Determination of Tetrodotoxin by HILIC-MS/MS

EUROPEAN UNION REFERENCE LABORATORY FOR MARINE BIOTOXINS

Ed 01 06 2017

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1. Summary

Tetrodotoxin (TTX) and its analogues are produced by marine bacteria and have been detected in marine bivalves and gastropods from European waters.

As a result of discussions among the EU Commission WG on Bivalve molluscs, regarding the risk of the presence of TTX in marine bivalves and gastropods in the EU, the European Commission asked EFSA for a scientific opinion on the risks to public health related to the presence of TTX and TTX analogues in the named seafood.

The Panel on Contaminants in the Food Chain reviewed the available literature but did not find support for the minimum lethal dose for humans of 2 mg, mentioned in various reviews. Some human case reports describe serious effects at a dose of 0.2 mg, corresponding to 4 µg/kg body weight (bw). However, the uncertainties on the actual exposure in the studies preclude their use for derivation of an acute reference dose (ARfD). Instead, a group ARfD of 0.25 µg/kg bw, applying to TTX and its analogues, was derived based on a TTX dose of 25 µg/kg bw at which no apathy was observed in an acute oral study with mice, applying a standard uncertainty factor of 100. Estimated relative potencies for analogues are lower than that of TTX but are associated with a high degree of uncertainty. Based on the occurrence data submitted to EFSA and reported consumption days only, average and P95 exposures of 0.00–0.09 and 0.00–0.03 lg/kg bw, respectively, were calculated. Using a large portion size of 400 g bivalves and P95 occurrence levels of TTX, with exception of oysters, the exposure was below the group ARfD in all consumer groups. A concentration below 44 µg TTX equivalents/kg shellfish meat, based on a large portion size of 400 g, was considered not to result in adverse effects in humans. Liquid chromatography with tandem mass spectroscopy (LC–MS/MS methods are the most suitable for identification and quantification of TTX and its analogues, with LOQs between 0.1 and 25 µg/kg.

(Scientific opinion on the risks for public health related to the presence of tetrodotoxin (TTX) and TTX analogues in marine bivalves and gastropods." (2017) EFSA Journal, 15(4), 4752-4817)

The recommendations included in the EFSA Opinion on TTX are summarized below:

Recommendations

• More occurrence data on TTX and its analogues in edible parts of marine bivalves and gastropods from different EU waters are needed to provide a more reliable exposure assessment.

• Occurrence data on marine gastropods are needed from different EU Member States.

• Data on concentrations of TTX and its analogues should be obtained using EU approved and validated chemical-analytical methods. In addition, certified standards and reference materials for TTX and analogues are needed.

• Information on the fate of TTX and its analogues during cooking is needed to refine exposure assessments.

• Studies on the sources and critical factors leading to the accumulation of TTX in marine bivalves and gastropods are needed.

• Further information on toxicokinetics of TTX and its analogues is needed.

• Further information on the acute oral toxicity of TTX and its analogues is needed. Chronic

effects should also be investigated.

• Given the high uncertainties associated with derivation of the relative potencies of TTX analogues, adequate and well described evidence is needed to estimate their relative potencies, preferentially after oral exposure.

• As STX and TTX exert similar toxic effects via a similar mode of action, the possibility to combine STX and its analogues together with TTX and its analogues in one HBGV should be explored.

FOREWORD

The EURLMB WG on LC-MS met in Brussels on June 7th 2017 and the Conclusion and recommendations of the EFSA Opinion was evaluated.

The EURLMB proposed LC-MS/MS as the method to be used for the evaluation studies on TTX incidence in bivalves and gastropods in the EU.

The EURLMB presented the studies carried out on the Single Laboratory Validation (SLV) of LC-MS/MS for the analysis of TTX in mussels and agreed to the group that a summary on these results as well as the analytical procedure used on this SLV will be distributed among the Group to help with the method development with the aim of organizing a future intercomparison study among the NRLs interested in this intercomparison, taking into account the restrictions imposed by the lack of naturally contaminated samples as well as standards and reference materials for TTX analogues.

This Document summarizes the analytical protocol carried out at the EURLMB for the intralaboratory validation of HILIC- LC-MS/MS for TTX in mussels.

The conditions used in this SLV validation were based on those proposed by Turner on a pre-trial study for the validation of PSP and TTX by HILIC- LC-MS/MS (see references).

The summary of the results of the EURLMB- SLV are included in ANNEX

.

2. Chemical and physical properties

Table 1: Chemical and physical properties

Property name	**Property value**
Molecular weight	319,27 g/mol
Exact mass	319,102 g/mol
Solubility	Highly soluble in water (10^6 mg/L at 25ºC) (US EPA 2004), diluted acetic acid, partially soluble in alcohol and ether, insoluble in acetonitrile, acetone (O'Neil 2006).
Stability	Stable to boiling except in an alkaline solution.
CAS Number	4368-28-9

3. Equipment

3.1. LC-system equipped with MS/MS detector

3.2. Boiling water bath

3.3. Centrifuge, operating at ≥ 3000 g for this method (normally run 4500 rpm)

3.4. Micro-centrifuge

3.5. Vortex

3.6. Vacuum Manifold for manual SPE, if required

3.7. Micropipettes

3.8. Polypropylene centrifuge tubes

3.9. Polypropylene 700 µL autosampler vials

3.10. Waters Acquity UPLC Glycan BEH Amide HILIC Column 130 Å 1.7 µm, 2.1 x 150 mm

3.11. Supelco SupelcleanTM ENVI-Carb 250 mg/3 mL

4. Reagents

4.1. Primary Reagents

4.1.1. Acetonitrile (MeCN), LC-MS and HPLC grade

4.1.2. Methanol (MeOH), LC-MS

4.1.3. Water, Deionised (Milli-Q) or LC-MS

4.1.4. Formic acid 98-100% LC-MS

4.1.5. Glacial Acetic acid (HAc)

4.1.6. Ammonium Hydroxide LC-MS additive, (25% as NH_3)

4.2. HILIC LC Mobile Phases

4.2.1. Mobile Phase A1:

500 mL water (LC-MS) + 75 µL formic acid + 300 µL ammonium hydroxide

(Mix well between and after formic acid and ammonium hydroxide additions)

Important: due to NH_4OH volatility and low concentration in Mobile Phase A1, this mobile phase must be prepared freshly (every day).

4.2.2. Mobile Phase B1:

700 mL acetonitrile (LC-MS) + 300 mL water (LC-MS) + 100 µL formic acid and mix well

4.2.3. Mobile Phase A2 (for shutdown):

200 mL water (LC-MS) + 1 mL formic acid and mix well

4.2.4. Mobile Phase B2 (for shutdown):

Methanol (LC-MS)

4.3. Sample Preparation Reagents

4.3.1. 1% $_{v/v}$ HAc : 1000 mL water (deionised) + 10 mL acetic acid

4.3.2. 20%$_{v/v}$ MeCN with 1% $_{v/v}$ HAc: 200 mL acetonitrile (HPLC) + 800 mL Milli-Q + 10 mL HAc

4.3.3. 0,025% $_{v/v}$ NH_3: 500 mL Milli-Q + 500 µL 25% NH_3

4.3.4. Standard Dilution Solvent, 80% $_{v/v}$ MeCN with 0,25% $_{v/v}$ HAc : 80 mL acetonitrile + 20 mL Milli-Q + 250 µL HAc

4.4. Standard

4.4.1. Validation studies were carried out using tetrodotoxin (TTX), 1 mg (Tocris-Bioscience, Bristol UK), Batch 43B, MW: 319,27g/mol ($C_{11}H_{17}N_3O_8$)

4.4.2. Certified standards for TTX are available in CIFGA

4.4.3. Other standards of TTX are commercial available, which information are provided on EURLMB webside.

5. Analytical procedure

5.1. Sample Extraction

Accurately weigh 5,0 ± 0,1 g of tissue into 50 mL polypropylene centrifuge tubes. Add 5,0 mL 1% $_{V/V}$ HAc (4.3.1), and mix thoroughly on a vortex for 90 s. Cap and place in a boiling water bath for 5 min. Remove from water bath, and cool until achieve room temperature. Remix on a vortex for 90 s. Centrifuge at ≥ 4000 g for 10 min. Transfer 1 mL of supernatant to a 1.5 mL centrifuge tube and add 5 µL of 25%$_{V/V}$ NH_3 (4.1.6), and vortex. Centrifuge at ≥ 10000 g for 1 minute. Clean-up extract with Graphitised Carbon SPE.

Observation: Ammonium hydroxide improves TTX retention on carbon stationary phase and produces protein precipitation in shellfish extract. TTXs retention in HILIC column are strongly affected by proteins.

5.2. Graphitised Carbon SPE Clean-up

Condition an ENVI-Carb 250 mg/3mL cartridge with 3 mL of 20% MeCN + 1% $_{V/V}$ HAc (4.3.2), followed by 3 mL of 0,025% $_{V/V}$ NH_3 (4.3.3). Approximate flow of 6 mL/min (or 1 or 2 drops/s). Elute both to level of top frit and discard to waste.

Add 400 µL of the sample extract to the cartridge. Approximate flow of 6 mL/min. Elute to top frit and discard to waste.

Wash the cartridge with 700 µL Milli-Q water (4.1.3) (**Critical step:** High water quantity or fast flow rate in wash step produces TTXs elution and consequently yields low recovery values). Elute to dryness and discard to waste.

Elute cartridge with 2 mL of 20% $_{v/v}$ MeCN 1% $_{v/v}$ HAc (4.3.2). Approximate flow of 3 mL/min (1 drop/s approximately). Elute to dryness and collect in a polypropylene tube. Mix on a vortex.

Dilute collected samples by transferring 100 µL of eluent and adding 300 µL of MeCN (4.1.1) in a polypropylene 700 µL LC-MS auto-sampler vial. Mix on a vortex.

Observation: diluted extract must be filtered using 0,22 µm membrane filter in order to avoid the chromatographic column blockage.

5.3. Matrix match (MMS) preparation

Acetic shellfish extract purified by SPE-ENVI-Carb (3.11) must be diluted ¼ with MeCN (4.1.1).

5.4. Standard solutions preparation

In this validation study, commercial TTXs standard from Tocris was used (4.4.1).

5.4.1. This commercial standard must be dissolved into 1 mL of 0,03 M acetic acid (commercial standard stock solution).

5.4.2. Commercial standard stock solution (1 mg TTX/mL) must be diluted 1/10 in acetic acid 0,03 M (working solution).

5.4.3. Prepare 6 standard solutions from working solution (5.4.2) using matrix match (MMS) (5.3).

Range: 10µg/kg - 10mg/kg (mussel matrix match); 10µg/kg - 100µg/kg (oyster matrix match).

Observations: TTXs standard solutions are stable in 1 week when preserved at 4ºC or less (standards must be prepared in polypropylene vials, not glass vials).

6. Instrument Conditions

6.1. LC instrument conditions

Table 2: LC instrument conditions

Parameter	Description
Column	Waters Acquity UPLC Glycan BEH Amide HILIC Column 130 Å 1.7 µm, 2.1 x 150 mm
Injection volume	2 µL
Runtime	11 min
Column temperature	60 ºC
Sample compartment	4 ºC

6.2. Run LC System

6.2.1. Turn on LC pump HILIC column connected to LC system with low flow rate (0,1 mL/min); 50% of mobile phase A1 and 50% of mobile phase B1.

6.2.2. Increase slowly flow rate until achieve 0,4 mL/min.

6.2.3. When column backpressure is stable, run UPLC Start-Up HILIC Method for column conditioning (Table 3).

6.2.4. Load UPLC HILIC Method and equilibrate column with mobile phase (inject solvent 2 times or more) (Table 4).

6.2.5. Inject four times lowest standard concentration in MMS and check retention times and peak areas reproducibility. RSD (Relative Standard Deviation) must be <1% for retention time and ≤10% for peak area.

6.2.6. Run standards and samples randomly, and between inject solvent for column cleaning.

6.2.7. Run Cleaning Column Method (inject solvent) (Table 6).

6.2.8. Run UPLC Shutdown HILIC Method (Table 5).

Table 3: UPLC Start-Up HILIC Method

Time (min)	**Flow Rate (mL/min)**	**% A1**	**% B1**
0	0,3	50	50
4	0,3	50	50
6	0,4	50	50

15	0,4	50	50
16	0,5	2	98
17	0,4	2	98
17,5	0,4	2	98

Table 4: UPLC HILIC Method (11 min run time)

Time (min)	Flow Rate (mL/min)	% A1	% B1
0	0,4	2	98
5	0,4	2	98
7,5	0,4	50	50
9	0,5	50	50
9,5	0,5	5	95
9,8	0,8	2	98
10,6	0,8	2	98
11	0,4	2	98

Time (min)	Flow Rate (mL/min)	% A1	% B1
0	0,1	100	0
15	0,3	100	0
30	0,3	100	0
31	0,3	0	100
35	0,3	0	100

Table 5: UPLC Cleaning Column HILIC Method

Table 6: UPLC Shutdown HILIC Method

Time (min)	Flow Rate (mL/min)	% A2	% B2
0	0,3	100	0
4	0,3	100	0
8	0,3	0	100
9	0,3	0	100
11	0,4	0	100
15	0,4	0	100

6.3. MS instrument conditions

Table 7: Source parameter for a 1290 Infinity Agilent LC-MS/MS System (iFunnel Jet Stream Ion Source, 6495 Triple Quad, Agilent)

Source Parameters		
Gas Temperature (ºC)	150	

Gas flow (L/min)	12		
Nebulizer (psi)	45		
Sheath Gas Heater (ºC)	400		
Sheath Gas Flow (L/min)	12		
Capillary (V)	4000		
V Charging (V)	300		
Ion Funnel Parameters			
Positive High Pressure RF	150	Negative High Pressure RF	90
Positive Low Pressure RF	60	Negative Low Pressure RF	60

Other parameters:

- Dwell: 20mS
- Frag: 380V
- Cell Acc (V): 5
- Polarity: Positive
- Acquisition mode: static
- Delta EMV: 400

Table 8: MRM transitions for TTX and analogues (Jang et al. 2010; Boundy et al. 2015)

Compound	**Precursor Ion**	**MS1Res**	**Product Ion**	**MS2Res**
TTX/4-epi-TTX	320,1	Unit/enh(6490)	302,1	Unit/enh(6490)
TTX/4-epi-TTX	320,1	Unit/enh(6490)	162,1	Unit/enh(6490)
11-deoxy-TTX/5-deoxy-TTX	304,1	Unit/enh(6490)	286,1	Unit/enh(6490)

11-deoxy-TTX/5-deoxy-TTX	304,1	Unit/enh(6490)	162,1	Unit/enh(6490)
4,9- Anhydro TTX	302,1	Unit/enh(6490)	284,1	Unit/enh(6490)
4,9- Anhydro TTX	302,1	Unit/enh(6490)	162,1	Unit/enh(6490)
6,11-dideoxy-TTX	290,1	Unit/enh(6490)	272,1	Unit/enh(6490)
6,11-dideoxy-TTX	290,1	Unit/enh(6490)	162,1	Unit/enh(6490)
5,6,11-trideoxy-TTX	272,1	Unit/enh(6490)	254,1	Unit/enh(6490)
5,6,11-trideoxy-TTX	272,1	Unit/enh(6490)	162,1	Unit/enh(6490)

Note: Validation studies were carried out for TTX. Concentration value is available only for TTX (Tocris standard (4.4.1)).

7. Observations

7.1. General recommendations

- Ion source in mass spectrometer should be optimized for each instrument. Parameters must be adjusted in order to achieve highest sensitivity of MS/MS system (Table 7).
- Mass spectrometer optimization: Mass spectrometer parameters should be adjusted for each instrument controlling fragmentation, collision energy, temperature, gas flow, etc. Adjust each parameter in order to achieve highest detection sensitivity of MS/MS system (Table 7 and Other parameters (6.3)).
- Sodium co-elution saturating ESI source suppressing signal by controlling sodium formiate cluster, recommendation proposed by Turner *et al*. 2015. When intense signal is observed, cleaning ion source and HILIC column are required.
- Salts and other matrix contents such us proteins, amino acids, etc affect strongly retention process of TTX in HILIC stationary phase. Sample clean-up must be done properly.
- For LC-MS/MS mobile phases use only LC-MS grade reagents.
- Storage standard and samples properly during the analysis at least < 4ºC.
- Attention during the analysis to the possible mobile phase degradation. Mobile phase A1 (4.2.1.) has low stability.

7.2. Recommendations for improving chromatographic performance

HILIC is a sensitive form of chromatography, particularly in comparison to reverse-phase LC. It is extremely important that this chromatography is approached in a systematic manner.

- Once the sequence is started, keep it running. Do not allow the system to pump mobile phase at starting conditions.

- If analysis has already started, and samples are not ready on the instrument, keep instrument running by several injections of matrix match and methanol solvents, in order to maintain the equilibrium between stationary phase and mobile phase (UPLC HILIC Method (Table 4)).

7.3. Regarding HILIC column

7.3.1. Drift retention times (early or later elution).

a. Clean the column using UPLC Cleaning Column HILIC Method (Table 5).
b. Run UPLC Shutdown HILIC Method (Table 6).
c. UPLC Start-Up HILIC Method (Table 3).
d. Inject two or more times solvent using UPLC HILIC Method (Table 4).
e. Inject low concentration standard, and check the retention time and peak area reproducibility.

7.3.2. Boardning chromatographic peaks.

a. Clean column (see 7.3.1).
b. Prepare fresh mobile phases and use startup and equilibration protocol as describe in 7.3.1.

7.3.3. Equilibration: Between injections allows to column equilibrate during 5 min with initial conditions of UPLC HILIC Method (Table 4).

7.3.4. Solvents composition changes: When solvents composition are changed in LC-system, purge channels very well and start with low flow rate (0,1 mL/min) and increase slowly until run flow rate achieved.

8. References

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quadruple mass spectrometer. *Food Additives & Contaminants: Part A*, *33*(11), 1728-1740.

- Boundy, M. J., Selwood, A. I., Harwood, D. T., McNabb, P. S., & Turner, A. D. (2015). Development of a sensitive and selective liquid chromatography–mass spectrometry method for high throughput analysis of paralytic shellfish toxins using graphitised carbon solid phase extraction. *Journal of Chromatography A*, *1387*, 1-12.

- EFSA CONTAM Panel (EFSA Panel on Contaminants in the Food Chain), Knutsen HK, Alexander J, Barreg_ard L, Bignami M, Brüschweiler B, Ceccatelli S, Cottrill B, Dinovi M, Edler L, Grasl-Kraupp B, Hogstrand C, Hoogenboom L, Nebbia CS, Oswald IP, Rose M, Roudot A-C, Schwerdtle T, Vleminckx C, Vollmer G, Wallace H, Arnich N, Benford D, Botana L, Viviani B, Arcella D, Binaglia M, Horvath Z, Steinkellner H, van Manen M and Petersen A, (2017). Scientific opinion on the risks for public health related to the presence of tetrodotoxin (TTX) and TTX analogues in marine bivalves and gastropods. *EFSA Journal, 15*(4), 4752-4817.

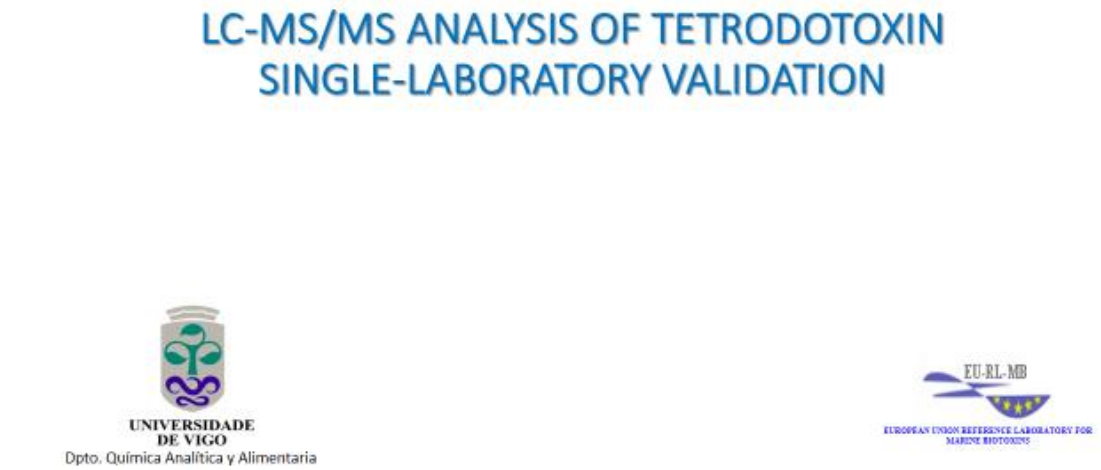
- Jang, J. H., Lee, J. S., & Yotsu-Yamashita, M. (2010). LC/MS analysis of tetrodotoxin and its deoxy analogs in the marine puffer fish Fugu niphobles from the southern coast of Korea, and in the brackishwater puffer fishes Tetraodon nigroviridis and Tetraodon biocellatus from Southeast Asia. *Marine drugs*, *8*(4), 1049-1058.

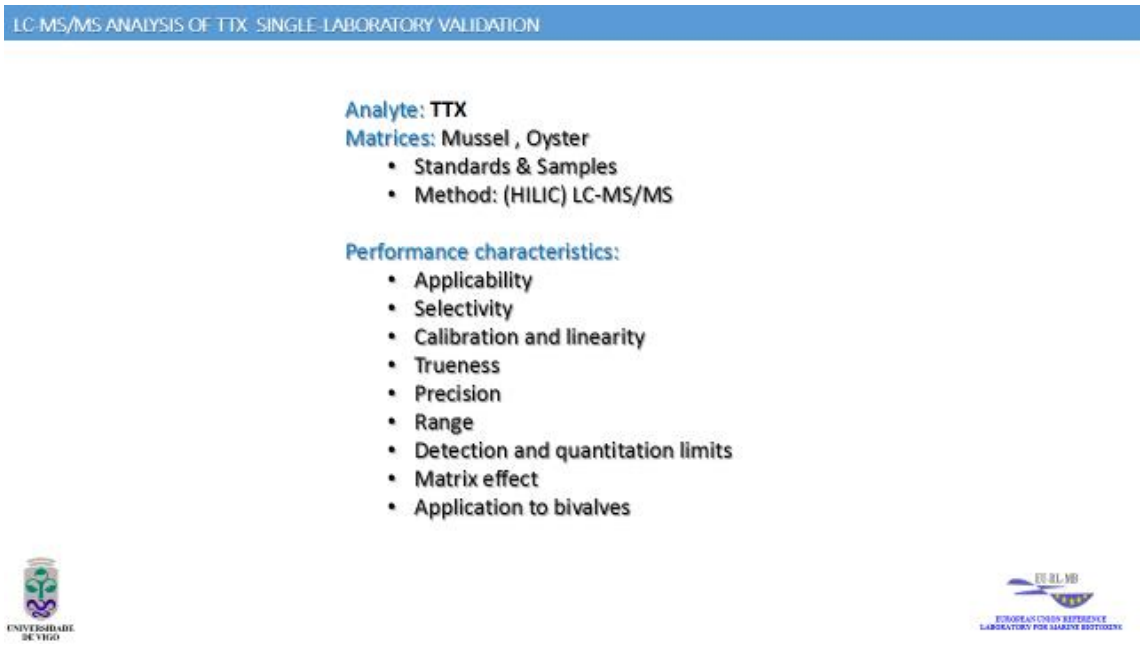
- Turner, A. D., McNabb, P. S., Harwood, D. T., Selwood, A. I., & Boundy, M. J. (2015). Single-laboratory validation of a multitoxin ultra-performance LC-hydrophilic interaction LC-MS/MS method for quantitation of paralytic shellfish toxins in bivalve shellfish. *Journal of AOAC International*, *98*(3), 609-621.

- Turner, A. D., Boundy, M. J., & Rapkova, M. D. (2017). Development and Single-Laboratory Validation of a Liquid Chromatography Tandem Mass Spectrometry Method for Quantitation of Tetrodotoxin in Mussels and Oysters. *Journal of AOAC International, 100*(5), 1-14.

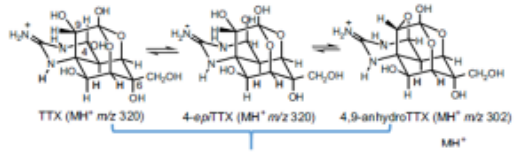
9. Appendix

Results from internal validation studies carried out in EURLMB for TTX's in mussel and oyster are attached in this document.

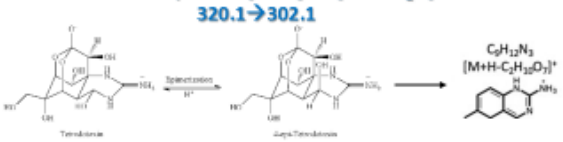




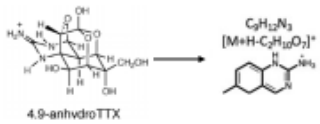
LC-MS/MS ANALYSIS OF TTX SINGLE-LABORATORY VALIDATION



TTX and 4-epi-TTX: $[M+H]^+ \rightarrow [M+H-H_2O]^+$
320.1→302.1



TTX and 4-epi-TTX: $[M+H]^+ \rightarrow [M + H-C_3H_{10}O_7]^+$
320.1→162.1



4,9-anhydroTTX: $[M+H]^+ \rightarrow [M + H-C_3H_{10}O_7]^+$
302.1→162.1

Compound	Percursor Ion	Product ion 1 (Q)	Product Ion 2 (q)
TTX/ 4 epi TTX	320,1	302,1	162,1
4,9 anhydro TTX	302,1	284,1	162,1

LC-MS/MS ANALYSIS OF TTX SINGLE-LABORATORY VALIDATION: STANDARDS & SAMPLES

Standards

- Validation studies were carried out using tetrodotoxin (TTX), 1 mg (Tocris-Bioscience, Bristol UK), Batch 43B, MW: 319,27g/mol ($C_{11}H_{17}N_3O_8$)
- Certified standards for TTX are available in CIFGA
- Other standards of TTX are commercial available, which information are provided on EURLMB webside.

Samples

- Raw mussel tissue, uncontaminated mussel from Spain
- Raw oyster tissue, uncontaminated oyster from Spain.

LC-MS/MS ANALYSIS OF TTX: SINGLE-LABORATORY VALIDATION: METHOD

Sample Extraction

(Turner et al. (2017) Journal of AOAC International, Vol 100 No 5; Turner et al. (2015), Journal of AOAC International, Vol 98, No 5, pp 609-621)

5.0 mL 1%$_{(v/v)}$ HAc : 5,0g homogenized shellfish tissue
(Boiling water bath, 5 min)
Centrifudge (10 min, 2500rpm)
Raw extract

Purification

(Turner et al. (2015), Journal of AOAC International, Vol 98, Nº3, pp 609-621; Boundy et al. (2015), Journal of Chromatography A, Vol 13 87, pp 1-12)

Extract sample pre-SPE: 5µL of NH_3 25%$_{(v/v)}$ was added to 1 mL acetic acid extract, mix and centrifuge 10000rpm (1min)

SPE-ENVI-CARB Conditions:

Conditioning: 3 mL AcN/Water/HAc (20:80:1)$_{v/v}$
3 mL NH_3 0,025% $_{(v/v)}$
Load: 400 µL of Extract
Washing: 700 µL of Ultrapure water
Elution: 2 mL AcN/Water/HAc (20:80:1)$_{v/v}$
Sample dilution: Eluate: AcN (1:4) $_{v/v}$

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TTX SINGLE-LABORATORY VALIDATION: METHOD

(HILIC) LC-MS/MS

(Boundy et al. (2015), Journal of Chromatography A, Vol 1387, pp 1-12; Jang et al. (2010), Marine Drugs, vol. 8, pp. 1049-1058)

HILIC Chromatography conditions

	Solvent	Water	AcN	Formic acid	Ammonium Hydroxide
Mobile Phase A	Vol (mL)	500.000	0.000	0.075	0.300
	C (mM)			3.26	4.28
	C (% $_{v/v}$)			0.015	0.06
Mobile Phase B	Vol (mL)	300.000	700.000	0.100	0.000
	C (mM)			2.17	0.000
	C (% $_{v/v}$)			0.100	0.000

ACQUITY UPLC ***GLYCAN BEH AMIDE HILIC*** Column, 130Å, 1.7 µm, 2.1 mm X 150 mm, **Lot#0140350431**, Waters IRELAND

Time (min)	A (%)	B (%)	Flow Rate (mL/min)
0.0	2.0	98.0	0.4
5.0	2.0	98.0	0.4
7.5	50.0	50.0	0.4
9.0	50.0	50.0	0.5
9.5	5.0	95.0	0.5
9.8	2.0	98.0	0.8
10.6	2.0	98.0	0.8
11.0	2.0	98.0	0.4

Column temperature: 60ºC
Injection Volume: 2µL
Run: 9.0 min
Post Run: 2.0 min

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LC-MS/MS ANALYSIS OF TTX. SINGLE-LABORATORY VALIDATION: METHOD

MS/MS conditions

LC System 1290 Infinity Agilent
iFunnel Jet Stream Ion Source, 6495 Triple Quad, Agilent

Source Parameters			
Gas Temp (ºC)	150		
Gas Flow (L/min)	12		
Nebulizer (psi)	45		
Sheath Gas Heater (ºC)	400		
Sheath Gas flow ((L/min)	12		
Capillary (V)	4000		
V Charging (V)	300		
Ion Funnel Parameters			
Pos High Pressure RF	150	Neg High Pressure RF	90
Pos Low Pressure RF	60	Neg Low Pressure RF	60

Dwell: 20mS
Frag: 380 V
Cell Acc (V): 5
Polarity: Positive
Delta EMV: 400

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LC-MS/MS ANALYSIS OF TTX. SINGLE-LABORATORY VALIDATION: METHOD

Bane et al. (2016), FOOD ADDITIVES & CONTAMINANTS: PART A, VOL. 33, NO. 11, pp. 1728–1740, Jang et al. (2010), Marine Drugs, vol. 8, pp. 1049-1058 , Boundy et al. (2015), Journal of Chromatography A, Vol 1387, pp 1-12

Compound	Precursor Ion	MS1Res	Product Ion	MS2Res
TTX/4-epi-TTX	320.1	Unit/enh(6490)	302.1	Unit/enh(6490)
TTX/4-epi-TTX	320.1	Unit/enh(6490)	162.1	Unit/enh(6490)
11-deoxy-TTX/5-deoxy-TTX	304.1	Unit/enh(6490)	286.1	Unit/enh(6490)
11-deoxy-TTX/5-deoxy-TTX	304.1	Unit/enh(6490)	162.1	Unit/enh(6490)
4,9- Anhydro TTX	302.1	Unit/enh(6490)	284.1	Unit/enh(6490)
4,9-Anhydro TTX	302.1	Unit/enh(6490)	162.1	Unit/enh(6490)
6,11-dideoxy-TTX	290.1	Unit/enh(6490)	272.1	Unit/enh(6490)
6,11-dideoxy-TTX	290.1	Unit/enh(6490)	162.1	Unit/enh(6490)
5,6,11-trideoxy-TTX	272.1	Unit/enh(6490)	254.1	Unit/enh(6490)
5,6,11-trideoxy-TTX	272.1	Unit/enh(6490)	162.1	Unit/enh(6490)

Quantitative transition (Q): 320.1 for TTX and 302.1 for 4,9-anhidro TTX

Qualitative transition (q): all TTX compounds 162.1

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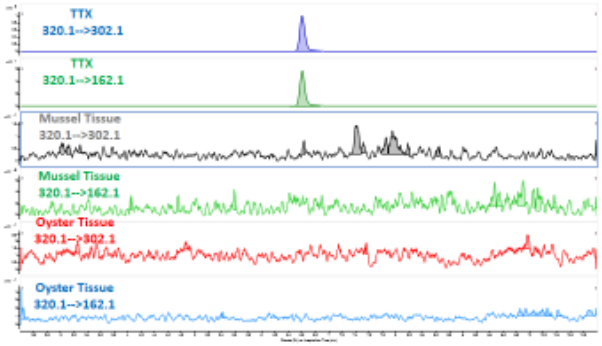
EU-RL-MB EUROPEAN UNION REFERENCE LABORATORY FOR MARINE BIOTOXINS

LC-MS/MS ANALYSIS OF TTX SINGLE-LABORATORY VALIDATION: APPLICABILITY

Analysis of Tetrodotoxin (TTX) in raw Mussels and Oysters

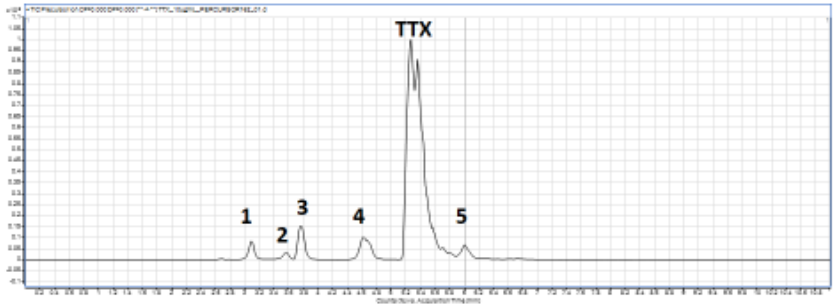
LC-MS/MS ANALYSIS OF TTX SINGLE-LABORATORY VALIDATION: SELECTIVITY



Uncontaminated mussel tissue
Uncontaminated oyster tissue

Absence of TTX chromatographic peak (no matrix interferences)

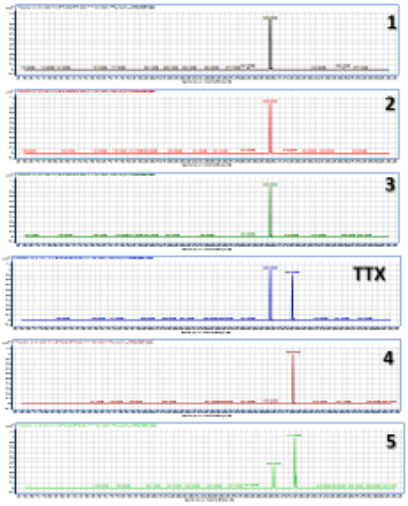
LC-MS/MS ANALYSIS OF TTX SINGLE-LABORATORY VALIDATION: SELECTIVITY



Determination of precursors ions from mass fragment m/z= 162.1 (common fragment ion for TTXs compounds)
Precursor Ion Chromatogram (CE: 25eV, FG:380V, Mass range: 100-400), 2µL of 500ng/mL of TTX Std from TOCRIS

Compound	Product ion (m/z)	Percursor ion (m/z)
1	302.1	162.1
2	302.1	162.1
3	302.1	162.1
4	320.1	162.1
5	320.1	162.1

LC-MS/MS ANALYSIS OF TTX SINGLE-LABORATORY VALIDATION: SELECTIVITY



Compound	Product ion (m/z)
1	Related with 4,9-anhydro-TTX mass (302.1)
2	Related with 4,9-anhydro-TTX mass (302.1)
3	4,9- anhydro-TTX
4	4-epi-TTX
5	Related with TTX mass (320.1)

Good selectivity for TTXs separation using LC (HILIC) conditions above mentioned, TTX and other TTX analogues shows good chromatographic resolution (Rs)

LC-MS/MS (HILIC):

✓ **Selective**

✓ **Specific**

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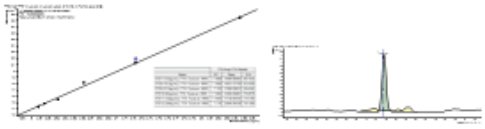
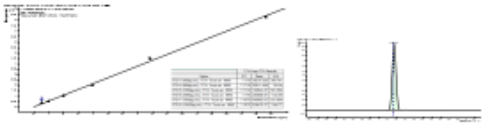
TTX SINGLE-LABORATORY VALIDATION: CALIBRATION and LINEARITY

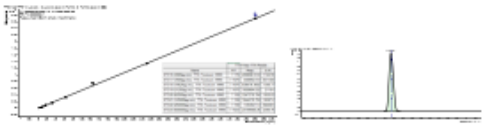
Calibration range from **0.16** to **4.8** µg of TTX /Kg (mussel)



Calibration range from **10.0** to **300.0** µg of TTX /Kg (mussel)



Calibration range from **80.0 µg** of TTX /Kg to **10,0 mg** of TTX /Kg (mussel)



Linearity studies carried out using mussel matrix match for standard preparation

Range (ng/mL)	Range (µg/Kg)	Slope	Intercept	R^2	SET
0.008 - 0.24	0.20 - 4.8	59382,7921	14046,3199	0,9969	6
0.50 - 15.00	10.0 - 300	53927,8579	2431,9902	0,9981	6
4.0 - 500	80.0 - 10 000.0	53982,3948	74602,2882	0,9993	8
	Average	55764,34827			
	STD DEV	2558,723041			
	RSD(%)	4,6			

Linearity range with correlation coefficient higher then 0,99 are demonstrated between 0,16 µg/Kg to 10,0 mg TTX/Kg

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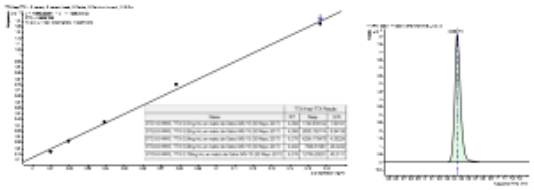
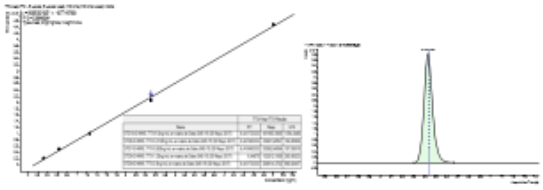
TTX SINGLE-LABORATORY VALIDATION: CALIBRATION and LINEARITY

Calibration range from **0,20** to **3,12** µg of TTX /Kg (oyster)



Calibration range from **10.0** to **100.0** µg of TTX /Kg (oyster)



Linearity studies carried out using oyster matrix match for standard preparation

Range (ng/mL)	Range (µg/Kg)	Slope	Intercept	R^2	SET
0.01 – 0,16	0,2 – 3,1	74998,2356	1259,3131	0,9959	5
0.31 - 5.00	6.0 – 100.0	90895,6216	-8077,4377	0,9984	5

TTX SINGLE-LABORATORY VALIDATION: TRUENESS

CRM material is not available for assessing the trueness of the method. The studies were carried out by external standard addition to the uncontaminated samples

Recovery

Levels of TTX added to **mussel** matrix: 0,01 - 0,025 - **0,044** - 0,100 - 0.900 - 4.000 - 10.000 mg/Kg

Recommendation Level: 44µg TTX/Kg equivalent 0.044 mgTTX/Kg

Number spike/per level: 3, Number SPE-ENVI-CARB/per extract:3, Number of Operators: 2, TOTAL: 126 analysis

Spike Level	10µg/Kg	25µg/Kg	44µg/Kg	100µg/Kg	900µg/Kg	4.0 mg/Kg	10.0 mg/Kg
Recovery (%)	91.6	92.7	102.3	94.9	109.5	104.7	95.7
STD. DEV.	6.6	1.8	3.6	2.1	5.3	3.6	8.5
N	3	3	3	3	3	3	3
RSD(%)	17.2	2.0	3.3	2.3	4.9	3.4	8.9

Range	R (%)	STD DEV	n	RSD(%)	N=n-1	t(95%)	R(%) Lowest	R (%) Highest
10µg/Kg-10mg/Kg	98,8	6,3	7	6,4	6	2,447	93,0	104,6

LC-MS/MS ANALYSIS OF TTX SINGLE-LABORATORY VALIDATION: TRUENESS

Levels of TTX added to **Oysters** matrix: 10,0 – 25,0 – 44,0 – 100,0 µg/Kg

Recommendation Level: 44µg TTX/Kg equivalent 0.044 mgTTX/Kg

Number spike/per level: 3, Number SPE-ENVI-CARB/per extract:3, Number of Operators: 2, TOTAL: 72 analysis

Spike Level	10µg/Kg	25µg/Kg	44µg/Kg	100µg/Kg
Recovery (%)	69,3	75,5	79,6	71,9
STD. DEV.	8,7	4,0	3,2	2.4
N	3	3	3	3
RSD(%)	12,5	5,3	4,0	3,0

Range	R (%)	STD. DEV	n	RSD(%)	N=n-1	t(95%)	R(%) Lowest	R (%) Highest
10µg/Kg-100µg/Kg	74,1	3,9	4	5,2	3	3,102	67,1	81,1

LC-MS/MS ANALYSIS OF TTX SINGLE-LABORATORY VALIDATION: PRECISION

The precision was generated from the analysis of several replicates, at different concentration levels, and in different days.

i) Relative standard deviation obtained for several injections of different sets of calibration solution

LC-MS/MS instrument

TTX ng/mL	TTX µg/Kg	n	RT RSD(%)	Response RSD(%)
1	*20*	4	0,2	4,2
5	*100*	6	0,1	4,3
15,625	*312,5*	4	0,1	2,3
62,5	*1250*	5	0,3	3
125	*2500*	4	0,1	1,6
140	*2800*	6	0,1	5,5
500	*10000*	6	0,2	6,3

i) Relative standard deviation results obtained for recovery studies at different spiked levels and at different days used for the recovery precision evaluation.

TTX LC-MS/MS method

TTX µg/Kg	n	Mussel RSD(%)	Oyster RSD(%)
10	3	17,2	12,5
25	3	2	5,3
44	3	3,3	4
100	3	2,3	3
900	3	4,9	
4000	3	3,4	
10000	3	8,9	

LC-MS/MS ANALYSIS OF TTX: SINGLE-LABORATORY VALIDATION: PRECISION

iii) Relative standard deviation result for chromatographic retention times at different days

Retention times reproducibility

TTX ng/mL	May 5th	May 10th	May 25th	May 31st	June 3rd
RT (min)	7,17	5,03	6,47	6,48	5,79
STD DEV	0,01	0,02	0,02	0,04	0,02
n	6	14	6	17	20
RSD(%)	0,1	0,3	0,4	0,6	0,3

TTX SINGLE-LABORATORY VALIDATION: RANGE

IUPAC definition: The validate range is the interval of analyte concentration within *which the method* can be regarded as validated.

Calibration curve range	***Mussel***	*0,20µg/Kg to 10mg/Kg*
	Oyster	*0,2µg/Kg to 100µg/Kg*
Recovery studies range	***Mussel***	*10µg/Kg to 10mg/Kg*
	Oyster	*10µg/Kg to 100µg/Kg*

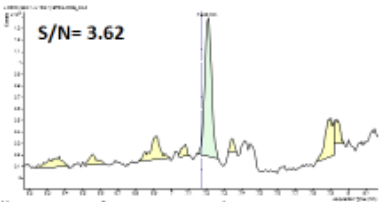
Method range:

MUSSELS: 10 µg/Kg to 10 mg/Kg
OYSTERS: 10 to 100 µg/Kg

LC-MS/MS ANALYSIS OF TTX SINGLE-LABORATORY VALIDATION: DETECTION and QUANTITATION LIMITS

6 mussels and oysters samples were spiked at low level concentration (0.4 and 0.8 µgTTX/Kg) and submitted to complete method of analysis (Extraction, Cleanup, and measurement)

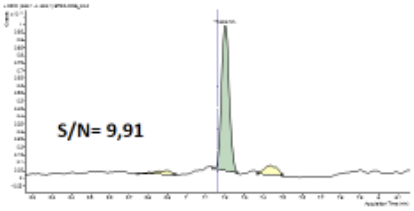
LOD: Small amount of TTX prepared in matrix match injected into LC-MS/MS system which produces signal to noise ratio >3 (qualitative transition 320.1→162.1)



Oyster matrix
LOD= 0,36±0,11µg/Kg, n=6

Mussel matrix
LOD= 0 ,31±0,12µg/Kg, n=6

LOQ: Small amount of TTX prepared in matrix match injected into LC-MS/MS system which produces signal to noise ratio >10 (quantitative transition 320.1→302.1)



Oyster matrix
LOQ= 0,86±0,13µg/Kg, n=6

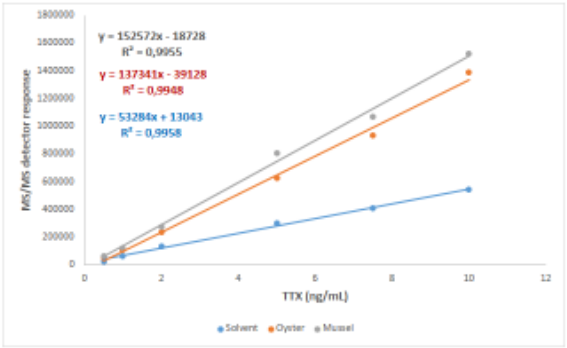
Mussel matrix
LOQ= 1,03±0,34µg/Kg, n=6

LC-MS/MS ANALYSIS OF TTX SINGLE-LABORATORY VALIDATION: MATRIX EFFECT

Six standard solutions equivalent to 2 to 200 µg/Kg level of TTX in shellfish were prepared in:

- **Solvent: AcN- 1% HAc (4:1)$_{v/v}$**
- **Oyster Matrix**
- **Mussels Matrix**

Matrix effect: Slope ratio



	Slope	Ratio (MMS/Solvent)
AcN/HAc	53284	1,0
Mussel MMS	152572	2,9
Oyster MMS	137341	2,6

LC-MS/MS analysis carried out using standards prepared in matrix match

Protocol:

Operator 1 spike mussels samples at 88 µg of TTX/kg level and oyster samples at 440 µg TTX/Kg (Triplicate studies)

Operator 2 applied internal validated method to the analysis of mussels samples

Operator 3 applied internal validation protocol to the analysis of oyster samples

SPE-ENVI-CARB Recovery values for mussels: 98.8%
SPE-ENVI-CARB Recovery value for Oysters: 74.1%

Samples	n	TTX (LC-MS/MS)	RSD(%)	Add. Level	Recovery	Correction Level	Accuracy	
							Syst. Error (µg/Kg)	BIAS (%)
Mussels	3	80,3µg TTX/Kg	4,3	88,0µg TTX /Kg	98,8%	81,3µg TTX/Kg	-6,7	7,7
Oysters	3	300,2µg TTX/Kg	1,1	440µg TTX/Kg	74,1%	405,1µg TTX/Kg	-34,9	7,9