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New approach to the direct detection of known and new diarrhoeic shellfish toxins in mussels and phytoplankton by liquid chromatography–mass spectrometry

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Abstract

A new approach using combined liquid chromatography–mass spectrometry (LC–MS) with ionspray ionization is proposed for the direct detection of known and new toxins in mussels and phytoplankton. A first stage reversed-phase, negative ion mode, selected ion monitoring (SIM) LC–MS analysis was performed in order to detect DSP toxins in the same chromatographic run with a total run time of 20 min. The toxins analysed included yessotoxin (YTX), okadaic acid (OA) and four of its analogues, dinophysistoxins (i.e. DTX-1, DTX-2, DTX-2B, DTX-2C), and pectenotoxins (PTXs), involving PTX-2, two PTX-2 secoacids (PTX-2SAs), PTX-2SA, 7-*epi*-PTX-2SA, and AC1, the three isomeric toxins structurally related to PTX-2 recently identified in Irish phytoplankton. Positive samples can, therefore, be analyzed through reversed-phase, positive ion mode SIM LC–MS, in order to perform complete chromatographic separations of the structurally related toxins within the OA and PTX groups. Detailed toxin profiles of a number of toxic phytoplankton and shellfish, from different marine areas, were easily obtained through the new approach. PTX-2SAs and AC1 were found in phytoplankton and shellfish from Ireland as well as in Italian shellfish. Moreover, for the first time there was evidence of the presence of PTX-2 in Irish phytoplankton. YTX was present in Italian shellfish. Four isomeric OA toxins were detected in samples from Ireland with OA, DTX-2 and DTX-2B present in shellfish, and OA, DTX-2 and DTX-2C in phytoplankton. In contrast, OA was the only toxin from this group to be detected in Italian mussels.

Keywords: Shellfish poisoning; *Mytilus edulis*; Toxins

1. Introduction

Bivalve shellfish can be vectors of phytoplanktonic toxins accumulated in their organisms by filter-feeding mechanisms. Among the phycotoxin-related toxic phenomena, diarrhoeic shellfish poisoning (DSP), a severe human gastrointestinal illness associated with consumption of toxic shellfish [1] that have been feeding on dinoflagellates, such as *Dinophysis* spp., *Prorocentrum* spp. [2], *Protoceratium reticulatum* [3], *Lingulodinium polyedrum* [4,5], represents a serious threat to both public health and the shellfish industry [6,7].

Following the first episodes of DSP, the polyether

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lipophilic compounds okadaic acid (OA) and its methyl derivative, dinophysistoxin-1 (DTX-1) [6,8], were identified in toxic shellfish. Congeners of these toxins, such as the OA isomers, DTX-2 [9], DTX-2B [10] and DTX-2C [11], and a number of 7-*O*-acyl derivatives of OA, DTX-1 and DTX-2 [12,13], were subsequently identified either in phytoplankton or in shellfish. Other toxins, usually coexisting with OA analogues, but differing from them in chemical structures and biological activities, were also identified in toxic molluscs:

(1) Polyether lactones, pectenotoxins (PTXs), including PTX-1-7 [12,14,15,16] and three isomeric toxins closely related to PTX-2, but containing an open carboxylic acid rather than a lactone ring, i.e. PTX-2 seco acid (PTX-2SA), 7-*epi*-PTX-2 seco acid (7-*epi*-PTX-2SA) and AC1 [17,18].

(2) Brevetoxin-type polyethers, yessotoxins (YTXs), including yessotoxin (YTX), 45-hydroxyyessotoxin (45-OH YTX) [19], 45,46,47-trinor-YTX, homo-YTX and 45-hydroxyhomo YTX [16,20].

While toxins of the OA group have been proved to induce diarrhoea [21,22] and to have a powerful tumor promoting activity [23], no diarrhoegenic properties have been reported for PTXs [21] and YTXs [19,24,25], although hepatotoxic [21] and cardiotoxic effects [25], respectively, have been shown. It has, therefore, been suggested that PTXs and YTXs should not be regarded as 'diarrhoeic' toxins [24,26], although their monitoring in seafood should be continued [26].

The most common method for DSP toxin monitoring is the biological mouse bioassay, involving either Yasumoto's method [1] or its variations [14,27], both based on the *i.p.* injections of shellfish extracts in mice and observation of their survival times. Unfortunately, the mouse bioassay is widely recognized to be affected by a number of drawbacks, in addition to the ethical issues opposing the use of in vivo assays for regulatory purposes. Thus, the biological methods – implementing different toxin extraction and/or purification procedures, resulting in different selectivity and specificity – suffer from poor reproducibility, low sensitivity, possible interference from either matrix or contaminants other than DSP toxins, and leads to false positive and false negative results [28,29]. Moreover, the adoption of different biological methods and different times of observation for recording deaths of injected mice results in the current disparity of criteria for mollusc sanitary control within the EU countries [26].

The development of alternative methods for DSP monitoring in phytoplankton and shellfish is therefore required.

A number of factors, such as the lack of analytical standards for all the DSP toxins, the continuous identification of new DSP toxins, the complexity and variability of the biological marine material, the availability – in many cases – of only small amounts of samples for the analytical determination, the coexistence of multiple toxins in samples, combine to present serious hurdles to the development of suitable analytical methodologies. Nevertheless, several alternatives to the in vivo assays have been proposed for DSP toxin detection, both in shellfish and phytoplankton, and they have recently been reviewed [7,30].

The most promising alternatives appear to be the chemical instrumental methods, such as the highly sensitive and specific quantitative liquid chromatography–fluorimetric detection (LC–FLD) method following derivatization with 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD), for determination of YTX, 45-OH YTX and norYTX [31], as well as with 9-anthryldiazomethane (ADAM), for the determination of OA, DTXs [32], PTX-6 and PTX-7 [33]. The methods are currently effective tools for confirmation of the biological assay results in monitoring and research activities. However, sanitary control of DSP toxins in molluscs relying exclusively upon conventional LC methods will be possible only when standards of all the DSP toxins are commercially available.

On the other hand, LC coupled with mass spectrometry and tandem mass spectrometry (LC–MS, LC–MS–MS) using atmospheric-pressure ionization (API) proved a powerful approach for direct determination of DSP toxins of the OA class [10,34–39], PTXs [40,41] and YTXs [29]. Recent developments, involving both improved design and lower costs of LC–MS instruments, are making this technique a viable analytical tool in many laboratories [42]. The LC–MS approach has thus been recently proposed as a universal method for marine toxins [43].

LC–MS and MS–MS methods that have so far been proposed for DSP toxin determination in marine biological materials are based on multiple LC–MS analyzes adopting different chromatographic and/or mass spectrometric conditions, for targeted determination of OA, PTXs or YTXs.

In our renewed efforts focused on the use of LC–MS methods for the analysis of DSP toxins, we investigated the possibility of developing a new approach using LC–MS with ionspray ionization for the direct determination of known and new DSP toxins in mussels and phytoplankton.

2. Experimental

2.1. Reagents

All solvents were HPLC or analytical grade and were purchased from Farmitalia Carlo Erba (Milan, Italy). OA and DTX-1 were purchased from Calbiochem-Novabiochem (San Diego, CA, USA). YTX, DTX-2, DTX-2B, DTX-2C, PTX-2 and its analogues PTX-2SAs and AC1, were isolated following the previously reported procedures (YTX [19]; DTX-2, DTX-2B [37,44]; DTX-2C [11]; PTX-2 [12]; PTXSAs [17,18]).

Individual DSP toxin standard stock solutions at 5.0 μg/ml were obtained by dissolving pure toxin in methanol.

2.2. Samples

Irish toxic mussels (*Mytilus edulis*) were collected in 1996 from coastal areas of southwest Ireland. Italian mussels (*Mytilus galloprovincialis*) were collected from northern coastal areas of the Adriatic sea in 1997 and were positive for DSP toxins upon a preliminary analysis with Yasumoto's biological assay. The digestive glands (hepatopancreases) were cut from mussels and stored at −20°C prior to extraction.

Phytoplankton samples were collected from regions near the sites of toxic mussel production. Procedures for the extraction of toxins from mussel hepatopancreases and phytoplankton were carried out as previously described [35].

2.3. Liquid chromatography–mass spectrometry

Analysis was performed on a Phoenix 20 CU LC pump (Fisons, Milan, Italy) liquid chromatograph. A Valco (Valco, Houston TX, USA) injection valve, equipped with interchangeable loops (1 or 0.2 μl), was used in flow injection analysis (FIA)–MS and LC–MS experiments, respectively. FIA–MS were carried out on the individual toxin solutions.

The mobile phase was acetonitrile–water (80:20, v/v), containing 0.1% TFA, for positive ion mode experiments and acetonitrile–water (80:20, v/v), containing 2 m*M* ammonium acetate, for negative ion mode experiments. The flow rate was 40 μl/min.

Separations were performed on a microcolumn packed with Supelcosil LC18-DB (Bellefonte, PA, USA) (300 mm×1 mm, 5 μm) at room temperature, under isocratic conditions.

Mass spectral analysis was performed on a PE-SCIEX API III plus triple-quadrupole (PE-Sciex, Thornhill, Canada). The mass spectrometer was equipped with an API source and an ionspray interface. MS setting for positive ion-mode FIA–MS experiments was the same as previously described [10]. In negative-ion mode experiments, ionspray interface voltage was set at −4000 V and an orifice potential voltage (OR) of −90 V was adopted. Full-scan mass spectra were acquired in single MS negative-ion mode and positive-ion mode, over the mass range m/z 500–1300.

Data acquisition for LC–MS analyzes in negative-ion mode was performed by selected ion monitoring (SIM) on the ions at m/z, 803 for OA, DTX-2, -2B, -2C, at m/z 817 for DTX-1, at m/z 857 for PTX-2, at m/z 875 for PTX-2SAs and AC1, and at m/z 1141 for YTX.

Data acquisition for LC–MS analyzes in positive-ion mode was performed by SIM on the ions corresponding to the protonated molecules, $[M+H]^+$, of the analytes, at m/z 805 for OA, DTX-2, DTX-2B and DTX-2C, at m/z 819 for DTX-1, at m/z 859 for PTX-2 and at m/z 877 for PTX-2SAs and AC1.

3. Results and discussion

The aim of this research was the development of a

reliable method, using LC–MS with ionspray ionization, for the direct determination of three groups of DSP toxins in mussels and phytoplankton.

The observation of molecular mass information is one of the most important criteria for identification of the analytes. The very mild form of ionization in the ionspray LC–MS technique usually guarantees the selective formation of molecule-related ions of each mixture component in addition to the corresponding retention time, this provides sensitive and specific detection of the analytes for confirmatory work.

Previous experience in our laboratory showed that positive-ion LC–MS promoted the selective and sensitive formation of protonated molecules as well as producing good chromatographic performances for a number of polyether DSP toxins, such as OA, DTXs [10,11] and PTXs [5,18]. On the other hand, as no significant signal for YTX was evidenced in positive ion mode, negative-ion LC–MS was regarded as more appropriate for YTX detection [29]. Accordingly, negative-ion LC–MS mode was implemented in this study in order to investigate the possibility of direct determination of DSP toxins belonging to the OA, PTX and YTX groups, in mussels and phytoplankton.

First experiments were carried out by FIA–MS on individual solutions of the toxins, by adopting a mobile phase of acetonitrile–water (80:20, v/v), containing 2 m*M* ammonium acetate. The obtained FIA–MS spectra showed the exclusive presence of deprotonated molecules, $[M-H]^-$, at m/z, 803 for OA and for DTX-2, -2B, -2C, at m/z 817 for DTX-1, at m/z 857 for PTX-2 and at m/z 875 for PTX-2SA and 7-*epi*-PTX-2SA (data not shown). On the other hand, the negative ion FIA–MS spectra for YTX was dominated by the intense signals at m/z 1163, and at m/z 1141, due to $[M-Na]^-$ and to the $[M-2Na+H]^-$ ions, respectively, according to previous observation [29].

The negative ion LC–MS approach was then implemented by using a reversed-phase Supelcosil LC18-DB microcolumn and a mobile phase of acetonitrile–water (80:20, v/v), containing 2 m*M* ammonium acetate. The toxins eluted at the following retention times: 4.3, 5.5, 5.6, 6.1, 6.2, 6.8, 6.9, 9.8 and 10.1 min for YTX, OA, DTX-2, -2B, -2C, AC1 and PTX-2SAs, DTX-1 and PTX-2, respectively (Fig. 1a). The use of the above chromatographic conditions allowed a complete separation for YTX, eluting before other DSP toxins, whereas a significant overlapping was observed for OA and its isomers, DTX-2, -2B and -2C, which eluted around the same retention times as the unresolved PTX-2 analogues, PTX-2SAs and AC1. Moreover, incomplete separation was obtained for DTX-1 and PTX-2. While complete separation of isomeric toxins is required, for analytes that were monitored in the same m/z selected ion chromatograms (i.e. OA, DTX-2, -2B and -2C, and PTX-2SA, 7-*epi*-PTX-2SA and AC1), toxins with different molecular masses were monitored by SIM of the deprotonated molecules $[M-H]^-$ (i.e. DTX-1, at m/z 817, PTX-2, at m/z 857), which allowed their unambiguous identification even if they were chromatographically unresolved. Successive attempts to improve chromatographic separation by varying the mobile phase composition were unsuccessful. The above approach was however considered appropriate as a first stage analysis for unambiguous identification of YTX, DTX-1 and PTX-2, as well as for screening for OA isomers and the three PTX-2 analogues requiring 20 min for total run time.

In order to achieve complete chromatographic separations of the isomeric toxins within the OA and PTX groups, the reversed-phase, positive ion SIM LC–MS analysis was then implemented using the same chromatographic column and a mobile phase of acetonitrile–water (80:20, v/v), containing 0.1% TFA (Fig. 1b). Although the separation of the analytes was not excellent under these conditions, with toxins eluting at 8.2, 8.8, 9.3, 9.4, 9.7, 9.8, 9.9, 10.2 and 10.3 min for AC1, PTX-2SA, OA, 7-*epi*-PTX-2SA, DTX-2, PTX-2, DTX-2B, DTX-1 and DTX-2C, respectively, the specificity of LC–MS reduced the need for complete chromatographic resolution of individual compounds, allowing their unambiguous identification in different m/z selected ion monitoring chromatograms.

Detailed toxin profiles of a number of toxic phytoplankton and shellfish, from different marine areas, were finally obtained through the new approach.

Figs. 2 and 3 show the representative LC–MS chromatograms from the analysis of Irish phytoplankton and Italian mussels, respectively. PTX-

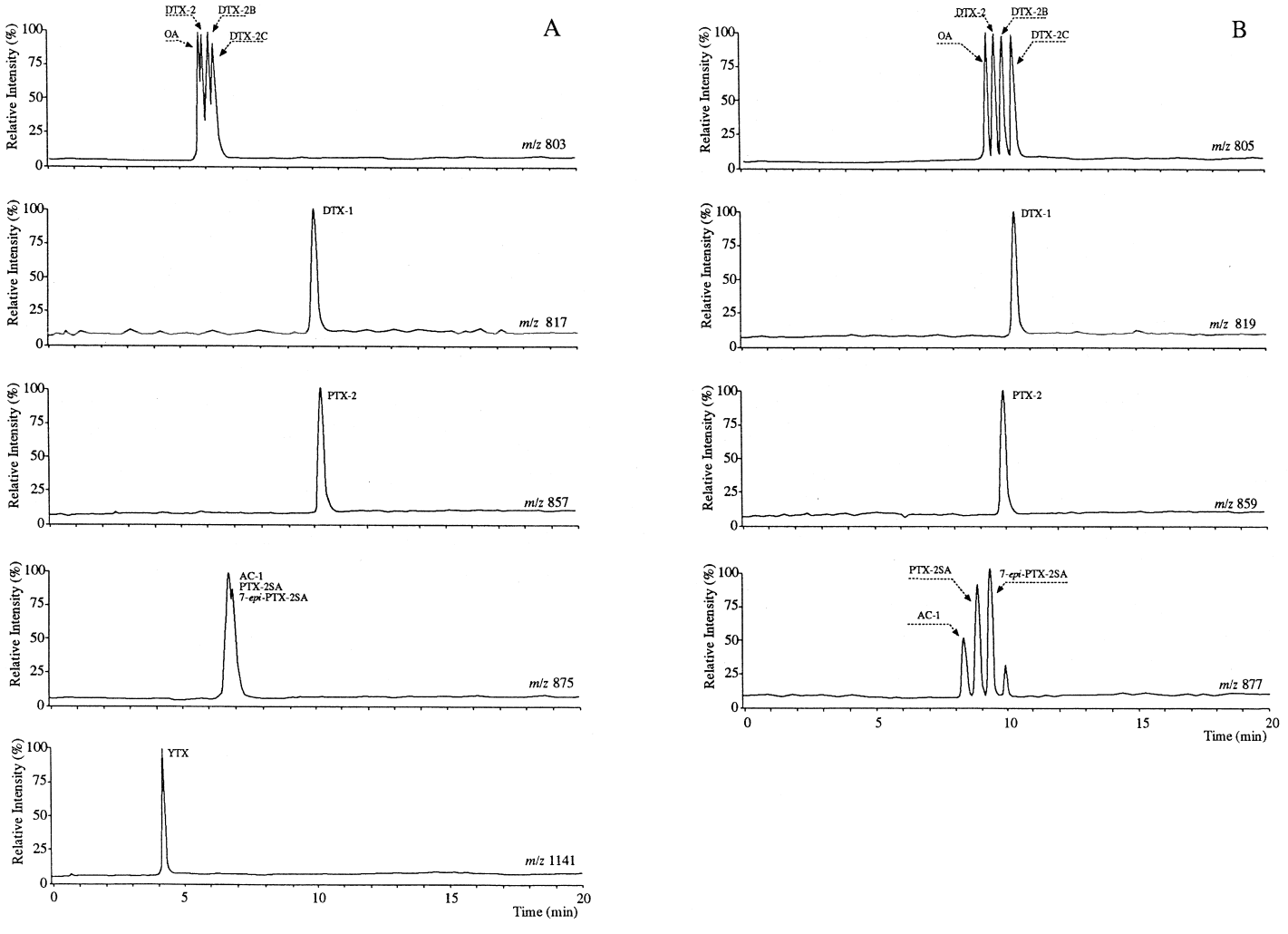

Fig. 1. SIM LC–MS analysis of DSP toxins in negative ion mode (A) and positive ion mode (B). The signal of the protonated molecules, $[M+H]^+$, of AC1, PTX-2SA and 7-*epi*-PTX-2SA was detected in the m/z 877 trace.

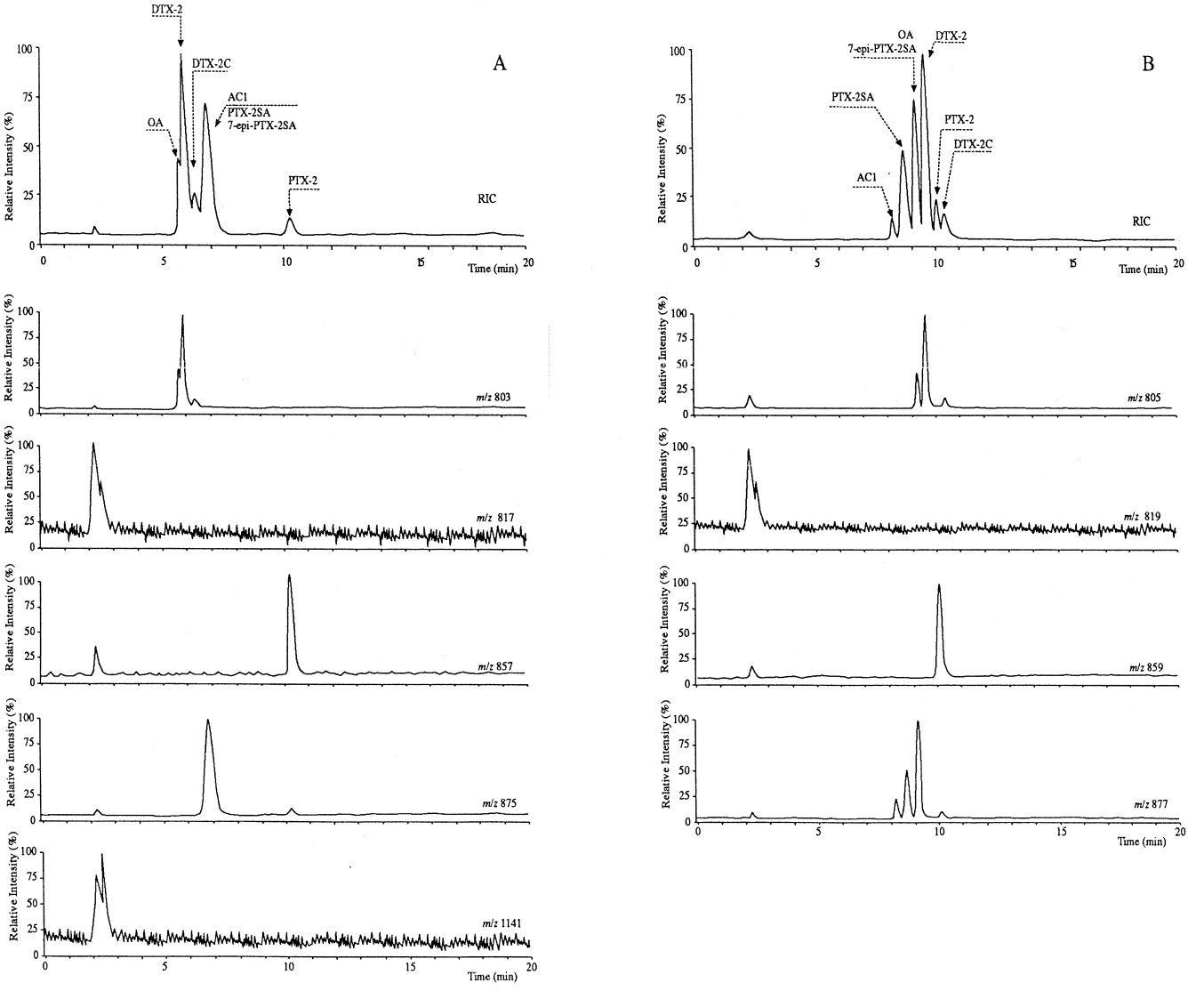

Fig. 2. Reconstructed ion current (RIC) profile and extracted ion current profiles of ionspray SIM LC–MS analysis of Irish phytoplankton in negative ion mode (A) and positive ion mode (B).

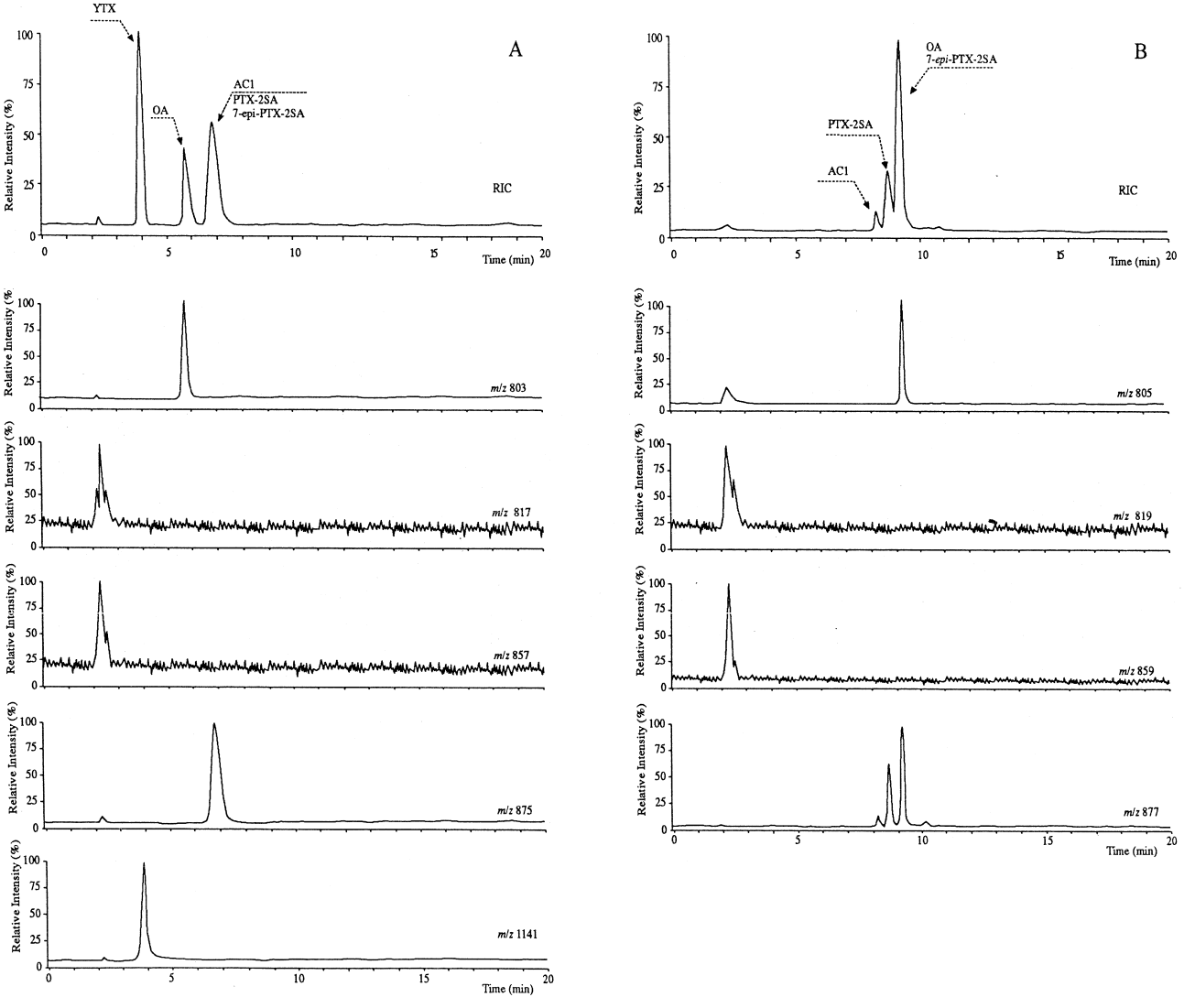

Fig. 3. Reconstructed ion current (RIC) profile and extracted ion current profiles of ionspray SIM LC–MS analysis of Italian mussels in negative ion mode (A) and positive ion mode (B).

2SAs and AC1 were found in phytoplankton and shellfish from Ireland as well as in Italian shellfish. Moreover, there was unprecedented evidence of PTX-2 in Irish phytoplankton. YTX was present in Italian shellfish. Four isomeric OA toxins were detected in samples from Ireland with OA, DTX-2 and DTX-2B present in shellfish, and OA, DTX-2 and DTX-2C in phytoplankton. In contrast, OA was the only toxin from this group to be detected in Italian mussels.

The new proposed approach allows, for the first time, the direct detection of known and new toxins in mussels and phytoplankton, with a high degree of sensitivity and specificity, due to the use of the LC–MS technique both in positive and negative ion modes. Although, at this stage, a full validation of the method was hampered by the limited availability of a number of pure toxins, this assay represents an effective alternative to the mouse bioassay for analysis of known and new DSP toxins in marine biological materials in monitoring and research work.

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