Food Chemistry 269 (2018) 166–172

Contents lists available at ScienceDirect

Food Chemistry

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

Rapid analysis of paralytic shellfish toxins and tetrodotoxins by liquid chromatography-tandem mass spectrometry using a porous graphitic carbon column

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ARTICLE INFO

Keywords:
Shellfish matrices
PSTs
GCs
TTXs
Hypercarb
LC-MS/MS

ABSTRACT

Although paralytic shellfish toxins (PSTs) have traditionally been analyzed by liquid chromatography with either pre- or post-column derivatization, and these methods have been validated successfully through inter-laboratory studies, mass spectrometry methods have also been described in literature for use in monitoring programs. However, methods using liquid chromatography coupled with mass spectrometry (LC-MS) need to be improved in terms of sensitivity, analyte recovery and retention time stability because of undesirable matrix effects. Furthermore, tetrodotoxin (TTX) has been found in northern European bivalves, so it is important to analyze TTX compounds alongside PSTs because characteristics of their toxicity are similar. This paper describes, for the first time, a chemical method that allows determination of PSTs, both hydrophilic and hydrophobic, alongside TTX and its analogue 4,9-anhydro tetrodotoxin (4,9-anhTTX) with LC-MS/MS using a Hypercarb® column. The method was validated for 13 hydrophilic PSTs and TTXs and was able to discriminate six hydrophobic PSTs in 20 min. The method was developed for four shellfish matrices: mussel (*Mytillus galloprovincialis*), clam (*Ruditapes decussatus*), scallop (*Pecten maximus*) and oyster (*Ostrae edulis*). Clean-up procedure used in this work allowed us to obtain good results for validation parameters for both PSTs and TTXs. No standards were available so strains of *Gymnodinium catenatum* (*G. catenatum*) were used instead.

1. Introduction

Paralytic shellfish poisoning (PSP) is a common seafood toxicity illness worldwide. Toxic dinoflagellates are ingested and concentrated by filter-feeding mollusks, which promotes entry of paralytic shellfish toxins (PSTs) into the food chain. Although mollusks are the traditional exposure pathway for these toxins, mainly via consumption of mussels and clams, they are not the only vectors and reports about other sources are increasing, such as marine gastropods, crustaceans, and some fish (Deeds, Landsberg, Etheridge, Pitcher, & Longan, 2008).

The most prevalent paralytic shellfish toxins (PSTs) can be categorized as three families, based on structural differences in their functional groups: the carbamate family includes saxitoxin (STX), neosaxitoxin (NEO) and the gonyautoxins (GTX1-4); the decarbamoyl family includes decarbamoyl-gonyautoxins (dcGTX1-dcGTX4) and decarbamoyl-saxitoxins (dcSTX, dcNEO); the *N*-sulfocarbamoyl toxins include GTX5, GTX6 (also called B1 and B2, respectively) and C toxins (C1-4). In addition, a novel group of PSTs [GC toxins(GC1-6)], containing a hydroxybenzoate side-chain, have been identified in strains of *G. catenatum* (Negri et al., 2007), which are found in Australian, Chinese, Portuguese, Uruguayan and Spanish waters.

The official method for determining PSTs in shellfish in most countries is the mouse bioassay (AOAC, 2005). This method gives quantitative information about the toxicity of samples, which is calculated based on the time of death in mice injected with acidic extracts of shellfish. This method has several limitations, however, particularly ethical concerns related to the use of live animals, and there have been great efforts in establishing an alternative method. These include a receptor binding assay (RBA), enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR). In addition to these assays, there are also analytical methods, separating individual toxin congeners, which can then be identified and quantified (Etheridge, 2010).

High performance liquid chromatography with fluorescence

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https://doi.org/10.1016/j.foodchem.2018.07.008
Received 2 May 2017; Received in revised form 22 June 2018; Accepted 1 July 2018
Available online 02 July 2018
0308-8146/

detection (HPLC-FLD) methods have been validated for PSTs through inter-laboratory studies (Lawrence, Niedzwiadek, & Menard, 2005; Van de Riet et al., 2010). However, as the STX analogues do not contain a chromophore, they have to be oxidized before detection. This derivatization is performed with either pre- or post-column oxidation. The pre-column method (Lawrence et al., 2005), also called the Lawrence method, has been accepted as an alternative for PSTs detection in shellfish samples and is used for regulatory purposes in the European Union (EU) (Anon, 2006). The post-column method (PCOX) (Van de Riet et al., 2010) was accepted by the Association of Official Analytical Chemists (AOAC) in 2011, as an alternative method for PSTs (AOAC, 2011), when it was validated through an inter-laboratory study using mussels, clams, oysters and scallops. There are advantages and disadvantages with both methods (DeGrasse, Van de Riet, Hatfield, & Turner, 2011; Rourke et al. 2008), meaning the right choice depends on the environment.

Mass spectrometry methods have also been described in literature (Dell'Aversano, Hess, & Quilliam, 2005; Diener, Erler, Christian, & Luckas, 2007; Jaime, Hummert, Hess, & Luckas, 2001) for the analysis of PSTs in shellfish monitoring programs. Dell'Aversano et al. (Dell'Aversano et al., 2005) improved liquid chromatography with mass spectrometry (LC-MS) detection, by establishing a method based on hydrophilic interaction liquid chromatography. It was more sensitive than previous approaches. In 2007, Diener et al. (2007) published a new LC-MS/MS method that used a zwitterionic (ZIC) hydrophilic interaction chromatography (HILIC) column for the separation of the three groups of PSTs in a single chromatographic run. Other authors have reported different HILIC-MS/MS methods for the determination of PSTs, although method sensitivity, analyte recovery, and retention time stability were compromised due to significant matrix effects (Turrell, Stobo, Lacaze, Piletsky, & Piletska, 2008). The number of interfering peaks, when shellfish extracts are analyzed with LC-MS/MS, suggests that extensive sample clean-up is needed to remove either salts or similar leading to the noise.

Some authors have investigated different types of clean-up procedures, such as solid phase extraction (SPE) (Sayfritz, Aasen, & Aune, 2008; Turrell, et al., 2008). More recently, Boundy, Selwood, Harwood, McNabb, & Turner (2015) proposed a new sample clean-up procedure, based on the use of graphitized carbon SPE cartridges, which eliminated matrix interference in partnership with HILIC-MS/MS analysis. This provided a sensitive, selective and rapid analysis for PSTs in a variety of samples and this method has been subjected to single-laboratory validation, and is being used for regulatory testing in New Zealand (Turner, McNabb, Harwood, Selwood, & Boundy, 2015).

Although tetrodotoxin (TTX) is not a PST, it elicits the same symptoms (Bane, Lehane, Dikshit, O'Riordan, & Furey, 2014) and co-occurrence of TTX and PSTs has been reported previously(Shiu, Lu, Tsai, Chen, & Hwang, 2003). The mouse bioassay has been used to establish toxicity, but their presence in seafood can be misdiagnosed because TTXs block sodium channels much like saxitoxin (STX) (Wingerd, Vetter, & Lewis, 2012). Generally, LC-MS and LC-MS/MS are best to analyze TTX and related compounds (Bane et al., 2014).

In addition to using porous graphitic carbon in sample clean-up, analysis of PSTs and TTX using a HPLC-FLD post-column method with a porous graphitized column has been described previously (Rey, Botana, Álvarez, Antelo, & Botana, 2016). This column has very powerful properties related to separation performance and robustness. Thus, in this work, sample clean-up was achieved using graphitized carbon SPE cartridges, which helped to reduce matrix interference.

In this study, we describe a new LC-MS/MS method, based on the use of a LC porous graphitized column coupled with mass spectrometry. The method is capable of discriminating the majority of PSTs, both hydrophilic and hydrophobic, and TTX and its analogue 4,9-anhydro tetrodotoxin (4,9-anhTTX). The clean-up procedure developed previously, using SPE porous graphite cartridges, was also tested to determine its suitability for bivalve samples analyzed using LC-MS/MS (Rey et al., 2016).

2. Materials and methods

2.1. Chemicals

HPLC grade methanol and acetonitrile, analytical reagent grade trifluoroacetic acid (TFA), dichloromethane, tetrahydrofuran, propionic acid, 2-propanol and ammonium acetate were all purchased from Panreac Química S.A. (Barcelona, Spain).

Trichloroacetic acid (TCA) and triethylamine were purchased from Sigma Aldrich (Madrid, Spain). HPLC grade water was used to prepare solutions.

Certified reference materials provided by Cifga S.A. (www.cifga.es, Lugo, Spain) were: saxitoxin (STX), neosaxitoxin (NEO), decarbamoylsaxitoxin (dcSTX), gonyautoxin 5 (GTX5), gonyautoxins 1 and 4 (GTX1,4), gonyautoxins 2 and 3 (GTX2,3), decarbamoylgonyautoxins 2 and 3 (dcGTX2,3), *N*-sulfocarbamoyl-gonyautoxins 2 and 3 (C1,2), tetrodotoxin (TTX) and 4,9-anhydro tetrodotoxin (4,9-anhTTX). Decarbamoylneosaxitoxin (dcNEO) was obtained from NRC (Halifax, Nova Scotia, Canada).

Decarbamoylgonyautoxins 1 and 4 (dcGTX1,4) were obtained by hydrolysis under basic pH from the GTX1,4 certified reference material. *N*-sulfocarbamoyl-gonyautoxins 1 and 4 (C3,4), gonyautoxin 6 (GTX6) and hydroxybenzoate toxins (GCs) were obtained from strains of *G. catenatum* provided by Cifga S. A.

Commercial toxin-free shellfish (1 kg), specifically mussels*(Mytillus galloprovincialis)*, clams*(Ruditapes decussatus)*, scallops*(Pecten maximus)* and oysters*(Ostrae edulis)*acquired from the local market; they were from aquaculture farms on the northwestern Spanish coast(mussels and oysters from Mariscos Ría de Vigo, S. L.; clams from Industrias y Cultivos Marinos, S. A.; scallops from Pescanova, S. L.).

2.2. Instrumentation

The HPLC equipment consisted of an Alliance 2695 Separation Module (Waters, Barcelona, Spain) with: a quaternary pumps system, an autoinjector with refrigerated rack, degasser, column oven and system controller. A post-column system 515 HPLC pump from Waters was used. This system was coupled to a triple quadrupole mass spectrometer Quattro Micro™ API equipped with an electrospray (ESI) source (Waters, Barcelona, Spain). The separation and identification of toxins was achieved in a Hypercarb® column (i.d. 2.1 mm × 100 mm, particle size 3 μm) from Thermo (Fisher Scientific, Madrid, Spain).

2.3. Samples extraction and clean-up

Samples were extracted according to the official procedure for PSTs described in the PCOX method (AOAC, 2011): 5 g of shellfish homogenate were extracted with 5 mL of 0.1 M HCl. The solution was mixed using a vortex mixer and the pH adjusted to between 2 and 4, preferably 3. When necessary, the pH was adjusted by adding dropwise either 5 M HCl or 0.1 M NaOH. Samples were placed into a boiling water bath for 5 min and then allowed to cool to room temperature. The pH was checked and adjusted to 3, as necessary. The mixture was centrifuged at 3.000 × *g* for 10 min, and the supernatant decanted.

The samples clean-up procedure was as described in the literature (Rey, et al., 2016): 500 μL of the supernatant was deproteinated by adding 25 μL 30% (w/v) TCA, mixed with a vortex and centrifuged at 16.000 × *g* for 5 min; 40 μL of 1.0 M NaOH were added, the solution mixed and centrifuged again at 16.000 × *g* for 5 min(AOAC, 2011.

After, 500 μL of dichloromethane were added to a deproteinated samples, which were mixed with a vortex and the aqueous and organic phases separated by gravity. The aqueous phase was cleaned using a Hypersep Hypercarb® SPE cartridge (200 mg, 3 mL) with porous graphitic carbon (PGC) (Thermo Fisher Scientific, Madrid, Spain): 500 μL

of sample extract were loaded onto the conditioned cartridges and washed out with 11 mL of 0.1 M ammonium acetate. The cartridges were conditioned previously with 3 mL of dichloromethane/methanol 80/20 (v/v) followed by 3 mL of methanol and 3 mL of 0.1 M ammonium acetate. Toxins were eluted with 500 µL of 0.1 M citrate buffer (pH = 4.0) in 10% acetonitrile (v/v) (Rey et al., 2016).

2.4. Matrix effect evaluation

Possible interference caused by the compounds present in the matrices were investigated: mussel, clam, scallop and oyster extracts were used to establish interferences using LC-MS/MS. The standards were diluted in each of the extracts and chromatographic separation carried out under the optimized conditions described for this study (see "Methods" section). Matrix effects were calculated according to:

$$\%ME = [[(A+ B)-B]/A]*100$$

where % ME = percentage of matrix effect, A = area of the toxin standard, B = area of shellfish extract and A + B = area of shellfish extract spiked with the toxin standard.

A value of ME = 1 meant there was no matrix effect; if ME was > 1 ionization has been enhanced and, therefore, a positive matrix effect was present; if ME was less than 1, ionic suppression and a negative matrix effect were noted (Rossignoli, Mariño, Martín, & Blanco, 2015).

To calculate the matrix effects, samples were first cleaned-up: 1) shellfish extracts were deproteinated by adding TCA and passed through a 0.2 µm syringe filter, as described in PCOX method (AOAC, 2011); 2) shellfish extracts were deproteinated by adding TCA and the extract was purified using a Hypersep Hypercarb® (PGC) SPE cartridge, as previously described (see "Samples extraction and clean-up" section); 3) extracts were deproteinated by adding TCA, extracted with dichloromethane and purified using a Hypersep Hypercarb® (PGC) SPE cartridge, as previously described (see "Samples extraction and clean-up" section).

2.5. Methods

An Alliance 2695 Separation Module coupled to a Quattro Micro mass spectrometer equipped with an ESI source was used for sample analysis. The HPLC system was equipped with a Hypercarb® column (i.d. 2.1 mm × 100 mm, particle size 3 µm) in a column oven at 25 °C; the injection volume was 10 µL (partial loop with needle overfill mode). Mobile phase composition was: 0.1% (v/v) trifluoroacetic acid in water for solvent A, solvent B was acetonitrile and solvent C was water. The flow rate was set at 0.250 mL min^{-1}. Chromatographic separation was performed by gradient elution starting with 100% A, 0% B and 0% C for 3 min, then decreasing to 50% A and increasing to 5% B and 45% C in one minute. The next 6 min decrease A to 30% and increase B to 10% and C to 60%. At 10.5 min the proportions were: 60% A, 35% B and 5% C. For the next 7 min, decrease A up to 30%, increase B up to 50% and C up to 20%. This condition was hold for 3 min and increasing afterward to 100% A over 0.5 min. This last proportion was maintained for 9 min, until the next injection to equilibrate the system.

The gradient conditions for PSTs and TTX are summarized in Table 1.

A post-column solution composed of propionic acid/2-propanol 75/25 (v/v) was mixed with the LC eluent through a tee-union, before reaching the electrospray capillary; the flow rate of the post-column solution was 0.075 mL min^{-1}. The Quattro Micro mass spectrometer was operated with the following optimized source-dependent parameters (ESI source): capillary voltage 2.7 kV, desolvation temperature 500 °C, desolvation gas flow 850 L/h N_2, cone gas flow 30 L/h N_2, source temperature 150 °C, collision Argon pressure 0.3 Pa (3×10^{-6} bar). The cone voltages and collision energies were optimized for each toxin by standard infusion. MassLynx 4.1 with QuanLynx software (Waters, Manchester, UK) was used for data processing. Fig. 1 shows the ion chromatogram obtained for the separation of PSTs, both hydrophilic and hydrophobic, together with TTX and 4,9-anhTTX.

Table 1
Gradient conditions for separation of PSTs (hydrophilic and hydrophobic toxins) and TTXs.

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0	100	0	0
3	100	0	0
4	50	5	45
10	30	10	60
10.5	60	35	5
17.5	30	50	20
20.5	30	50	20
21	100	0	0
30	100	0	0

The mass spectrometer operated in MRM (multiple reaction monitoring), detecting in positive mode, and analyzed two product ions per PST and TTX (Table 2): one for quantification (the most abundant) and another for confirmation. Collision energies, cone voltages and dwell times were optimized for maximum sensitivity.

2.6. Validation parameters

2.6.1. Linearity

Linearity was calculated by injecting each PST and TTX standards, at different concentrations in the solvent, and calculating peak areas. Instrument response was plotted against the respective concentration and the curves were obtained using the least square linear regression method. Equations and the correlation coefficients (r) for each toxin were obtained to verify the linearity of response of the LC system to the matrix fortified PST standard, as shown in Table 1S (Martin-Smith & Rudd, 1990).

2.6.2. LODs and LOQs

Limits of detection (LODs) for matrix-free toxins were determined from data for samples extrapolated to a signal to noise ratio of 3. Five blank replicate extracts for each matrix (mussel, clam, scallop and oyster) were analyzed and repeated over 6 days (n = 30). The results are expressed as µg STX diHCl eq kg^{-1} where the toxicity equivalent factors (TEF) were taken from Oshima (1995) for PSTs; for TTX and its analogue, the results were expressed as µg kg^{-1}. Limits of quantification (LOQs) were determined multiplying signal to noise ratio by 10 (Eurachem, 2014) (Table 3).

2.6.3. Repeatability

Repeatability was calculated for each matrix at 3 different concentrations: 200 µg STX·diHCl kg^{-1}, 800 µg STX·diHCl kg^{-1} and 1,600 µg STX·diHCl kg^{-1} for PSTs; 3,225 µg kg^{-1}, 806 µg kg^{-1} and 202 µg kg^{-1} for TTX; 375 µg kg^{-1}, 94 µg kg^{-1} and 23 µg kg^{-1} for 4,9-anhTTX. Six replicates were analyzed and repeated over 8 days (n = 48), for each matrix at each concentration. Relative standard deviation percentages (%RSD) are presented in Table 2S.

2.6.4. Recovery

Recovery was determined using known concentrations of spiked blank samples; six replicates were made for each of the four matrices under study. The concentrations and recovery are summarized in Table 4.

3. Results and discussion

3.1. Optimized chromatography and MS/MS method

The percentage of TFA has a significant influence on the retention

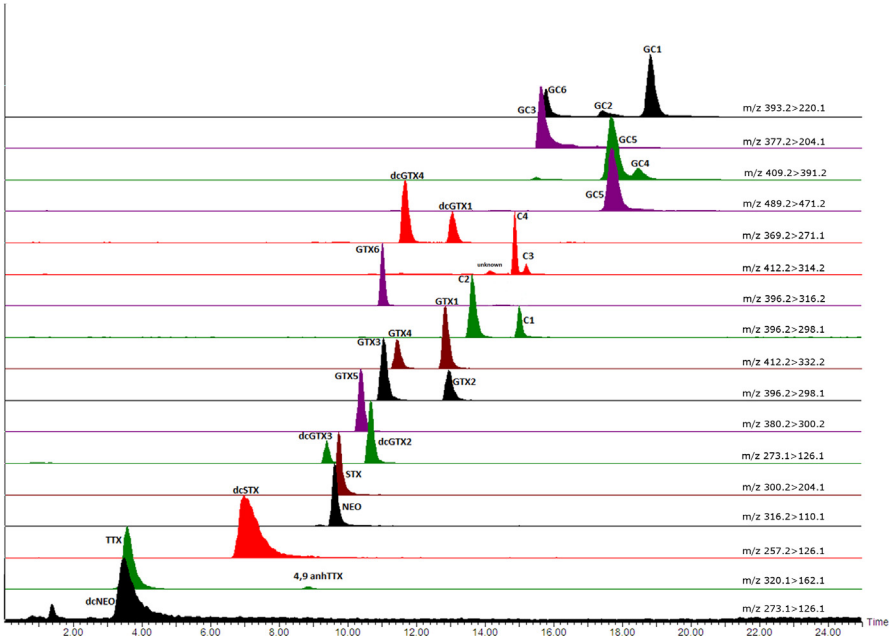

Fig. 1. Representative ion chromatogram for PSTs and TTXs analysis. Concentrations used for the standards (as $\mu g\,g^{-1}$): 0.634 for dcNEO, 1.613 for TTX, 1.219 for dcSTX, 0.188 for 4,9anhTTX, 0.500 for dcGTX3, 1.281 for NEO, 1.223 for STX, 1.375 for GTX5, 2.194 for dcGTX2, 0.513 for GTX3, 0.444 for GTX4, 1.669 for GTX1, 1.388 for GTX2, 0.719 for C2 and 2.506 for C1.

Table 2
MS/MS parameters for the analysis of paralytic shellfish poisoning toxins, tetrodotoxin and its analogue.

Analogue	RT, min	ESI + transition	CE, eV	Cone, Volts
dcNEO	3.6	**273.1** > **126.1**, 255.2	**20**, 20	**35**, 35
TTX	3.8	**320.1** > **162.1**, 302.1	**25**, 25	**40**, 40
dcSTX	7.2	**257.2** > **126.1**, 138.1	**20**, 20	**35**, 35
4,9-anhTTX	8.8	**302.1** > **162.1**, 150.1	**40**, 40	**40**, 40
dcGTX3	9.3	**353.2** > **255.2**, 273.1 > 255.2	**20**, 20	**20**, 35
NEO	9.6	**316.2** > **110.1**, 177.1	**40**, 25	**35**, 35
STX	9.7	**300.2** > **204.1**, 179.1	**25**, 25	**35**, 35
GTX5	10.4	**380.2** > **300.2**, 300.2 > 204.1	**20**, 25	**20**, 35
dcGTX2	10.6	**273.1** > **126.1**, 353.2 > 255.2	**20**, 20	**35**, 20
GTX6	10.9	**396.2** > **316.2**, 298.1	**20**, 20	**25**, 25
GTX3	11.1	**396.2** > **298.1**, 316.2	**20**, 20	**25**, 25
dcGTX4	11.4	**369.2** > **271.1**, 289.1 > 271.1	**20**, 20	**20**, 35
GTX4	11.5	**412.2** > **314.2**, 332.2 > 314.2	**20**, 20	**15**, 35
GTX1	12.8	**412.2** > **332.2**, 332.2 > 314.2	**20**, 20	**15**, 35
dcGTX1	12.8	**289.1** > **126.1**, 289.1 > 271.1	**20**, 20	**35**,35
GTX2	12.9	**396.2** > **316.2**, 298.1	**20**, 20	**25**, 25
C2	13.9	**396.2** > **298.1**, 316.2	**20**, 20	**25**, 25
C4	14.9	**412.2** > **314.2**, 332.2 > 314.2	**20**, 20	**15**, 35
C1	15.1	**396.2** > **316.2**, 298.1	**20**, 20	**25**, 25
C3	15.2	**412.2** > **332.2**, 332.2 > 314.2	**20**, 20	**15**, 35
GC3	15.7	**377.2** > **204.1**, 263.1	**30**, 30	**35**, 35
GC6	15.9	**393.2** > **220.1**, 375.1	**30**, 30	**35**, 35
GC2	17.5	**473.2** > **375.2**, 220.1	**15**, 30	**15**, 15
GC5	17.7	**489.2** > **471.2**, 341.2	**15**, 15	**15**, 15
GC4	18.4	**409.2** > **391.2**	**15**	**35**
GC1	18.9	**393.2** > **220.1**, 375.1	**30**, 30	**35**, 35

RT: Retention time.
CE: Collision energy.
Bold font indicates primary quantitative MRM transition.

time for PSTs, TTX and its analogue, reducing polar retention because by acting as a competitive modifier. Therefore, the first step was to select the right concentration of TFA in the mobile phase: different percentages of TFA for solvent A were tested (0.025%, 0.050%, 0.075% and 0.1%). Both 0.075% or 0.1% gave good resolution. In the case of 0.075% TFA, the first toxins eluted were earlier than when 0.1% TFA was used, and peaks shape was less good: dcNEO and dcSTX eluted 1 min earlier and TTX and 4,9-anhTTX eluted 0.5 min earlier than with 0.1% TFA. Above 0.1%, there was a delay in retention time for all the toxins, especially those eluting earlier. At 0.1%, none of the toxins exhibited significant variation in retention times. Therefore, 0.1% was chosen for subsequent experiments.

Acetonitrile and methanol were also tested as organic modifiers for solvent B. Although both have similar benefits (Scientific, 2007), column pressure problems associated with the use of methanol meant it was necessary to decrease the flow rate to $0.2\,mL\,min^{-1}$. No delay occurred in retention times for dcNEO, dcSTX and TTX when methanol was used, but significant variations were observed for all the other toxins and peak shape was not as good as with the use of acetonitrile; C1 did not elute at all. Consequently, acetonitrile was used subsequent experiments.

MS signal suppression, due to TFA, was also examined. This loss in ion signal can be attributed to the formation of a strong TFA ion-pairing with the analytes (Chan, Bolgar, Dalpathado, & Lloyd, 2012), reducing ionization efficiency (Kuhlmann, Apffel, Fischer, Goldberg, & Goodley, 1995). Several authors have developed methods to increase the signal (Chan et al., 2012; Kuhlmann et al., 1995) using post-column addition of propionic acid/2-propanol (75%:25%) at a ratio 1:2 (propionic acid/2-propanol:TFA) (Kuhlmann et al., 1995), or a solution of 1% NH_4OH in methanol (v/v) to the column flow in ratios ranging from 0.5:1 to 50:1 (NH_4OH:TFA) (Chan et al., 2012). In this work, both solutions were tested.

A 75:25 (v/v) post-column solution of propionic acid/2-propanol was used and four flow rates were tested: 0.05, 0.075, 0.1 and $0.125\,mL\,min^{-1}$. The column flow rate was $0.250\,mL\,min^{-1}$. A standard mixture was injected and dcNEO and dcGTX3 had the greatest signal when the post-column flow rate was $0.05\,mL\,min^{-1}$ but, for the remainder of the toxins, signal intensity was the lowest. dcSTX, NEO, STX, GTX5 and C1 had better results than previously when the flow rate was $0.075\,mL\,min^{-1}$. $0.1\,mL\,min^{-1}$ was only useful for GTX3, *C2*, TTX and 4,9-anhTTX and $0.125\,mL\,min^{-1}$ was suitable for dcGTX2, GTX2, GTX4 and GTX1. Therefore, a post-column flow rate of $0.075\,mL\,min^{-1}$ was adopted, as the optimal because five of the toxins achieved their best signal intensities at this rate; no toxin was at its lowest intensity with this flow rate.

Post-column solutions of 1% and 5% NH_4OH in methanol (v/v) were also tested, as described in previously (Chan et al., 2012). Several post-column flow rates were assayed for each solution: 0.01, 0.05 and $0.1\,mL\,min^{-1}$. All the results showed less signal intensity than in the case for propionic acid:2-propanol; therefore 75:25 (v/v) propionic acid:2-propanol, at a flow rate of $0.075\,mL\,min^{-1}$, was chosen.

Table 3
Limits of detection (LOD) and quantification (LOQ) for each toxin in each matrix, expressed as μg STX·diHCl kg^{-1}.

		dcNEO	dcSTX	dcGTX3	STX	NEO	GTX5	dcGTX2	GTX3	GTX4	GTX1	GTX2	*C2*	C1	TTX[a]	4,9- anhTTX[a]
Mussel	LOD	38.373	43.525	6.504	15.253	44.589	1.127	2.098	10.048	8.102	22.799	9.474	1.559	0.387	6.245	12.432
	LOQ	77.643	58.329	19.574	60.405	122.468	2.975	6.685	26.048	19.426	35.228	29.849	4.300	1.322	12.452	24.947
Clam	LOD	36.286	42.007	4.495	7.415	55.844	1.242	1.529	10.003	10.985	25.384	11.370	1.697	0.413	8.007	13.778
	LOQ	70.684	53.268	12.877	34.277	159.984	3.359	4.788	25.900	29.038	43.846	36.168	4.759	1.408	18.325	29.433
Scallop	LOD	33.322	41.449	7.697	11.264	41.510	1.005	2.673	8.637	9.390	30.560	9.431	1.633	0.446	10.841	16.752
	LOQ	60.806	51.409	23.550	47.102	112.203	2.568	8.603	21.347	23.719	61.099	29.704	4.546	1.518	27.771	39.345
Oyster	LOD	54.707	41.071	4.568	8.995	44.002	1.367	1.787	8.476	8.286	21.761	10.791	1.621	0.413	8.840	15.143
	LOQ	132.088	50.147	13.118	39.545	120.510	3.774	5.648	20.809	20.039	31.768	34.240	4.509	1.407	21.101	33.984

[a] : values expressed as μg kg^{-1}

Column temperature did not only influence retention times for the toxins or peak signals. Therefore, the optimal column temperatures tested for all toxins ranged from 25 °C (minimal oven temperature) to 50 °C. Above 50 °C, peaks underwent splitting and broadening. 25 °C and 30 °C produced similar results regarding peak height, but 25 °C provided the best resolution between adjacent peaks. Thus, 25 °C was chosen as the optimal column temperature.

3.2. Shellfish matrix interferences and investigative sample clean-up

Evaluation of matrix effects on the quantitative analysis of toxins in shellfish is an important and sometimes overlooked aspect of assay validation (Rogatsky & Stein, 2005). In this work, the chromatographic interference from the matrices under study were investigated. Samples were cleaned-up using three different procedures (see "matrix effect evaluation" section) and the specificity of the method was evaluated.

Chromatographic interference from the matrices were investigated through comparison of chromatograms for both blank and spiked matrices: 1) analysis of mussel, clam, scallop and oyster spiked samples after PCOX clean-up procedure showed important matrix effects for most toxins (except for dcNEO and GTX3) and high variability in matrix effects for most toxins regardless of the species; 2) solid phase extraction sample clean-up with Hypercarb cartridges was effective in reducing matrix effects. However, matrix effects still were > 15% for some toxins in some matrices, although the variability in matrix effect regarding species was less it was still high; 3) clean-up procedure with deproteinization, dichloromethane extraction and solid phase extraction with Hypercarb cartridges reduced matrix effects to less than 15% for all PSTs and there was little variation between matrices. Results summarized in Table 5 show the matrix effect (%) for each clean-up procedure. Although the difference between clean-up procedures (2) and (3) was only extraction with dichloromethane, this difference was investigated, taking into account our previous results (Rey et al., 2016) for the separation of PSTs and TTX by HPLC. Including dichloromethane (procedure 3) removed lipophilic compounds, which made the matrix clearer for the investigation of toxins. Although the matrix effects did not follow a pattern, when procedure 2 was used, signal suppression was higher in most cases (43 out of 60 results were higher than 15%) meaning the impact on sample toxicity would be remarkable. If procedure 3 was used, the matrix effect diminishes considerably: only 16 out of 60 results showed signal suppression, and then always less than 15%.

3.3. Validation parameters

An appropriate calibration model is necessary for reliable quantification. Therefore, the relationship between analyte in a sample and the corresponding response was investigated. Table 1S shows linearity results for each PST and TTX. Regression for each toxin was calculated using at least 10 points and all the correlation coefficients were > 0.99. All the toxins had a wide range of linearity, except C1, which beyond 0.313 mg kg^{-1} was not linear.

Limits of detection (LODs) were determined from the noise signal for each toxin, multiplying it by 3 and converting values to μg STX.diHCl kg^{-1}. TEFs values were those taken from Oshima (1995). Limits of quantification (LOQs) were calculated by multiplying the noise signal by 10 and converting values to μg STX.diHCl kg^{-1}(Eurachem, 2014). In case of TTX and 4,9-anhTTX, both LOD and LOQ were expressed as μg kg^{-1}. Table 3 shows LODs and LOQs for PSTs and TTXs in the four matrices.

Both limits, for each toxin, were calculated from the most intense characteristic transition. LOD values ranged from 0.387 to 55.844 μg STX·diHCl kg^{-1} and LOQs values ranged from 1.322 to 159.984 μg STX·diHCl kg^{-1} (Table 3). All LOQs were less than 1/10 of the existing regulatory limit for PSTs, 800 μg STX.diHCl kg^{-1}, except for NEO which was 1/6. These data suggest the method has potential to be used for routine monitoring analysis of shellfish toxicity, with the existing regulatory limits.

LOD and LOQ values for TTX in the different matrices ranged from 6.245 to 10.841, and from 12.452 to 27.771 μg kg^{-1}, respectively. In case of 4,9-anhTTX, LODs ranged from 12.432 to 16.710 and LOQs ranged from 24.947 to 39.345 μg kg^{-1}, respectively. In all cases, the lowest result was derived with the mussel matrix and the highest value associated with the scallop matrix. Although these results are higher

Table 4
Percentage of recovery (3 days, 6 replicates, n = 18) for each toxin in mussel, clam, scallop and oyster matrices.

		dcNEO	dcSTX	NEO	STX	GTX5	dcGTX3	dcGTX2	GTX3	GTX2	GTX4	GTX1	*C2*	C1	TTX	4,9-anhTTX
	μg g^{-1}	0.63	0.760	0.799	0.381	0.701	0.030	0.128	0.259	0.685	0.221	0.830	0.718	2.505	1.488	0.117
Mussel	Recovery%	79.71	109.31	109.76	88.65	73.04	122.15	78.51	72.24	75.45	79.82	92.50	73.57	119.58	119.31	85.77
	SD%	3.54	1.87	2.31	4.14	5.05	5.80	5.83	4.11	4.36	4.41	3.46	3.80	3.44	4.36	4.39
Clam	Recovery%	81.20	88.68	86.53	74.19	77.22	117.71	118.93	73.12	83.41	73.32	93.79	73.98	111.22	118.21	82.84
	SD%	4.23	3.06	2.87	4.14	3.38	3.59	3.23	3.54	4.66	5.25	2.38	5.51	4.42	4.86	5.47
Scallop	Recovery%	78.93	112.11	118.68	87.58	75.84	118.56	115.24	89.29	112.38	117.41	107.66	112.80	109.30	111.24	77.35
	SD%	3.52	2.27	5.72	2.07	3.77	4.23	2.32	5.22	4.99	3.47	4.06	4.94	4.88	5.60	5.60
Oyster	Recovery%	77.65	111.36	79.02	83.08	89.82	93.24	111.22	81.12	84.61	73.73	89.29	80.56	78.76	90.01	74.14
	SD%	4.78	3.59	2.73	4.97	4.51	4.83	2.64	5.21	5.25	5.13	3.15	4.76	5.09	5.12	5.12

Table 5
Percentage of matrix effect obtained for each toxin standard dissolved in matrix extract after different clean-up procedures.

		dcNEO	dcSTX	NEO	STX	GTX5	dcGTX3	dcGTX2	GTX3	GTX2	GTX4	GTX1	C2	C1	TTX	4,9-anhTTX
1	Mussel	4.04	37.81	35.42	33.33	−65.70	−4.68	−50.53	−59.61	−77.99	–32.67	−86.54	29.97	−30.10	−47.17	28.58
	Clam	2.27	13.77	−4.57	15.71	−41.45	−26.81	−36.42	−3.20	−62.59	−24.55	−83.65	29.23	−44.59	−60.21	8.45
	Scallop	0.99	22.56	17.28	9.61	−21.14	5.95	−15.02	4.38	−64.04	−16.38	−35.37	−4.86	27.44	−44.86	−61.29
	Oyster	6.03	−7.57	4.67	3.31	−48.27	−40.12	−37.06	−2.25	−83.59	–23.31	−83.14	−61.50	−5.59	−42.70	−41.60
2	Mussel	−9.68	−3.99	−2.69	−11.48	2.02	−12.82	0.20	−39.71	−9.97	−31.16	−8.49	1.48	−29.43	−10.55	0.54
	Clam	−6.70	−6.91	0.97	5.28	3.11	−8.89	0.59	−38.80	8.67	−26.21	1.56	38.65	−43.66	−10.12	−2.36
	Scallop	−10.52	−28.72	4.96	−5.98	−12.14	−11.12	−7.82	−20.09	−15.27	−9.17	3.62	−4.81	17.01	−17.29	2.63
	Oyster	−9.97	–23.82	−9.78	−10.82	−12.99	−26.97	−26.94	−26.67	−59.23	−16.62	−29.12	−26.42	5.79	−18.78	4.14
3	Mussel	2.92	−10.84	6.05	2.27	1.86	−1.51	2.58	8.41	0.23	−4.14	12.54	−0.62	−6.49	1.71	7.65
	Clam	5.85	−4.75	1.38	2.91	5.01	−1.72	2.23	2.72	3.91	−6.15	4.98	5.22	8.38	−0.29	−0.94
	Scallop	−0.94	8.51	8.91	−2.07	2.14	3.02	1.80	9.02	7.71	8.39	7.33	2.78	7.40	2.05	3.51
	Oyster	10.94	−6.08	6.28	6.71	1.66	−5.50	3.80	2.06	−1.02	−2.17	8.14	8.26	7.36	−10.63	0.90

1) PCOX clean-up; 2) PCOX clean-up and hypercarb cartridge and 3) PCOX clean-up, dichloromethane extraction and hypercarb cartridge.

than others obtained with LC-MS/MS, there are no result for bivalve matrices in literature only other marine species (Bane et al., 2014).

Results for repeatability are summarized in Table 2S. RSD percentages obtained for all toxins were within the acceptable range, as indicated by IUPAC (Horwitz, Albert, & Deutsch, 2000), and were relatively consistent. Only 4,9-anhTTX showed values > 10%: 11.22% in clams and 10.91% in oysters at the lowest concentration, and 10.17% in mussel at an intermediate concentration.

Recovery values were obtained for each matrix at one concentration. Matrix blanks were spiked with known amounts of toxins; clean-up was performed as described previously and aliquots injected in to the system. Recovery (%) is summarized in Table 4. All values were within the acceptable range (70–120%), except for dcGTX3, which was outside this range only for mussel (122.15%) (Alder et al., 2000).

3.4. Identification of hydroxybenzoate PSTs

Saxitoxin analogues were isolated and characterized from an Australian strains of *G. catenatum* containing a hydrophobic side chain and designated GC toxins (Negri et al., 2003). These Australian strains contained 12–63% GC toxins, although other strains also make an important contribution to sources of toxins in Portugal, Spain and China (Negri et al., 2007). Therefore, in areas where *G. catenatum* is a toxin source, it is important to have shellfish monitoring programs that include them (Etheridge, 2010). HPLC methods will miss these compounds due to their hydrophobicity: they are retained by C18 resins (Negri et al., 2003) and will be removed when extracts are passed through C18 cartridges during solid-phase extraction (SPE). They can also have longer retention times than hydrophilic analogues, meaning they do not appear in HPLC methods setup for known hydrophilic PSTs. GCs are also partially hydrolyzed in strong alkaline media used typically for oxidation in these methods (Negri et al., 2003). Several researchers have used hydrophilic interaction liquid chromatography tandem mass spectrometry (HILIC-MS/MS) to determine the presence of these emerging PSTs (Costa, Robertson, & Quilliam, 2015; Vale, 2008). The structural information obtained from the MS/MS ion product spectra revealed at least three diagnostic ion fragments for each toxin (Costa et al., 2015).

In this work, the analysis of extracts from *G. catenatum* with the Hypercarb® column in scan mode highlighted the presence of several compounds that could be related to hydroxybenzoate toxins. The gradient program detailed in Table 1 was used to elute these toxins. Although the initial gradient conditions were as for PSTs and TTXs, to accomplish optimal separation of these toxins alongside the hydrophilic PSTs and TTXs, slight changes were made: hydrophilic PSTs and TTXs can be eluted in a run time of approximately 15 min but GCs require 20 min (Fig. 1). This was not a remarkable change but, nevertheless, it is valuable for the separation and identification of GCs with PSTs and TTXs.

The lack of commercial standards for GCs meant it was not possible to undertake a validation study. Limited knowledge about their toxicity, however, makes it necessary to investigate further the fate of these compounds.

HPLC-FLD methods are the most widely-used to detect PSTs. These methods are accepted widely for routine monitoring of those toxins. However, in recent years, there has been increased use of liquid chromatography coupled to mass spectrometry for such analyses. The disadvantages of high detection limits, salts in shellfish extracts, matrix peaks and mobile phases used in HPLC-FLD that are not compatible with mass spectrometry detection (Gerssen, 2014) have also been solved. As a consequence, this is that LC-MS/MS methods can be a good alternative to HPLC-FLD. In this work, the mobile phase used is similar to those use by HPLC-FLD methods (Rey et al., 2016), although it was necessary the use a post-column solution as well. LOD and LOQ for TTX were improved (Rey et al., 2016), because LC-MS/MS allows optimization of the operating parameters for each target molecule. The important contribution of GC toxins detection is remarkable, because their analysis is not possible using HPLC-FLD methods: SPE C18 cartridges are often used and GC toxins can be retained by these resins due to their hydrophobicity. Hydrophobic analogues also have longer retention times and might not appear over the duration of common HPLC-FLD methods (Negri et al., 2003). Therefore LC-MS/MS is a good tool for detection of GC toxins with PSTs and TTXs provided the correct clean-up approaches are used and the run time lengthened.

4. Conclusions

A LC-MS/MS method with Hypercarb® column was optimized, for the first time, to quantify hydrophilic PSTs, TTX, and 4,9-anhTTX, allowing a discrimination of all relevant PSTs and TTXs in a single run. This method should be of interest in the analysis of PSTs and TTXs for regulatory purposes. It also included the identification of some PSTs that contain a hydrophobic side chain, namely GC toxins, something that has not yet been achieved to date. The time to separate all the toxins was 20 min. It is the first time that PSTs, both hydrophilic and hydrophobic, and TTX and its analogue 4,9-anhTTX have been separated in one short (20 min) run. Previous research in this field failed to analyze these compounds simultaneously, making this a remarkable improvement in the study of these toxins.

A sample clean-up procedure using Hypercarb® cartridges showed excellent results for the retention and elution of PSTs and TTXs from complex bivalves, minimizing matrix effects for this LC-MS/MS method.

Acknowledgements

The research leading to these results has received funding from the following FEDER cofunded-grants. From CDTI and Technological Funds, supported by Ministerio de Economía, Industria y Competitividad, AGL2014-58210-R, AGL2016-78728-R (AEI/FEDER, UE), ISCIII/PI16/01830 and RTC-2016-5507-2. From CDTI under ISIP Programme, Spain, IDI-20130304 APTAFOOD and ITC-20161072. From European Union POCTEP 0161-Nanoeaters -1-E-1, and Interreg Atlantic Area Transnational Cooperation Programme 2014-2020: EAPA_317/2016, ALERTOX-NET.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.07.008.

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