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Direct detection of yessotoxin and its analogues by liquid chromatography coupled with electrospray ion trap mass spectrometry

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Abstract

A liquid chromatography mass spectrometry (LC–MS) method is proposed for the sensitive, specific and direct detection of yessotoxin and its analogues, marine biotoxins which are associated with diarrhetic shellfish poisoning (DSP) and which have been found in the North Adriatic sea since 1995. The LC–MS method provided a detection limit of 70 pg for yessotoxin in full scan mode and was applied to determine the toxic profiles of a number of extracts or partially purified fractions of toxic mussels collected along the Emilia Romagna coasts (Italy) in the period 1995–1999. Detection of a desulfo-yessotoxin derivative from *Mytilus galloprovincialis* collected in 1998 is also reported.

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1. Introduction

The group of diarrhetic shellfish poisoning (DSP) toxins, produced by some dinoflagellates associated with the genera *Dinophysis*, *Prorocentrum*, and *Protoceratium* includes three different classes of lipophylic polyether compounds [1]: (i) okadaic acid (OA) and its derivatives named dinophysistoxins (DTXs), (ii) pectenotoxins (PTXs), and (iii) yessotoxins (YTXs). Shellfish which accumulate these toxins are responsible for thousands of cases of human intoxication frequently characterized by vomiting and diarrhea. The poisoning is caused by ingestion of bivalves, like mussels or scallops, and poses serious problems to both public health and shellfish industries worldwide. In Italy, human gastroenteritis due to the consumption of contaminated mussels collected from the Northern Adriatic sea have occurred since 1989 [2]. A research program was initiated by our group in 1990 to carefully examine the toxin profiles in mussels from the Northern Adriatic sea. Initially, DSP phenomena

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were related to the presence of OA [3,4] and DTX1 [5]. In 1995, yessotoxin (YTX) was indicated as the main causative agent [6]. Since then, besides yessotoxin, a number of YTX analogues have been recently isolated and characterized by our group, some of which seem to be unique to the northwestern Adriatic sea [7–11].

An accurate determination of toxin profiles is of critical importance for assessment of health risks. In fact, the three groups of toxins differ toxicologically: the OA group causes diarrhea and tumor promotion [12], the PTXs injure the liver [13], while very limited data are available regarding the effects of YTXs on cellular systems. Yessotoxin deviates from the other DSP toxins in being non-diarrheagenic while histopathological analysis revealed that it causes myocardial injuries at high doses [14]. An involvement of the nervous system in YTX toxicity has also been hypothesized on the basis of structural analogy of YTX with brevetoxins and cyguatoxins, both responsible for neurological and cardiovascular symptoms [1]. However, yessotoxin's effects on humans as well as those of its analogues have not been proven yet.

Routine monitoring of shellfish for DSP toxins is generally carried out using mouse bioassay. The mouse lethality of YTX by intraperitoneal injection (0.1 mg/kg) is the strongest among all the DSP toxins, but its oral toxicity is the weakest as can be deduced by considering that the maximum oral dose of 1 mg/kg does not kill the mouse [15]. The presence of YTX in shellfish may, therefore, lead to overestimation of risk of DSP in consumers when the standard mouse bioassay is used. Thus, instrumental methods are required to individuate the nature of the implied toxins.

The most common methods for specific detection of DSP toxins provide for derivatization of each toxin with an appropriate auxiliary reagent for fluorescence labeling followed by HPLC analysis. Unfortunately, there is no reagent which fits all DSP toxins. 9-Anthryldiazomethane (ADAM) is used for OA, DTXs and PTXs, [16] while YTXs are derivatized with a dienophile reagent, 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD) [17]. It has to be noted, however, that, for the application of the latter method, the presence of a conjugated diene functionality in the side chain of YTX-like compounds is a prerequisite. Thus, YTX, homoYTX, 45-OHYTX, 45-OHhomoYTX, and 45,46,47-trinorYTX can be detected, while the method is not reliable for detection of 42,43,44,45,46,47,55-heptanor-41-oxohomoYTX, carboxyYTX, carboxyhomoYTX, and adriatoxin, which lack conjugated double bonds in the side chain (Fig. 1). Currently there is no method for the direct and combined determination of all YTX-like compounds.

Recently, Quilliam et al. [18,19] have reported the

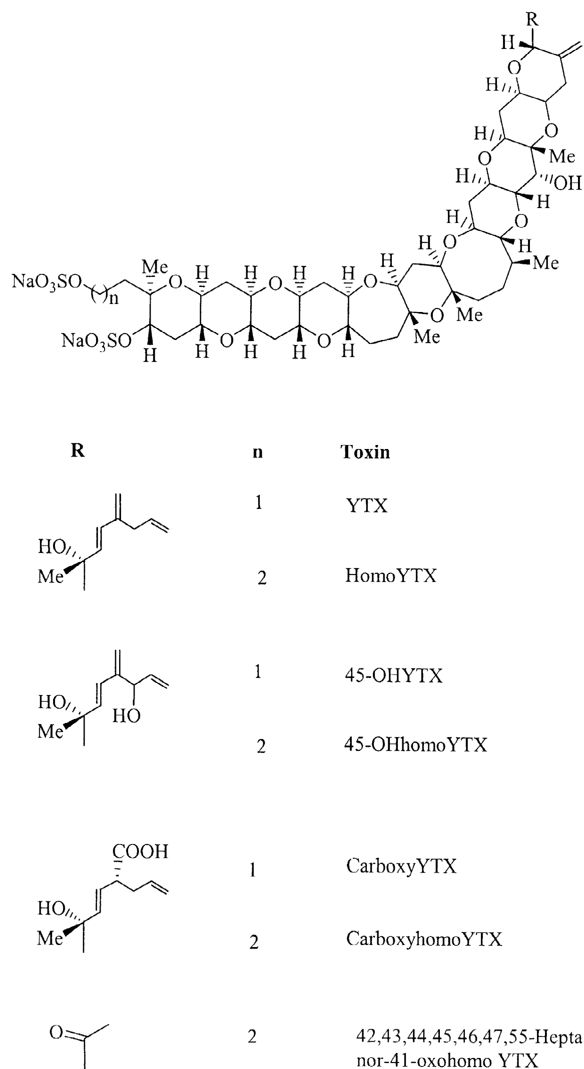

Fig. 1. Structures of various yessotoxins.

application of LC coupled with ion-spray mass spectrometry (LC–ISP-MS) for the detection of lipophylic toxins, such as OA, DTXs and PTXs in plankton and shellfish samples. YTX, 45-OHYTX and homoYTX have also been effectively determined by LC–MS [20,21], but other yessotoxin derivatives remain untested. The advantage of the LC–MS approach is that it is possible to detect intact, underivatized toxins and related compounds in relatively crude extracts.

In this paper we have examined the suitability of the LC–MS method proposed by Quilliam et al. [18,19] to separate and univocally detect all yessotoxins isolated so far. For this purpose standard solutions at known concentration of YTX and OA as well as solutions of a number of YTX analogues from mussels of the North Adriatic sea were employed. Unlike Quilliam et al., who developed the method for DSP using a triple quadrupole MS system as the LC detector, we report herein the coupling of HPLC to an electrospray ion trap mass spectrometer. Compared to a triple quadrupole mass spectrometer, the ion trap has the advantage that full-scan spectra can be recorded without any loss of sensitivity [22]. Due to the storage function of the trap, the sensitivity can be increased by collecting ions over a long period, thus resulting in detection limits in the full scan mode which are 1–2 orders of magnitude lower than for quadrupole systems [23]. For this reason, LC–MS analyses were conducted in full scan mode. However, we are aware of the drawbacks of the ion trap technique, consisting mainly of the difficulty in achieving linear calibration curves, but, fortunately, this was not a problem for YTX within the tested concentration range.

2. Experimental

2.1. Chemicals

All organic solvents were of distilled-in-glass grade (Carlo Erba, Milan, Italy) and were filtered and degassed before use. Water was distilled and passed through a Milli-Q water purification system (Millipore Ltd., Bedford, MA, USA). Formic acid (95–97%, laboratory grade), ammonium formate (analytical reagent grade) and OA standard were purchased from Sigma–Aldrich (Steinheim, Germany). Yessotoxin was purchased from the Institute of Environmental Science and Research Limited (Wellington Science Center, New Zealand) and individual standard stock solutions containing 14.1, 4.7, 1.6, 0.52, 0.17, 0.057 μg/ml were obtained from the pure toxin by dilution with methanol. 45-OHYTX [8], 45-OHhomoYTX [10], carboxyYTX [9], carboxyhomoYTX [10], and 42,43,44,45,46,47,55-heptanor-41-oxohomoYTX [11] were obtained from Italian contaminated mussels as previously reported.

2.2. Sampling and extraction procedures

The toxic mussel (*Mytilus galloprovincialis*) samples from the Adriatic sea were collected in coastal areas of the Northern Adriatic sea (Cesenatico, Emilia Romagna, Italy) when routine control tests had shown mussels to be positive for DSP toxicity [24,25]. After collection the mussels were stored frozen at −20 °C until extraction. Samples were extracted according to procedures reported previously [8–11]. Briefly, digestive glands were homogenized with a Waring blender and extracted with acetone (twice) at room temperature. After evaporating acetone, the aqueous concentrate was extracted twice with EtOAc. The organic layer after removal of the solvent gave a residue, which was dissolved in MeOH–water (8:2) and extracted with *n*-hexane. The hydromethanolic layer was further partitioned between MeOH–water (4:6) and CH_2Cl_2. The dichloromethane-soluble material was successively chromatographed by MPLC on a Büchi 861 apparatus using a Develosil ODS column (Nomura Chemical Co., Japan) and a solvent gradient system from 60 to 100% of methanol. The toxic fraction, eluting with 100% of methanol, was purified on a Toyopearl HW-40 SF column (Tosoh Corporation, Tokyo, Japan), using 100% methanol as eluent. The presence of YTXs in the dichloromethane extract and in the eluates was checked by mouse bioassay [24,25], by NMR on a Brüker AMX-500 spectrometer [8–11], and by LC–MS.

2.3. Flow injection analysis (FIA)–MS and LC–MS

High-pressure pump SP model P 4000 (ThermoFinnigan Separation Products, San Jose, CA, USA) equipped with a rheodyne injection valve with interchangeable loops (10–200 μl), was used for FIA–MS and LC–MS experiments. FIA–MS experiments were carried out on the individual yessotoxin standard solution (14.1 μg/ml) at 200 μl/min flow-rate. Separations were performed on a column packed with Hypersil C_8 BDS, 50×2.00 mm, 3 μm (Phenomenex, Torrance, CA, USA) at room temperature. Eluent A was water and B was acetonitrile–water (95:5), both eluents containing 3.5 m*M* ammonium formate and 50 m*M* formic acid. A gradient elution (from 10 to 100% B in 10 min then 100% B for 15 min) was required for combined analysis of OA and YTXs. The flow-rate was 200 μl/min. A sample injection volume of 5 μl was used in most cases.

All the mass spectral analyses were performed on a Finnigan MAT LCQ mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an electrospray ionization source in the negative ion mode. Full scan spectra were collected from *m/z* 500 to 1500, using a capillary temperature of 150 °C, a capillary voltage of −10 V, a tube lens offset of −60 V, a spray voltage of 4.5 kV, a sheath gas and an auxiliary gas flow of 80 and 20 (arbitrary units), respectively. Total microscans was set at 3 and maximum injection time at 200 ms. Extracted ion chromatograms were obtained selecting ions reported in Table 1. Subsequent MS–MS experiments were obtained by resonance excitation which was performed by applying supplementary voltages (collision energy, CE) corresponding to 35–45% of the maximum value (5 V peak-to-peak) on the $[M-2Na+H]^-$ and $[M-2Na-SO_3+H]^-$ ions, respectively. The He pressure inside the trap was kept constant; the pressure directly read by ion gauge (in the absence of N_2 stream) was $2.8 \cdot 10^{-5}$ Torr (1 Torr = 133.322 Pa).

3. Results and discussion

First experiments were carried out by FIA–MS on the individual standard solution of YTX. The obtained full scan spectra showed the exclusive presence of pseudomolecular ion at *m/z* 1141.5 due to $[M-2Na+H]^-$. All source parameters (capillary voltage, capillary temperature, gas flow, etc.) were optimized by constantly adding YTX to the HPLC flow via rheodyne valve injector. The negative ion LC–MS was then implemented by using a reversed-phase Hypersil C_8 BDS column and a mobile phase containing ammonium formate and formic acid as suggested by Quilliam et al. [18,19] for analysis of DSP toxins. The standard methanolic solution of YTX at various concentrations (14.1, 4.7, 1.6, 0.52, 0.17, 0.057 μg/ml) was analyzed at six dose levels

Table 1
Retention times and main ions observed in Full Scan (FS) MS and MS–MS experiments for YTX and its derivatives[a]

Toxin	t_R (min)	RRT[b]	$[M-2Na+H]^-$	$[M-2Na-SO_3+H]^-$ [c]
YTX	8.98	0.86	1141.5	1061.5
HomoYTX	9.01	0.86	1155.5	1075.5
45-OHYTX	8.73	0.84	1157.5	1077.5
45-OHhomoYTX	8.76	0.84	1171.5	1091.5
CarboxyYTX	8.18	0.79	1173.5	1093.5
CarboxyhomoYTX	8.21	0.79	1187.5	1107.5
42,43,44,45,46,47,55-Heptanor-41-oxohomoYTX	8.04	0.77	1061.5	981.5

[a] LC conditions: Hypersil C_8 BDS, 50×2.00 mm, 3 μm column; gradient HPLC analysis over 25 min using a mobile phase: acetonitrile–water containing 3.5 m*M* ammonium formate and 50 m*M* formic acid from 9.5:90.5 to 95:5 for 10 min and 95:5 for the final 15 min; flow-rate 200 μl/min; injection volume 5 μl.

[b] RRT, relative retention times. Retention time relative to okadaic acid eluting at 10.38 min under the same LC–MS conditions. $RRT = t_{R,x}/t_{R,OA}$.

[c] Fragment ions obtained in LC–MS–MS experiments carried out at a collision energy of 35% using $[M-2Na+H]^-$ as precursor ion.

in triplet. The average of triplicate measurements was used for plotting. Peak areas were used to express peak intensity. The minimum detection level for matrix-free toxin on column was thus estimated from the data to be 70 pg ($S/N=3$). A good linearity ($r^2>0.998$) was observed between the amounts of injected toxin and the peak areas within the tested concentration range.

On the basis of the good detection limit for YTX, it was considered worthwhile to test the suitability of the existing method for detection of all YTXs isolated so far. Therefore, we used a number of YTX analogues we had isolated and identified in the course of our ongoing studies in the period 1995–1999. The expected toxins were easily detected and eluted at the retention times reported in Table 1. As shown, most of the investigated toxins overlap and any attempt to improve chromatographic separation by varying the mobile phase composition was unsuccessful. However, toxins with different molecular masses were monitored by extracted ion chromatograms (XIC) of the $[M-2Na+H]^-$ ions, thus allowing their unambiguous identification even if they were chromatographically unresolved.

The above approach seems to be appropriate for unambiguous identification of all YTXs and represents the first step toward their quantitative determination. For quantitation of YTX analogues, preparation of their appropriate standards is required; unfortunately the low purity and the poor amount of available material prevented us from performing quantitative studies.

Since YTXs are often coexisting with other DSP toxins such as OA whose standard is commercially

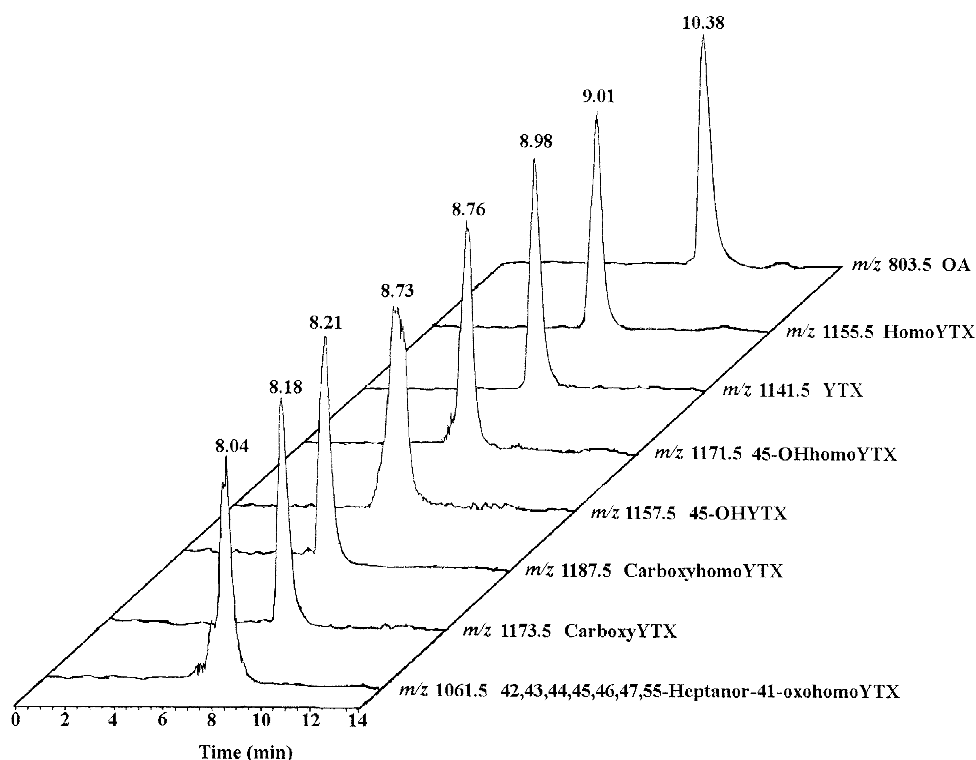

Fig. 2. LC–electrospray ionization (negative ion) ion trap MS analysis of a wide range of yessotoxins in a blend of mussel tissue extract added to OA standard solution. Selected monitoring of yessotoxins with different molecular masses was carried out by extracted ion chromatograms (XIC) of the $[M-2Na+H]^-$ ions. For OA the XIC is relevant to the $[M-H]^-$ ion. LC conditions: Hypersil C_8 BDS, 50×2.00 mm, 3 μm column; gradient HPLC analysis over 25 min using a mobile phase: acetonitrile–water containing 3.5 m*M* ammonium formate and 50 m*M* formic acid from 9.5:90.5 to 95:5 for 10 min and 95:5 for the final 15 min; flow-rate 200 μl/min; injection volume 5 μl.

available, we tested the suitability of the method to detect all these toxins at the same time. Fig. 2 shows reversed-phase gradient elution LC–MS analysis of a wide range of yessotoxins in a blend of contaminated mussel tissue extracts added to OA standard. YTXs were eluted 1.3–2.3 min before OA, which can be used as reference peak. Retention times of YTXs relative to OA are also reported in Table 1.

MS–MS experiments were carried out for further confirmation. In the MS–MS experiments the protonated molecular ion $[M-2Na+H]^-$ was fragmented. The fragmentations in this experiment are known to occur by losing neutral species and fragment ions derive from the "western part" of the molecule in which a negative charge is localized by the presence of the sulfate esters [26]. As shown in Fig. 3 at a collision energy (CE) of 35%, all investigated disulfated YTXs gave an intensive product ion at $[M-2Na+H-80]^-$ corresponding to loss of SO_3. Product ion chromatograms of all YTX and homoYTX derivatives are also presented in Fig. 3. As expected from the high selectivity of the MS–MS technique, no interference with the analytes was observed in the product ion chromatograms.

Further MS–MS experiments were carried out by increasing collision energy (CE, 45%) and using $[M-2Na+H-80]^-$ ion as precursor ion. In Fig. 4, we report, by way of example, MS–MS spectra of

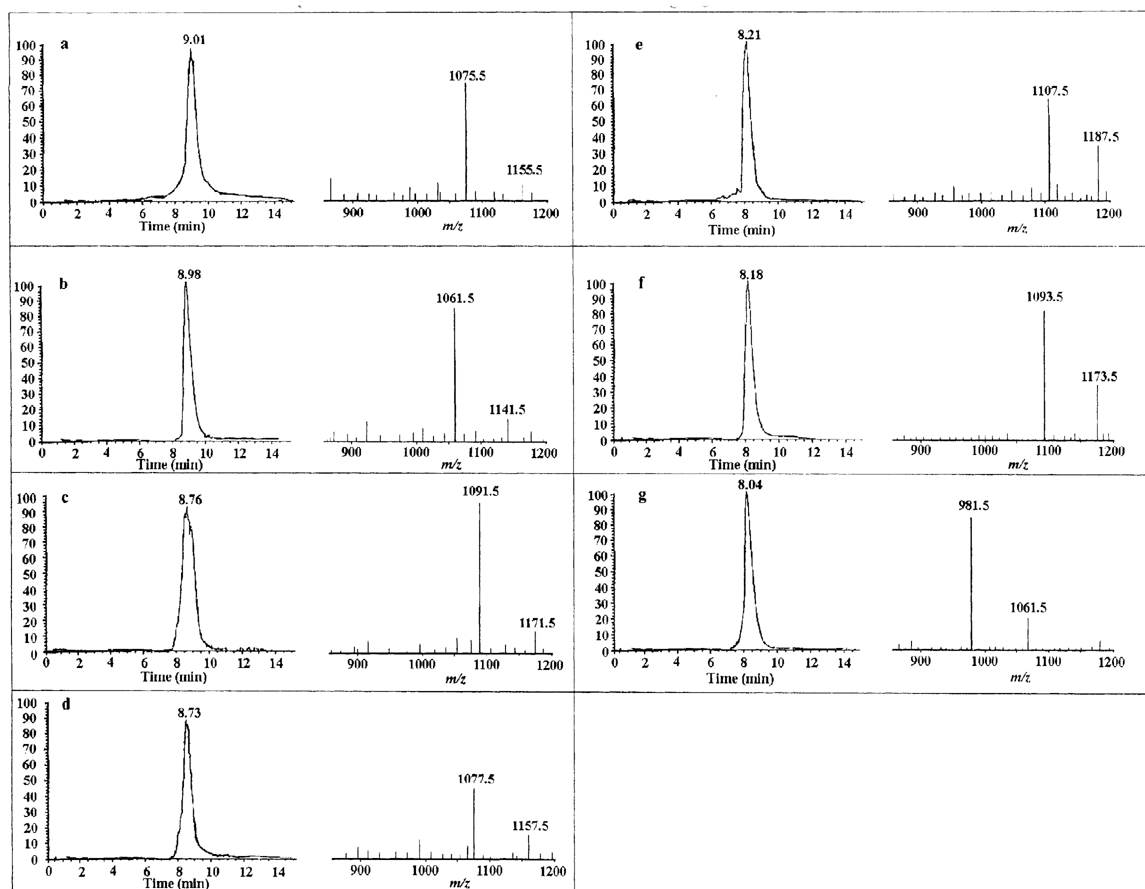

Fig. 3. Product ion chromatograms and relevant MS–MS spectra of homoYTX (a), YTX (b), 45-OHhomoYTX (c), 45-OHYTX (d), carboxyhomoYTX (e), carboxyYTX (f), and 42,43,44,45,46,47,55-heptanor-41-oxohomoYTX (g), using $[M-2Na+H]^-$ as precursor ions and a collision energy of 35%. Fragment ion $[M-2Na-80+H]^-$ corresponding to loss of SO_3 group is observed for each derivative. For LC conditions see Fig. 2.

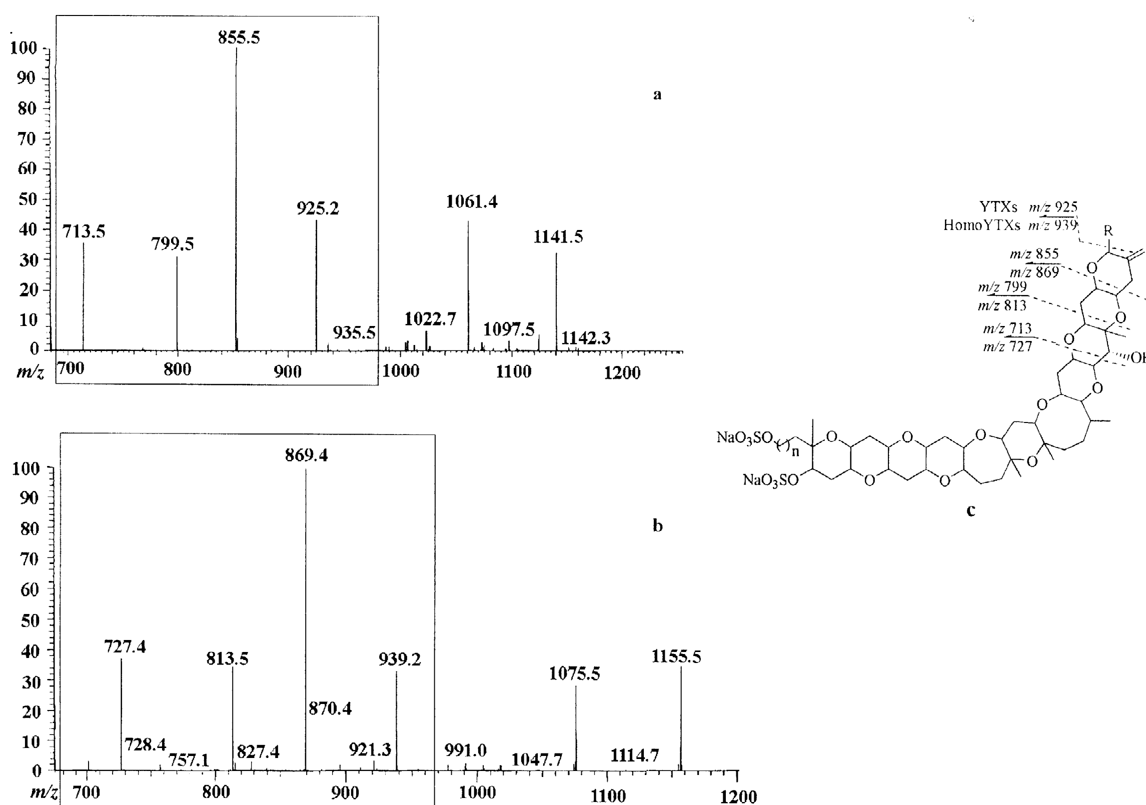

Fig. 4. (a) MS–MS spectrum at a collision energy of 45% for YTX. $[M-2Na-SO_3+H]^-$ ion at m/z 1061.5 was used as precursor ion. Characteristic ions relative to fragmentation of the polycyclic backbone skeleton for all YTX-like compounds are contained in the frame. (b) MS–MS spectrum at a collision energy of 45% for homoYTX. $[M-2Na-SO_3+H]^-$ ion at m/z 1075.5 was used as precursor ion. Characteristic ions relative to fragmentation of the polycyclic backbone skeleton for all homoYTX-like compounds are contained in the frame. (c) Characteristic fragment ions observed for YTX- and homoYTX-like compounds in the mass range 700–1200 u of tandem mass spectra. All the fragmentations were accompanied by loss of sulfonate (SO_3) which denotes that fragment ions linked one of the sulfate esters.

YTX and homoYTX. The fragmentation patterns of the two molecules were very similar, all the peaks differing for only 14 mass units in the two spectra. It has to be noted that the region of the spectra under m/z 950 contains ions relative to the fragmentation of the polycyclic backbone skeleton. Since fragmentation occurs at specific sites of the ether rings, the same product ions were observed for all YTX- or homoYTX-like compounds that we analyzed. Consequently, this part of the MS–MS spectrum can be considered as a fingerprint, thus allowing YTX and homoYTX analogues to be distinguished from each other.

The above method was finally tried out by analyzing toxic extracts or partially purified fractions of mussels (see Experimental), collected in the period 1995–1999 along Cesenatico coasts, at our disposal. Each mixture was thus separately investigated by LC–MS. The results of the analyses fully agreed with those we had previously obtained through a more complex and time consuming analysis implying the isolation and the successive structure determination of the individual compounds by NMR and MS [7–11].

By way of example, we report herein the results of the analysis of the toxic mixture obtained from *M. galloprovincialis* collected in 1998. LC–MS data were quite interesting, since, besides the already known composition [11], consisting of homoYTX, 45-OHhomoYTX, carboxyhomoYTX, and 42,43,44,

45,46,47,55-heptanor-41-oxohomoYTX, they revealed that a novel YTX analogue was present in the mixture on the basis of the following evidence. Extracted ion chromatograms (XIC) of the ion at m/z 1061.5 showed the presence of two peaks eluting at 7.46 and 8.05 min, respectively (Fig. 5). Assignment of the peak at 8.05 min to 42,43,44,45,46,47,55-heptanor-41-oxohomoYTX was an easy task, comparing the retention time and mass spectrum with those of an authentic sample, whereas the peak at 7.46 min could not be associated to any of the samples at our disposal. So, LC–MS–MS experiments (CE, 45%) were carried out using ion at m/z 1061.5 as precursor ion. The peak at 8.05 min, as expected, gave product ions at m/z 981 $[M-2Na-SO_3+H]^-$ and m/z 939 corresponding to loss of the eastern side chain thus confirming its identification as 42,43,44,45,46,47,55-heptanor-41-oxohomoYTX. An inspection of the MS–MS spectrum of the peak at 7.46 min revealed the typical fragmentation pattern of the backbone skeleton of yessotoxin (Fig. 4a), but no sulfonate loss was observed for this peak, thus suggesting the presence of only one sulfate ester group in the molecule. All the data suggested that the peak under investigation was due to a desulfo-YTX, the only uncertainty being in the desulfated position. NMR experiments are required to unambiguously assign 1-desulfo- (Fig. 6) [27] or the alternative 4-desulfo-YTX to the above peak. However, to the best of our knowledge, this is the first report of a desulfo-YTX derivative from Italian mussels.

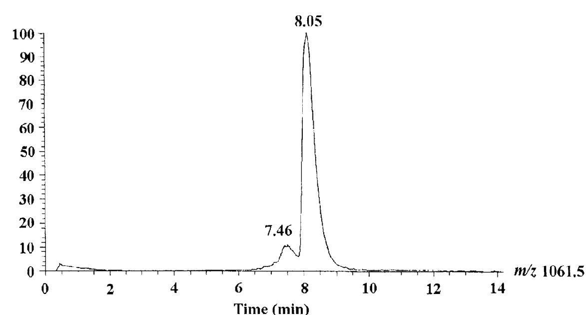

Fig. 5. Extracted ion chromatogram for ion at m/z 1061.5 in the LC–electrospray ionization ion trap MS analysis of a toxic mixture obtained from *M. galloprovincialis* collected in 1998. Based on retention times and subsequent MS–MS experiments, peak eluting at 7.46 min was assigned to a desulfo-YTX derivative, whereas peak at 8.05 min was assigned to 42,43,44,45,46,47,55-heptanor-41-oxohomoYTX. For LC conditions see Fig. 2.

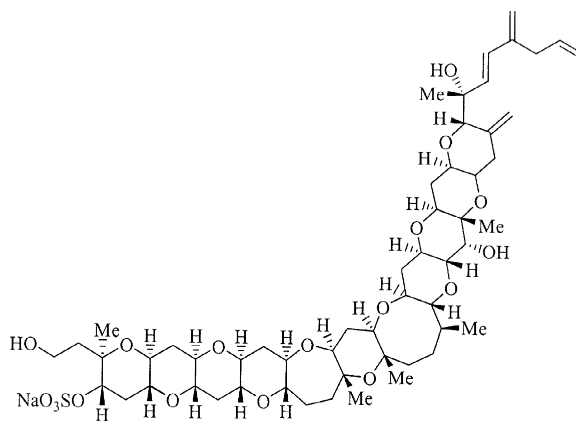

Fig. 6. Structure of 1-desulfo-yessotoxin.

4. Conclusion

The proposed method, based on the LC–MS technique in negative ion mode, allows the direct detection of known YTXs with a high degree of sensitivity and specificity. Although at this stage a full validation of the method was hampered by the limited availability of a number of pure toxins, this assay provides useful information on toxin composition. Furthermore, together with rapid detection of known compounds, the method allows highlighting of the possible presence of new analogues, thus presenting natural products chemists with the potential to discover new YTX analogues useful for structure–bioactivity relationship studies.

In conclusion, our results allow the method developed by Quilliam for lipophylic biotoxins to be applied also to YTXs, thus providing an effective alternative to the mouse bioassay currently in use, as well as a valuable tool for scientists involved in both regulatory and research fields.

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