^2 is used for identification and confirmation of PLTX, *m/z* 906.81 ($[M + H + Ca]^{3+}$ or $[M + 2H + K]^{3+}$) and 1359.71 ($[M + Ca]^{2+}$ or $[M + H + K]^{2+}$); and 42-OH-PLTX, *m/z* 912.15 ($[M + H + Ca]^{3+}$ or $[M + 2H + K]^{3+}$) and 1367.72 ($[M + Ca]^{2+}$ or $[M + H + K]^{2+}$). The LOD and LOQ for PLTX are 190 and 650 ng/mL, respectively (*M Fraga, et al.* Anal Chim Acta 2016; 903:1-12. doi: 10.1016/j.aca.2015.09.040).

1.3.10.   Identification and quantification of Ciguatoxins (CTXs) by UPLC-MS/MS

Chromatographic separation is performed at 35°C, the injection volume is 5 µL and flow rate of 0.4 mL/min using a column AQUITY UPLC BEH C18 (2.1 × 100 mm, 1.7 µm, Waters, Manchester, UK). Mobile phases A and B are water and acetonitrile: water (95:5), respectively, both acidified with 50 mM formic acid and 2mM ammonium formate. Chromatographic separation was performed by gradient elution starting with 50% B for 2.5 min, then increasing to 100% B for 4.5 min., this condition was hold for 4.5 min and reducing afterward to 50% B over 0.1 min. MS detection is performed in SIM mode. The efficacy of the method was studied in different matrix species after an extraction protocol Data showed no loss of toxin in each step. The results agree with the efficiency achieved in the method previously described (>95% for P-CTX-1B). The limit of detection (LOD) is 1.125 µg/Kg and a limit of quantification (LOQ) is 3.75 µg/Kg (Otero *et al.* Anal Chem. 2010;82(14):6032-9 and *M Silva*, et al. Toxins 2015; 7(9):3740-57. doi: 10.3390/toxins7093740).

1.3.11.   Identification and quantification of Ciguatoxins (CTXs) by UPLC-MS-IT-TOF

Toxin separation is performed with an ACQUITY UPLC Phenyl-Hexyl column (2.1 × 100 mm, 1.7 µm particle size, Waters, Spain). Mobile phases A and B were water and acetonitrile: water (95:5), respectively, both acidified with 50 mM formic acid and 2 mM ammonium formate. Chromatographic separation is performed by gradient elution starting with 50% B for 2.5 min, then increasing to 100% B for 4.5 min., this condition is hold for 4.5 min and reduced afterward to 50% B over 0.1 min. This proportion is maintained 2.4 min. until the next injection to equilibrate the system. The mobile phase flow rate is 0.4 mL/min, the injection volume is 5 µL and the temperature is maintained at 35°C for a total run time of 14 min. The MS method is operated in positive ESI source mode. CTXs are analyzed using MS^1 data and identified by $[M+H]^+$, $[M+K]^+$, $[M+Na]^+$, $[M+NH_4]^+$ ions and several water loses (*M Silva*, et al. Toxins 2015; 7(9):3740-57 and *I Rodriguez et. al*. Org Lett. 2015 May 15;17(10):2392-5).

1.3.12.   Identification and quantification of brevetoxins (PbTx) by UPLC-MS-IT-TOF

Toxin separation is performed with an UPLC BEH C18 (2.1 × 100 mm, 1.7 µm particle size, Waters, Spain). Mobile phases A and B were water and methanol, respectively, both acidified with 0.1% acetic acid. Chromatographic separation is performed by gradient elution starting with 50% B to 100% B. The mobile phase flow rate is 0.4 mL/min, the injection volume is 5 µL and the temperature is maintained at 35°C for a total run time of 14 min. The MS method is operated in positive ESI source mode. PbTx

are analyzed using MS[1] data and identified by [M+H]+, [M+K]+, [M+Na]+, [M+NH4]+ ions and several water loses.

1.3.13. Detection of pinnatoxins by UPLC-MS/MS (EU reference method for lipophilic toxins).

The toxins are separated using a column AQUITY UPLC BEH C18 (2.1 x 100 mm, 1.7µm) at 40°C. Mobile phase A was 100% water and B acetonitrile-water (95:5), both containing 50 mM formic acid and 2 mM ammonium formate. The gradient program with a flow rate of 0.4 mL/min was started with 30% B and then a linear gradient to 70% B in 3 min. After an isocratic hold time linear of 1.5 min at 70% B and return to the starting conditions of 30% B in 0.1 min. Finally, 30% B was kept for 1.99 min before the next injection. MS detection is performed in MRM mode selecting three transitions per toxin [M+H]+> 572, [M+H-H_2O]+, 164).

1.3.14. Detection of cianotoxins by UPLC-MS/MS

The toxins are separated using a column AQUITY UPLC HSS T3 (2.1 x 100 mm, 1.7µm) at 35°C. Mobile phase A was 100% water and B acetonitrile, both containing 0.05% formic acid. The gradient program with a flow rate of 0.4 mL/min was started with 0% B for 4 min, and then a linear gradient to 70% B in 4 min. After an isocratic hold time linear of 2 min at 70% B and then return to the starting conditions of 0% B in 0.5 min. Finally, 0% B was kept for 2.5 min before the next injection. MS detection is performed in MRM mode selecting two transitions per toxin (*I Rodriguez, et. al.* Environ Toxicol Chem. 2017 Mar;36(3):645-654).

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2. University of Porto/CIIMAR

2.1. Detection of Tetrodotoxin and Paralytic Shellfish Toxins by LC-MS/MS using Porous Graphitic Carbon Column

Based on the method of Rey *et al*. (2018) using Hypercarb column as stationary phase, here in a 20 min run all relevant hydrophilic and hydrophobic PSTs analogs and TTXs are discriminated. Toxins were extracted with HCl followed by a TCA deproteination and a dichloromethane partion, prior to cleaning step with Hypercarb SPE cartridges proofed to minimize matrix effect enabling the application of this method to different shellfish species.

2.2. Detection of Lipophilic Toxins by LC-MS/MS using BDS-Hypersil-C8 Column

This method based on Otero *et al*. (2011) allows analyzing simultaneously Spirolides (13,19-SPX; 13-SPX; 20G-SPX), Azaspiracids regulated and not regulated, Okadaic acid and its congeners and the Yessotoxin group in an 18 min run. Based in a methanolic extraction followed by a double partition with dichloromethane, the method showed good results when tested with different benthic matrices - unpublished work.

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3. Fundación Azti (AZTI).

3.1. Zebrafish embryo test for the detection of marine toxins

The procedure is based on the evaluation of the effect of toxins on the model vertebrate zebrafish (*Danio rerio*). The procedure is an adaptation of a method employed for evaluating the effect of other chemical contaminants (Barranco et al, Journal of Applied Toxicology 2017; 37:253-264). It consists of extracting toxins from contaminated shellfish using standard methods and to expose zebrafish embryos to the extracted solution during 24-48h. After the exposure, acute toxicity is visually detected by evaluating embryos' death and malformations. Also, subacute toxicity is evaluated by differential expression of selected genes.

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4. National University of Ireland Galway (NUIG).

4.1. Structural Analysis of Toxins

4.1.1. NMR

The NMR experiments are performed on either a 500 MHz spectrometer (Varian) or 600 MHz spectrometer (Agilent) equipped with a cryoprobe. In order to assess the full structure of a pure toxin, at least one milligram is necessary. The bigger the mass of the toxin, the more quantity required. 500 µl of deuterated solvent will be added to the sample and then the solution will be transferred in either a 3 or 5 mm NMR tube. Depending on the purity of the sample assessed by the ^{1}H NMR analysis, it will be decided to go further with 1D and 2D experiments (^{13}C, COSY, TOCSY, HSQC, HMBC and ROESY).

4.1.2. LC-MS/MS

High-resolution mass spectra (HRESIMS) are obtained from an Agilent 6540 mass spectrometer coupled with a UPLC (Agilent). Depending on the size and the nature of the compound, the method will be decided. The Marine Biodiscovery Lab possesses all types of reverse phase columns (C8, C18, Amide, Cyano, HILIC, PFP, T3, Phenylhexyl, Phenyl).

4.1.3. Chiroptical techniques

4.1.3.1. Electronic Circular Dichroism

Electronic Circular Dichroism data are obtained on a Chirascan V100 spectrophotometer (Applied Photophysics). This technique requires a submilligram quantity of a sample and allows the assignment of its absolute configuration by comparison with theoretical spectra. It is necessary that the molecule analysed possesses both a chiral centre and a chromophore nearby. The analysis follows an in-house Standard Operating Procedure.

4.1.3.2. Vibrational Circular Dichroism

Vibrational Circular Dichroism data are obtained on a Chiral*IR-2X*$^{\text{TM}}$ spectrometer (Biotools). The technique requires at least 5 mg of sample and allows the assignment of its absolute configuration by comparison with theoretical spectra. On the contrary to the ECD technique, the VCD only requires the presence of at least one chiral centre. The analysis follows an in-house SOP.

5. Institute Français de Recherche pour l´Exploitation de la Mer (IFREMER), France.

5.1. LC-MS/MS and LC-HRMS methods for ciguatoxins in seafood.

A LC-MS/MS method and a LC-HRMS method for the detection of ciguatoxins in seafood (Sibat et al., 2018) was developed. Although no formal validation has been performed, the LOD/LOQ for CTX1B of the optimized LC-MS/MS method is in the same height of order than EFSA and US guidelines (i.e. 0.010 µg CTX1B eq. /kg), even if it still requires improvement for multiple analogues present. The use of HRMS is still hampered by a lack of sensitivity but proved useful to detect false positive samples or to confirm the identity of the most concentrated P-CTX-contaminated ones. The method is used in seafood (sea urchin, gastropod, fishes) and extraction protocol was developed which is as follows. Extraction: Acetone on lyophilized tissue (10:150, w,v) repeated twice, dried extract reconstituted in 90% MeOH, defatted twice with hexane, dried and reconstituted in a ethyl acetate/MeOH (90:10, v:v) before a two-step SPE clean-up (Florisil, Sep-pak 500 mg, Waters and C18 prevail, 500 mg, Alltech). The purified dried extract was reconstituted in MeOH before analysis. For the LC-MS/MS

5.1.1. Analysis of LC-MS/MS: Zorbax Eclipse Plus C18 column (50 x 2.1 mm, 1.8 µm, Agilent) with mobile phase A: water, B: MeOH, both with 2 mM ammonium formate and 50 mM formic acid. Flow: 0.4 mL/min. V: 5 µL and T: 40 °C. Gradients: 78 – 88% B in 10 min, hold 4 min and equilibration (total run 20 min). MS/MS: Scheduled MRM detection (+MS, QTRAP 4000, ABSciex) for 12 ciguatoxins. Quantitation against P-CTX3C (Wako) and P-CTX1B and in matrix-matched calibration curve (fish matrix). LOD: 0.006 µg/kg (P-CTX1B) ; 0.050 and µg/kg (P-CTX3C). LOQ: 0.013 µg/kg (P-CTX1B) and 0.150 µg/kg (P-CTX3C) (matrix effect 85-115%). Validation: Not performed

5.1.2. Analysis of LC-HRMS: C18 Kinetex column (50 x 2.1 mm, 1.7 µm, Phenomenex) with mobile phase A: water, B: ACN 95% all with 2 mM ammonium formate and 50 mM formic acid. Flow: 0.4 mL/min. V: 5 µL and T: 40 °C. Gradient: 10 – 90% B in 5 min, held 2 min and equilibration (total run 13 min). HRMS: full scan (m/z 100 – 1700) and targeted MS/MS (m/z 150 – 1700) (QTOF 6550, Agilent). Quantitation against P-CTX3C (Wako) and P-CTX1B and in matrix-matched calibration curve (fish matrix). LOD: 0.075 µg/kg (P-CTX1B) and 0.100 µg/kg (P-CTX3C). Validation: Not performed.

5.2. LC-MS/MS method for ovatoxins in marine organisms.

Due to recurrent blooms of *Ostreopsis* cf. *ovata* in the NW Mediterranean Sea (Villefranche-sur-Mer), we have developed an LC-MS/MS method for the quantitation of ovatoxins in marine organisms to assess accumulation in seafood (Brissard et al., 2014). This method has been optimized over several years and the column has been replaced by the Poroshell 120-EC C18 (100 x 2.1 mm, 2.7 µm, Agilent) to get satisfactory resolution between analogues. We have now two LC-MS/MS methods for the identification of palytoxin, isobaric palytoxin and 11 ovatoxins (a to f and g to k) as dwell time had to be maximized for better sensitivity. No formal validation has been performed. The method is used in digestive tube and remaining flesh of marine organisms (sea urchin, gastropod, fish) and extraction protocol was developed which is as follows. Extraction: 90% MeOH (2:3, w/v) repeated thrice and volume adjusted to 10 mL before ultrafiltration (0.2 µm). Analysis: LC-MS/MS. C18 Gemini column (150 x 2 mm, 3 µm, Phenomenex) or Poroshell 120-EC C18 (100 x 2.1 mm, 2.7 µm, Agilent). A: water and B: 95% ACN both with 2 mM ammonium formate and 50 mM formic acid. Flow: 0.2 mL/min. V: µL and T: 22 °C. Gradient: 20 – 100 % (B) in 10 min, held for 4 min and equilibration (total run 20 min).

MS: MRM detection (+MS, QTRAP 4000, ABSciex) with 3 transitions/toxin for isobaric PLTX and 6 ovatoxines (a – f) Quantitation against PLTX standard (Wako) assuming same molar response. LOD: 15 ng/mL for the PLTX standard. LOQ: 50 µg/kg (sea urchin matrix). Validation: Not performed

5.3. LC-MS/MS method for pinnatoxins in mollusks.

Following atypical toxicity with the mouse bioassay, we have developed an LC-MS/MS method for the quantitation of pinnatoxins and pteriatoxins in mollusk matrices that allowed us to pinpoint Pinnatoxin G as the causative toxin in bivalves from a French Mediterranean lagoon (Hess et al., 2013). This method is fast and highly sensitive thus suitable for the monitoring of pinnatoxins in shellfish and is still used to date, with only minor changes (e.g. use of another LC column for complex profiles and addition of transitions for portimine, PnTX H and I). However, no formal validation has been performed. Samples: homogenized digestive glands and whole flesh of mussels and clams. Extraction: MeOH (1:9, w/v) repeated twice and volume adjusted to 20 mL before ultrafiltration (0.2 µm)

Analysis: LC-MS/MS. Hyperclone MOS C8 column (50 x 2 mm, 3 µm, Phenomenex) with the C8 guard column. Mobile phases: A: water and B: 95% ACN both with 2 mM ammonium formate and 50 mM formic acid. Flow: 0.2 mL/min. injection volume: 5 µL and T: 25 °C. Gradient: 30 – 95 % (B) in 2.5 min, held for 5 min and equilibration (total run 10 min). MS: MRM detection (+MS, QTRAP 4000 or 5500, ABSciex) with 3 transitions/toxin for 7 pinnatoxins (A – G) and 3 pteriatoxins (A – C). Quantitation against PnTX G standard (NRC) assuming same molar response. LOD: 0.05 ng/mL for the PnTX G standard. LOQ: 0.5 ng/mL corresponding to 5 µg/kg (matrix effects: 68-89% and 79-110% recovery in digestive gland and whole flesh tissues). Validation: Not performed

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6. Laboratorio Cifga S.A. (CIFGA).

6.1. Simultaneous detection of Tetrodotoxins and Paralytic Shellfish Toxins by UPLC-HILIC-MS/MS

CIFGA developed and uses an internal standard operating procedure for the detection of a wide range of PSTs: STX, dcSTX, NEO, dcNEO, GTX5, GTX6, dcGTX1&4, dcGTX2&3, GTX1&4, GTX2&3, C3&4 and C1&2, including TTX and analogues. Chromatographic separations is achieved by a Waters (Milford, MA, USA) ACQUITY UPLC BEH Amide column (100 x 2.1 mm, 1.7 µm) or (150 x 2.1 mm, 1.7 µm). Second larger column gives a baseline resolution for GTX2/dcGTX2 and GTX3/dcGTX3 pairs of toxins (Rs=1.5). *Not published, internal CIFGA method.*

These methods are used simultaneously with that from *Turner et al. (2015)*, since inter-comparative trial was also implemented for the detection of PSPs and TTX.

Extraction method from *Boundy et al. (2015)* is used in order to carry out UPLC-HILIC-MS/MS analysis.

6.2. Simultaneous detection of Tetrodotoxins and Paralytic Shellfish Toxins by HPLC-Hypercarb-MS/MS.

Another method for less sensitive equipments was developed and validated at CIFGA and USC (*Rey et al., 2018*). The method involves Hypercarb column (100 mm × 2.1 mm, ID 3 µm; Thermo Fisher Scientific). Two PGC columns were tested, having used each one previously for a period of 8-10 years. This method allows determination of PSTs, both hydrophilic and hydrophobic (namely GC toxins), along with TTX and its analogue 4,9-anhydro tetrodotoxin (4,9-anhTTX) with LC-MS/MS. The method was validated for 13 hydrophilic PSTs and TTXs and is able to discriminate 6 hydrophobic PSTs in 20 min.

Samples extraction and clean-up is carried out according to the extraction protocol for analysis of PSTs by HPLC-FLD using Hypercarb (*Rey et al., 2016*).

6.3. Detection of analogues of Azaspiracids by LC-MS/MS.

Reverse-phase UHPLC-MS/MS method under acid elution condition was developed by CIFGA for simultaneous detection of 27 AZAs analogues. In addition, neutral elution was performed to evaluate the presence of 37-epimers of the regulated AZA1-3. *Not published, internal CIFGA method.*

Marine Lipohilic official extraction method was implemented for the analysis of these toxins.

6.4. Detection of brevetoxins and cyclic imines by LC-MS/MS.

Reverse-phase UPLC-MS/MS method under acid elution condition was develop for analysis of 11 brevetoxins. SIR experiments were developed and a relative response factor, RRF=1 to Brevetoxin-3 is assumed. *Not published, internal CIFGA method.*

Marine Lipohilic official extraction method was implemented for the analysis of these toxins.

6.5. Detection of cyclic imines by LC-MS/MS.

Reverse-phase UPLC-MS/MS method under acid elution condition was develop for the analysis of 17 spirolides, gymnodimine and 8 pinnatoxins. *Not published, internal CIFGA method.*

Marine Lipohilic official extraction method was implemented for the analysis of these toxins.

6.6. Detection of gambierone and 44-methyl-gambierone by LC-MS/MS.

Reverse-phase UPLC-MS/MS method under acid elution condition was develop for analysis of these toxins associated to *Gambierdiscus*. *Submitted (not published yet), internal CIFGA method.*

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7. Marine Institute (MI).

7.1. Detection and quantification of toxins by UPLC-MS/MS (EU reference method for lipophilic toxins).

Toxin Groups: Lipophilic Toxins Group.

Analogues Monitored: *Diarrheic Shellfish Poisoning Toxins* (DSP) including:Okadaic Acid,(OA) Dinophysis toxin 1 (DTX-1), Dinophysis toxin 2 (DTX-2) and their esters by hydrolysis. *Azaspiracid Shellfish Poisoning* (AZP) including: Azaspiracid 1 (AZA-1), Azaspiracids 2 (AZA-2) and Azaspiracids 3 (AZA-3). *Yessotoxin Group* toxins including: TX, Homo YTX, 45 homo OH YTX and 45 OH, YTX, *Pectenotoxins:* PTX-1 and PTX-2.

Brief Description:

Samples are extracted in 100% methanol. Total DSP toxins are detected by conversion of their esters to OA, DTX1 and DTX-2. Hydrolysis is carried out on a sub-sample of the extract with Sodium Hydroxide. Samples (hydrolysed and un-hydrolysed) are analysed by UPLC MS/MS XEVO using either alkaline or UPLC-Xevo G2-S MS-ToF acidic methods

UPLC MS/MS XEVO Toxins are separated using EU harmonised method for the determination of lipophilic toxins by LC-MS/MS : Analytical Column – Acquity BEH C18 2.1x100mm 1.75µm particle size or equivalent: Column temperature: 40 ºC : Injection Volume: 5µl. Mobile phase A: 50ml 2mM Ammonium bicarbonate, (pH 11±0.5), with7.5ml ammonium hydroxide solution (28-30%) made up 500mL Deionised water: Mobile phase B: 50ml 2mM Ammonium bicarbonate (pH 11±0.5), made up 500ml with MeCN: Flow rate of 0.6ml/min: 0 mins 75:25 A:B; 1.5 mins 50:50 A:B; 1.7 mins 25;75 A:B; 4.0 mins 100:0 A:B: 7.2mins 75:25.

UPLC-Xevo G2-S MS-ToF. A gradient method is applied to separate and elute the toxins in a separate positive and negative run. The method employs an acidic mobile phase
Analytical Column – Acquity BEH C18 2.1x100 mm 1.75 µm particle size or equivalent: Column temperature: 30 ºC : Injection Volume: 2µl. Mobile phase A: 50 mL of 1M formic acid, 40mM ammonium formate in 1 L distilled H2O: Mobile phase B 50 mL of 1M formic acid, 40mM ammonium formate in 1 L MeCN: Flow rate of 0.3ml/min
NEG 4.0mins run-time: 0 mins 95:5 A:B; 1 mins 50:50 A:B; 2 mins 10: 90 A:B; 3.01 mins 95:5 A:B; POS 6.5 mins run-time: 0 mins 70:30 A:B; 5 mins 10:90 A:B; 5.51 mins 70: 30 A:B.

7.2. Detection and quantification of AZA analogues.

Toxin Group: *Azaspiracid Shellfish Poisoning* (AZP) by UPLC-Xevo G2-S MS-ToF.

Analysis is performed on an Acquity UPLC coupled to a Xevo G2-S QToF operated in MS^e (100–1200 *m/z*) mode. Leucine encephalin is used as the reference compound.

Brief Description Cone voltage 40 V: Collision energy is 50: Cone gas flow rate 100 L/h : Desolvation gas flow rate 1000 L/h : Source temperature is 120 °C: injection volume is 2 µL: Sample temperature 6 C: Binary gradient elution is used: phase A water and phase B of 95% acetonitrile in water (both containing 2 mM ammonium formate and 50 mM formic acid): Column type 50 mm × 2.1 mm i.d., 1.7 µm, Acquity UPLC BEH C18: Column temperature 25 °C: The gradient is from 30–90% B over 6 min at

0.3 mL/min, held for 0.5 min, and returned to the initial conditions and held for 1 min to equilibrate the system.

7.3. Detection and quantification of AZA analogues Paralytic toxins by (EU reference method for lipophilic toxins): Lawrence method

Toxin Group: Paralytic Shellfish Poisoning (PSP) by Liquid Chromatography with fluorescence detection (UHPLC FLD).

Analogues Monitored: dcGTX-2, 3, dcSTX, GTX-2, 3, GTX-5, STX, C-1, 2, GTX-1, 4, NEO and dcNEO

Brief Description: Samples are extracted by heating with 1% acetic acid and cleaned up using solid phase extraction (SPE) C18 cartridges. The screening method involves periodate oxidation of samples, quality control materials and high and low level standards followed by analysis using reverse phase Liquid Chromatography (UPLC) with fluorescence (FLD) detection. Positive samples can then be fully quantified through either peroxide oxidation of the SPE C18 extract or further cleaned using a COOH SPE step before periodate oxidation, depending on the analogues identified in the screening method. Results are expressed as µg STX diHCl equivalents per kg using toxic equivalents factors.

Analytical Column with guard – Supelcosil C18 15 x 4.6mm, 5µm:column temperature 30C: Mobile phase A(0.1M ammonium formate, 100% H_2O): Mobile phase B (0.1M ammonium formate, 5% MeCN): automated Gilson XL4 SPE system: injection volume 50µL periodate oxidations and 20 µL peroxide oxidations: 13 min run time: : Flow rate of 2.0/min: 0 mins 100:0 A:B; 5 mins 95:5 A:B; 9 mins 30: 70 A:B; 10 mins 100:0 A:B.

7.4. Detection and quantification of Tetrodotoxin toxins by UPLC MS/MS XEVO XS (Boundy *et. al*., 2005 Lawrence *et al*.2017)

Toxin Groups: Tetrodotoxin (TTX) by UPLC MS/MS XEVO XS

Analogues Monitored: TTX and 4, 9-anhydro TTX and 4-epiTTX

Brief Description: Sample extraction with (1:100 v/v) acetic acid/water followed by clean up with SPE Supelco ENVI-Carb cartridge**.** Tetrodotoxin (TTX) and its analogues are determined by reverse phase Liquid Chromatography (UPLC) coupled to Mass Spectrometry MS/MS XEVO XS.
A gradient method is applied to separate and elute the toxins in a single chromatographic run : XBridge BEH Amide, 2.1x150mm and a 2.5µm particle size: 0.6ml/min flow rate; Column temperature 40 C sample temperature 5 C; injection volume 5uL:Mobile phase A 100% water with 75ul formic acid+300ul ammonium hydroxide: mobile phase B acetonitrile: water (70:30)+100ul formic acid: Run time 4.0 mins: Flow rate of 0.6ml/min: 0 mins 5:95 A:B; 1.33 mins 10:90 A:B; 2.5 mins 50;50 A:B; 3.17 mins 5:95 A:B

7.5. PCR method for the detection of *Azadinium spinosum* for rapid routine monitoring

Toxin Groups: Azadinium species

Brief Description: An in-house validated and accredited method (ISO 17025) for the detection of *A. spinosum* in seawater samples. The PCR molecular technology detects specific DNA target regions of toxic producing phytoplankton n species. It is has a high sample through-put, is highly specific to low numbers and has a rapid turnaround time. Cultured or field samples are extracted using the TaqMan

hydrolysis probe assay: The method has now been expanded as a multi-plex assay for both *A. spinosum* and *A.poporum*.

8. The Queen's University of Belfast (QUB)

8.1. Cytotoxicity studies by High Content Analysis (HCA)

In vitro cell-based multi-parametric cytotoxicity imaging tool for quantitative analysis of single cells using automated fluorescence microscopy and advanced imaging software. HCA has been used in QUB as an unvalidated research tool to assess the synergistic cytotoxicity of single toxins and multiple toxin combinations including marine toxins, mycotoxins, microcystins and other contaminants (e.g. pesticides, heavy metals).

> Assessing the combined toxicity of the natural toxins, aflatoxin B1, fumonisin B1 and microcystin-LR by high content analysis.
> J.P. Meneely, *et al.* Food and Chemical Toxicology **121** (2018) 527-540
> http://dx.doi.org/10.1016/j.fct.2018.09.052

> Challenging conventional risk assessment with respect to human exposure to multiple food contaminants in food: A case study using maize.
> R. Clarke, *et al.* Toxicology Letters **238** (2015) 54–64
> http://dx.doi.org/10.1016/j.toxlet.2015.07.006

> High content analysis: A sensitive tool to detect and quantify the cytotoxic, synergistic and antagonistic effects of chemical contaminants in foods.
> R. Clarke, *et al.* Toxicology Letters **233** (2015) 278–286
> http://dx.doi.org/10.1016/j.toxlet.2015.01.011

8.2. Mycotoxin determination by UPLC-MS/MS

Triple-quadrupole UPLC-MS/MS method employing QuEChERS extraction for detection and quantification of 77 mycotoxins and other fungal metabolites. Validated to conventional in-house (single laboratory) standards as a research tool for use with animal feed ingredient DDGS.

> Determination of the mycotoxin content in distiller's dried grain with solubles using a multianalyte UHPLC–MS/MS method.
> M. Oplatowska-Stachowiak, *et al.* J. Agric. Food Chem. **63** (2015) 9441–9451
> http://dx.doi.org/10.1021/acs.jafc.5b03844

8.3. Cyanotoxin determination by UPLC-MS/MS

Triple-quadrupole UPLC-MS/MS method employing solid phase extraction for detection and quantification of freshwater cyanotoxins: microcystins-LR, -YR, -RR, -LA, -LY and -LF, nodularin, cylindrospermopsin, anatoxin-a and the marine diatom toxin domoic acid in brackish and freshwater samples. Validated to conventional in-house (single laboratory) standards as a research tool.

> A validated UPLC–MS/MS method for the surveillance of ten aquatic biotoxins in European brackish and freshwater systems.
> B. Greer, *et al.* Harmful Algae **55** (2016) 31–40
> http://dx.doi.org/10.1016/j.hal.2016.01.006

Microcystins-LR, -YR, -RR, -LA, -LY and -LF, nodularin, cylindrospermopsin, anatoxin-a and masked microcystins also measured in fish muscle, liver and eggs by UPLC-MS/MS method employing solid phase extraction. Validated in-house (single laboratory) as a research tool for fish muscle using the core principles of EC Decision 2002/657.

Detection of freshwater cyanotoxins and measurement of masked microcystins in tilapia from Southeast Asian aquaculture farms.
B. Greer, *et al.* Anal. Bioanal. Chem. **409** (2017) 4057–4069
http://dx.doi.org/10.1007/s00216-017-0352-4

8.4. Tetrodotoxin determination by UPLC-MS/MS

Triple-quadrupole UPLC-MS/MS method employing solid phase extraction for detection and quantification of tetrodotoxin and estimation of several TTX analogues in shellfish tissues. Basic in-house validation only as a research tool. Not published.

8.5. Marine and freshwater biotoxin detection by planar waveguide immunobiosensor

The MBio SnapEsi® LS System biosensor is an rapid immunoassay instrument based on planar waveguide with fluorescence detection capable of portable multiplex detection of biotoxins. Used by QUB as a research tool to demonstrate methods for detection of paralytic shellfish toxins in marine algae[a]; microcystins in freshwater[e] and cyanobacterial extracts[b]; domoic acid, okadaic acid, saxitoxin, cylindrospermopsin and microcystins in algal/cyanobacterial cultures and seawater[c]; cylindrospermopsin in freshwater[e]; and azaspiracids in mussel and oysters[d]. All validation to conventional in-house (single laboratory) standards.

a. Development and validation of an ultrasensitive fluorescence planar waveguide biosensor for the detection of paralytic shellfish toxins in marine algae.
J.P. Meneely, *et al.* Biosensors and Bioelectronics **41** (2013) 691–697
http://dx.doi.org/10.1016/j.bios.2012.09.043

b. Next generation planar waveguide detection of microcystins in freshwater and cyanobacterial extracts, utilising a novel lysis method for portable sample preparation and analysis.
S. Devlin, *et al.* Analytica Chimica Acta **769** (2013) 108– 113
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c. Development of a Planar Waveguide Microarray for the Monitoring and Early Detection of Five Harmful Algal Toxins in Water and Cultures.
S.E. McNamee, *et al.* Environ. Sci. Technol. **48** (2014) 13340–13349
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d. Detection of azaspiracids using an immunosensor.
M. Subrt, *et al.* Poster 298, (2018) 18th International Conference on Harmful Algae, Nantes, France, 21-26 October 2018.

e. An Innovative Portable Biosensor System for the Rapid Detection of Freshwater Cyanobacterial Algal Bloom Toxins.
S.R. Bickman, *et al.* Environ. Sci. Technol. **52** (2018) 11691-11698
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ALERT SYSTEMS FOR SAFER SEAFOOD PRODUCTS

9. Instituto Tecnológico para el control del medio marino de Galicia (INTECMAR)

9.1. Determination of lipophilic marine toxins by LC-MS/MS

In-house validation of determination of the lipophilic marine toxins according the "EU -Harmonised Standard Operating Procedure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS (Version 5, January 2015)".The instrumental method is based in the work "Automated on-line solid-phase extraction coupled to liquid chromatography–tandem mass spectrometry for determination of lipophilic marine toxins in shellfish" ([1]Regueiro et al. Food Chemistry 129 (2011) 533–540) without on-line SPE step.

9.2. Determination of emerging azaspiracid by UPLC-MS/MS

"Presence of azaspiracids in bivalve molluscs from Northern Spain" ([2]J. Blanco et al. Toxicon, 137 (2017) 135–143). With this method we can identify 31 emerging azaspiracids easily.

9.3. Determination of Tetrodotoxin by HILIC-MS/MS.

This method is for determination of TTX and TTX analogues in marine bivalves and gastropods. ([3]European Union Reference Laboratory for Marine Biotoxins, Ed. 01 06 2017).

Table 1: Summary of the proposed methods

Proposed method	Marine toxins	Matrix	Reference method	Chromatographic system
9.1	AO; DTX1; DTX2; PTX1; PTX2; AZA1; AZA2; AZA3; YTX; Homo-YTX; 45OH-YTX; 45OH-HomoYTX; GYM; 13 -desmethyl SPX C; 13,19-didesmethyl SPX C; 20 -methyl SPX G; Pinatoxin G	Bivalve molluscs	[1]Regueiro et al. Food Chemistry 129 (2011) 533–540	UPLC-MS/MS (TQ) without on-line SPE step
9.2	AZA-1,2,3,4,5,6,7,8,9,10,11,12; AZA-33,34,35,36,37,38,39,40,41,42,43; AZA-54,55,56,57,58; Me-AZA2; AZA2 phosphate; AZA11phosphate	Bivalve molluscs	[2]J. Blanco et al. Toxicon, 137 (2017) 135–143	UPLC-MS/MS (TQ)
9.3	TTX; 4epi-TTX; 11deoxy-TTX; 5deoxy-TTX; 4,9 Anhydro-TTX; 6,11dioxy-TTX; 5,6,11trideoxy-TTX	Mussel, Oyster	[3]European Union Reference Laboratory for Marine Biotoxins, Ed. 01 06 2017.	UPLC-MS/MS (TQ)

[1]Automated on-line solid-phase extraction coupled to liquid chromatography–tandem mass spectrometry for determination of lipophilic marine toxins in shellfish, Regueiro et al. Food Chemistry 129 (2011) 533–540.

[2]Presence of azaspiracids in bivalve molluscs from Northern Spain, J. Blanco et al. Toxicon, 137 (2017) 135–143.

[3]Determination of Tetrodotoxin by HILIC-MS/MS, European Union Reference Laboratory for Marine Biotoxins, Ed. 01 06 2017.

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10.Commisariat à lÉnergie Atomique (CEA).

10.1. Mass spectrometry-based methods for detection of freshwater and marine neurotoxins

10.1.1 MALDI-TOF analysis for anatoxin-a

We developed a matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) method for the detection of the low molecular weight anatoxin-a and homoanatoxin-a directly on lyophilized and fresh filaments of axenic strains of the genus Oscillatoria, using 2,5-dihydroxybenzoic acid (DHB) as matrix. To counteract the span of low molecular mass ions (< m/z 1000) generated by the matrix, we induced the matrix-suppression effect to obtain high quality anatoxin-a and homoanatoxin-a MALDI signals. MALDI desorption/ionization of the matrix-anatoxin-a and the matrix-homoanatoxin-a generated protonated molecules [M+H]+ at m/z 166.12322 and 180.1372, respectively. The method is rapid and straightforward: toxin standards (1μl) or emulsified cyanobacterial filaments (1μl) are mixed with 1 μl DHB directly on the stainless-steel MALDI-sample plate, the mixture is allowed to polymerize for some minutes prior to MALDI-TOF analysis (Aráoz et al. 2008a).

10.1.2 UPLC-MS/MS

A method for the simultaneous detection and quantification of gymnodimine A, 13,19-didesmethyl spirolide C, pinnatoxin A, 13-desmethyl spirolide C, 20-methyl spirolide G and pinnatoxin G by Ultra Performance Liquid Chromatography coupled to a Triple Quadrupole Detector (UPLC-TQD, Waters, MA, US) was developed. The samples are chromatographed through a BEH C18 column (Waters, 2.1 x 100 mm; 1.8 μm particle size) at a flow rate of 0.6 mL/min in a 6-min run. Data acquisition is performed by multiple reaction-monitoring mode (MRM) (Aráoz et al., 2012)

10.2. Receptor-based methods for detection of freshwater and marine neurotoxins

10.2.1 Filtration radioactive ligand-binding assay for freshwater cyanotoxins

This is the reference method for screening nicotinic acetylcholine receptor (nAChR) ligands. Briefly, the analyte is incubated with *Torpedo* electrocyte membranes in an appropriate buffer. An amount of radioactive tracer is added, and the whole is incubated to equilibrium. The analyte will compete with the radioactive tracer for binding to the acetylcholine binding site. Following radioligand displacement, the complex receptor-radioligand is adsorbed onto a glass fiber membrane by filtration. Unbound radioligand is removed by successive washing steps. Scintillation counting is performed from dried filters. In a study of the Cyanobacterial Collection of Pasteur Institute, five out of seventy-six axenic filamentous cyanobacterial strains were shown to inhibit 125I-α-bungarotoxin binding to Torpedo-nAChR (Aráoz et al., 2005).

10.2.2 Non-radioactive filtration binding assay

A non-radioactive filtration assay was developed for detecting cyanobacterial anatoxin-a and homoanatoxin-a. Competition experiments using *Torpedo*-electrocyte membranes rich in nAChR were performed at equilibrium by incubating *Torpedo* membranes with cyanobacterial samples and

biotinylated-α-bungarotoxin as toxin-tracer. The mixture was filtered and rinsed in a 48-well dot-blot device. Detection was performed by chemiluminiscence or by color precipitation (Aráoz et al. 2008b).

10.2.3   Non-radioactive Microplate-Receptor Binding Assay

We developed a method for the detection and identification of aquatic neurotoxins based on the high affinity of the toxins for their receptor targets and constitutes an alternative to antibody based ELISA methods. Advantageously, the microplate-receptor binding assay allows the detection of different families of neurotoxins directed against the same receptor or channel targets. The microplate-receptor binding assay (WO2012101378A1) and is commercialized by ABRAXIS. Torpedo marmorata-electrocyte membranes, rich in muscle-type nAChR, were immobilized and stabilized on the surface of 96-well microtiter plates. Biotinylated α-bungarotoxin (the toxin tracer) and streptavidin-horseradish peroxidase (the conjugate) enabled the detection and quantitation of anatoxin-a in surface waters that were obtained from different locations across the US (Rubio et al., 2014) and of cyclic imine toxins in shellfish extracts obtained from a French seafood market (Aráoz et al. 2012). When present in environmental samples, both, anatoxin-a and cyclic imine neurotoxins competitively inhibit biotinylated-α-bungarotoxin binding to *Torpedo*-nAChR in a concentration dependent manner. The method compares favorably to liquid chromatography tandem mass spectrometry and provides accurate results for anatoxin-a, and cyclic imine toxins monitoring. The microplate-receptor binding assay allows rapid detection of nanomolar concentrations of cyclic imine neurotoxins directly in shellfish samples The assay allowed the simultaneous analysis of up to 25 samples per triplicate within 3.5 h and it is well suited for in-situ or laboratory monitoring of low levels of toxins in drinking, surface, and ground water as well as in shellfish extracts. This functional assay is a high throughput method for rapid detection of nicotinic neurotoxins directly in environmental samples with minimal sample handling, high sensitivity, reduced matrix effect and low cross-reactivity. This method constitutes an alternative to the mouse bioassay for the survey of known and unknown neurotoxins that target nAChR from marine and freshwater organisms

10.2.4   Toxin-receptor lateral flow test « NeuroTorp »

We have developed a lateral flow test to detect fast-acting neurotoxins blocking nAChR based on a new concept for lateral flow tests: The high affinity of neurotoxins for their receptor targets. The prototype «NeuroTorp » is based on the immobilization of Torpedo-membrane lipid-vesicles, rich in nAChR, on high porosity borosilicate membrane filter support, and on the use of a biotinylated α-bungarotoxin as toxin-tracer. Visual detection is performed by enzymatic reaction or by using a nano-gold coated conjugate or fluorescent toxin-tracer. When a toxic sample is applied, the nicotinic toxin will displace the toxin tracer enabling its visual detection. In contrast to immunochromatographic tests that detect a given toxin, NeuroTorp could be applied to the detection of a series of toxin families acting on nicotinic receptors like the freshwater anatoxin-a and the cyclic imine toxins pinnatoxins, spirolides, gymnodimines, α-bungarotoxin (WO2012101378A1). NeuroTorp provides the means for early, rapid and quantitative detection of nicotinic toxins in the field by end-users.

10.2.5   Neurotoxin fishing

A methodology for discovering new ligands directed against nicotinic acetylcholine receptors from complex mixtures containing small size alkaloids or large peptides was developed. The methodology uses purified electrocyte membranes from *Torpedo marmorata in-solution* (Echterbille et al., 2017) or immobilized on the bottom of 96-wells microplates (Aráoz et al., 2012). The present method allows the direct capture of large marine snails' conopeptides or aquatic alkamoids of small size by *Torpedo*-nicotinic acetylcholine receptors *in-solution* or immobilized on the bottom of 96-well microplates. After washing and elution, the chemical nature of the captured toxin could be determined by mass spectrometry.

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11. Centre for Environment Fisheries and Aquaculture Science (CEFAS).

11. 1. Simultaneous detection of Tetrodotoxin and Paralytic Shellfish Toxins by UHPLC-HILIC-MS/MS

A method was developed (Boundy et al., 2015) and validated (Turner et al., 2015) at Cefas in collaboration with Cawthron Institute for the detection of a wide range of saxitoxins, including "M toxins" and other analogues which are emerging analogues currently not assessed through standard HPLC detection methods. The method also includes TTX, emerging as a significant risk in European bivalve molluscs. The method involves a simple, rapid single-dispersive extraction in 1% acetic acid, followed by desalting clean-up using carbon solid phase extraction, prior to dilution and analysis by UHPLC-HILIC-MS/MS, with a total run time of 11 mins. The method is capable of detection and quantifying all PST analogues available as reference standards and was shown to have acceptable performance characteristics following single laboratory validation in 12 species of bivalve mollusc shellfish. The method is currently undergoing international collaborative study, with the aim of achieving international acceptance.

11.2. Detection of multiple Tetrodotoxins by HILIC-MS/MS

The PST/TTX method has been modified to allow detection and quantitation of a range of TTX analogues, with a total run time of 7 mins. The TTXs method uses the same single dispersive extraction and carbon SPE clean-up as for PST, with a modified LC gradient to achieve separation between TTXs and interfering matrix co-extractives such as arginine. Turner et al., 2017. The method has been single-laboratory validated for mussels and oysters.

11.3. Detection of extended analogues of AZAs by LC-MS/MS

Reverse-phase UHPLC-MS/MS method developed for simultaneous detection of 40 AZA analogues, in addition to the regulated AZA1-3. The method has been validated and applied to the quantitation of AZAs in UK shellfish. Not yet published.

11.4. Detection of brevetoxins and cyclic imines by LC-MS/MS

Reverse-phase UHPLC-MS/MS method for detection of cyclic imines (spirolides and pinnatoxins) plus brevetoxins available as reference materials. Simultaneous detection enables rapid detection of a wide range of emerging toxins. Not published, internal Cefas method.

11.5. Detection of the cyanotoxins (microcystins and nodularins) in shellfishery products by LC-MS/MS

Method developed and single-laboratory validated for detection and quantitiation of microcystins and nodularins in water, algae and shellfish tissues. Currently includes all MC analogues available as commercial reference standards. The method is accredited to ISO17025 at Cefas and is routinely applied to monitoring of recreational waters as well as potential uptake of cyanotoxins in shellfish and fishery food products. Reference: Turner et al., 2018.

11.6. Ciguatoxins by LC-MS/MS and N2A cell toxicity

Method under development for detection of Pacific and Caribbean ciguatoxins. N2A method to be installed into lab Spring 2019. No reference available yet.

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