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Journal of Chromatography A, 976 (2002) 329–334

JOURNAL OF CHROMATOGRAPHY A

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Liquid chromatography with electrospray ion-trap mass spectrometry for the determination of yessotoxins in shellfish

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Abstract

Yessotoxins are a group of large polyether toxins, produced by marine dinoflagellates, which cause widespread contamination of filter-feeding shellfish. A new, sensitive liquid chromatography–mass spectrometry (LC–MS) method has been developed for the determination of yessotoxin (YTX) and 45-hydroxy-yessotoxin (45-OHYTX), a major metabolite in shellfish. The LC system was coupled, via an electrospray ionisation (ESI) source, to an ion-trap MS in negative mode. The molecular related ion species at m/z 1141 $[M\text{-}2Na+H]^-$ was used as the parent ion for multiple MS experiments. MS–MS and MS^3 gave major fragment ions at m/z 1061 $[1141\text{-}SO_3H]^-$ and m/z 945 $[1061\text{-}C_9H_{12}O]^-$. Predominant ions, that are due to the fragmentation of the backbone structure of YTXs, were observed at the MS^4 stage. Reversed-phase LC using a C_{16} amide column was preferable to C_{18} phases for the separation of YTX and 45-OHYTX. Optimum calibration and reproducibility data were obtained for YTX using LC–MS–MS; $r^2=0.9960$, RSD≤6.3% at 0.25 μg YTX/g ($n=5$). The detection limit ($S/N=3$) was 30 pg YTX on-column which corresponded to 3 ng/g shellfish tissue.

Keywords: Shellfish poisoning; Food analysis; Toxins; Yessotoxins

1. Introduction

Yessotoxin (YTX), first isolated in Japan from scallops (*Patinopecten yessoensis*) [1], was initially classified among the diarrhetic shellfish poisoning (DSP) toxin group because of its frequent co-occurrence with DSP toxins [2]. However, YTX does not induce diarrhea but cardiotoxic effects have been demonstrated in mice [3,4]. In recent years, the aquaculture industry in several European countries has experienced prolonged and unnecessary closures due to the presence of YTX in bivalve shellfish [5]. The remarkably high sensitivity of mice toward YTX has rendered the bioassay, used by most regulatory agencies, unsuitable because of its low relevance to human toxicological responses [4,6]. YTX and new analogues have been reported from shellfish in Norway, Chile, New Zealand and Italy [7–13]. Thus far, nine naturally occurring YTXs have been identified. The dinoflagellates, *Protoceratium reticulatum* [14] and *Lingulodinum polyedrum* (*Gonyaulax polyedra*) [15,16] have been identified as the organisms responsible for shellfish contamination by YTXs.

YTX has a large polycyclic ether skeleton, a diene side-chain and two sulphate moieties (Fig. 1). Recently, a sensitive fluorimetric liquid chromatography (LC) method has been developed for the determination of YTX in shellfish following derivatisation using the dienophile, DMEQ-TAD [17], but this method is time-consuming. Liquid chroma-

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)00946-9

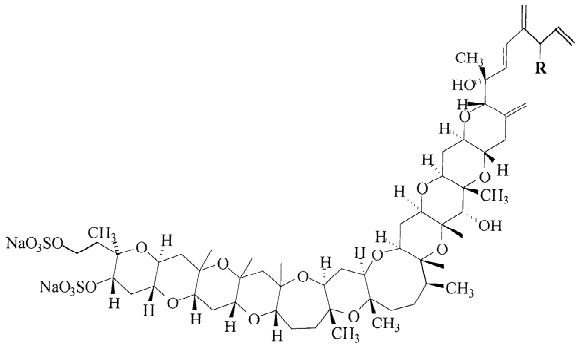

Fig. 1. Yessotoxin (R=H); 45-hydroxyyessotoxin (R=OH).

tography–mass spectrometry (LC–MS) has been proposed as a universal method for the analysis of marine biotoxins and offers both high sensitivity and rapid throughput of samples [18–20]. The polyether toxins belonging to the DSP and azaspiracid (AZP) classes have been determined by LC–MS–MS [21,22]. The analysis of YTX and homoYTX in mussels (*Mytilus galloprovincialis*) and phytoplankton was recently reported using an ionspray interface [16,19,23]. LC–MS–MS, with selected reaction monitoring (SRM), is a valuable technique for determining biotoxins in a complex matrix because of its high specificity compared to single stage MS. Triple stage quadrupole mass spectra of polyether marine toxins usually show major product ions due to the sequential loss of water molecules but few structurally diagnostic fragment ions are produced [21].

It has been demonstrated that negative ion fast atom bombardment (FAB) MS–MS represents a useful method for providing valuable structural information for molecules containing fused ether rings or repeating structural units [24–27]. The molecular related ion at m/z 1061 $[M\text{-}2Na+H\text{-}SO_3]^-$ was used as the precursor ion in MS–MS experiments on YTX [27]. This produced multiple ions from neutral losses of the side chain and fragmentation of each of the fused rings. The spectra derived from charge-remote fragmentations were very informative by providing data on the composition of each ring of YTX [27]. We now report the application of LC with electrospray ionization (ESI) multiple MS (MS^n), in the negative mode, for the determination of YTX and 45-OHYTX in shellfish.

2. Materials and methods

2.1. Materials

YTX and 45-OHYTX standards are commercially unavailable and were prepared from contaminated mussels harvested from Norway using procedures that have been described previously [10]. HPLC-grade acetonitrile and water were purchased from Labscan (Dublin, Ireland) and ammonium acetate from Sigma–Aldrich (Dorset, UK). Contaminated blue mussels (*Mytilus edulis*) were harvested in Skjer, Sognefjord, at the south-west coast of Norway.

2.2. Extraction of mussel tissues

Mussels (*M. edulis*) (≈50 g) were carefully divided into digestive glands (hepatopancreas) and remaining tissue. The shellfish tissues were separately homogenised (Ultra Turrax, IKA, Germany) for 1 min. The homogenate (≈1 g) was accurately weighed into a centrifuge tube (50 ml), 80% methanol (9 ml) was added and the mixture was homogenised for 1 min followed by centrifugation at 3000 *g* for 3 min. The supernatant was transferred to a test tube (12 ml) and evaporated to dryness in a centrifugal evaporator (RC 10.22, Jouan, Derbyshire, UK). The residue was reconstituted with methanol (500 μl) using sonication and vortex mixing, syringe filtered (0.45 μm) and transferred to an insert (200 μl) in an amber vial (2 ml). An aliquot (3–5 μl) was analysed by $LC–MS^n$.

2.3. $LC–MS^n$

The LC system was a Waters 2690 Alliance (Waters, Milford, MA, USA) which included a thermostated autosampler to maintain samples at 4 °C. This was linked to a Finnigan MAT LCQ Classic ion-trap mass spectrometer (Thermo-Finnigan, San Jose, CA, USA) using Xcalibur software. The mass resolution of this instrument is 0.1 amu but MS data are presented here using whole integer mass values for clarity. Isocratic chromatography was performed using acetonitrile–water (60:40, v/v) containing 0.5 m*M* ammonium acetate, at a flow-rate of 500 μl/min. The analytical column (Discovery

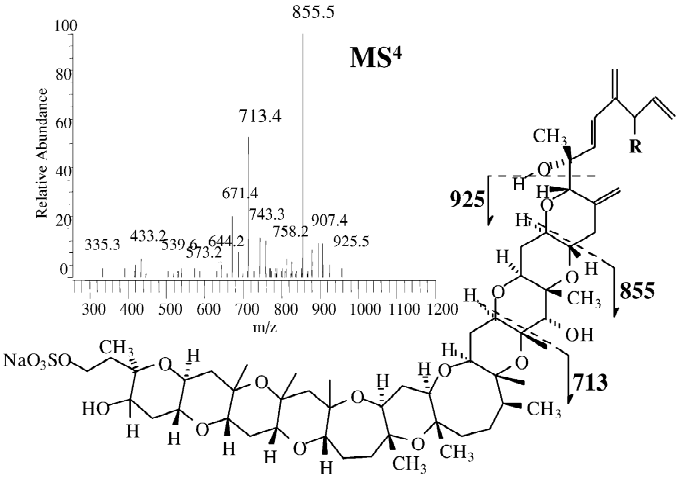

Fig. 2. Characteristic fragmentation observed using multiple MS of YTX (R=H) in negative mode. The primary product ion from the MS^3 stage, m/z 925, was trapped and fragmented to produce the MS^4 spectrum shown which contained the predominant ions, m/z 855 and 713, formed by the cleavage of the polyether backbone.

RP Amide C_{16}, 150×4.6 mm, 5 μm, Supelco, Dublin, Ireland) was operated at 35 °C. Using an automated sequence, the eluent flow was diverted to waste for 1 min after sample injection and MS detection was carried out between 1 and 12 min of the chromatography run, followed by a second divert to waste for 1 min prior to the next chromatographic sequence.

Mass spectrometric analysis was carried out at atmospheric pressure using an ESI source and data was acquired in negative mode. The MS was tuned using YTX standard solution (1 μg/ml) which was infused at 3 μl/min with monitoring of the $[M\text{-}2Na+H]^-$ ion at m/z 1141. The optimised instrument settings were as follows: capillary temperature (200 °C), spray voltage (7.5 kV) generating a spray current of 25 μA, capillary voltage (−16.0 V), tube lens offset voltage (−15.0 V) and with the arbitrary units for sheath gas flow and auxiliary gas flow set at 80 and 20, respectively.

Multiple tandem MS produced collision-induced dissociation (CID) spectra and were obtained by trapping the $[M\text{-}2Na+H]^-$ ion for each toxin which fragmented similarly giving major ions due to sulphonate loss $[M\text{-}2Na+H\text{-}SO_3]^-$ and cleavage of the unsaturated side chain. The optimised relative collision energies (RCEs) were 35% for MS–MS, 40% for MS^3. YTXs were determined using the following target parent and fragment ion combinations in the mass spectrometer: YTX: m/z 1141⇒1061⇒925; 45-OHYTX: m/z 1157⇒1077⇒925. The mass acquire window width used for trapping was 3 amu. Quantitative data for all yessotoxins were based on standard YTX. The optimised RCE was 30% for MS^4 experiments. Four C_{18} reversed-phase columns, Discovery C_{18} (Supelco), Inertsil (Jones Chromatography), Luna C_{18} (2) and Prodigy (Phenomenex), were examined for their efficacy in the analysis of YTX and 45-OHYTX but were found to be unsatisfactory.

3. Results and discussion

3.1. MS fragmentation studies on yessotoxins

The fragmentation of YTXs was examined using a quadrupole ion-trap MS with ammonium acetate as the buffer for negative ion mode operation. This study employed multiple MS which involved repeated trapping and fragmentation of ions. An advantage of the ion-trap spectrometer is the ability to switch between a full-scan MS and a CID scan of fragments without a significant loss in sensitivity. Using standard YTXs, the molecular-related ion species, $[M\text{-}2Na+H]^-$, at m/z 1141 (YTX) and m/z

1157 (45-OHYTX), were used as the parent ions for multiple MS experiments. The fragmentation sequence was elucidated using multiple MS. MS–MS gave major fragment ions at m/z 1061 [1141-$SO_3H]^-$ (YTX) and m/z 1077 [1157-$SO_3H]^-$ (45-OHYTX). The main product ion, [M-2Na+H-$SO_3]^-$, is most likely the loss of sulphonate from the C-4 position [27]. Significantly, MS^3 yielded the fragment ion, m/z 925, as the base peak for both toxins in their respective spectra. The latter is a diagnostic ion for YTX and several analogues that differ only in the unsaturated side-chain. This moiety is lost at the MS^3 stage to give a common structural entity (Fig. 2). Two major ions in the MS^4 spectra, m/z 855 and m/z 713, are due to the charge remote fragmentation of the polyether backbone of YTXs.

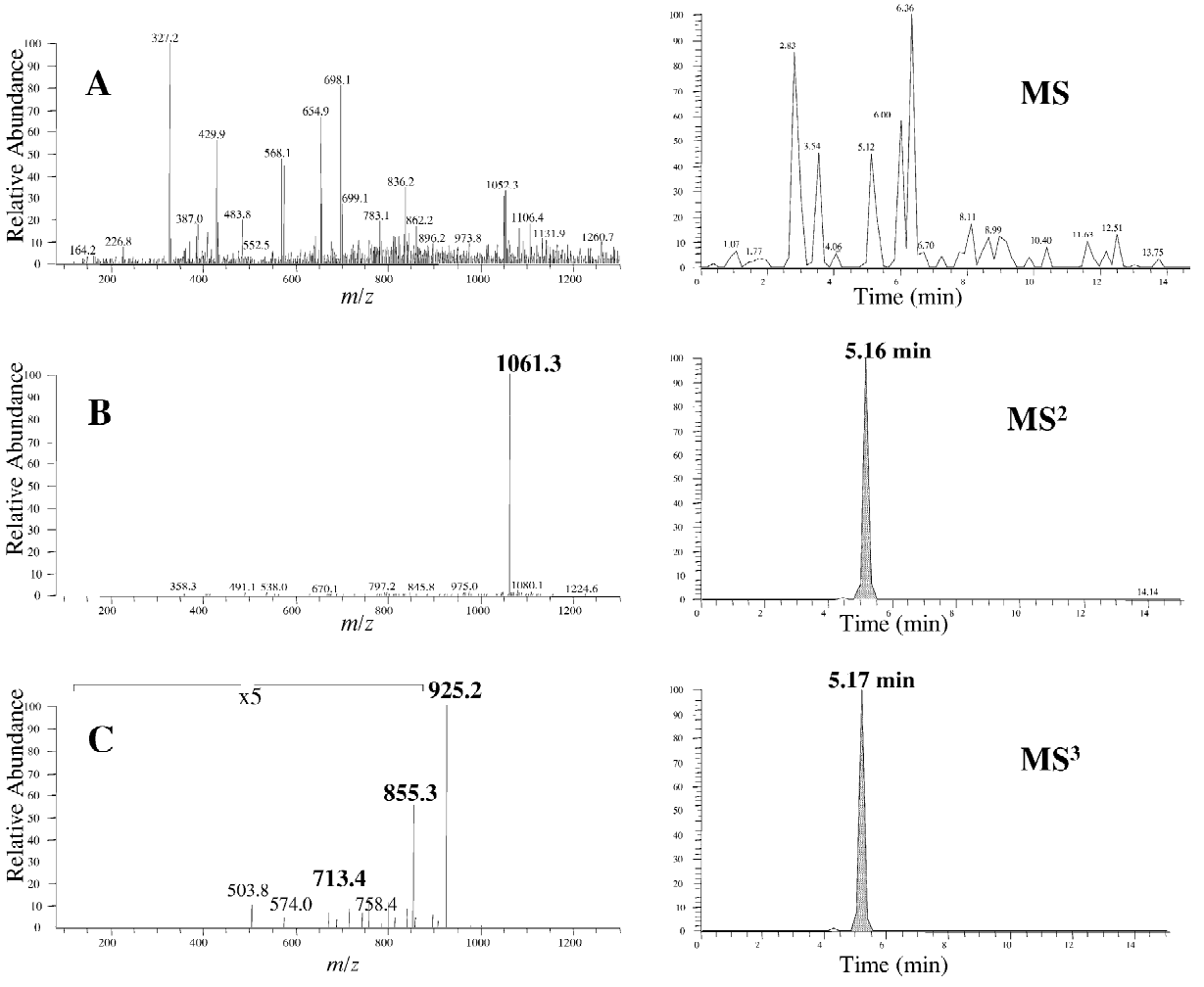

Fig. 3. CID mass spectra and chromatograms for YTX in an extract of naturally contaminated mussels from Norway. (A) LC–MS obtained in full scan MS, m/z=80–2000, with display of mass range (m/z=1140–1142) chromatogram. (B) LC–MS–MS; chromatogram of product ion obtained by specifying a mass range of m/z=1060.5–1061.5. Parent and product ions; m/z 1141⇒1061. (C) LC–MS^3; chromatogram of product ion obtained by specifying a mass range of m/z=924–926. Parent and product ions; m/z 1141⇒1061⇒925. Chromatographic conditions: Discovery RP Amide C_{16} column (150×4.6 mm, 5 μm) at 35 °C; mobile phase was acetonitrile–water (60:40, v/v) containing 0.5 m*M* ammonium acetate; flow-rate was 200 μl/min.

3.2. LC–ESI–MS of yessotoxins

A recent study involving the determination of biotoxins in shellfish matrices, demonstrated that better calibration and sensitivity data could be obtained using either LC–MS–MS or LC–MS^3 rather than LC–MS [28]. This is attributed to a dramatic reduction in background signal in multiple MS modes typically observed using quadrupole ion-trap spectrometry [29]. Obviously, selectivity is also enhanced when multiple MS modes are used. Six scan events were performed simultaneously: (a) full scan MS, (b) MS–MS YTX, (c) MS–MS 45-OHYTX, (d) MS^3 YTX, (e) MS^3 45-OHYTX, (f) MS^4. The predominant ion was m/z 925 in the MS^3 of YTX and 45-OHYTX. This ion was generated by trapping and fragmenting the product ions from the MS–MS stage, m/z 1061 (YTX), m/z 1077 (45-OHYTX).

The calibration data for standard YTX solutions were: LC–MS–MS, 0.125–5.0 μg/ml (r^2=0.9960, n=3); LC–MS^3, 0.25–5.0 μg/ml (mean r^2=0.9898, n=3). Using blank shellfish extracts spiked with YTX, the detection limit (S/N=3) for LC–MS–MS was 30 pg on-column (3 ng/g), and for LC–MS^3, it was 50 pg on-column (5 ng/g). Calibration data in LC–MS–MS mode were linear for spiked shellfish matrix extracts in the range, 0.25–5.0 μg YTX/ml (mean r^2=0.9813, n=3). Relative standard deviation (RSD) values were less than 6.3% (0.25 μg YTX/ml, n=5). Although valuable structural information was acquired at the MS^3 and MS^4 stages, reproducible quantitative data were not obtained with shellfish matrices. Fig. 3 shows the chromatograms and spectra obtained for mussels (*M. edulis*) that were cultivated in Norway. This illustrates the importance of using LC–MS–MS (B) or LC–MS^3 (C) compared to LC–MS (A). Using LC–MS, the analyte peak was indiscernible from background signals due to extensive matrix interference. Data were obtained in full-scan mode with specific mass ranges selected post-acquisition.

3.3. Chromatographic phases for YTXs

YTX is bioconverted slowly to 45-OHYTX in shellfish tissue [30] and it is probable that this is a detoxification process since 45-OHYTX is less toxic than YTX [31,32]. It was difficult to achieve satisfactory chromatographic separation of YTXs using C_{18} phases although this does not prohibit their use due to the mass selectivity. Poor peak shapes, low or variable retention and poor resolution were observed for the analysis of YTXs in crude shellfish extracts using four C_{18} columns from different manufacturers. However, good separation of YTX and 45-OHYTX was achieved on an amide reversed-phase column (Discovery RP Amide C_{16}) with isocratic LC. The chromatograms (Fig. 4) show well resolved peaks for YTXs; 4.1 min (45-OHYTX) and 5.3 min (YTX).

This analytical method for the determination of

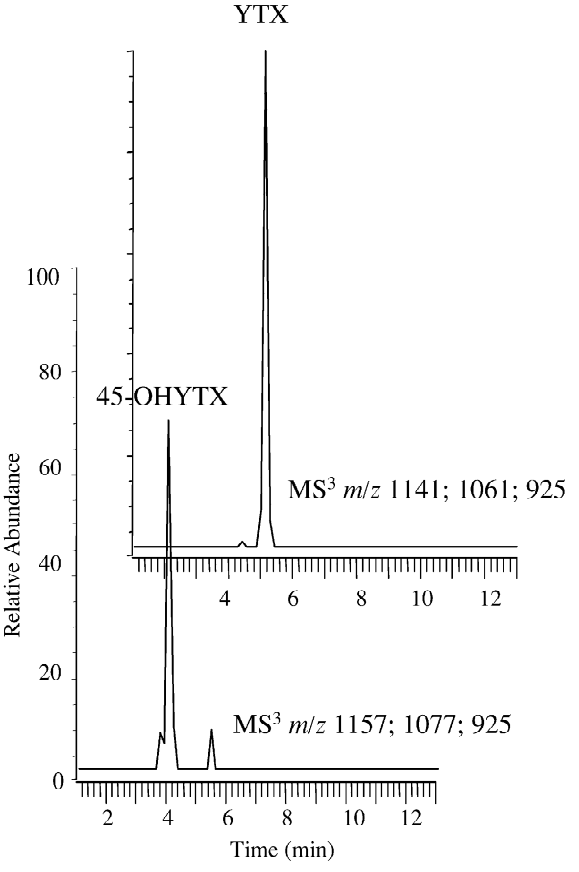

Fig. 4. The chromatograms for YTX (0.20 μg/g) and 45-OHYTX (0.11 μg/g) in mussel tissues obtained using LC–MS^3. Chromatographic conditions as in Fig. 3.

YTXs does not require any cleanup of extracts, is rapid and readily automated, and could be applied for the regulatory control of these toxins in shellfish.

Acknowledgements

Funding from the Higher Education Authority (Programme for Research in Third-Level Institutions) is gratefully acknowledged.

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