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Development of a method for the identification of azaspiracid in shellfish by liquid chromatography–tandem mass spectrometry

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Abstract

Azaspiracid is the main toxin responsible for a number of recent human intoxications in Europe resulting from shellfish consumption. The first micro liquid chromatography–tandem mass spectrometry (micro-LC–MS–MS) method was developed for the determination of this novel shellfish poisoning toxin in mussels. The analyte was extracted from whole mussel meat with acetone and chromatographed on a C_{18} reversed-phase column (1.0 mm I.D.) by isocratic elution at 30 μl/min with acetonitrile–water (85:15, v/v), containing 0.03% trifluoroacetic acid. The toxin was ionised in an ionspray interface operating in the positive ion mode, where only the intact protonated molecule, $[M+H]^+$, was generated at m/z 842. This served as precursor ion for collision-induced dissociation and three product ions, $[M+H-nH_2O]^+$ with $n=1–3$, were identified for the unambiguous toxin confirmation by selected reaction monitoring LC–MS–MS analysis. A detection limit of 20 pg, based on a 3:1 signal-to-noise ratio, was achieved for the analyte. This LC–MS–MS method was successfully applied to determine azaspiracid in toxic cultivated shellfish from two regions of Ireland. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Shellfish poisoning; Food analysis; Azaspiracid; Toxins

1. Introduction

Azaspiracid, formerly named Killary Toxin-3 (KT-3), is a marine toxin responsible for a new toxic syndrome, azaspiracid poisoning (AZP). This occurred for the first time in November 1995 in The Netherlands after consumption of cultured mussels from the west coast of Ireland (Killary Harbour) [1,2]. The associated human symptoms of nausea, vomiting, severe diarrhoea and stomach cramps, could be correlated to the presence of diarrhoetic shellfish poisoning (DSP) toxins. However, only insignificant levels of okadaic acid (OA) and dinophysistoxin-2 (DTX-2) were detected in the mussels and the known toxic phytoplankton were not observed in water samples collected at that time [1]. In February 1996 KT-3 was isolated from blue mussels, *Mytilus edulis* from Killary Harbour, which also contained other toxins in lower quantities [1].

Studies carried out by high-resolution (HR) fast atom bombardment mass spectrometry (FAB-MS) and nuclear magnetic resonance (NMR) on the isolated toxin suggested a molecular formula of $C_{47}H_{71}NO_{12}$ (M_r 841) [2,3]. Since the toxin struc-

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ture is characterised by a trispiro assembly, a rare azaspiro ring structure fused with a 2,9-dioxabicyclo[3.3.1]nonane ring, and a carboxylic acid, the toxin was re-named azaspiracid. It differs from any of the previously known nitrogen-containing toxins from shellfish or dinoflagellates, e.g., prorocentrolide, pinnatoxin, gymnodimine and the spirolides [3].

In 1997, azaspiracid was again responsible for a toxic incident which occurred in Arranmore Island, Ireland, with more than 12 human intoxications [4]. We have also recently identified azaspiracid in mussels and oysters harvested in 1998 from several other regions of Ireland (Clew Bay and Bantry) and in imported mussels in Italy and these data will be published elsewhere. Up to now, the lack of routine analytical methods has hampered widespread studies aimed at the detection of azaspiracid in marine biological material.

The impact of azaspiracid on human health is almost unknown. The minimum lethal dose (mouse, i.p.) was estimated to be 0.2 mg/kg [3]. Mice administered high doses of azaspiracid died in short periods showing paralytic shellfish poisoning (PSP)-like symptoms. At low dose levels, mice died of progressive paralysis within 2–3 days while diarrhoea was not observed [1]. Acute morphopathological changes in mice induced by azaspiracid are distinctly different from other marine toxins such as DSP, PSP and amnesic shellfish poisoning (ASP) toxins [5], the main target organs being the liver, pancreas, thymus, spleen and digestive tract. Also, azaspiracid has been supposed to inhibit protein synthesis [5].

Although the biogenetic origin of azaspiracid has not been identified yet, the toxin is thought to be produced by a dinoflagellate on account of its highly oxygenated polyether structure, the pattern of methylation in the molecule and its seasonal occurrence [3].

Unlike OA and DTXs, natural depuration of azaspiracid was very slow and persistence of mussel toxicity was observed for about eight months [1,4].

The development of analytical methods able to determine azaspiracid in seafood represents a priority research topic in order to support both basic scientific research and seafood monitoring. It should be noted that the Yasumoto DSP mouse bioassay [6], which is the basis of monitoring programs in most European countries, including Ireland and Italy, produces a positive result only at high levels of azaspiracid in shellfish and is probably not suitable for detecting this toxin due to the low extraction efficiency (10–40%) of total azaspiracid present in mussels (unpublished).

Liquid chromatography coupled with mass spectrometry and tandem mass spectrometry (LC–MS, LC–MS–MS), using an ionspray interface has proved to be an excellent analytical approach for direct determination of marine toxins responsible for DSP [7–23], ASP [7,24–26], neurotoxic shellfish poisoning (NSP) [24], PSP [7,24], ciguatoxins and maitotoxins [27,28]. The LC–MS approach has thus been recently proposed as a universal method for marine toxins [29].

The aim of this research was to investigate for the first time the possibility of using ionspray LC–MS and LC–MS–MS for the identification of azaspiracid in shellfish.

2. Experimental

2.1. Reagents

All solvents were HPLC or analytical grade and were purchased from Carlo Erba (Milan, Italy). OA was purchased from Calbiochem–Novabiochem (San Diego, CA, USA). Azaspiracid standard was isolated from Irish contaminated mussels as described by Satake et al. [1]. Individual OA and azaspiracid standard stock solutions at 1.0 μg/ml were obtained by dissolving pure toxin in methanol. Standard azaspiracid calibration solutions in the range 0.1–1 μg/ml for azaspiracid were prepared by dilution of the individual standard stock solutions with methanol.

2.2. Samples

Mussel (*Mytilus edulis*) samples were collected from two regions of Ireland, Killary Harbour and Arranmore Island (Fig. 1), in February 1996 and in October 1997, respectively, following reports of incidents involving human intoxications from shellfish. The mussels, tested with the Yasumoto DSP

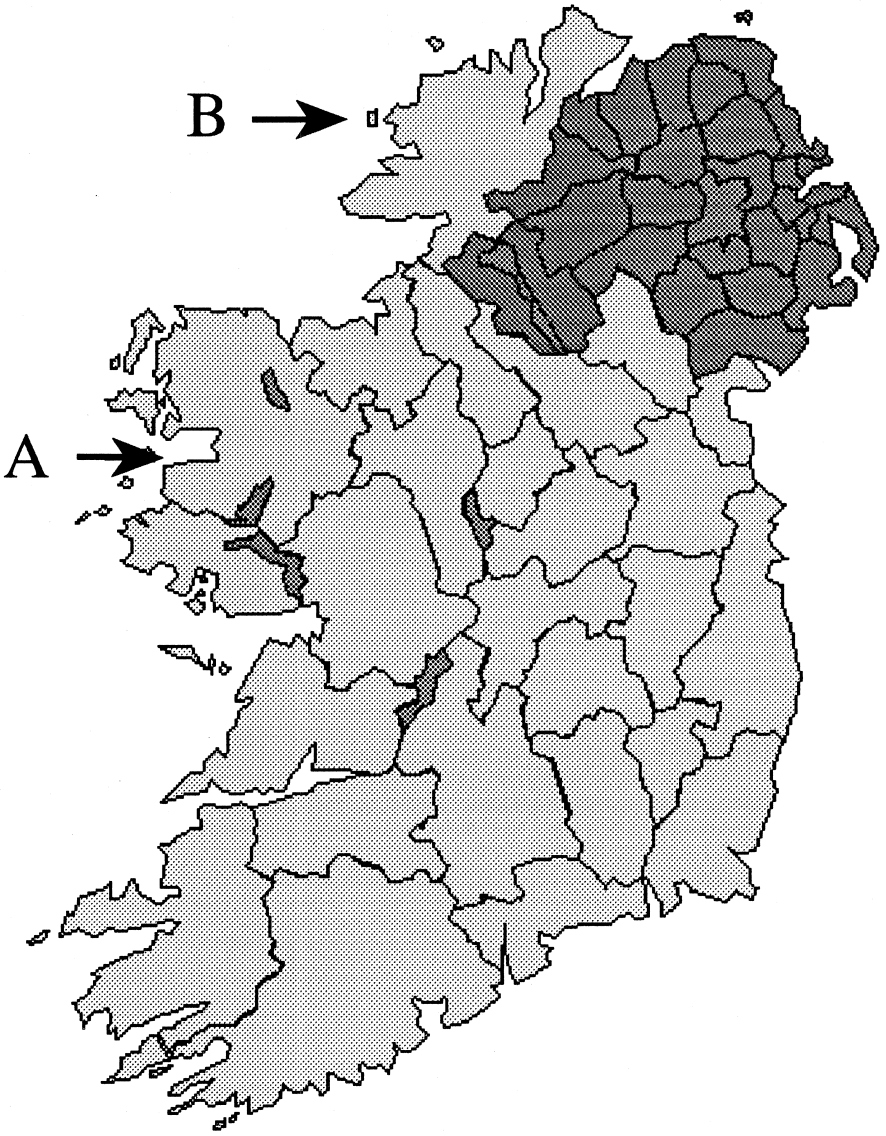

Fig. 1. Map of the sampling sites along the Irish coast: (A) Killary Harbour Bay and (B) Arranmore Island.

mouse bioassay [6], were positive for toxins. The samples were stored at −20°C until extraction. Whole mussel meat (2 g) was homogenised and centrifuged at 1000 *g* for 15 min three times with 10, 5 and 5 ml of acetone, respectively. The extracts were collected and evaporated to dryness in a rotary evaporator with a temperature controlled bath (40°C) and the residue was reconstituted in 2 ml of methanol before LC–MS and LC–MS–MS analyses. Extracts showing high levels of azaspiracid were appropriately diluted before injection into the LC–MS–MS system.

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 a 1-μl loop, was used for injections in flow injection analysis (FIA) MS, FIA–MS–MS, LC–MS and LC–MS–MS experiments. FIA–MS and FIA–MS–MS were carried out on toxin solutions containing 1 μg/ml of azaspiracid.

Chromatographic separations were performed on a microcolumn packed with Vydac 218TP51 (Separation Group, Hesperia, CA, USA) (250 mm×1 mm, 5 μm) at room temperature, under isocratic conditions, with a mobile phase of acetonitrile–water (85:15, v/v), containing 0.03% trifluoroacetic acid (TFA) and a flow-rate of 30 μl/min.

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 atmospheric pressure ionization (API) source and an ionspray interface set at a voltage of 5500 V. Ultra-high-pure (UHP) nitrogen was used as the curtain gas and nebulizer gas in the ionspray interface. For positive ion mode experiments an orifice potential voltage (OR) of 55 V was adopted. The standard software packages (PE-Sciex) were used for instrument control data acquisition and data elaboration.

Full-scan mass spectra were acquired in single MS positive-ion mode over the mass range m/z 700–900.

Data acquisition for LC–MS analyses was performed by selected ion monitoring (SIM) on the ions corresponding to the protonated molecules, $[M+H]^+$, of the analytes, at m/z 842 and at m/z 805 for azaspiracid and OA, respectively.

Full-scan collision-induced dissociation (CID) spectra were acquired by colliding the quadrupole 1 (Q1) selected precursor ion with argon gas (gas thickness $300 \cdot 10^{13}$ molecules cm^{-2}) in Q2 operated in radiofrequency (RF)-only mode and scanning the second quadrupole mass spectrometer, Q3, in the range m/z 50–900.

The protonated molecule of both toxins (m/z 842 for azaspiracid and at m/z 805 for OA) was the precursor ion for CID (collision energy was set at 40 eV) and diagnostic product ions for both analytes were identified to carry out selected reaction monitoring (SRM) micro-LC–MS–MS analysis. This was implemented using the precursor→product ion combinations of m/z 842→824, m/z 842→806 and m/z 842→788 for azaspiracid and m/z 805→751 for OA.

3. Results and discussion

Ionspray LC–MS is the most powerful technique for the identification of known and new toxins differing both in chemical structure and biological action [7–28]. The very mild ionization in the ionspray LC technique usually guarantees the selective formation of molecule-related ions of the toxin in addition to the corresponding retention time, providing sensitive and specific detection of the analyte for confirmatory purposes. Moreover, the LC–MS and LC–MS–MS approach is frequently the only method for obtaining structural information due to the small amounts of marine natural products available for analysis.

The aim of this research was to investigate for the first time the suitability of the ionspray LC–MS and LC–MS–MS in order to unambiguously detect azaspiracid in shellfish.

Preliminary experiments were carried out by FIA on azaspiracid standard solution in order to screen the solvent system and ionisation parameters to obtain suitable ionisation of the compound. Negative ion FIA–MS experiments were initially implemented on azaspiracid standard solutions by adopting a mobile phase of acetonitrile–4 m*M* ammonium acetate (80:20, v/v) and a flow-rate of 30 μl/min; these experiments were, however, unsuccessful since no significant signal for azaspiracid was obtained.

Positive-ion FIA–MS was then performed by adopting a mixture of acetonitrile–water acidified with TFA as mobile phase, promoting the selective and highly sensitive formation of protonated molecules, as well as good chromatographic performances during LC–MS analyses, for a number of polyether DSP toxins [14,19,21,22]. Fig. 2 shows the positive-ion full-scan (mass range m/z 700–900) FIA–MS spectrum, for azaspiracid standard solution by adopting a mobile phase of acetonitrile–water (85:15, v/v), containing 0.03% TFA and a flow-rate of 30 μl/min. The exclusive formation of the protonated molecule, $[M+H]^+$, at m/z 842 previously identified by HR-FAB-MS [3] was observed for the analyte without any evidence of fragmentation.

Following experiments were then performed to obtain spectra with maximum intensities of the protonated molecule of the azaspiracid by SIM FIA–MS analyses at m/z 842. The effect of varying orifice potential voltage was investigated between the

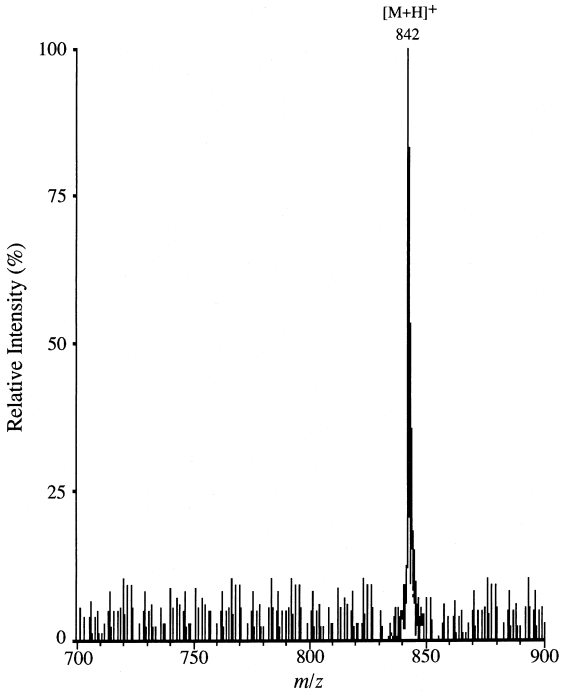

Fig. 2. Positive ion mass spectrum of azaspiracid. Conditions: FIA; mobile phase, acetonitrile–water (85:15, v/v), containing 0.03% TFA; flow-rate, 30 μl/min, 1 μl injection (1 μg/ml); OR was set at 55 V.

range 35–60 V. At the end an OR of 55 V was adopted.

Further structural information on azaspiracid was obtained by CID MS–MS experiments, which were carried out by selecting the precursor ion $[M+H]^+$ at *m/z* 842. A typical positive product ion mass spectrum (range *m/z* 50–900) of an azaspiracid standard solution acquired by FIA–MS–MS is shown in Fig. 3. Significant fragment ions observed at *m/z* 824, *m/z* 806 and *m/z* 788 could be assigned to the loss of up to three successive losses of H_2O molecules from the protonated molecule, *m/z* 842. This positive-ion MS–MS spectrum of the protonated molecule, $[M+H]^+$, of the azaspiracid was structurally informative, as product ions corresponding to successive losses of water from the protonated molecule are typically produced by low-energy collision fragmentation for several different marine polyether toxins [10,12,22,23,27].

Micro-LC–MS was then performed using a micro-column packed with Vydac 218TP51 (Separation Group) (250 mm×1 mm, 5 μm) with a mobile phase of acetonitrile–water (85:15, v/v), containing 0.03% TFA and a flow-rate of 30 μl/min. Fig. 4A shows the SIM LC–MS chromatogram obtained from the analyses of the standard mixture of azaspiracid and OA, monitoring the $[M+H]^+$ ions of the analytes at *m/z* 842 and at *m/z* 805, for azaspiracid and OA, respectively. Under the adopted conditions good signal and separation were obtained for both toxins which eluted at 6.0 min and 6.2 min, respectively. Specificity of the SIM LC–MS method was also proved by analysis of blank and spiked control samples. No interference was noticed around the OA and azaspiracid retention times (data not shown).

In order to achieve targeted analyses and maximum sensitivity as well as for quantitative purpose, the SRM micro-LC–MS–MS analysis was then implemented. On the basis of the MS–MS fragmentation of azaspiracid and OA, the combinations precursor→product ions of *m/z* 842→824, *m/z* 842→806 and *m/z* 842→788 for azaspiracid and *m/z* 805→751 for OA were considered suitable for confirmatory analyses. A representative chromatogram from the SRM LC–MS–MS analysis of standard solutions of azaspiracid and OA is shown in Fig. 5A. External standard SRM LC–MS–MS calibration curves, for azaspiracid covering the range 0.1–1 μg/ml were obtained for azaspiracid. A good linearity was observed, with a correlation coefficient (r^2) better than 0.995.

An instrumental SRM LC–MS–MS detection limit of ca. 20 pg was estimated for azaspiracid which is considerably lower than the limit of the mouse bioassay (i.e., about 2.8 μg of azaspiracid). This limit theoretically corresponds to a method detection limit of ca. 20 ng of azaspiracid per gram of whole mussel. An improvement of the method detection limit could however be hypothesised by concentrating the sample extracts, perhaps including a suitable clean-up procedure.

A full validation study including intra- and inter-day assays was hampered at this stage by the lack of availability of the azaspiracid standard which was necessary for recovery experiments.

Toxin profiles of the mussels (*Mytilus edulis*), sampled from Killary Harbour and Arranmore Island (Ireland) during AZP toxicity phenomena, were finally obtained through the SIM LC–MS method. Fig. 4B shows the SIM LC–MS chromatogram of a mussel sample which was positive by mouse bioas-

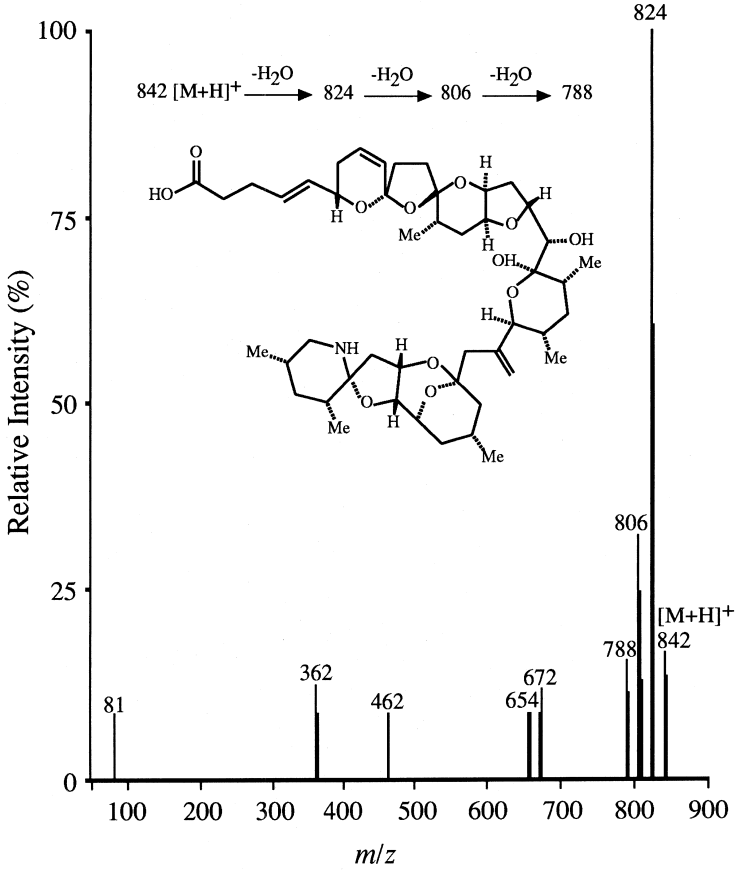

Fig. 3. Positive product ion mass spectrum of azaspiracid with the $[M+H]^+$ ion, at m/z 842 as precursor. Conditions: FIA; mobile phase, acetonitrile–water (85:15, v/v), containing 0.03% TFA; flow-rate, 30 μl/min, 1 μl injection (1 μg/ml); OR was set at 55 V. Argon was used as the collision gas. CID was carried out with a collision energy of 40 eV.

say. Azaspiracid and OA were detected in this sample by the chromatographic peaks which eluted at retention times of authentic standards in the SIM trace at m/z 842 and at m/z 805 for azaspiracid and OA, respectively. SIM LC–MS analyses on these samples were also carried out but other DSP toxins were not detected.

For confirmatory and quantitative purposes SRM LC–MS–MS analyses of the Irish mussels previously assayed by the SIM LC–MS method were carried out.

Fig. 5B shows the SRM LC–MS–MS chromatogram of the above mussel samples. Both azaspiracid and OA were observed, thus confirming the SIM LC–MS results. The determined amounts of toxins of 0.2 μg of OA and 6.4 μg of azaspiracid per gram of whole mussel (data not corrected for recovery) demonstrated the positive results in the mouse bioassay to be essentially due to the high level of azaspiracid.

The micro-LC–MS–MS method was able to unambiguously confirm azaspiracid in all of the assayed Irish samples, at concentrations ranging from 3.1 to 6.4 μg per gram of whole mussel, clearly accounting for the lethality on mouse bioassay.

In conclusion, the developed method provided analytically useful MS and MS–MS data for the determination of azaspiracid in biological materials which included the identification of the analyte based on retention time, molecular mass, and structural

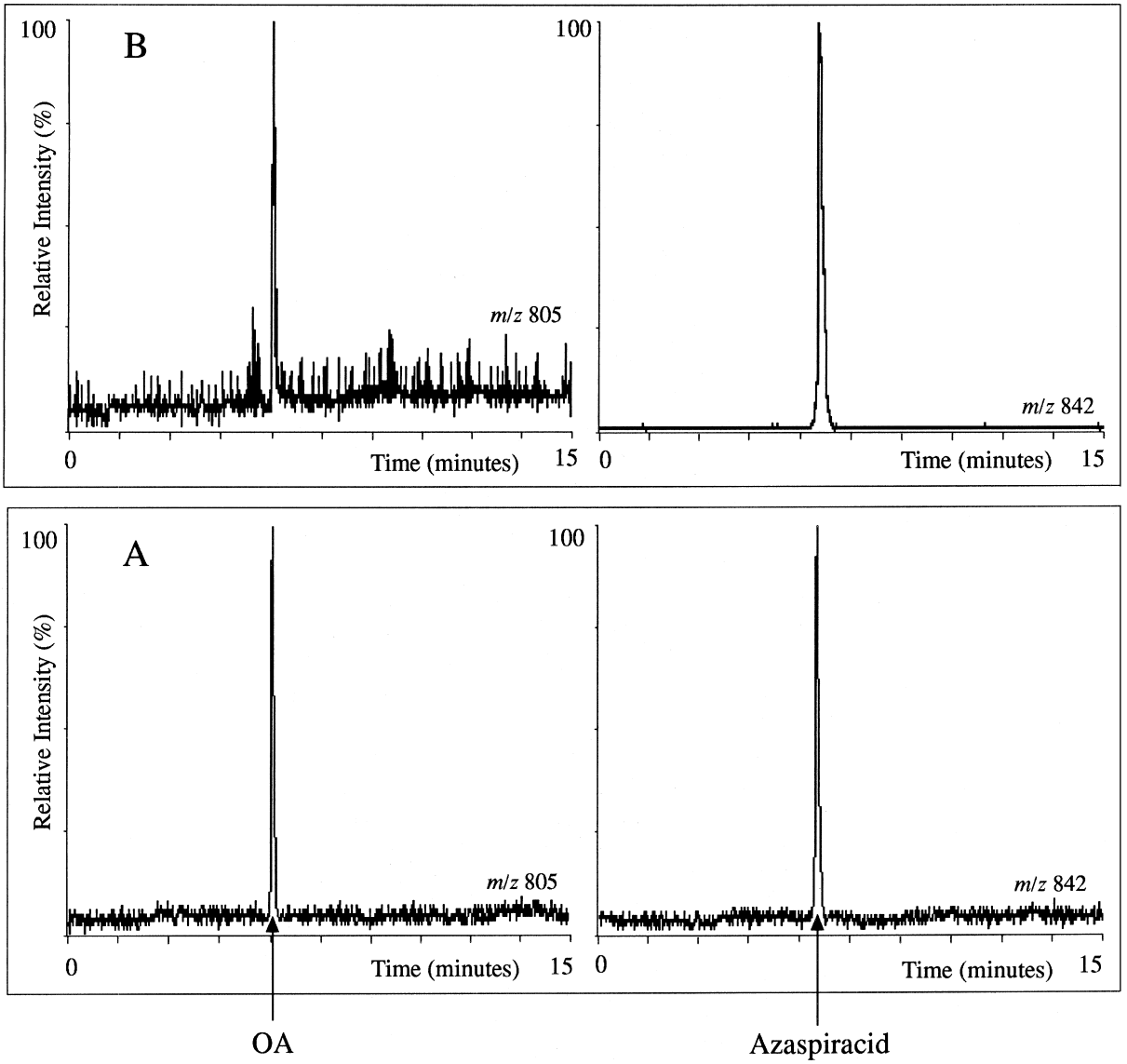

Fig. 4. SIM LC–MS chromatograms of: (A) standard mixture containing OA (0.5 μg/ml) and azaspiracid (0.1 μg/ml); (B) acetone extract of Irish toxic mussel sample containing OA and azaspiracid. $[M+H]^+$ ions of OA and azaspiracid were monitored at m/z 805 and at m/z 842, respectively. Conditions: isocratic HPLC analysis; microcolumn packed with Vydac 218TP51 (250 mm×1 mm, 5 μm); mobile phase, acetonitrile–water (85:15, v/v), containing 0.03% TFA; flow-rate, 30 μl/min, 1 μl injection; OR was set at 55 V.

information, such as the presence of three product ions for azaspiracid, thus achieving a confidence in identification higher than 99.9% [30].

The proposed method could also be feasible to identify the toxin in algae materials. The knowledge of the biogenetic origin of the azaspiracid represents a priority research topic as this is the basis for a targeted phytoplankton monitoring in order to assure safety of seafood and to study the structural modification of biotoxins.

Research is currently in progress in order to control azaspiracid in shellfish from different marine

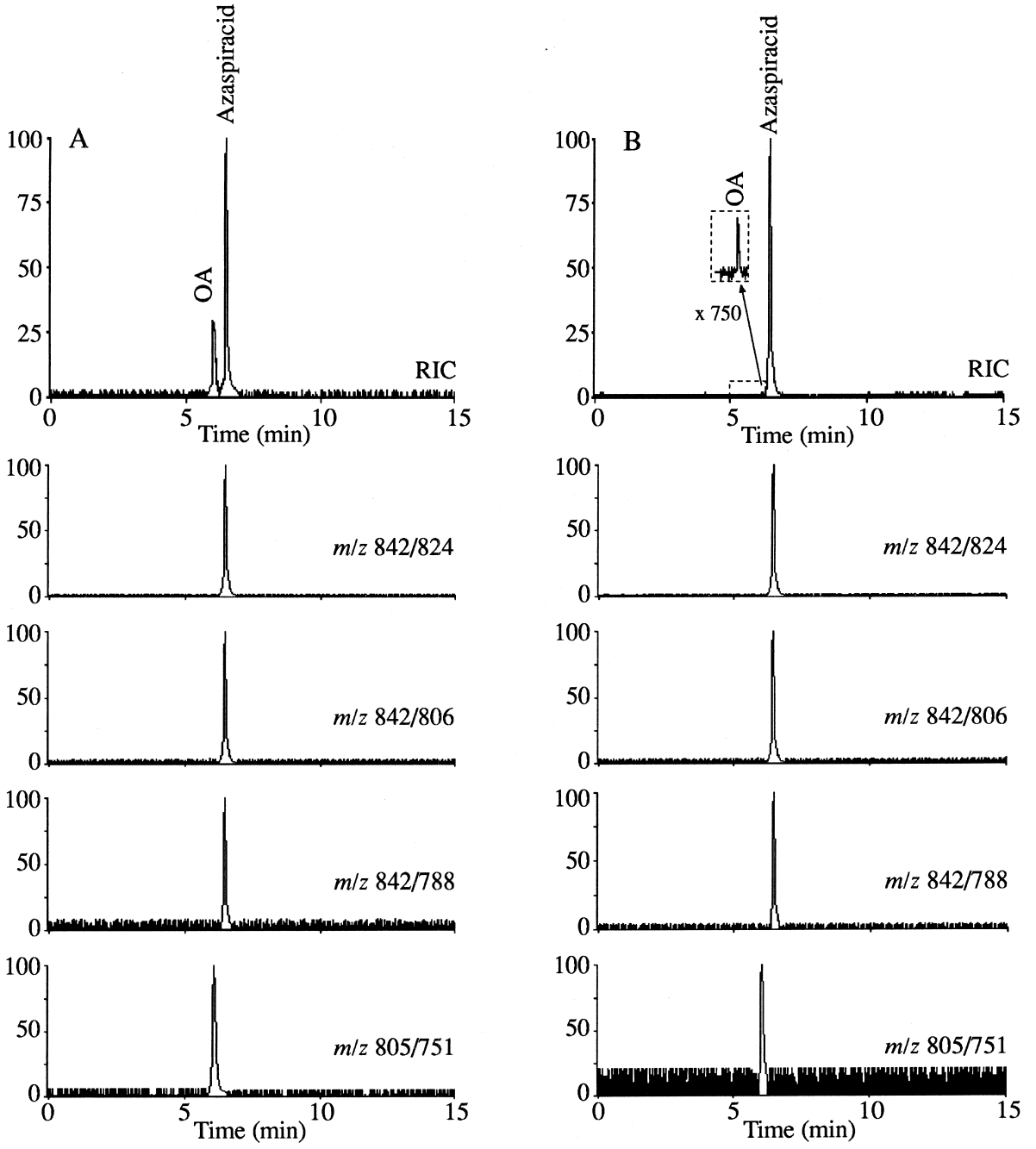

Fig. 5. SRM LC–MS–MS chromatograms of: (A) standard mixture containing OA (0.5 μg/ml) and azaspiracid (0.1 μg/ml); (B) acetone extract of Irish toxic mussel sample containing OA (0.2 μg/g) and azaspiracid (6.4 μg/g). Precursor–product ion combinations used in SRM detection are shown. Conditions: isocratic HPLC analysis; microcolumn packed with Vydac 218TP51 (250 mm×1 mm, 5 μm); mobile phase, acetonitrile–water (85:15, v/v), containing 0.03% TFA; flow-rate 30 μl/min, 1 μl injection; OR was set at 55 V. Argon was used as the collision gas. CID was carried out with a collision energy of 40 eV.

areas in Europe, as well as to identify the azaspiracid producing organism.

References

[1] M. Satake, K. Ofuji, K.J. James, A. Furey, T. Yasumoto, in: B. Reguera, J. Blanco, M.L. Fernández, T. Wyatt (Eds.), Proceedings of the 8th International Conference on Harmful Algae, Vigo, Xunta de Galicia and I.O.C. of UNESCO, 25–29 June 1997, p. 468.

[2] T. Yasumoto, M. Satake, in: B. Reguera, J. Blanco, M.L. Fernández, T. Wyatt (Eds.), Proceedings of the 8th International Conference on Harmful Algae, Vigo, Xunta de Galicia and I.O.C. of UNESCO, 25–29 June 1997, p. 461.

[3] M. Satake, K. Ofuji, H. Naoki, K.J. James, A. Furey, T. McMahon, J. Silke, T. Yasumoto, J. Am. Chem. Soc. 120 (1998) 9967.

[4] K. Ofuji, M. Satake, T. McMahon, J. Silke, K.J. James, H. Naoki, Y. Oshima, T. Yasumoto, Nat. Toxins, in press.

[5] E. Ito, K. Terao, T. McMahon, J. Silke, T. Yasumoto, in: B. Reguera, J. Blanco, M.L. Fernández, T. Wyatt (Eds.), Proceedings of the 8th International Conference on Harmful Algae, Vigo, Xunta de Galicia and I.O.C. of UNESCO, 25–29 June 1997, p. 588.

[6] T. Yasumoto, Y. Oshima, M. Yamaguchi, Bull. Jpn. Soc. Sci. Fish. 44 (1978) 1249.

[7] M. A Quilliam, B.A. Thomson, G.J. Scott, K.W.M. Siu, Rapid Commun. Mass Spectrom. 3 (1989) 145.

[8] S. Pleasance, M.A. Quilliam, J.C. Marr, Rapid Commun. Mass Spectrom. 6 (1992) 121.

[9] J.C. Marr, T. Hu, S. Pleasance, M.A. Quilliam, J.L.C. Wright, Toxicon 30 (1992) 1621.

[10] R. Draisci, L. Lucentini, L. Giannetti, P. Boria, A. Stacchini, Toxicon 33 (1995) 1591.

[11] R. Draisci, L. Lucentini, L. Giannetti, P. Boria, Al. Stacchini, R. Poletti, Riv. Soc. Ital. Sci. Alim. 1 (1996) 7.

[12] M.A. Quilliam, J. AOAC Int. 78 (1995) 555.

[13] K.J. James, A.G. Bishop, M. Gillman, S.S. Kelly, C. Roden, R. Draisci, L. Lucentini, L. Giannetti, P. Boria, J. Chromatogr. A 777 (1997) 213.

[14] R. Draisci, L. Lucentini, L. Giannetti, P. Boria, R. Poletti, Toxicon 34 (1996) 923.

[15] T. Suzuki, T. Mitsuya, H. Matsubara, M. Yamasaki, J. Chromatogr. A 815 (1998) 155.

[16] R. Draisci, L. Giannetti, L. Lucentini, E. Ferretti, L. Palleschi, C. Marchiafava, Rapid Commun. Mass Spectrom. 12 (1998) 1291.

[17] R. Draisci, E. Ferretti, L. Palleschi, C. Marchiafava, R. Poletti, A. Milandri, A. Ceredi, M. Pompei, Toxicon 37 (1999) 1187.

[18] K.J. James, E.P. Carmody, M. Gillman, S.S. Kelly, R. Draisci, L. Lucentini, L. Giannetti, Toxicon 35 (1997) 973.

[19] R. Draisci, L. Lucentini, L. Giannetti, P. Boria, K.J. James, A. Furey, M. Gillman, S.S. Kelly, J. AOAC Int. 81 (1998) 1.

[20] R. Draisci, L. Giannetti, L. Lucentini, C. Marchiafava, K.J. James, A.G. Bishop, B.M. Healy, S.S. Kelly, J. Chromatogr. A 798 (1998) 137.

[21] R. Draisci, L. Palleschi, L. Giannetti, L. Lucentini, K.J. James, A.G. Bishop, M. Satake, T. Yasumoto, J. Chromatogr. A 847 (1999) 213.

[22] K.J. James, A.G. Bishop, R. Draisci, L. Palleschi, C. Marchiafava, E. Ferretti, M. Satake, T. Yasumoto, J. Chromatogr. A 844 (1999) 53.

[23] R.W. Dickey, S.C. Bobzin, D.J. Faulkner, F.A. Bencsath, D. Andrzejewski, Toxicon 28 (1990) 371.

[24] M.A. Quilliam, in: D. Barcelo (Ed.), Applications of LC–MS in Environmental Chemistry, Elsevier, Amsterdam, 1998, p. 415.

[25] M.A. Quilliam, M. Xie, W.R. Hardstaff, J. AOAC Int. 78 (1995) 543.

[26] L. Fritz, M. A Quilliam, J.L.C. Wright, A.M. Beale, T.M. Work, J. Phycol. 28 (1992) 439.

[27] R.J. Lewis, M.J. Holmes, P.F. Alewood, A. Jones, Nat. Toxins 2 (1989) 56.

[28] R.J. Lewis, A. Jones, Toxicon 35 (1997) 159.

[29] M.A. Quilliam, in: B. Reguera, J. Blanco, M.L. Fernández, T. Wyatt (Eds.), Proceedings of the 8th International Conference on Harmful Algae, Vigo, Xunta de Galicia and I.O.C. of UNESCO, 25–29 June 1997, p. 509.

[30] S. Porter, R. Patel, I. Kay, in: N. Haagsma, A. Ruiter (Eds.), Proceedings of the EuroResidue III, Veldhoven, Utrecht University, Faculty of Medicine Veterinary, 6–8 May 1996, p. 795.