Food Sci. Biotechnol. Vol. 18, No. 4, pp. 842 ~ 848 (2009)

Food Science and Biotechnology

Investigation of Domoic acid in Shellfish Collected from Korean Fish Retail Outlets

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Abstract The purpose of this study was to determine the extent of domoic acid (DA) a potent neurotoxin, responsible for the syndrome amnesic shellfish poisoning (ASP) contamination of various species of bivalve shellfish purchased from fish market in Korea and the implications for food safety. Liquid chromatography (LC) methods were applied to quantify DA in shellfish after sample clean-up using solid-phase extraction (SPE) with strong anion exchange (SAX) cartridges. Toxin detection was achieved using photodiode array ultraviolet (LC-UV) and electrospray ionization-mass (LC-ESI-MS). DA was identified in 4 bivalve shellfishes of 872 shellfishes collected from March, 2006 to October, 2007 in Korea. DA amount of 3 surf clams (*Mactra veneriformis*) collected at Seoul, Daejeon, and Daegu were 4.13, 1.99, and 1.94 mg/kg, respectively. DA amount of 1 pink butterfly shell (*Peronidia venulosa*) collected at Seoul was 3.02 mg DA/kg. The amounts of DA that were present in 4 bivalve shellfishes were within EU guideline limits for sale of shellfish (20 mg DA/kg).

Keywords: amnesic shellfish poisoning, domoic acid, shellfish toxin, food safety, liquid chromatography

Introduction

Amnesic shellfish poisoning (ASP), also known as domoic acid poisoning (DAP) because amnesia is not always present, was first recognized in 1987 in Prince Edward Island, Canada. At this time, ASP caused 3 deaths and 105 cases of acute human poisoning following the consumption of blue mussels (*Mytilus edulis*) (1). The causative toxin (excitatory amino acid, domoic acid or DA, Fig. 1) was produced by the diatom species *Pseudonitzschia pungens* f. *multiseries* (=*Nitzschia pungens* f. *multiseries*) (2). This was the first recorded human intoxication, the symptoms produced included nausea, disorientation, temporary amnesia, and in more serious cases, especially, elderly people and/or those with gastric lesions, persistent short term memory loss, and/or coma resulted (3).

Following the Canadian toxic outbreak, DA has been detected in a variety of shellfish throughout the world. Reports of DA in shellfish have included USA (4), New Zealand (5), Mexico (6), and several European countries (7-10). The environmental impacts of this toxin has also included large-scale animal mortalities involving birds (6,11,12), sea lions (13,14), and whales (15). The Canadian authorities imposed an action limit of 20 mg DA/kg shellfish tissue. Programs to monitor the DA concentrations in shellfish are in place in many countries worldwide and numerous incidents of DA contamination have been recorded in a wide variety of shellfish species (3).

However, there were not any monitoring reports and regulation limit of ASP in Korea. Therefore, we now report the monitoring results of DA contamination of various species of bivalve shellfish sold in fish market in Korea from March, 2006 to October, 2007 and the implications for food safety.

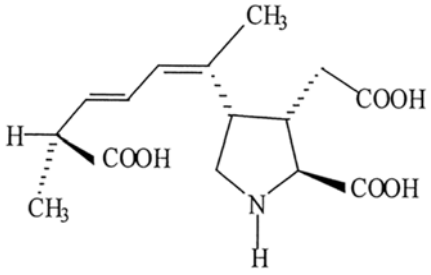

Fig. 1. Structure of domoic acid.

Materials and Methods

Shellfishes Eight-hundred-seventy-two (872) shellfishes, blue mussel (*Mytilus edulis*), oyster (*Crassostrea gigas*), scallop (*Patinopecten yessoensis*), short-neck clam (*Ruditapes philippinarum*), ark shell (*Scapharca subcrenata*), hard clam (*Meretrix lusoria*), venus clam (*Gomphina melanaegis*), surf clam (*Mactra veneriformis*), pink butterfly shell (*Peronidia venulosa*), and others were purchased from fish market in Seoul, Busan, Daegu, Daejeon, Kwangju, and Gangneung in Korea from March 2006 to October 2007 (Table 1). The shellfish samples were collected at 5 big cities (Seoul, Busan, Daegu, Daejeon, and Kwangju) and Gangneung where is the biggest city in eastern part of Gangwon province. We tried to collect the main shellfishes, such as blue mussel, oyster, scallop, short-neck clam, and ark shell, at each city and every month all we can. The samples were transported to laboratory in 5°C of icebox and kept below −18°C.

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Received March 4, 2009; Revised March 3, 2009;
Accepted March 12, 2009

Table 1. Domoic acid (DA) in shellfish purchased from fish market in Korea from March 2006 to October 2007

Shellfish	2006		2007		Total		
	Domestic	Imported	Domestic	Imported	Domestic	Imported	Total
Blue mussel	0/13[1)]	0/1	0/42	0/23	0/55	0/24	0/79
Oyster	0/8	-	0/36	-	0/44	-	0/44
Scallop	-	0/10	0/24	0/40	0/24	0/50	0/74
Short-neck clam	0/37	0/6	0/32	0/14	0/69	0/20	0/89
Ark shell	0/35	0/1	0/29	0/5	0/64	0/6	0/70
Hard clam	0/23	0/42	0/23	0/30	0/46	0/72	0/118
Venus clam	0/10	0/29	0/14	0/19	0/24	0/48	0/72
Surf clam	2/17	1/6	0/26	0/16	2/43	1/22	3/65
Pink butterfly shell	-	1/5	0/18	0/14	0/18	1/19	1/37
Others	0/55	0/48	0/38	0/83	0/93	0/131	0/224
Total	2/198	2/148	0/282	0/244	2/480	2/392	4/872

[1)]Number of positive sample/number of tested sample; -, not tested.

Chemicals Certified domoic acid (DA) (1 mg), acetonitrile, trifluoroacetic acid (TFA), and water were liquid chromatography (LC) grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Working solution Certified DA (1 mg) was dissolved in 10 mL of 90% acetonitrile for 100 ppm of stock solution and stored at −80°C. Stock solution (100 ppm) was diluted with 90% acetonitrile for making of 10 to 0.001 ppm working solution.

Shellfish samples preparation Samples were extracted according to a procedure described by Quilliam *et al.* (16). Briefly, 10 g of shellfish homogenized by Waring blender (Waring Commercial, Torrington, CT, USA) was accurately weighed into a graduated centrifuge tube. Methanol-water (1:1, 40 mL) were added and homogenized (2 min at 10,000 rpm) by Waring blender (Waring Commercial). The resulting slurry was then centrifuged at 3,000×g for 10 min. The supernatant was filtered through a 0.45-μm filter into a screw-capped vial. For clean-up and concentration, the extracts (5 mL) were subjected to a solid-phase extraction (SPE) method with a strong anion-exchanger (SAX) cartridge (3 mL, J.T. Baker, Deventer, Netherland), conditioned with methanol-water (1:1, 3 mL). Filtered supernatant (5 mL) was loaded onto the cartridge and washed with 5 mL acetonitrile-water (1:9). A 0.1 M formic acid (5 mL, pH 2.92) solution was added to the cartridge, the first fraction (0.5 mL) was discarded and the following fractions (3.0 mL) were collected, and 20 μL aliquot was injected for high performance liquid chromatography (HPLC) and LC/MS analyses.

Liquid chromatography-ultra violet (LC-UV) LC-UV analysis was carried out using an aliquot (20 μL) from the SPE stage. The LC system was an Agilent 1100 series (Agilent Tech., Palo Alto, CA, USA) which consisted of a binary pump, a thermostatically controlled autosampler (4°C), and a UV photodiode array detector. Isocratic chromatography was performed using acetonitrile/water (1:9, v/v) with 0.1% TFA, at a flow rate of 0.8 mL/min, with a reversed phase column (Waters Symmetry C18; 25 cm×4.6 mm, 5 μm, Waters, Milford, MA, USA) at 40°C.

Liquid chromatography (LC)-electrospray ionization-mass (LC-ESI-MS) determination of DA LC of DA was performed with a Agilent 1100 Series Quaternary Pump and Agilent 1100 Series autosampler (Agilent Tech, Germany). The chromatographic separation was carried out on a reversed-phase column (Symmetry C_{18} 5 μm, 3.9×150 mm i.d., Waters, Ireland) using isocratic elution [acetonitrile/water (1:9 v/v) with 0.1% TFA]. The flow rate was 0.5 mL/min, throughout. MS measurements were performed using an Agilent LC/MSD Trap VL mass spectrometer with an atmospheric pressure ionization (API) source operating in electrospray (ES) mode (Agilent Tech, Germany). The eluate from the LC column was transferred to the MS device using a split ratio of 5:1 (volume to waste/volume transferred), and nitrogen (heated to 325°C, 10 L/min) was applied to dry the ion-spray aerosol. Nitrogen was also used nebuliser gas with 40 psi. The ionization voltage of the ES interface was set to 3.5 kV. The MS system was operated in positive multiple ion detection (MID) mode to give highest sensitivity. Mass range was scanned *m/z* 250-350 (DA, 312.2).

Results and Discussion

Determination of DA in shellfish using LC-UV Sample preparation and analysis of DA in shellfish was performed using a procedure similar to that previously described (16). LC-UV is currently the preferred analytical technique for the determination of DA in shellfish and a method is available, formally validated for mussels in an AOAC collaborative study (17). The detection of DA is facilitated by its strong absorbance at 242 nm. The LC-UV detection limit for DA is about 10-80 ng/mL, depending on the sensitivity of the UV detector that is used. The detection limit in tissue is dependent upon the method of extraction and clean-up. If crude extracts (either acidic or aqueous methanol) are analyzed without clean-up, the practical limit for quantitation is about 1 μg/g (18). This is suitable for most regulatory laboratories concerned with detecting contamination levels greater than 20 μg/g. However, interferences are commonly encountered that can give false positives with crude extracts. For example, it has been shown that tryptophan and some of its derivatives are often

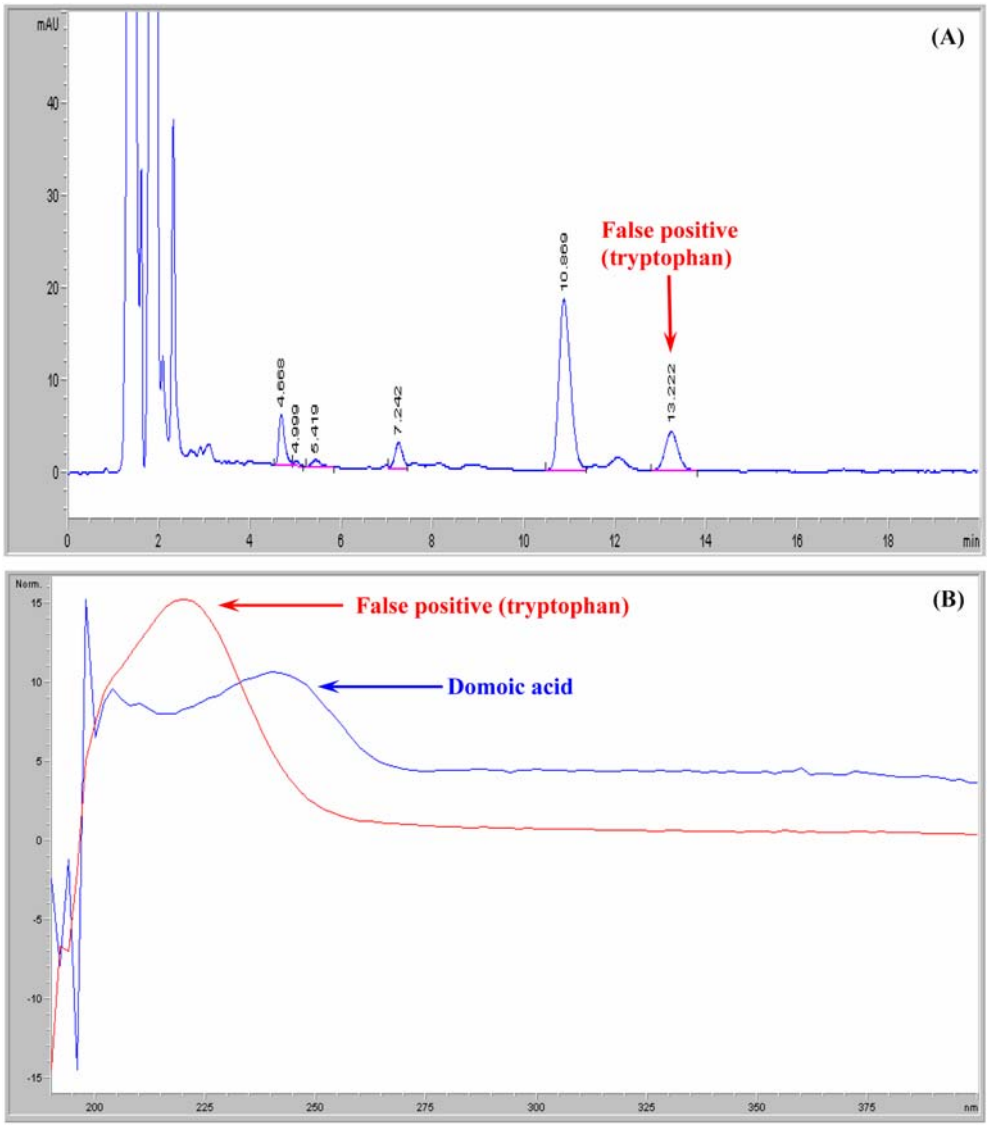

Fig. 2. Chromatogram from the LC-UV analysis (A) and UV spectrum of false-positive (tryptophan) results of domoic acid (B).

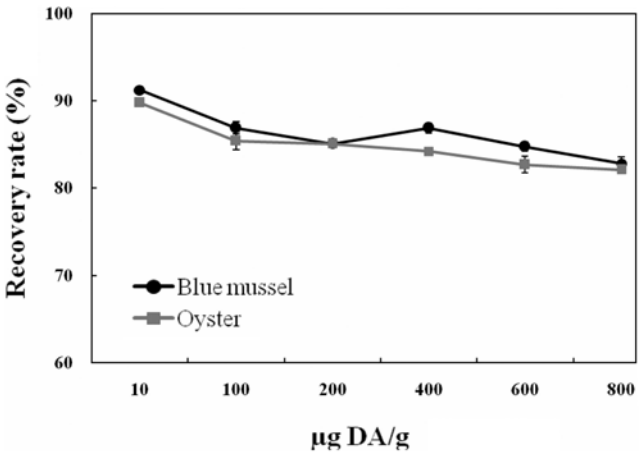

Fig. 3. Recovery of domoic acid (DA) from extracts of blue mussel and oyster digestive glands using anion exchange solid phase extraction (SPE).

present in substantial concentrations in shellfish and finfish tissues and that these compounds elute close to DA. Therefore, we identified false-positive results with UV spectrum (Fig. 2). A SPE method with SAX cartridge has been recommended for sample clean-up of extracts from mussels (16) and scallops (10). The average recoveries of DA from the reference standard blue mussel and oyster material, using SPE, was 86.3 and 84.9%, respectively (n=6). However, they were observed that the recoveries of DA from blue mussel and oyster digestive gland were inconsistent and studies revealed that the recovery was dependent on the concentration of DA. The data from this SPE study are summarized in Fig. 3 and they were shown that there was reduced extraction efficiency at higher levels of DA. The average DA recovery (n=6) from blue mussel hepatopancreas was 91.3%, at a concentration of 10 µg DA/g, but diminished to 82.9% at 800 µg DA/g. It is necessary therefore to retest sample extracts that had higher levels of DA using LC-MS without SPE to obtain reliable

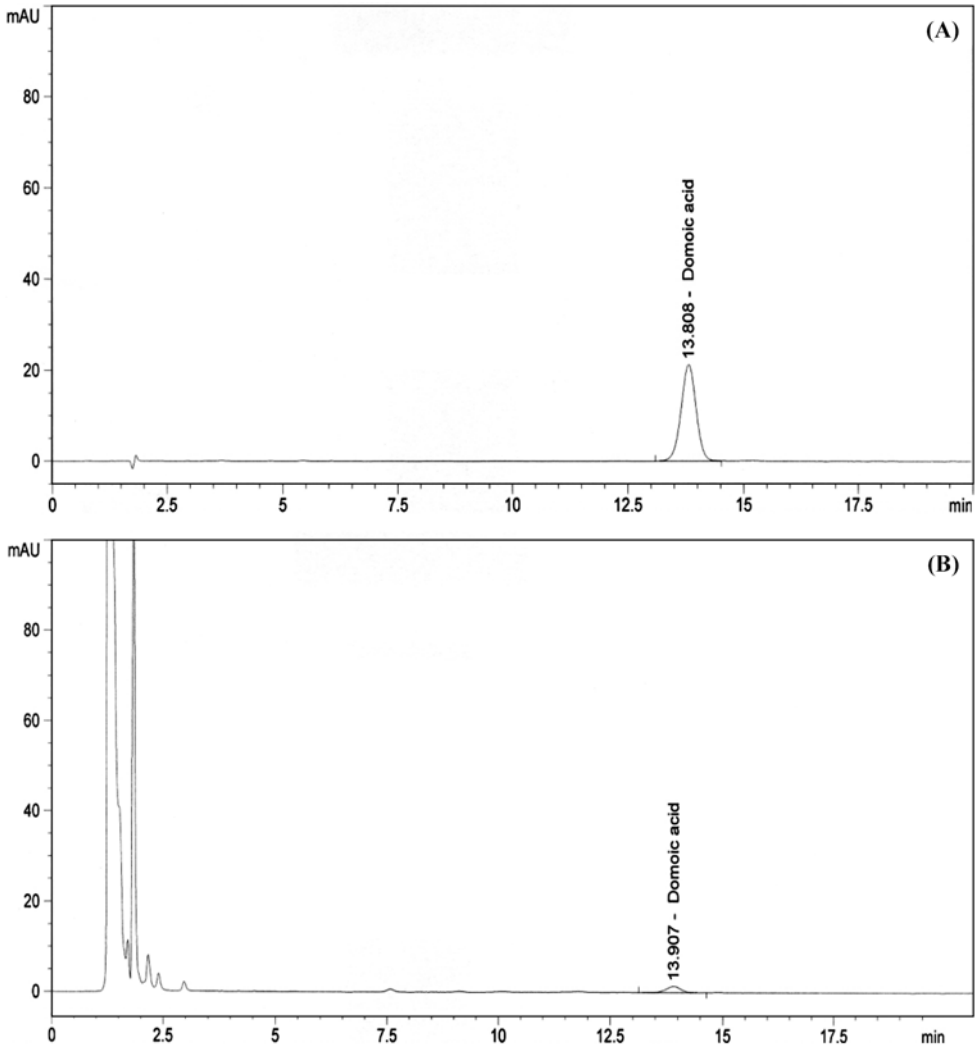

Fig. 4. Chromatogram from the LC-UV/DAD analysis of DA from reference standard (A) and DA in surf clam total tissue (B). The concentration of domoic acid was 10 μg/g in reference standard.

quantitative data. Representative chromatograms (LC-UV) from DA reference standard and an extract of surf clam were shown in Fig. 4. The retention time of DA was 13.808 min.

Determination of DA in shellfish using LC-ESI-MS LC-MS techniques have been employed in the past for the analysis of the neurotoxin DA (19-22). Quilliam *et al.* (20) has used both fast atom bombardment (FAB)-MS and FAB-MS-MS spectrometry to analyze this toxin and its isomers. Full mass spectra from these experiments shows the molecular ion for the toxin at *m/z* 312 $[M+H]^+$, a peak at *m/z* 623 is indicative of the dimer ion [2 M+H] . For this study, Agilent LC/MSD Trap VL mass spectrometer was used to determine DA in shellfish tissue. The MS was equipped with an ESI interface and operated in positive ion mode. ESI/MS of an extract from surf clam (total tissues) collected at fish market in Seoul, July 2006 showed ion peaks at *m/z*=312 which correspond with $(M+H)^+$ of DA, indicating that the DA in samples are identical (Fig. 5).

Domic acid in various shellfish species From March 2006 to October 2007, 872 shellfishes (480 domestic and 392 imported shellfishes) were purchased from fish market in Korea for DA monitoring. The main bivalve molluscs that were studied included, blue mussel, oyster, scallop, short-neck clam, ark shell, hard clam, venus clam, surf clam, and pink butterfly shell. DA was detected from 4 shellfishes (2 domestic and 2 imported shellfishes) that are 3 surf clams and 1 pink butterfly shell (Table 1). In July 2007, this research group first detected DA in surf clam and the toxin levels found were 4.13 μg/g of total tissues (about a quarter of the regulatory limit). In studies of Lewis *et al.* (23), the highest growth rates for source organism of ASP, such as *Pseudonitzschia pungens* f. *multiseries,* were observed at 20 and 25°C. The highest stationary phase cell concentrations occurred at 5 to 15°C and decreased at 20 to 25°C. From this study, it is supposed ASP will be occurred from late of spring to early of summer. The toxin was found mainly at northern coast of England, western Bay of Fundy, Canada, and Pacific coast of America. During the period of monitoring, DA was detected at trace levels in 3 surf clam and 1 pink butterfly shell with 1.94-4.13 μg/g. The generally applied guideline value of 20 mg DA/kg

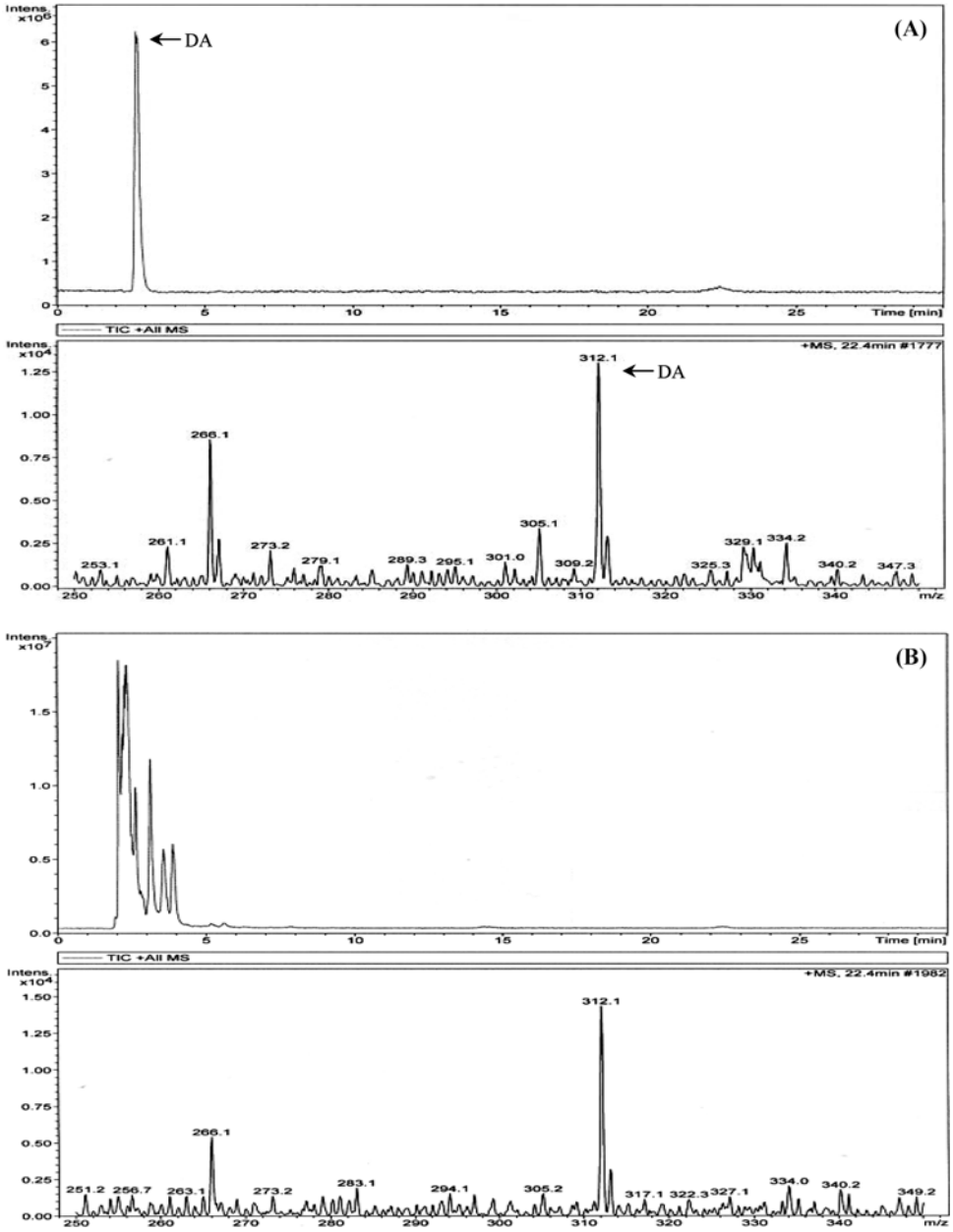

Fig. 5. Chromatogram of DA from reference standard with detection using LC–ESI-MS (MS). Retention time of DA was 2.69 min and the MS spectrum generated by targeting the *m/z* 312 ion of DA (A). Chromatogram and MS spectrum of DA from surf clam (B).

mussels is derived from an ASP incident in Canada (Prince Edward Island) and is taken on by several other countries. The guideline level of 20 mg DA/kg is equal to an intake of 0.03 to 0.1 mg DA/kg body weight/person with a body weight of 60 kg assuming that consumption of mussels is between 100 and 300 g/meal. The epidemiological data used to derive the guideline value, revealed mild gastrointestinal effects in humans at 1 mg DA/kg body weight. No observed adverse effect level (NOAEL) of amnesic shellfish poisoning (ASP) is 0.2-0.3 mg DA/kg body weight, while the lowest observed adverse effect level (LOAEL) of ASP was 0.9-2.0 mg DA/kg body weight and serious intoxications were recorded at 1.9 to 4.2 mg DA/kg body weight (24). There are no shellfishes exceeding the regulatory limit of 20 μg DA/g in this study and these did not constitute a threat to human health. Table 2 summarizes the data from this monitoring of DA in shellfish sold at fish market in Korea. Blue mussels were responsible for the initial fatal outbreak of ASP in Canada and very high levels of DA (up to 350 μg/g) were found in samples from Prince Edward Island (25). In late October and November 1991, razor clams (*Siliqua patula*) living in the surf zone on Pacific coast beaches in Washington and Oregon contained DA at levels as high as in the edible parts (i.e., foot, siphon, and mantle) as high as 154 μg/g (wet weight). Therefore recreational and commercial harvest of the clams was forbidden in America. In Japan, from 1991 onwards, ASP toxin screening of cultured bivalves and of diatoms has been carried out. DA has not been detected in industrially important shellfish (26). In Ireland, very high

Table 2. Domoic acid (DA) amount in shellfish purchased from fish market in Korea from March 2006 to October 2007 by LC–ESI-MS

Shellfish species	Collected time	Collected area	DA amount (μg/g)
Surf clam (imported)	July 2006	Seoul	4.17±0.04
Pink butterfly shell (imported)	July 2006	Seoul	3.04±0.10
Surf clam (domestic)	July 2006	Daejeon	2.05±0.24
Surf clam (domestic)	July 2006	Daegu	1.93±0.11

concentrations of DA, up to 3,000 μg/g in scallop hepatopancreas, were detected in December 1999. DA was detected in scallops from production areas on all Irish coasts. In other species no DA was detected (27).

After the 1987 ASP outbreak, the Canadian authorities imposed an action limit for DA in mussels of 20 μg DA/g mussel flesh, which when exceeded would result in closure of shellfish harvesting areas. This action limit was derived from a retrospective estimation of level of DA in mussels, which had caused illness in some consumers during the ASP outbreak (200 μg DA/g of mussel flesh) and incorporation a 10 fold safety factor (28). The action limit employed by Canada has been adopted elsewhere and is the limit enforced in the European Union, United States of America, New Zealand, and Australia for DA in a variety of shellfish species.

From this study, we found that DA has not been detected in industrially important shellfish, such as blue mussel, oyster, and scallop in Korea. However, the monitoring of ASP should be continued to prevent food poisoning by ASP and to protect human health.

Acknowledgments

This research was supported by grant from Korea Food & Drug Administration (07052-34) and Brain Korea 21 Program of the Ministry of Education Science and Technology.

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