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Method Article

A data independent acquisition all ion fragmentation mode tool for the suspect screening of natural toxins in surface water

M. Picardo, O Núñez, M. Farré*

Water and Soil Quality Research Group, IDAEA-CSIC, c/Jordi Girona 18-26, Barcelona 08034, Spain

A B S T R A C T

Among natural freshwater pollutants, cyanotoxins, mycotoxins, and phytotoxins are the most important and less studied. Their identification in surface water is challenging especially cause of the lack of standards and established analytical parameters. Most target methods focus one or a single group of compounds with similar characteristics. Here we present an *AIF* fast method for the *tentative identification* of natural toxins in water. Respect to the previous method [1], it offers higher performances for the acquisition of unknown compounds at low levels for higher number of analytes.

The key aspects of the method are:

• The qualitative screening DIA-AIF workflow using *Q Exactive Orbitrap*. Both targeted and suspect screening bases have been combined with online databases and suspect list to retrieve candidates as suspect natural toxins and their metabolites or degradation products.

• The in-silico analysis of mass spectrums allowed a fast structural characterization.

• The workflow has been finally applied to real samples coming from the Czech Republic, Italy, and Spain allowing the determination of 17 suspect natural toxins, 4 of them confirmed. None toxin passed the limit of 1 µg/L taken from the legislation applied for microcystin LR and arbitrarily extended to all toxins.

A R T I C L E I N F O

Method name: Data Independent Acquisition All Ion Fragmentation mode
Keywords: AIF, DIA, HPLC-HRMS/MS, QExactive Orbitrap, Tentative identification
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* Corresponding author.
E-mail address: mfuqam@cid.csic.es (M. Farré).

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Specifications table

Subject Area	Chemistry
More specific subject area	*Environmental Analytical Chemistry*
Method name	Data Independent Acquisition All Ion Fragmentation mode
Name and reference of original method	*Picardo M., Sanchís, J., Nuñez O., Farré M. Suspect screening of natural toxins in surface and drinking water by high performance liquid chromatography and high-resolution mass spectrometry. Chemosphere Volume 261, December 2020, 127888*
Resource availability	*Compound Discoverer 3.1 (ThermoFisher); MzCloud; MetFrag; Xcalibur*

Method details

Common approaches for the analysis of natural toxins in surface waters rely on solid-phase extraction as a sample preparation protocol followed by target analysis with Data Dependent Acquisition methods for a limited number of compounds. Most methods are specifically designed for a group of toxins with similar parameters or a single compound, depending on its physic-chemical characteristics. However, the prioritization of natural toxins and their degradation products in the surface water environment is of increasing importance due to their different eco-toxicological properties [2].

The need for identification protocols is critical, especially considering the low availability of certified standards. Among them, targeted approaches are generally used to analyze known chemicals of interest while non-targeted approaches are more challenging. This is due to the need for identification and structure characterization protocols that require the use of multiple instruments (NMR and IR) which usually are not available or highly expensive.

High-Resolution Mass Spectrometry (HRMS) based on high-resolution instruments such as Orbitrap, QTOF, and FTICR are helping to fulfill the need for reliable identification methods, providing sensitive fragmentation spectrums (MS/MS) for the identification of known and known-unknown compounds [3]. HRMS provides a high amount of information for characterization and identification purposes (molecular formula, isotopic patterns, double bond equivalents) comparing the experimental results with online or in-house databases of chemical compounds. Tandem mass spectrometry and the consequent fragmentation spectra are mandatory to achieve a tentative structural characterization. In these regards, the data-acquisition methodology used to acquire MS/MS spectrums is of critical consideration that influences the type of data generated, and the choice of which method to use is largely dependent on the aim of the approach. Among them, Data-Dependent Acquisition (DDA), Single Reaction Monitoring (SRM), and Data-Independent Acquisition (DIA) are the most used (Fig. S1 of the Supporting Information).

This work aims to introduce the All Ions Fragmentation (AIF) acquisition approach as a suspect screening method for a wide range of natural toxins in surface water. The AIF acquisition for all theoretical fragment ions was used to acquire the entire MS/MS spectrum with no precursor preselection. Data processing and information extraction required the use of various bioinformatics tools to deconvolute complex mass spectra, using data from prior experiments in DDA mode to generate spectral libraries that were used in the interrogation of DIA data [4]. The objectives can be resumed in (**i)** develop a robust workflow for the determination of natural toxins in surface water samples using the AIF mode; (**ii)** provide a reliable workflow to describe how to process the acquired data, (**iii)** demonstrate the advantages to use this approach as a tentative identification protocol for the screening of natural toxins using real samples.

A Q-Exactive Orbitrap was used to obtain the full scan and MS/MS spectrums with the AIF mode. Data mining was then carried out using Compound Discoverer using a published suspect list with 2384 natural toxins [1] and the online databases Chemspider and MassBank [5] and also with fragmentation prediction tools such as MetFrag. The “Fish score” option was used to structurally characterize the MS/MS patterns. Finally, 24 natural toxins have been tentatively identified from surface water samples coming from three sampling sites in Europe.

Standard solutions

Table S1 of the supporting information reports the standards used for method optimization. Compounds **1–5, 7–12, 14, 15, and 22–26** were supplied from Merck (Darmstadt, Germany). Compound **6** was supplied from Santa Cruz Biotechnology (Dallas, TX, USA). **16–21** were from Cyano (Cyanobiotech GmbH, Berlin, Germany). Methanol (MeOH), acetone, and acetonitrile (ACN) HPLC grade were from Merck (Darmstadt, Germany). HPLC grade water was from Baker (Madrid, Spain). Fortified samples (5 mL each) with the 23 compounds (Table S1) at a concentration of 1 µg/L were prepared in both HPLC water and artificial freshwater (AFW) adding 10 % of MeOH, to reach the initial chromatographic conditions, and to simulate the presence of matrix interference. Samples were mixed with magnetic bar stirring at 200 rpm and letting set for an hour to ensure the good mixing of the standards. To prepare the AFW we followed the description of Lipschitz and Michel [6], the organic matter was simulated by adding 10 mg/L of humic acid of technical grade from Sigma-Aldrich (reference 53680). The method optimization was carried out analyzing the standard solution in pure HPLC and artificial water, previously mixed for an hour at 25 °C and processed as reported below.

Sample preparation

Sample preparation was previously reported by Picardo et al. [1]. Briefly, intracellular toxins were released by sonication for 20 min and further filtered with a glass fibre (GF/B) microfiber filter grade (Sigma Aldrich, Steinheim, Germany). Solid-phase extraction (SPE) consisted of a 3 mL cartridge filled with 200 mg of porous graphitized carbon (PGC) (Sigma Aldrich, Steinheim, Germany) and 200 mg of Polypropylene polymeric phase Bond-Elut PPL (PPL) (Agilent, Santa Clara, CA, USA) separated by a Teflon frit. The third sorbent was the Oasis HLB plus, 225 mg (Waters Corporations, Mil-ford, MA) connected at the end of the cartridge. Conditioning required 10 mL of methanol followed by 10 mL of water. 100 mL of sample was loaded at a constant flow rate (2 mL/min) using a vacuum manifold. After the procedure, analytes were eluted in backflush with 15 mL of water/methanol 20:80 (*v/v*), 15 mL of methanol, and 15 mL of acetone/methanol 1:1 (*v/v*). Solvents were warmed at 45 °C before elution. The eluate was concentrated to approximately 100 µL using a teardrop ampoule connected to a vacuum evaporator (rotavapor) and re-dissolved to 1 mL of mobile phase (approx. 0.9 mL of ACN 10% acidified at 0.1 % of formic acid) in a tared vial. Finally, 20 µL of samples were injected in the HPLC-HRMS/MS instrument.

Liquid chromatography-mass spectrometry

Chromatographic separation followed the same parameters reported by Picardo et al. [1]. Briefly, the separation was performed with an Acquity UPLC System (Waters, Milford, MA, USA) using a Lichrosphere column, 125 mm × 2 mm i.d., 5 μm (Merck, Barcelona, ES). 20 µL of samples were injected, the constant flow rate was 250 µL/min. Mobile phases were water (A) and acetonitrile (B) acidified with 0.1 % of FA. The gradient was 0–3 min, 10% of B; at 3–13 min B was increased to 90% and kept at a constant concentration from 13 to 15 min; 15-16 min B decreased to 10%; 16–20 min equilibration at 10% B. Total run time was 20 min. The analysis was performed using a Q-Exactive™ Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Samples were acquired in Full Scan and AIF mode in positive (+) mode in a range from 75 to 1100 *m/z*. Collision Induced Dissociation (CID) was obtained using a normalized collision energy of 35 eV. The mass spectrometer parameters are reported in Table 1**.**

Suspect screening workflow

After the acquisition, spectral data were further processed to tentatively identify suspect natural toxins with the screening approach as reported below.

In silico processing

No inclusion list of precursors ions was used in the acquisition method however, the suspect list reported by Picardo et al. [1] was used in combination with the in silico tools for the data

Table 1
Analyser acquisition parameters.

Tune Data	
Spray Voltage:	3250 V
Capillary Temperature (+ or +-):	300 °C
Sheath Gas (+ or +-):	40
Aux Gas (+ or +-):	25
Spare Gas (+ or +-):	0
Max Spray Current (+):	100
Probe Heater Temp. (+ or +-):	300 °C
S-Lens RF Level:	70
Ion Source:	HESI
Resolution FS	35000 FWHM
Resolution AIF	17500 FWHM
AGC target	$10e^5$
Max Inject time	100ms
Microscans	1

processing. Blanks of the entire procedure were processed to exclude background noise. Then, raw files from Orbitrap were uploaded to Compound Discoverer 3.1 (Thermo Fischer Scientific, San Jose, CA, USA) and processed with the Environmental Untargeted Metabolomics workflow. Here, peak alignment, unknown compound detection, compound grouping across all samples, elemental composition prediction, and chemical background hiding (using blank samples) were applied with a mass error of < 5 ppm. Finally, a tentative list of various compounds is displayed. Table 2 reports the parameters used for the compound identification with Compound Discoverer 3.1

Structural identification

The structure elucidation of the compounds in the candidate list was based on accurate mass data processing using Compound Discoverer 3.1 nodes. The molecular ions, potassium, sodium, and ammonium adducts and their transitions were used as identification parameters. Fish scoring node was applied to obtain the predicted structures of the transitions of each precursor selected in the full scan spectrum under the same retention time. MzLogic node was then used to compare the experimental and theoretical fragments contained in MzCloud. The spectrums have been submitted to MzCloud online search to obtain the corresponding similarity score (SS). Compounds with SS lower than 70% were discarded. Furthermore, MetFrag [7] was the last step for structural identification. Here, the MS/MS spectrums and the relative intensities have been uploaded. Here candidates have been retrieved using the molecular formula, neutral mass with a mass error of 5 ppm using the KEGG database. Only the first 10 candidates with a similarity score higher than 0.9/1.0 have been considered as valid candidates for the last step. Finally, each suspect natural toxin that fulfilled the requirements, was checked with Xcalibur (Thermo Fischer Scientific, San Jose, CA, USA) to control the elution profile and the retention times overlapping of the precursors and their transitions.

Confirmation

Confirmation was not possible for all the compounds due to the lack of standards. However, for the ones available the same procedure was carried out to obtain the AIF spectra from the standards to finally confirm the suspect compounds. (Level 1). Here identification levels from 1 to 5 were assigned to the suspect compounds, following what was previously reported by Schymanski et al. [8]. The lowest level 5 corresponded to the accurate mass, while level 4 was achieved using the spectral information to assign a molecular formula. Level 3 resulted at the end of the first identification step when the primary tentative candidate was proposed when existing some evidence to recognize a possible structure. Finally, levels 2 and 1 were achieved using databases reporting diagnostic evidence to assign an exact structure and using the standard respectively.

Table 2
Compound Discoverer 3.1 parameters for the peak alignment and identification.

Workflow node	Advanced Parameters	Parameter	Value
Select spectra	Spectrum filter	Min precursor Mass	75 Da
		Max precursor Mass	1100 Da
	Scan event filter	Polarity mode	Positive
		Min Collision Energy	0
		Max Collision Energy	70
		Scan type	Any
	Peak filter	S/N threshold	3
Align retention times	General settings	Adaptive alignment	
		Max shift	2 min
		Mass tolerance	5 ppm
Find expected compounds	General settings	Mass tolerance	5 ppm
		Intensity tolerance [%]	50
		Intensity threshold [%]	0.1
		S/N threshold	3
		Min peak Intensity	100000
Detect compounds	General settings	Mass tolerance	5 ppm
		Intensity tolerance [%]	40
		S/N threshold	3
		Min peak Intensity	100000
		Ions Checked	$[M+ACN+H]^+$, $[M+H]^+$, $[M+K]^+$, $[M+NH_4]^+$, $[M+Na]^+$
		Min Elements count	C, H, O
Group Compounds	Compound Consolidation	Mass tolerance	5 ppm
		RT tolerance	1 min
	Fragment data selection	Preferred Precursor Ions	$[M+ACN+H]^+$, $[M+H]^+$, $[M+K]^+$, $[M+Na]^+$ $[M+NH_4]^+$
Search ChemSpider	Search settings	Databases	MassBank, Toxin, Toxin-target database,
		Search Mode	By Formula and mass
		Mass tolerance	5 ppm
		Max results per compound	20
		Max predicted compounds	3
Search MzCloud	General settings	Compound classes	Natural toxins
		Library	Autoprocessed; Reference
	DIA Search	Use DIA scans for search	True
		Max isolation width	500
		Match activation type	False
		Match Activation energy	Any
		Activation energy tolerance	100
		Apply intensity threshold	False
		Match factor Threshold	10
Search Mass list	General settings	Mass List	In house suspect list
		Use retention times	False
		RT tolerance	-
		Mass Tolerance	5 ppm

Application on real samples

The procedure was then applied to real samples coming from different sites in Europe. Briefly, 2 samples were from Piave River 46°10′12.6"N 12°15′58.2"E (Belluno, Italy), 3 from Sykovec (Tri Studne, Czech Republic), Brno Dam (49°13′58.1"N 16°31′03.3"E, Czech Republic) and Jedovnice (49°20′04.2"N 16°45′58.7"E, Czech Republic), respectively. 1 from Cardener River (41°40′48.2"N 1°50′39.1"E Barcelona, Spain). 1 L of surface water sample was collected in each point. All samples were processed in triplicate. Sampling was carried out between July and August were the highest biological activity was expected in the cited areas.

Optimization and suspect screening using AFW standards solutions

Unlikely by what is generally applied with the ddMS^2, all the characteristic MS/MS transitions that can ensure a positive assignment were considered. Fig. S2 reports the mass spectrum of the umbelliferone standard $[M+H]^+$ m/z 163.0394 in artificial surface water after processing with Compound Discoverer 3.1 that matched the HR $[M+H]^+$ with the Chemspider and the in-house suspect list using the exact mass of the precursor ion 163.0195 m/z with a maximum error of 5 ppm algorithm. The picture reports the Full Scan and the AIF spectrum. As expected, the fragmentation spectra differ between the one obtained with DDA since also other transitions coming from interfering ions are displayed. As shown in Fig. 1**,** the sensitivity increased when the spectrum is acquired in AIF mode providing higher intensities for the same fragments under the same experimental conditions (CE energy, concentration, MS parameters). A similar result was also highlighted by Sentandreu et al., [9] who reported that the breakdown pattern of previously isolated compounds and AIF patterns cannot be comparable. An over-breakdown is generally observed when AIF is applied retrieving a higher transition intensity rather than the molecular ion at the same collision energy. The tentative list obtained after the first analysis of AFW samples resulted in a list where appeared both compounds of interest and interferents. The noise was further hidden from the background using blanks. The first tentative structure was obtained using the "*FISh scoring*" employed to elucidate the structure of each transition in the MS/MS spectrum to predict in silico fragments based on the structure of the parent compound using a list of expected fragments reported in online databases. Then, the "*mzLogic*" algorithm compares the fragmentation patterns and the structures with MzCloud. Here, umbelliferone had a full match (100%) from our suspect list and a partial match in Chemspider with a score of 86 %.

The most abundant fragment at 107.0492 *m/z* produced by the loss of –CO and –COH was observed followed in intensity by the 95.0492 and the 79.0180 *m/z*. However, during this experiment at least 5 positive fragments (green highlight in Fig. S3) were necessary to consider the compound as a tentative candidate. As a result of this processing, is possible to observe that even if there were 80 unmatched transitions produced by interference, 16 were recognized as structural fragments**.** Spectra comparison depends on the Collision Energy applied. Here at 35 eV, umbelliferone structure $C_9H_6O_3$ (7-hydroxycoumarine) was confirmed with a match score of 87.7 % which is over the threshold required to accept a candidate to be further investigated. The MS/MS spectrum was also investigated using MetFrag [7] to predict the fragmentation and assign a formula for each transition. Here 2 candidates were displayed (umbelliferone and 4-hydroxycoumarin). The mass spectrum obtained a final similarity score of 1.0 /1.0 for umbelliferone and 0.962/1.0 for the 4-hydroxycoumarin. A total of 29 fragments have been identified for the first compound and 27 for the second. Table 3 reports the structures, the formula, and the exact masses of the fragments considered for the tentative identification to level 2 of umbelliferone.

The final step to reach identification level 2 as reported by Schymansky et al. [8] was the manual check with XCalibur (Thermo Fischer Scientific, San Jose, CA, USA) to ensure the peak fitting under the same retention time of the precursor ion. Fig. 2 reports the MS^2 spectra with the fragments considered for the tentative identification of umbelliferone. The intensities are higher with respect to the noise originated by the interferences allowing a clear recognition of the peaks. The procedure resulted in the overlap of retention times with the same peak shape and intensities of the MS/MS spectrums confirming the good performances obtained in the identification of spectral patterns. Here three fragments were considered as qualitative ions, with a mass error under 5 ppm, briefly: $[C_8H_6O_2]^+$ 134.0368 m/z, 3.8 ppm; $[C_7H_7O]^+$ 107.0495 m/z, 3.8 ppm; $[C_6H_7O]^+$ 95.0495 m/z, 4 ppm; $[C_7H_7]^+$ 91.0546 m/z, 4.2 ppm; $[C_6H_7]^+$ 79.0546 m/z, 4.3 ppm. These steps were necessary to achieve the tentative identification level 2, however, the confirmation was only possible using standards.

Confirmation

The last step to confirm the suspect natural toxins to level 1, required the comparison with the standard. Fig. 3 shows the confirmation of the umbelliferone to the identification level 1. The standard solution at 1 µg/L in HPLC water was injected using the same acquisition method. As expected,

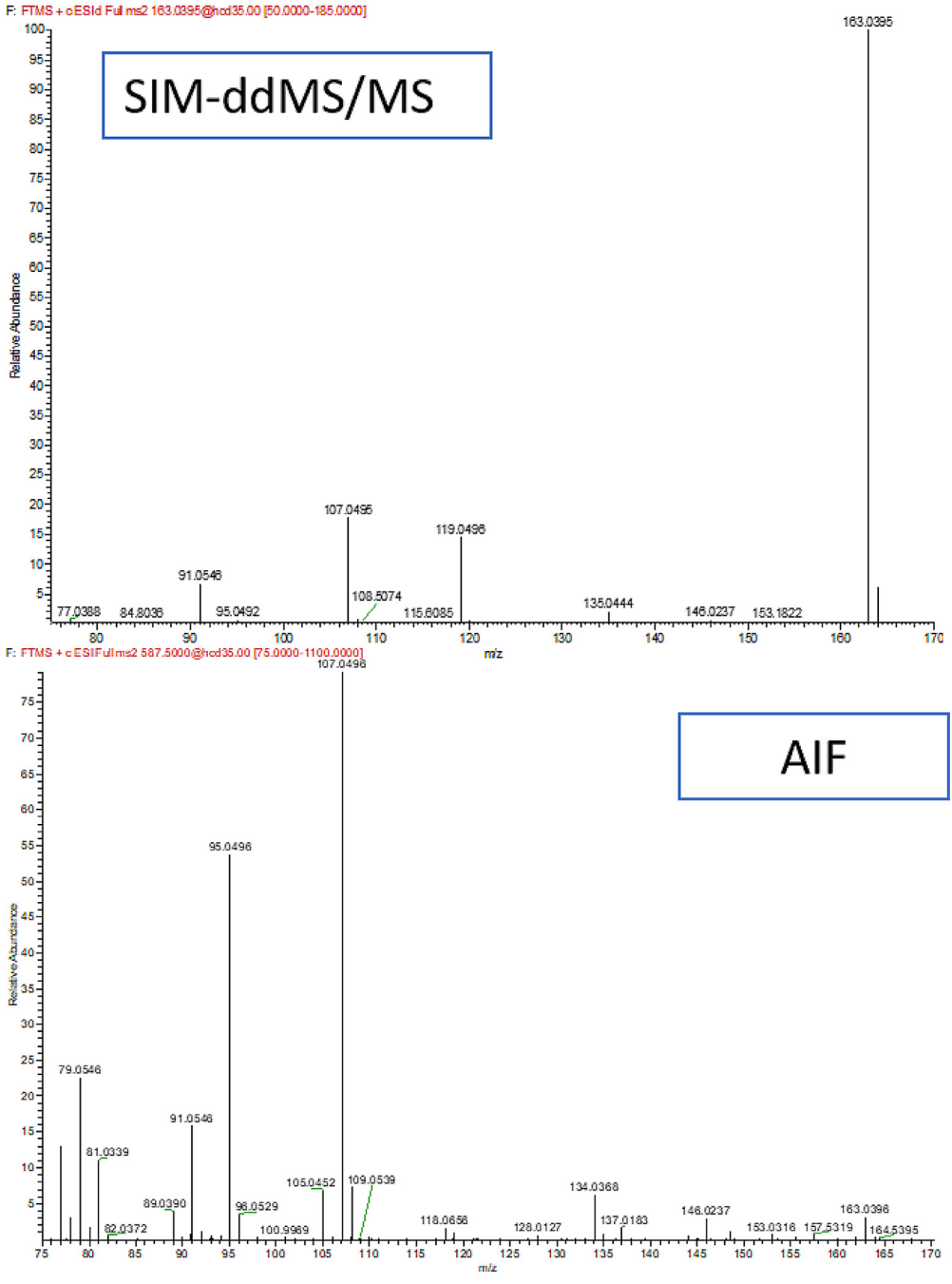
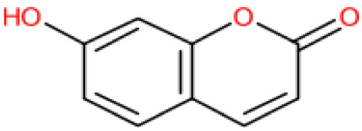
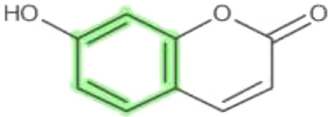
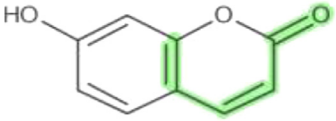
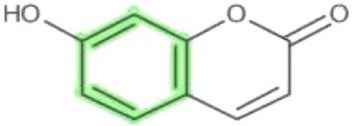
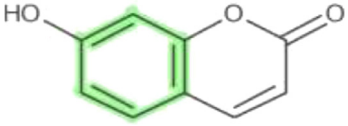
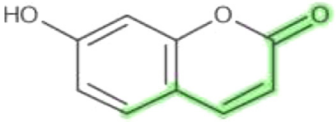
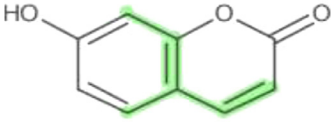
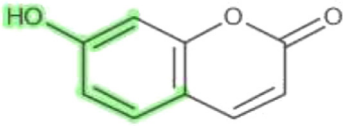
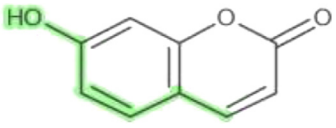


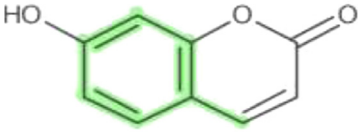
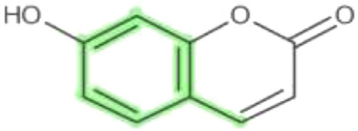
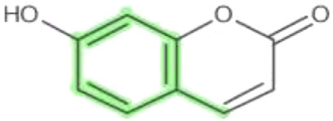
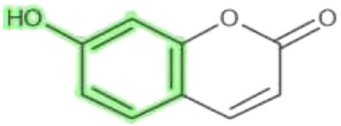
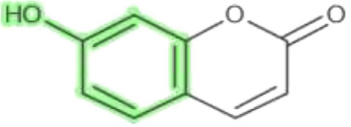
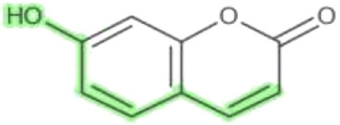
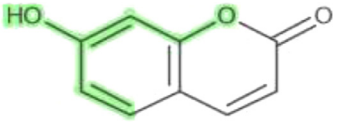
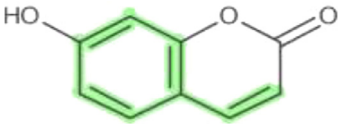
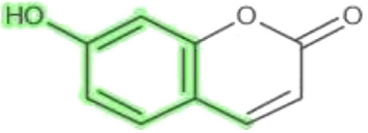
Fig. 1. Sensitivity comparison between SIM-ddMS/MS and AIF under the same experimental conditions.

Table 3
Fragmentation patterns recognised by MetFrag.

Precursor		
	**CompoundName** **Molecualr formula** **Identifier**	Umbelliferone C9H6O3 C9H6O3
Fragments		
	**Formula** **Mass** **Peak m/z**	$[C5H2O\text{-}H]^{1+}$ 77.00219 77.00256
	**Formula** **Mass** **Peak m/z**	$[C5H2O\text{-}H]^{+}$ 77.00219 77.00256
	**Formula** **Mass** **Peak m/z**	$[C6H3+H]+H^{+}$ 77.0386 77.03893
	**Formula** **Mass** **Peak m/z**	$[C6H3+2H]+H^{+}$ 78.04643 78.04679
	**Formula** **Mass** **Peak m/z**	$[C5H3O]^{+}$ 79.01785 79.01823
	**Formula** **Mass** **Peak m/z**	$[C6H4+2H]+H^{+}$ 79.05426 79.0546
	**Formula** **Mass** **Peak m/z**	$[C5H4O]+H^{+}$ 81.03351 81.03388
	**Formula** **Mass** **Peak m/z**	$[C5H4O+2H]+H^{+}$ 83.04917 83.04953

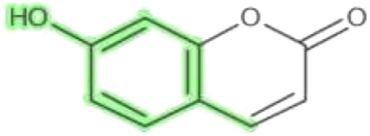
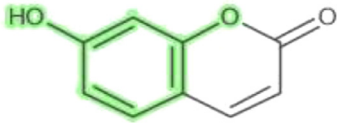
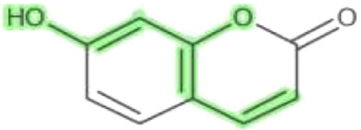
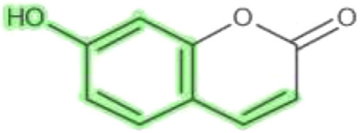
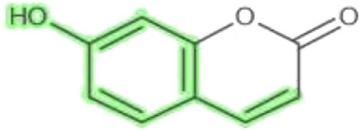
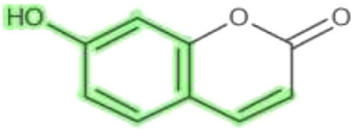
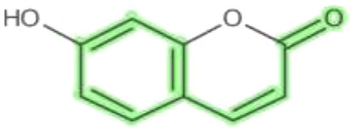
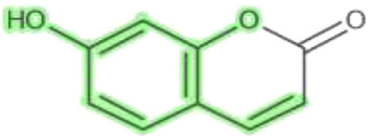
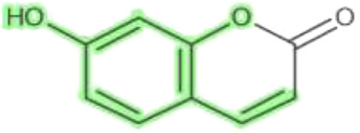
(*continued on next page*)

Table 3 (*continued*)

Precursor		
	**Formula** **Mass** **Peak m/z**	$[C7H4]+H^+$ 89.0386 89.03898
	**Formula** **Mass** **Peak m/z**	$[C7H4+H]+H^+$ 90.04643 90.0468
	**Formula** **Mass** **Peak m/z**	$[C7H4+2H]+H^+$ 91.05426 91.05464
	**Formula** **Mass** **Peak m/z**	$[C6H4O]+H^+$ 93.03351 93.03397
	**Formula** **Mass** **Peak m/z**	$[C6H4O+H]+H^+$ 94.04134 94.04169
	**Formula** **Mass** **Peak m/z**	$[C6H5O+H]+H^+$ 95.04917 95.04956
	**Formula** **Mass** **Peak m/z**	$[C5H4O2+H]+H^+$ 98.03625 98.03667
	**Formula** **Mass** **Peak m/z**	$[C8H5]^+$ 101.0386 101.03919
	**Formula** **Mass** **Peak m/z**	$[C7H5O]^+$ 105.03351 105.03397

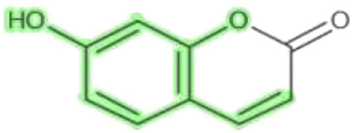
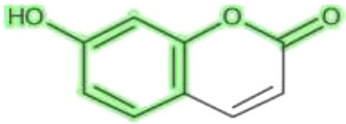
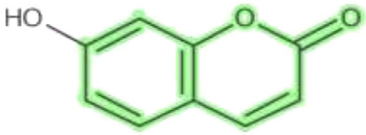
(*continued on next page*)

Table 3 (*continued*)

Precursor		
	**Formula** **Mass** **Peak m/z**	[C7H5O+H]+H$^+$ 107.04917 107.04959
	**Formula** **Mass** **Peak m/z**	[C6H4O2]$^+$ 108.02059 108.02084
	**Formula** **Mass** **Peak m/z**	[C6H4O2+2H]+H$^+$ 111.04408 111.04446
	**Formula** **Mass** **Peak m/z**	[C8H6O-H]$^+$ 117.03351 117.03391
	**Formula** **Mass** **Peak m/z**	[C8H6O]$^+$ 118.04134 118.04173
	**Formula** **Mass** **Peak m/z**	[C8H6O]+H$^+$ 119.04917 119.04971
	**Formula** **Mass** **Peak m/z**	[C9H5O+H]+H$^+$ 131.04917 131.04965
	**Formula** **Mass** **Peak m/z**	[C8H6O2-H] $^+$ 133.02842 133.0289
	**Formula** **Mass** **Peak m/z**	[C8H6O2] $^+$ 134.03625 134.0368

(*continued on next page*)

Table 3 (*continued*)

Precursor		
	**Formula** **Mass** **Peak m/z**	$[C8H6O2]+H^+$ 135.04408 135.04466
	**Formula** **Mass** **Peak m/z**	$[C7H4O3+H]+H^+$ 138.03116 138.03174
	**Formula** **Mass** **Peak m/z**	$[C9H5O2]^+$ 145.02842 145.02901

the typical fragment ions reported above and used as qualifier ions at m/z 134.0368, 107.0495, 95.0495, and 79.0546 were at the same retention time but with higher intensity. The signal was more intense due to the absence of interferents in the solution. The separation performance was comparable with the standards dissolved in AFW. The measured results were within the required limits for the identification of natural toxins in surface water samples. The same procedure was repeated with all the standards available. In Table 4 the results in AFW and HPLC water are reported. For each compound, more than 4 qualitative ions have been encountered in both AFW and HPLC water solutions. AFW samples presented as expected a lower signal suppressed by the most intense signals of the humic acids. However, the procedure allowed us to identify the standards and to validate the procedure for their determination. Quantitative validation was not included in this work since it is out of the aims.

Surface water samples analysis

Water samples coming from Italy, Spain, and Czech Republic were processed as described, performing the screening and the further identification and confirmation of different natural toxins. The pH was adjusted to 7.5 with formic acid 1.0 M, if necessary For the one in which standards were not available identification levels (ILs) system was applied [8]. This ILs method has been used by other authors to identify low molecular mass molecules when using data-independent acquisition [1,10]. 138 compounds have been proposed as suspect candidates in the first identification step. However, only 27 were reported as suspect natural toxins, 3 (cotinine, abscisic acid, and ptaquilosin B) were false positives and 4 (methoxycoumarin, MC-LR, abietic acid, and umbelliferone) were confirmed comparing by standards (Table 5). For the compounds that had previous literature with mass spectra under similar conditions, the MS/MS interpretation was less time-consuming. For instance, the mass spectrum of azelaic acid matched with the one reported in MassBank [5]. Comparing the common fragments m/z = 83.08897, 97.10339, 103.05256, and 125.09818 were found in both spectrums and the tentative identification level 2 was assigned. Then, the presence of suspect ptaquilosin B was also investigated. Ptaquiloside, a carcinogenic bracken fern toxin, is converted to the aglycone ptaquilosin B (PTB) in aqueous solutions due to the liberation of D-glucose to be then converted to pterosin B [11]. Here PTB was detected in the first identification step. However, the conversion rate of PTB depends on the temperature and the pH > 9. Here, since samples were frozen to -24 °C and the initial pH was 7.8 further investigation was required. Since D-glucose is released when converting ptaquiloside, its

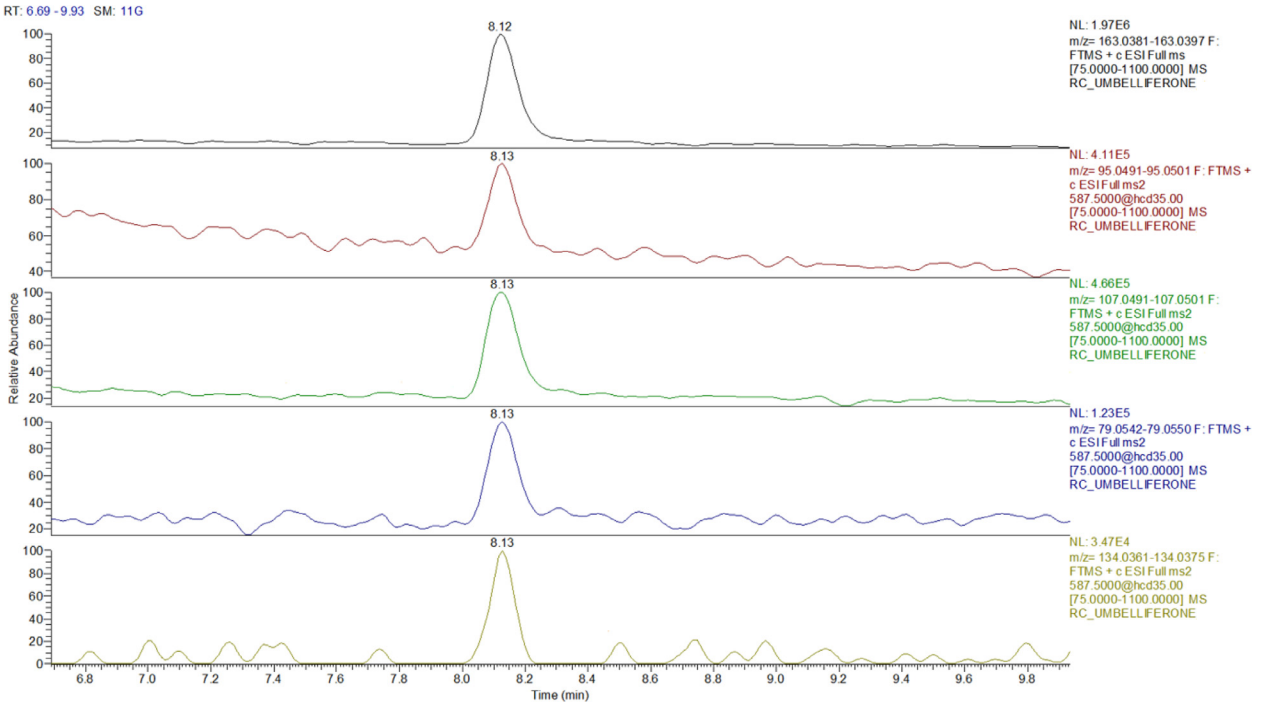


Fig. 2. Manual check with Xcalibur for peak shape and retention time fitting of precursor and transitions.

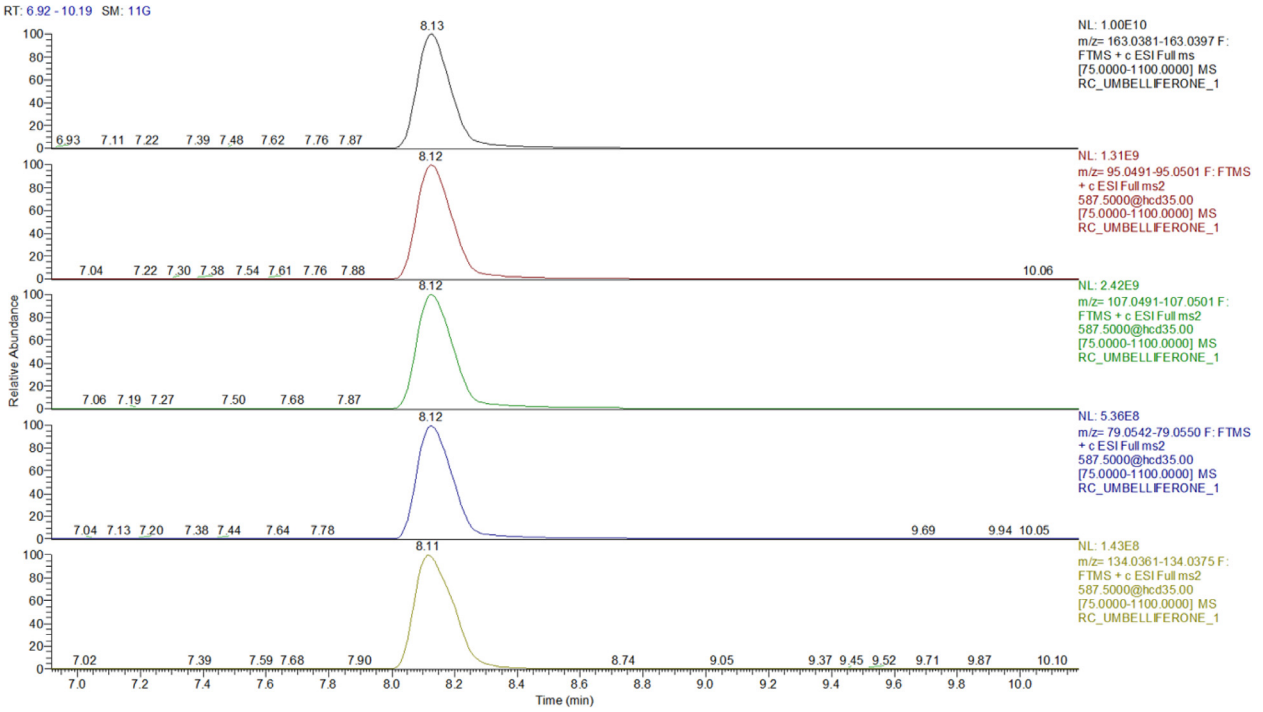


Fig. 3. Mass spectrums of the standard umbelliferone and its fragmentation products.

Table 4
Fragmentation patterns of the 23 natural toxins standards.

Compound	Rt	Precursor	Qi (1)	Structure	Qi (2)	Structure	Qi (3)	Structure	Qi (4)	Structure	Qi (5)	Structure
Ethoxycoumarin	11.2	191.0698	107.0492	C_7H_7O	95.0492	C_6H_7O	163.039	$C_9H_7O_3$	119.0492	C_8H_7O	91.0543	C_7H_7
Methoxycoumarin	10.1	177.0542	77.0386	C_6H_5	162.0310	$C_9H_6O_3$	106.0413	C_7H_6O	121.0647	C_8H_9O	134.0361	$C_8H_6O_2$
Abietic acid	1.5	303.2323	257.2269	$C_{19}H_{29}$	121.1014	C_9 H_{13}	147.1171	$C_{11}H_{15}$	287.2010	$C_{19}H_{27}O_2$	241.1954	$C_{18}H_{25}$
Aflatoxin B_1	9.8	313.0696	285.0763	$C_{16}H_{12}O_5$	269.0449	$C_{15}H_{10}O_5$	241.0499	$C_{14}H_9O_4$	214.0627	$C_{13}H_{10}O_3$	201.0913	$C_{12}H_8O_3$
Amygdalin	6.4	480.1483 [M+Na]+	85.0285	$C_4H_5O_2$	107.0492	C_7H_7O	325.11325	$C_{12}H_{21}O_{10}$	163.0602	$C_6H_{11}O_5$	127.0391	$C_6H_7O_3$
Anatoxin-a	1.6	166.1228	149.0964	$C_{10}H_{13}O$	95.0493	C_6H_7O	105.0700	C_8H_9	91.0544	C_7H_7	79.0544	C_6H_7
Atropine	6.8	290.1747	124.1120	$C_8H_{14}N$	93.06989	C_7H_9	103.0542	C_8H_7	260.1644	$C_{16}H_{22}NO_2$	142.1226	$C_8H_{16}NO$
B-Asarone	12.2	209.1166	179.0705	$C_{10}H_{11}O_3$	151.0756	$C_9H_{11}O_2$	121.0649	C_8H_9O	91.05446	C_7H_7	107.0493	C_7H_7O
Cinchonine	6	294.1733	79.0544	C_6H_7	184.0759	$C_{12}H_{10}NO$	130.0654	C_9H_8N	154.0653	$C_{11}H_8N$	142.0654	$C_{10}H_8N$
Cotinine	1.4	177.1029	80.0499	C_5H_6N	98.0606	C_5H_8NO	146.0609	C_9H_8NO	106.0657	C_7H_8N		
Cylindrospermopsin	1.5	415.1166	336.1675	$C_{15}H_{22}N_5O_4$	194.1293	$C_{10}H_{16}N_3O$	274.0864	$C_{10}H_{16}N_3O_4S$	318.1570	$C_{15}H_{20}N5O_3$		
Kojic Acid	2.3	143.0336	113.0234	$C_5H_5O_3$	126.0313	$C_6H_6O_3$	97.02863	$C_5H_5O_2$	87.00786	$C_3H_3O_3$		
Microcystin LA	11.5	910.4882	135.0808	$C_9H_{11}O$	227.0224	$C_9H_9N_2O_5$	299.0621	$C_{11}H_{15}N_4O_6$	155.0689	$C_6H_8N_3O_2$	297.0829	$C_{11}H_{15}N_4O_6$
Microcystin LF	12.3	986.5225	135.0808	$C_9H_{11}O$	213.0871	$C_9H_{13}N_2O_4$	258.1855	$C_{17}H_{24}N$ O	461.2398	$C_{23}H_{33}N_4O_6$	580.3016	$C_{32}H_{42}N_3O_7$
Microcystin LR	9.1	995.5575	135.0806	$C_9H_{11}O$	382.2089	$C_{17}H_{28}N_5O_5$	213.08728	$C_9H_{13}N_2O_4$	470.2729	$C_{20}H_{36}N_7O_6$	103.0544	C_8 H_7
Microcystin YR	11.7	1045.5355	135.0806	$C_9H_{11}O$	213.1364	$C_9H_{16}N_4O_2$	265.1609	$C_{19}H_{23}O$	323.1800	$C_{14}H_{24}N_6O_3$	466.2589	$C_{26}H_{36}N_4O_4$
Microcystin LY	11.2	1002.5353	135.0806	$C_9H_{11}O$	375.1918	$C_{20}H_{27}N_2O_5$	494.2616	$C_{28}H_{36}N_3O_5$	213.08723	$C_9H_{12}N_2O_4$	243.1343	$C_{11}H_{18}N_2O_4$
Nodularin	8.8	825.4505	135.080	$C_9H_{11}O$	227.103	$C_{10}H_{15}O_4N_2$	389.2074	$C_{21}H_{29}O_5N_2$	691.3768	$C_{29}H_{53}O_{12}N_7$	285.1668	$C_{11}H_{21}O_3N_6$
Ochratoxin-A	11.8	404.0885	358.0835	$C_{19}H_{17}ClNO_4$	257.0211	$C_{11}H_{10}ClO_5$	239.0105	$C_{11}H_8ClO_4$	120.0808	$C_8H_{10}N$	211.0157	$C_{10}H_8ClO_3$
P-Coumaric acid	7.8	165.0544	91.0543	C_7H_7	81.0336	C_5H_5O	81.03363	C_5H_5O	119.0492	C_8H_7O	147.0441	$C_9H_7O_2$
Scopolamine	6.21	304.1538	138.0912	$C_8H_{12}NO$	103.0542	C_8H_7	110.09641	$C_7H_{12}N$	103.0542	C_8H_7	121.0647	C_8H_9O
Thujone	12	153.1269	139.1120	$C_9H_{15}O$	97.0650	C_6H_9O	121.10143	C_9H_{13}	109.0651	C_7H_9O	135.1171	$C_{10}H_{15}$
Umbelliferone	8.12	163.0386	107.0492	C_7H_7O	95.0492	C_6H_7O	91.0546	C_7H_7	119.0493	C_8H_7O	134.0363	$C_8H_6O_2$

Table 5
Results of the suspect screneing with AIF acquisition in water samples.

Comp N°	Compound	Molecular formula	[M+H]+	Transition 1	Structure	Transition 2	Structure	Transition 3	Structure	Transition 4	Structure	Transition 5	Structure	Conf. Level
1	Aspidospermine	$C_{22}H_{30}N_2O_2$	355.2373	119.0491	C_8H_6O	107.0491	C_7H_6O	146.06	C_9H_9NO	228.1379	$C_{15}H_{18}NO$	152.1072	$C_9H_{15}NO$	2
2	O-Acetyltropine	$C_{10}H_{17}NO_2$	184.1329	108.0807	$C_7H_{11}N$	109.0648	$C_7H_{11}O$	127.0754	$C_7H_{11}O_2$	140.1068	$C_8H_{14}NO$	138.0913	$C_8H_{13}NO$	2
3	Microcystin LR	$C_{49}H_{74}N_{10}O_{12}$	995.5545	135.0805	$C_9H_{11}O$	382.2089	$C_{17}H_{28}N_5O_5$	213.0872	$C_9H_{13}N_2O_4$	265.1585	$C_{19}H_{21}O$	103.0544	C_8H_7	1
4	Heliotridine	$C_8H_{13}NO_2$	156.1018	120.0808	$C8H_{11}N$	122.0965	$C_8H_{11}N$	124.0758	$C_7H_{10}N_O$	110.0601	C_6H_9NO	108.0808	C_7H_9N	2
5	4-Heptyloxybenzoic acid	$C_{14}H_{20}O_3$	237.1481	105.0699	C_8H_{11}	133.1012	$C_9H_{11}O$	147.0854	C10H13O	123.0848	C8H12O	161.0961	$C_{11}H_{15}O$	False Ptaquilosin B
6	Hypoglicine A	$C_7H_{11}NO_2$	142.0861	107.0492	C_7H_8O	126.0550	$C_6H_9NO_2$	111.0441	C6H7O2	125.0597	C7H9O2	108.0808	$C_7H_{10}N$	2
7	Salsolinol	$C_{10}H_{13}NO_2$	180.1017	105.0700	C_8H_{10}	107.0493	C_7H_5O	118.0652	$C_8H_{10}N$	144.0810	$C_{10}H_{11}N$	162.0916	$C_{10}H_{12}NO$	2
8	Fumigaclavine C	$C_{23}H_{30}N_2O_2$	367.2374	105.0699	C_8H_9	119.0855	C_9H_{12}	243.1375	$C_{16}H_{17}O_2$	130.0653	C_9H_6N	144.0808	$C_{10}H_{11}N$	2
9	4-hydroxymellein	$C_{10}H_{10}O_4$	195.065	149.0234	$C_8H_6O_3$	121.0285	$C_7H_6O_2$	181.0496	$C_9H_7O_4$	163.0756	$C_{10}H_8O_2$	141.0543	$C_7H_7O_3$	2
10	(R)-reticuline	$C_{19}H_{23}NO_4$	330.1695	121.0285	$C_7H_6O_2$	111.0441	$C_6H_4O_2$	135.0805	C_9H_9O	125.0598	$C_7H_7O_2$	138.0914	$C_8H_{10}NO$	2
11	Apiol	$C_{12}H_{14}O_4$	223.0961	109.0648	C_7H_9O	135.0440	$C_8H_7O_2$	151.0754	$C_9H_9O_2$	147.0805	$C_{10}H_9O$	163.0391	$C_9H_8O_3$	2
12	Conhydrine	$C_8H_{17}NO$	144.1382	107.0856	C_8H_{11}	123.0805	$C_8H_{11}O$	138.0915	$C_8H_{12}NO$	111.0805	$C_7H_{11}O$	100.112	$C_6H_{14}N$	2
13	5-(N-Methyl-4,5-dihydro-1H-pyrrol-2-yl)pyridin-2-ol	$C_{10}H_{12}NO_2$	177.1019	120.0327	$C_6H_4N_2O$	107.0494	C_7H_4O	91.05438	C_7H_6	89.03877	C_7H_6-H	80.0496	C_5H_4N	False cotinine
14	Ridentin	$C_{15}H_{20}O_4$	265.1434	163.0757	$C_{10}H_{11}O_2$	123.0805	$C_8H_{11}O$	191.0709	$C_{11}H_{11}O_3$	207.1388	$C_{13}H_{19}O_2$	163.0757	$C_{10}H_{11}O_2$	False abscissic acid
15	Abietic acid	$C_{20}H_{30}O_3$	303.2317	257.226	$C_{19}H_{29}$	121.10145	C_9H_{13}	147.1171	$C_{11}H_{15}$	173.1328	$C_{13}H_{17}$	95.0945	C_6H_7O	1
16	Jervine	$C_{27}H_{39}NO\bullet$	426.2997	187.1120	$C_{13}H_{16}O$	191.1438	$C_{13}H_{19}O$	215.14362	$C_{15}H_{18}O$	219.1745	$C_{15}H_{22}O$	121.0650	$C_8H_{11}O$	2
17	Umbelliferone	$C_9H_6O_3$	163.0387	107.0492	C_7H_7O	119.0493	C_8H_7O	119.0493	C_8H_7O	135.0441	$C_8H_7O_2$	147.0441	$C_9H_7O_2$	1
18	Vincaminorein (Aspidospermine)	$C_{22}H_{30}N_2O_2$	355.2373	270.1859	$C_{18}H_{24}NO$	107.0491	C_7H_6O	119.0491	C_8H_6O	145.0652	$C_{10}H_{10}O$	98.0603	C_5H_8NO	2
19	Swainsonine	$C_8H_{15}NO_3$	174.1128	86.0603	C_4H_8NO	87.0443	$C_4H_7O_2$	124.0761	$C_7H_{12}NO$	140.0712	$C_7H_{12}NO_2$	138.0918	$C_8H_{13}NO$	2
20	Salsoline	$C_{11}H_{15}NO_2$	194.1173	91.0545	C_7H_8	179.1069	$C_{11}H_{14}O_2$	163.0759	$C_{10}H_{12}O_2$	107.0494	C_7H_5O	96.0809	C_6H_9N	2
21	Methoxycoumarin	$C_{10}H_8O_3$	177.0545	91.0546	C_7H_6	149.0239	$C_8H_5O_3$	163.0395	$C_9H_5O_3$	134.0361	$C_8H_6O_2$	121.0647	C_8H_9O	1
22	Azealic acid (Aspionene)	$C_9H_{16}O_4$	189.112	75.0439	$C_3H_5O_2$	83.0857	C_6H_{12}	97.0650	$C_6H_{10}O$	101.0599	$C_5H_9O_2$	143.1071	$C_8H_{15}O_2$	2
23	Aspergillic acid	$C_{12}H_{20}N_2O_2$	225.1602	98.0604	C_5H_8NO	124.0761	$C_7H_{10}NO$	209.1290	$C_{11}H_{17}N2O2$	152.0712	$C_8H_{11}NO_2$	86.0603	C_4H_6NO	2
24	Coniferyl acetate	$C_{12}H_{14}O_4$	223.0961	91.0546	C_7H_7	149.0238	$C_8H_7O_3$	121.0288	$C_7H_5O_2$	137.0602	$C_8H_8O_2$	177.0911	$C_{11}H_{10}O_2$	2

molecular ion m/z 181.07066 was searched. No *D*-glucose was found besides, the total absence of its precursor ptaquiloside brought to discard this compound as a tentative candidate. Finally, fragment analysis of 61 peaks revealed a strong similarity (1.0/1.0) with the 4-Heptyloxybenzoic acid [12] a carboxylic acid used with different purposes with no environmental importance for this work. Finally, 24 compounds have been detected and tentatively identified as suspect natural toxins. However, the confirmation to level 1 through mass spectrums comparison was carried out for 4 compounds with standards available (MC-LR, abietic acid, methoxycoumarin, and umbelliferone). Samples coming from the Czech Republic were collected in a blooming area which was characterized by green algal slime. This was the first signal to further investigate the presence of algal toxins such as microcystins. Here the tentative candidate microcystin LR was detected with the typical molecular ion at m/z= 995.5545. The doubly charged ion at 498.2822 *m/z* was also encountered at T_r 9.12 min with the typical higher intensity respect to the molecular ion [13]. Finally, the MS/MS spectra revealed the presence of the typical fragment at 135.0803 *m/z* which is the exact mass of the ADDA fragment part of all the microcystins structure. After manual analysis of the MS/MS spectra, the precursor and 3 common fragments were found to be consistent with the MC-LR structure. Finally, the MC-LR was confirmed to level 1 using the standard solution that revealed the presence of the qualitative fragment ions in both mass spectrums. The same confirmation procedure was applied for methoxycoumarin, abietic acid, and umbelliferone while 20 structures were proposed as suspect natural toxins with an identification level 2.

Declaration of Competing Interest

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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2021.101286.

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