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Development of an MS/HRMS library for rapid quantitation, confirmation and dereplication of marine toxins using all-ion high resolution mass spectrometry (Q-TOF)

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Overview

A Q-TOF All-ion MS/MS method was developed for non-targeted analysis of marine biotoxins
Quantitation and confirmatory analysis of known and regulated toxins was carried out with this method
Non-targeted results were dereplicated using an in-house developed library

Introduction

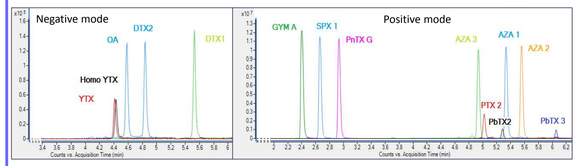
Marine biotoxins are algal metabolites that can harm humans through consumption of contaminated fish or shellfish or through direct contact exposure. The rapid identification of known compounds present in environmental and seafood samples, also referred to as dereplication, is crucial to the fast discovery of novel or emerging compounds. Monitoring programs aim at protecting consumers from intoxication or exposure. However, food poisoning or exposure of beachgoers may occur due to unidentified or emerging organisms and their toxins. We propose an MS/HRMS database screening approach for rapid and unambiguous dereplication of known and unknown marine or emerging toxins.

Method

All data were acquired on an Agilent Infinity 1290 UHPLC system (Agilent Technologies), coupled to either an Agilent 6540 or 6550 iFunnel Q-ToF mass spectrometer. Separation was achieved on a Agilent poroshell EC-C18 (100x2.1mm; 2.7µm) maintained at 40°C with mobile phases consisting of water and 95% acetonitrile, both containing 2mM ammonium formate and 50mM formic acid. An MS/HRMS library was constructed using pure compounds or, when these were not available, using extracts of microalgae and mussels. Names, structures and CAS number were transferred to instrument provider software (Agilent Masshunter PCDL manager) and linked to MS/HRMS spectra acquired at different collision energies. For dereplication, data files were processed in Masshunter using the "Molecular-Feature-Extraction"- or the "Find-by-Formula"-algorithm, followed by a database screening with fragment confirmation.

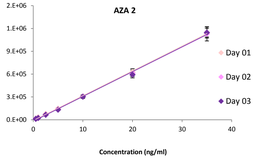
Evaluation of a quantitative HRMS/MS method

An All-ion MS/MS method was developed in positive and negative mode to detect regulated and non regulated lipophilic phycotoxins in a 9 min run. The data were acquired using different conditions : 1) with a low energy 2) with high energy values (30 and 50V).



Validation experiments

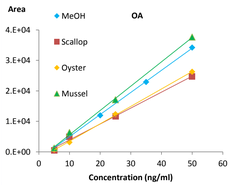
A linearity, repeatability and reproducibility study was carried out for four replicates and over 3 days using a range of seven concentrations (0.5 to 35 ng/ml in positive and 2.5 to 50 ng/ml in negative mode). As shown for Azaspiracid 2 (AZA 2), the regression line was linear (R^2>0.99) and the within-laboratory between-day reproducibility was less than 15% for all 14 toxins at each calibration level.



AZA 2 Concentration level (ng/ml)	Day 01 RSD (n=4)	Day 02 RSD (n=4)	Day 03 RSD (n=4)	Reproductibility RSD (n=12)
0.5	16%	12%	8%	12%
1	13%	13%	11%	12%
2.5	10%	9%	9%	9%
5	10%	8%	8%	9%
10	7%	7%	7%	7%
20	7%	7%	7%	7%
35	6%	8%	5%	7%

Different seafood matrices (mussel, scallop, oyster) were tested to determine the specificity of the All-ion MS/MS method . The matrix effect was evaluated by comparing the amount of toxin spiked to the extract with the amount of toxin found after analysis. The ionization yield depended on toxin-matrix combinations and ranged from 70 to 140%.

Shellfish	Ionization yield (%)											
	OA	DTX2	DTX1	YTX	Homo-YTX	PTX2	AZA 1	AZA 2	AZA 3	GYM A	SPX1	PnTX G
Mussel level 1	96	109	124	70	65	131	146	145	152	138	136	134
Mussel level 2	119	122	142	71	66	134	110	111	115	107	97	98
Mussel level 3	107	120	128	76	72	129	119	123	127	112	105	111
Mussel level 4	110	127	151	82	74	130	119	118	126	114	101	107
Oyster level 1	82	69	61	74	60	111	93	110	92	96	95	84
Oyster level 2	74	92	99	72	63	136	81	90	91	85	82	77
Oyster level 3	81	85	91	70	65	117	85	88	92	85	79	78
Oyster level 4	79	79	89	80	69	116	85	85	93	89	76	78
Scallop level 1	74	68	90	67	66	110	84	88	86	86	86	79
Scallop level 2	99	87	83	73	65	90	77	82	83	80	76	72
Scallop level 3	77	85	83	69	63	96	79	81	85	79	78	73
Scallop level 4	74	84	80	72	64	96	79	78	86	79	73	71



Several approaches for determining detection limit (LOD) and quantitation limit (LOQ) are possible, in this study, the calculation based on statistical formula:

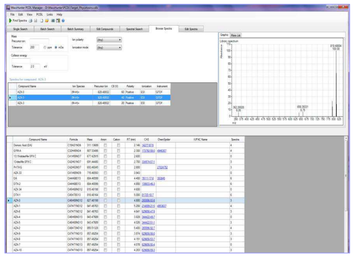
LOD = b + 3*S(b) / a
LOQ = b +10*S(b) / a

Toxin	LOD (µg/Kg)	LOQ (µg/Kg)
OA	51	101
DTX2	46	80
DTX1	33	110
GYM A	7	31
SPX1	10	39
AZA 1	8	34
PnTX G	10	40
AZA 2	11	37
AZA 3	8	34
PTX2	12	39
YTX	181	578
homo YTX	183	586

Personal Compound Database and Library (PCDL) for marine biotoxins

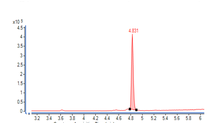
Toxin family	Toxin group	Number Compound	Number Molecular structure
ASP	DA	10	10
DSP	OA+DTX	60	13
	AZAs	40	32
	PTXs	17	15
	YTXs	31	16
PSP	STXs	64	60
FAT	TTXs	21	21
	GYMs	7	5
	SPXs	11	11
	PnTXs+PtenTXs	12	11
NSP	Imine cyclic	7	6
	PLTXs	11	4
	MTX	1	1
	PbTXs (BTX)	17	12
	Goniodomin A	1	1
	KBTX	10	4
	Tamulamide	2	2
	Gambiertoxins	7	6
	P-CTXs	27	27
	C-CTXs	2	2
CYANO	Oscillatoxin	11	11
	Anatoxin	12	12
	Cylindrospermocin	3	3
	Microcystins	14	14
	Aplysiatoxins	6	6
	Curacins	4	4
	Jamaicamides	3	3
	Antillatoxins	2	2
	Nodularin	7	7
	Kalkitoxin	1	1
	BMAA	1	1
	Trichotoxin	1	1
OTHERS	Karlotoxins	6	3
	Calyculins	14	14
	Gliotoxins	2	2
	Polycavernosides	5	5
	Amphidinols	12	12
	Amphidinolides	39	39
	portimine	1	1
	Nakijiquinone	4	1
	precursor C-CTXs	4	/

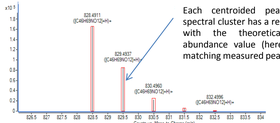
The ***PCDL library*** currently contains information for over 500 toxins with 400 structures and spectra for approximately 70 compounds (including all regulated toxins in the US and Europe). The library was constructed from existing databases, literature and from spectra acquired on the 6540 and 6550 Agilent Q-ToF instruments.
One of the main inconveniences of non-targeted HRMS data is the large size of raw-data files and time required for data handling and reprocessing. The MassHunter PCDL manager is a module in the MassHunter software suite that allows both for easy visualisation of compounds, spectra and structures in a library collection and for rapid interfacing with other Agilent software modules, i.e. rapid data processing. For instance, a data file from 10 min All-ion HRMS acquisition only requires a few seconds for dereplication against this library.



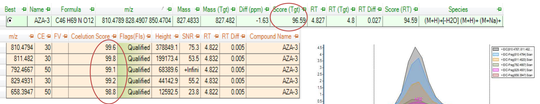
Qualitative screening using All-ions MS/MS

The modified ***find by formula*** algorithm searches compounds based on database entry in the low energy domain. The algorithm then uses All-ion spectra as source of fragments to look for in the high energy domain and compare elution profiles.





Azaspiracid 3 (AZA 3) was found with 5 valid qualifier fragments (Score > 99) from the PCDL spectrum

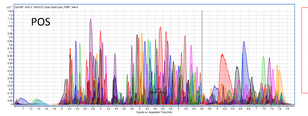


Overlay of the extracted ion chromatograms (EIC) with the coelution plots shows excellent agreement between the products and precursor ion. All fragment ions exhibit ratios of approximately 1 across the middle of the precursor peak, compared to the library spectrum.

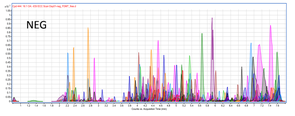
This matching process provided thus excellent confirmation for identification of AZA 3 in the shellfish sample.

Dereplication using Molecular Feature Extraction (MFE) followed by database screening

The ***Molecular Feature extraction*** (MFE) algorithm of MassHunter software was applied on raw mass spectral data generating lists of chemically qualified features. As an example, a freeze-dried mussel tissue (FDMT) reference material certified for multiple algal toxins was measured in negative and positive All-ion MS/MS and a MFE was operated with a peak filter of more than 10 000Cts, and a quality score >80.



Results
+ Positive mode:
1957 features in 832 groups
- Negative mode:
1078 features in 572 groups



Screening of FDMT against the library (510 entries) with a score >60 allowed for the detection of all certified compounds, normally detected in either negative (DA, YTX, 45-OH YTX, COOH-YTX, OA, DTX1, DTX2) or in positive mode (13-desmethyl SPX-C, PTX2, AZA1, AZA2 AZA3). An additional 10 compounds were identified using the library and tentatively confirmed.

Compound	Formula	RT	Mass	Mass (Tgt)	Diff (Tgt, ppm)	Score (Tgt)	Area	Flags (Tgt)
Cpd 41: Domoic Acid (DA)	C15 H21 N O6	2.146	311.1379	311.1369	3.13	95.19	8826653	multiple IDs
Cpd 136: OA	C44 H68 O13	4.117	804.468	804.466	2.53	94.2	278992	multiple IDs
Cpd 135: DTX-2	C44 H68 O13	4.305	804.4675	804.466	1.9	98.94	586263	multiple IDs
Cpd 140: DTX-1	C45 H70 O13	5.265	818.4755	818.4816	-7.5	70.78	1364202	No H adduct
Cpd 59: YTX	C55 H82 O21 S2	4.042	1142.4791	1142.479	0.08	97.08	91300	
Cpd 60: 45-OH-YTX	C55 H82 O22 S2	3.364	1158.4732	1158.4739	-0.63	90.67	23513	
Cpd 61: COOH-YTX	C55 H82 O23 S2	3.437	1174.467	1174.4688	-1.58	90.62	59179	
Cpd 157: AZA 1	C47 H71 N O12	4.764	841.5002	841.4976	3.04	87.27	9205611	multiple IDs
Cpd 171: AZA-2	C48 H73 N O12	4.931	855.5154	855.5133	2.43	94.16	2460885	multiple IDs
Cpd 148: AZA-3	C46 H69 N O12	4.472	827.4831	827.482	1.36	97.17	2563978	
Cpd 163: AZA-4	C46 H69 N O13	3.815	843.4779	843.4769	1.21	98.31	1321321	multiple IDs
Cpd 161: AZA-5	C46 H69 N O13	4.024	843.4777	843.4769	0.97	97.61	197345	multiple IDs
Cpd 158: AZA 6	C47 H71 N O12	4.649	841.4993	841.4976	1.96	86.41	546145	multiple IDs
Cpd 177: AZA-9	C47 H71 N O13	3.982	857.4939	857.4925	1.63	97.77	347008	multiple IDs
Cpd 138: AZA-25	C46 H67 N O11	4.66	809.4738	809.4714	2.89	82.64	157513	
Cpd 165: AZA 36	C46 H71 N O13	5.15	845.4937	845.4925	1.33	97.98	458276	
Cpd 179: PTX2	C47 H70 O14	4.399	858.4789	858.4766	2.69	95.7	615677	
Cpd 189: PTX2sa	C47 H72 O15	4.253	894.5224	876.4889	1.98	94.87	577725	multiple IDs
Cpd 192: 7-epi-PTX2sa	C47 H72 O15	4.003	876.4905	876.4871	3.91	98.15	1116927	multiple IDs
Cpd 111: 13-desMe-SPX C	C42 H61 N O7	2.834	691.4432	691.4448	-2.29	80.01	6467889	
Cpd 116: SPX C	C43 H63 N O7	2.845	705.4608	705.4604	0.47	67.61	348736	multiple IDs
Cpd 115: 20-Me-SPX G	C43 H63 N O7	2.96	705.4608	705.4604	0.55	97.2	1253074	multiple IDs

Conclusion

- ✓ The All-ion MS/MS method developed provides an integrated approach using HPLC-Qtof providing both accurate full scan HRMS and MS/HRMS data.
- ✓ Validation experiments demonstrated the usefulness of UHPLC-HRMS as a fully quantitative method for known analogues (linearity, matrix effect, LOD &LOQ) using All- ion MS/MS with high energy (CE= 30 and 50V).
- ✓ We developed an in-house database and spectral library for marine biotoxins containing over 500 compounds with spectra for all regulated toxins in the EU and US.
- ✓ We successfully used dereplication with the MFE-algorithm and database screening identified all known toxins as well as non-characterized toxins present in a freeze-dried mussel tissue reference material.