Method Article

Analysis of highly polar marine biotoxins in seawater by hydrophilic interaction liquid chromatography coupled to high resolution mass spectrometry

Cristina Bosch-Orea [a], Josep Sanchís [b,c], Marinella Farré [a,*]

[a] *Water and soil quality research group, Department of Environmental Chemistry, IDAEA-CSIC, Barcelona, Spain*
[b] *Catalan Institute for Water Research (ICRA), Parc Científic i Tecnològic de la Universitat de Girona, Spain*
[c] *Universitat de Girona, Spain*

A B S T R A C T

The monitoring of marine biotoxins (MBTs) in seawater is presented as an alternative strategy to determine their presence and the possible implications in the ecosystem. For this, an analytical method based on hydrophilic interaction liquid chromatography coupled to high resolution mass spectrometry (HILIC-HRMS) has been developed to identify and quantify some hydrophilic MBTs in seawater: saxitoxin (STX), decarbamoyl-saxitoxin (dcSTX), neosaxitoxin (NeoSTX), gonaytoxin-2,3 (GTX-2,3) and tetrodotoxin (TTX), which are responsible of gastrointestinal and central nervous system distress in humans when are consumed via seafood. Particulate and filtrate portion were analyzed separately in order to characterize the extracellular toxins dissolved in the water and those present in the particulate. Ultrasound assisted solid-liquid extraction with methanol was used for the isolation of the MBTs from particulate and solid phase extraction using silica cartridges for the filtrate. Extraction procedure was the most critical step during the analytical method due to the high polarity of the toxins and the absolute recoveries obtained ranged from 15 to 47 % in the filtrate and 26 to 71 % in the particulate portions. Limits of detection of the method ranged from 0.5 to 5 µg/L in the filtrate portion and from 3.1 to 62 µg/L in the particulate portion.

- Saxitoxins and tetrodotoxins have been analysed by using HILIC-HRMS.
- UAE with methanol and SPE with silica cartridges have been employed for the extractions of the polar MBTs from seawater.

* Corresponding author.
  E-mail address: mfuqam@cid.csic.es (M. Farré).

Specifications Table

Subject Area:	Environmental Science
More specific subject area:	*Marine biotoxins analysis*

*Method details

Chemicals and reagents

Certified Reference Materials (CRMs) of marine biotoxins were: gonyautoxin-2,3 (22.2 ± 1.5 µg/g and 8.2 ± 0.6 µg/g ≥97 % purity and reference CRM-00-GTX2&3); tetrodotoxin and 4,9-anhydro tetrodotoxin (25.9 ± 1.3 µg/g and 2.99 ± 0.16 µg/g, >96 % purity and reference CRM-03-TTXs); neosaxitoxin dihydrochloride (20.5 ± 1.1 µg/g, ≥99 % purity and reference CRM-00-NEO); decarbamoylsaxitoxin (19.5 ± 1.1 µg/g, ≥99 % purity and reference CRM-00-dcSTX); saxitoxin dihydrochloride (20.5 ± 1.5 µg/g, ≥99 % purity and reference CRM-00-STX), and were purchased from Cifga laboratory (Lugo, Spain). Chemical structures of the certified hydrophilic MBTs of this work represented in the Fig. 1. Solvents and reagents as ammonium formate, ammonium acetate, HPLC-grade methanol, ultra-pure water, acetonitrile, and formic acid were supplied by Merck (Darmstadt, Germany). Cartridges employed for the optimization were Silica 2g-Isolute from Biotage (Uppsala, Sweden) and OASIS HLB-500mg, Sep-Pak Diol-1g and Sep-Pack Aminopropyl (NH2)-500mg from Waters (Massachusetts, United States). And 0.2-µm pore size hydrophilic nylon membrane filters Millipore were supplied by Merck (Darmstadt, Germany).

Sampling

Seawater samples were collected from the surface of different locations in the Mar Menor at the coast of Murcia (South-eastern Spain, Mediterranean Sea), including marinas and beaches. The sampling took place during two different sampling campaigns in July 2018 and April 2019. Samples were kept frozen at -20°C in amber glass bottles until the analysis. Details of the sampling points are summarized in the *figure S1* of the supporting information.

Sample pre-treatment

Pre-treatment of samples were previously optimised and is explained in Section 1.6. This treatment consisted in a first step of filtration through a 0.2-µm pore size hydrophilic nylon membrane filter. Particulate and filtrate were extracted and analysed separately. For the particulate in the filters, an UAE was performed using 5-mL methanol during 5-min, in three cycles. The filtrate was subjected to a SPE using 2-g Silica Isolute cartridges. 0.5-mL of the filtered sample was added to 4.5 mL ACN and acidified at pH 3 with formic acid, and then loaded into the cartridge. The washing step was performed with 2 mL of ACN-water (9:1) at pH 3. The elution was carried out by gravity with 12-mL methanol. Final extracts of particulate and filtrate were evaporated under a N_2 stream in a Reacti-Vap III- PIERCE (Rockford, USA) to concentrate the sample and reconstituted to the 250 µL of the initial conditions of the mobile phase, 9:1 acetonitrile-water 5 mM ammonium formate at pH 3.2.

HILIC-HRMS analysis

Optimization of the instrumental analysis is detailed in Section 1.6. Chromatographic separation was carried out in an Acquity[TM] Ultra High Performance Liquid Chromatograph (UHPLC) system from
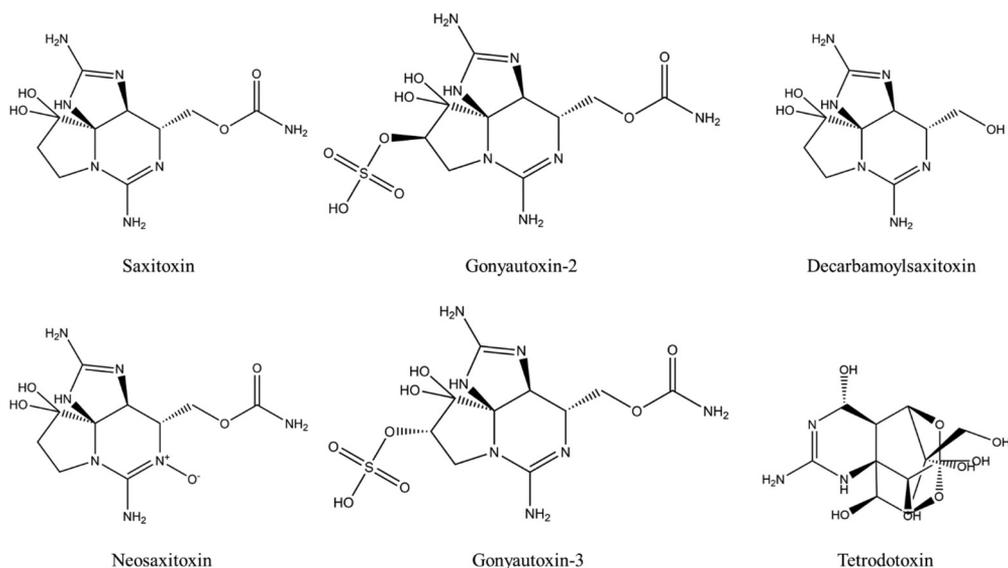
Fig. 1. Chemical structures of the target hydrophilic MBTs: saxitoxin (STX), decarbamoylsaxitoxin (dcSTX), neosaxitoxin (NeoSTX), gonautoxin-2.3 (GTX-2,3) and tetrodotoxin (TTX).

Waters (Massachusetts, United States). Then, the stationary phase employed was a HILIC column (HILIC LUNA® 150 mm × 2 mm, 3 μm, 200 Å from Phenomenex (Torrance, United States). Mobile phase was composed by (A) 5 mM ammonium formate-formic acid buffer in acetonitrile and (B) 5 mM ammonium formate-formic acid buffer in water, both with pH adjusted to 3.2. The elution gradient was programmed as following: 90 % A (0-3 min), 50 % A (3-10 % B), 90 % A (10-15 min) and 90 % A (15-20 min). A total time of 20 min was established for the chromatographic run, considering the last 5 min as a stabilisation step of the column. The flow rate was established at 0.3 mL/min and the volume of injection was 20 μL.

A heated electrospray ionisation source HESI-II from Thermo Fisher Scientific (San Diego, CA, USA) was used as interface between liquid chromatography and mass spectrometry. The source worked with the following parameters: sheath gas; 60 a.u., auxiliary gas; 15 a.u., sweep gas; 2 a.u., heater temperature; 350°C, capillary temperature; 320°C, S-lens RF level; 60 % and spray voltage; 3.5 kV working in positive mode.

Mass spectrometry was fulfilled in a Thermo Scientific QExactive mass spectrometer from Thermo Fisher Scientific with an Orbitrap analyser. The full scan data acquisition was in the range of 50-800 m/z at 70,000 full width at half maximum (FWHM) of resolution. In parallel, MS/MS spectrum of each compound was recorded at 35,000 FWHM by normalized collision energy (NCE). The most intense fragment was employed for the quantification and the rest as confirmation. Also, the ratio between the fragments of each toxins was used as an extra parameter of confirmation. In Table 1 are summarized the fragmentation pattern of the toxins at the optimal conditions.

Method validation and QA/QC

The validation of the developed method was accomplished with the evaluation of the selectivity, linearity, precision, detection and quantification limits and recoveries. These parameters are summarized in Table 2 and Table 3.

Selectivity and linearity of the method were evaluated by analysing the six analytes in solvent and seawater extracts. Linearity was measured in the concentration range of 0.1 to 100 μg/L by the Spearman coefficient R^2.

Table 1
Fragmentation pattern of the MBTs studied at optimal normalized collision energy (NCE). The relative standard deviation is given in parentheses.

Compound	Chemical formula	Molecular ion	m/z calculated	Product ion 1	m/z calculated	Relative abundance	Fragment ion ratio	Product ion 2	m/z calculated	Relative abundance	Fragment ion ratio	NCE (%)
Neosaxitoxin	$C_{10}H_{17}N_7O_5$	$[M + H]^+$	316.1364	$[C_{10}H_{16}O_4N_7]^+$	298.1257	30	2.53 (0.292)	$[C_9H_{13}O_2N_6]^+$	237.1093	12	8.84 (3.07)	40
Decarbamoyl saxitoxin	$C_9H_{16}N_6O_3$	$[M + H]^+$	257.1357	$[C_5H_8ON_3]^+$	126.0662	26	5.53 (1.27)	$[C_9H_{15}O_2N_6]^+$	239.1247	16	4.66 (1.03)	35
Tetrodotoxin	$C_{11}H_{17}N_3O_8$	$[M + H]^+$	320.1088	$[C_{11}H_{16}O_7N_3]^+$	302.0976	100	0.339 (0.0339)	$[C_8H_8ON_3]^+$	162.0658	38	0.500 (0.0593)	60
Gonyautoxin-2,3	$C_{10}H_{17}N_7O_8S$	$[M + H]^+$	396.0932	$[C_{10}H_{16}O_4N_7]^+$	298.1254	100	0.192 (0.0581)	$[C_{10}H_{16}O_7N_7S]^+$	378.0817	58	0.375 (0.0789)	10
Saxitoxin	$C_{10}H_{17}N_7O_4$	$[M + H]^+$	300.1432	$[C_{10}H_{16}O3N_7]^+$	282.1326	30	4.82 (0.450)	$[C_9H_{15}O_2N_6]^+$	239.1247	20	20.3 (2.89)	35

Table 2

Analytical parameters for the target hydrophilic MBTs.

Compounds	Instrumental parameters						Particulate		Filtrate	
	Retention time (min) and RDS (n=16)	Linearity (R^2)	Intraday (n=6,%) (20 µg/L)	Interday (n=3,%) (20 µg/L)	iLOD (pg on column)	iLOQ (pg on column)	MLOD (µg/kg)	MLOQ (µg/kg)	MLOD (µg/L)	MLOQ (µg/L)
Neosaxitoxin	7.73 (0.04)	0.5-100 (0.996)	2.41	12.3	1	3	12.5	37.5	0.5	1.5
Decarbamoyilsaxitoxin	8.70 (0.07)	0.1-100 (0.995)	1.87	15.0	1	3	3.125	9.375	0.5	1.5
Tetrodotoxin	7.97 (0.06)	1-100 (0.996)	11.02	11.04	1	3	6.25	18.75	5	15
Gonyautoxin-2,3	7.71 (0.04)	10-100 (0.998)	3.74	5.14	1	3	62.5	187.5	5	15
	7.97 (0.04)		8.71	16.0			62.5	187.5	5	15
Saxitoxin	7.39 (0.08)	0.5-100 (0.997)	3.8	12.9	1	3	3.125	9.375	0.5	1.5

Table 3

Values of recovery and matrix effect of the target toxins in the particulate and the filtrate portion at 40 µg/L of concentration. All values are expressed in percentage, %.

Compound	Particulate					Filtrate	
	Recovery	Recovery per cycle			Matrix effect	Recovery	Matrix effect
		1st cycle	2nd cycle	3rd cycle			
Neosaxitoxin	44.45 ± 5.48	72.66	23.93	3.41	31.4	23.17 ± 4.66	60.7
Decarbamoylsaxitoxin	32.34 ± 9.62	73.59	24.10	2.30	71.1	15.66 ± 3.03	77.3
Tetrodotoxin	71.18 ± 9.62	74.79	22.83	2.38	70.7	47.14 ± 9.91	79.7
Gonyautoxin-2,3	25.57 ± 4.97	45.20	25.72	29.08	71.0	25.67 ± 5.04	78.9
Saxitoxin	26.97 ± 5.08	63.85	32.75	3.40	43.4	19.81 ± 2.24	90.1

Repeatability of the instrument was determined as the intra-day and inter-day precision by the consecutive measurements during the same day (n=6) and different days (n=3) at the same instrumental conditions. Limits of detection (LODs) and quantification (LOQs) of the instrument and the final method were estimated as following: ILODs were determined by gradual dilutions of the standard solution mix containing the six biotoxins, starting at 40 µg/L of concentration. Consequently, ILOQs were estimated as 10/3 times the ILODs. MLODs for each extraction method was determined by the analysis of the standard solution mix in the matrix resulting after UAE for the particulate and SPE for the filtrate. Progressive dilutions of concentration starting at 40 µg/L were analysed to determine the limits of detection experimentally. MLOQs were estimated as 10/3 times the MLODs.

Recovery and matrix effect have been the parameters pondered over to evaluate the efficiency of the different pre-treatment processes. For this, fortified samples were processed for both extraction methods and compared with blanks extracts passed by the same processes and fortified in the moment of the analysis. Matrix effect was assessed to determine the interferences during ionization for particulate and filtrate portions. For this, fortified solutions of the target toxins at 40 µg/L were compared by the formula:

$$ME(\%) = ([Area]extract/[Area]solvent) \times 100$$

Being [Area]extract the integrated area of fortified blank extracts of seawater and [Area]solvent the integrated area of fortified pure solvent.

Optimization of the method

UAE of the particulate portion

In order to optimize the extraction of the MBTs from the particulate phase, an ultrasounds bath was employed during the solid-liquid extraction for 5 min. The cycles of extraction were also tested as well as the extraction solvent. Filters were spiked with 100-µL mix toxins at 100 µg/L and extracted with MeOH in three conditions; acidified at 0.1% of formic acid, MeOH acidified at 0.1% of acetic acid and pure MeOH.

In Fig. 2 are represented the values of recovery obtained for the particulate extraction via UAE with the different conditions of MeOH. Mean values for all compounds are above the 20% and are no higher than the 45% unless for TTX which has values ranging from the 62% to 145%. In general, there is no a significant difference in using MeOH or MeOH acidified with formic or acetic acid. The extraction of TTX is better when using MeOH at 0.1% FA though the standard deviation is higher than for the other conditions. For GTX-2,3 and STX can be observed slightly higher recoveries when using MeOH 0.1% FA but for the rest, Neo and dcSTX when using neutral MeOH. MeOH seems to be a suitable solvent to extract all compounds at the maximum recovery or with less standard deviation.

In Table 3 are listened the values of recovery for each cycle of extraction assisted with ultrasounds for 5 min with 5-mL MeOH. For most of the compounds, the first cycle removes around the 70% of the total toxin content and the second cycle almost the 100%, unless for GTX-2,3, which are recovered in equal portions during every cycle, making necessary the three cycles extraction. In addition, 5 minutes
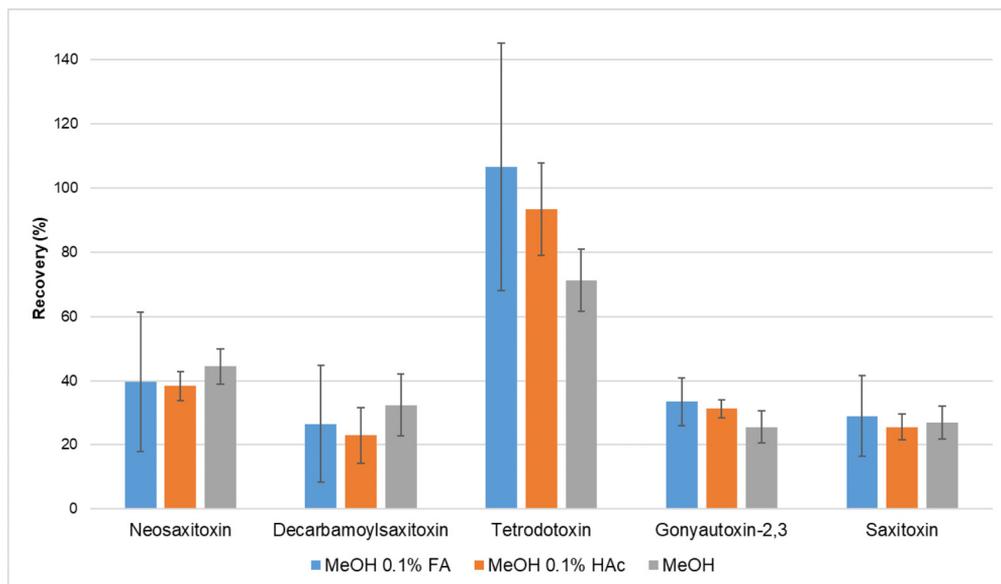
Fig. 2. Values of recovery for each MBT when extracted from the particulate using neutral MeOH, MeOH acidified at 0.1% FA and MeOH acidified at 0.1% AA by UAE.

of cycle extraction is enough time to ensure the lysis of the cells presents in the particulate and to separate the compounds from possible aggregates.

SPE of the filtrate portion

The optimization of this extraction included the test of different stationary phases, the conditioning of the sample and the volume.

The selected stationary phases were; divinylbenzene-pyrrolidone (HLB), silica (Silica), silica bonded with diol groups (Diol) and silica bonded with aminopropyl groups (NH2). In order to evaluate the retention capacity of the compounds into the stationary phases, a mass balance was carried out with 1-mL spiked sample/ACN (1:9) with 0.1% FA, measuring the content of the target toxins before and after the loading step, and after the elution. The elution was performed with MeOH and the extracts were evaporated and reconstituted to the initial conditions of the chromatography. Stationary phases were prepared in preparative tubes for SPE with 100 mg of each sorbent.

Conditions of the loading sample were tested for raw filtered samples and filtered samples conditioned with ACN (1:9) at 0.1% FA. For both conditions the samples were fortified with the mix of the six standards at 40 μg/L in volumes of 1, 5 and 10 mL.

In Fig. 3 are represented the moiety of the target toxins before and after the loading into the cartridges and after the elution. Results of mass balance confirmed the high hydrophilicity of these compounds and consequently the poor retention into the stationary phases. Best recoveries were obtained when using Silica and NH_2 cartridges, even considering the high loss of the compound. The loss of the compound when using the Silica cartridge goes from the 25% to the 56%, whereas recovery values are ranging from 34 to 58%. In the case of NH_2, higher recoveries for TTX, GTX-2,3 are achieved, 69 and 75%, respectively, but not for the rest of toxins.

According to the conditioning step of the filtrated sample, no recoveries were achieved when the samples were loaded without conditioning with ACN at 0.1% FA.

Acceptable recoveries were obtained in volumes of 1 and 5 mL of conditioned sample, but not for 10-mL samples. Then, the final SPE method selected was the one that includes the employment
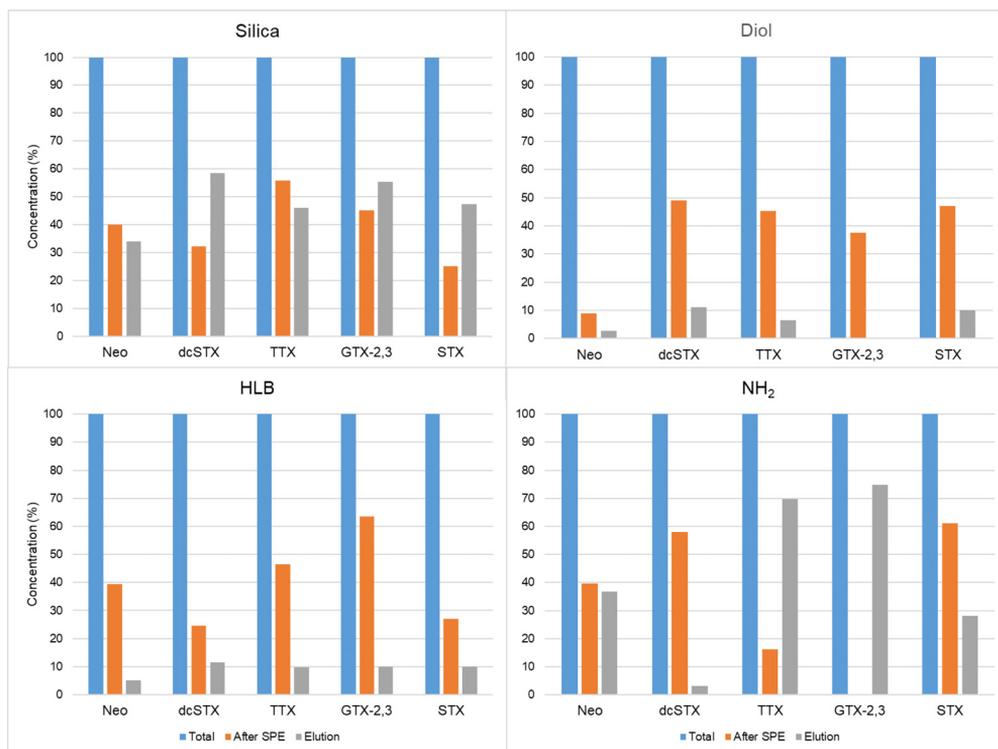
Fig. 3. Mass balance of the target toxins for the different stationary phases during the SPE. In blue colour is represented the concentration before the SPE, in orange colour the concentration that has not been retained in the cartridge and grey colour the concentration in the final extract of elution.

of silica cartridges for 5-mL sample conditioned with ACN 0.1% FA, and the recoveries are shown in Table 3.

HILIC-HRMS

Optimization of the chromatographic separation, was carried out with a HILIC column as stationary phase; silica surface covered with cross-linked diols groups phase HILIC LUNA® (150 mm x 2 mm, 3 µm, 200 Å) from Phenomenex (Torrance, United States).

For the mobile phase two different buffers of ammonium formate and ammonium acetate in ACN and water were tested; a buffer of ammonium formate adjusted at pH 3.2 with formic acid and the other of ammonium acetate at pH 5.8 with acetic acid. These salts were tested due to the high solubility in ACN and the compatibility with the mass spectrometry. The same mix of the corresponding MBTs was analysed in triplicate in the different conditions of mobile phases and flows.

Better resolution and peak shape were achieved when the mobile phase contained ammonium formate salts and FA. In Fig. 4 are represented the extracted ion chromatograms for each toxin at the different conditions of mobile phase. It was important to maintain the pH and the proportion of organic/aqueous phases in the mobile phase in order to avoid displacements in the retention times.

Mass spectrometry conditions were optimized by the direct injection of the standards into the mass spectrometer via an electrospray ionization (ESI) source. Positive and negative mode were tested to determine the best ionization for each analyte. Fragmentation parameters were evaluated for different values of NCE; 10,20,40,60 and 80 %.

Precursor ions type $[M-H]^+$ were the most intense for all the biotoxins and then, positive mode was the selected ionization mode. This group of biotoxins has hydropurine structures with amine
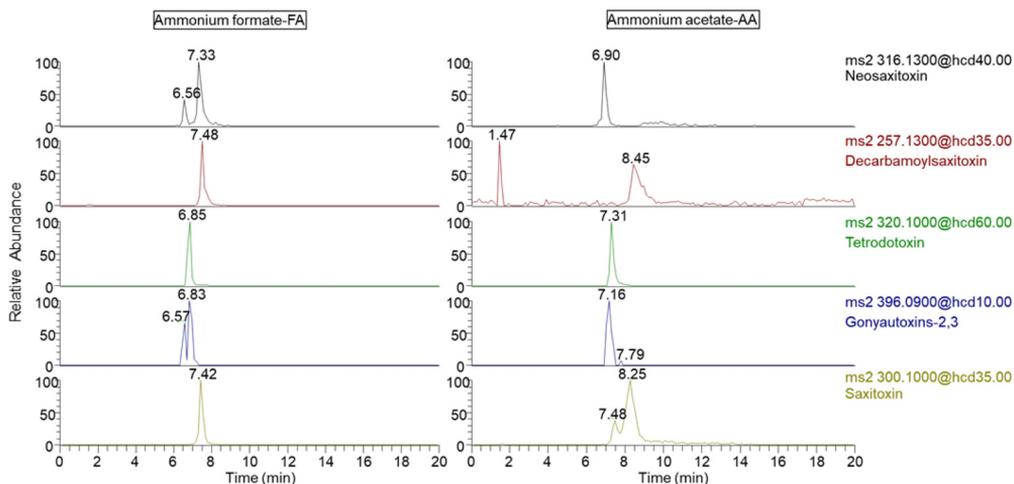
Fig. 4. Extracted ion chromatograms of the target MBTs when analysed by HILIC with different mobile phases composition: Ammoniun formate salt with FA and ammonium acetate salt with AA.

functional groups with strongest basic pKas ranging from 9.1 to 9.9. These groups are able to accept protons at the working conditions, with pH of 3, and then, are easily ionisable in positive mode.

The criterion to determine the most appropriated fragmentation pattern was considering the appearance of the maximum fragmentation ions and the precursor ion in the same mass spectrum. In figure S2 are represented the mass spectra for each biotoxin at the optimized NCE. Some toxins such as TTX required higher collision energies for the fragmentation, while some other such as GTX-2 and GTX-3 experimented fragmentation at the minimum energy employed.

Reconstitution of the final extract equal to the initial conditions of the chromatography was mandatory. Not only to ensure the good peak symmetry but also because ion suppression was observed when the extracts were containing more water content than in the initial conditions of the chromatography 90:10 (ACN/H2O 0.1% FA). In figure S3 is represented the loss of intensity when increasing the proportion of water in the injection vial.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2021.101370.