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LC-MS/MS method for the detection of multiple classes of shellfish toxins

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Abstract: Marine shellfish toxins are seafood safety problems of global concern. Herein the analysis of six shellfish toxins, regulated by European Union, with one single run by LC-MS/MS with acidic mobile phase was developed. After 80% methanol extraction of the shellfish toxins, the crude extract was subjected to HLB SPE cleanup before LC-MS/MS analysis. The method was validated according to Commission Decision 2002/657/EC. For azaspiracid-1 (AZA1), domoic acid (*DA*), dinophysistoxin-1 (DTX1), okadaic acid (*OA*), pectenotoxin-2 (PTX2), and yessotoxin (YTX) toxins the recovery rate was 99.4, 92.7, 114.1, 90.2, 115.2 and 87.8%, respectively. The intra-day relative standard deviation (RSD) was less than 5% for all of the shellfish toxins except *DA*. The inter-day RSD was less than 5% for AZA1, DTX1, PTX2, YTX, 7.85% for *DA*, and 14.63% for *OA*. The decision limit (CC_α) and detection capability (CC_β) for AZA1 were 13.6 and 14.8 ppb; for *DA* they were 1883 and 2051 ppb; DTX1 12.3 and 13.4 ppb; *OA* 8.0 and 8.7 ppb; PTX2 12.1 and 13.2 ppb; YTX 36.9 and 40.1 ppb.

Keywords: acidic mobile phase; diarrheic shellfish toxin; LC-MS/MS

Abbreviations: AZA1 – azaspiracid-1; CCα – decision limit; CCβ – detection capability; CRM – certified reference material; DA – domoic acid; DTX1 – dinophysistoxin-1; HAB – harmful algal bloom; OA – okadaic acid; PTX2 – pectenotoxin-2; RSD – relative standard deviation; YTX – yessotoxin

Shellfish are low saturated-fat, high-protein food with rich essential minerals (Prato *et al*. 2019). Due to global warming and anthropogenic activity, the occurrence of harmful algal bloom (HAB) has increased in many parts of the world (Botana 2016). Consumption of seafood contaminated by phycotoxins during HAB incidence would cause severe food poisoning and raise consumer concerns (Nicolas *et al*. 2017). The EU legislated the maximum permitted lipophilic toxin level of 160 µg/kg in okadaic acid (OA) equivalents for OA, dinophysistoxin (DTX) and pectenotoxin (PTX) together; 160 µg/kg for azaspiracid-1 (AZA), 1 mg/kg for yessotoxin (YTX) and 20 mg/kg for domoic acid (DA) (Alarcan *et al*. 2018).

Reducing the risk of shellfish poisoning requires routine monitoring of complex shellfish toxin profiles to provide an early warning of harmful algal toxin contamination (Rodríguez *et al*. 2017). Previously, shellfish toxins were checked by mouse bioassay, but it was not very specific and sensitive compared to LC-MS/MS (Suzuki *et al*. 2018).

For multiple shellfish toxin analysis, several LC/MS methods have been developed using acidic (McNabb *et al*. 2005; Braña-Magdalena *et al*. 2016), neutral (Stobo *et al*. 2005; McCarron *et al*. 2011),

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and basic (Gerrsen *et al*. 2009) pH mobile phases. Although the LC condition was important, proper sample cleanup was also important for successful LC-MS/MS analysis. These *et al*. (2009) found that SPE could eliminate the matrix effect in LC-MS/MS analysis of multiple shellfish toxins.

In the present study, the analysis of six EU regulated shellfish toxins with one single run by LC-MS/MS with acidic mobile phase is developed. After methanol extraction of the shellfish toxins, the crude extract was subjected to HLB SPE cleanup before LC-MS/MS analysis. The method was validated according to the guidelines given in Commission Decision 2002/657/EC.

MATERIAL AND METHODS

Reagents and chemicals. Water was deionized and passed through a water purification system to specific resistance greater than 18.0 MΩ/cm. Acetonitrile and methanol were of HPLC grade and were purchased from Tedia (USA). *n*-Hexane, ammonium acetate and formic acid for HPLC were purchased from Sigma-Aldrich (USA). OA (CRM-OA-c 14.3 ± 1.5 µg/ml), DA (CRM-DA-f 101.8 ± 2.1 µg/ml), PTX2 (CRM-PTX2 8.6 × 0.3 µg/ml), azaspiracid-1 AZA1 (CRM-AZA1 1.24 ± 0.07 µg/ml) were purchased from the National Research Council, Institute for Marine Biosciences (NRC-CNRC) (Canada). YTX (7.91 ± 0.64 µg/g) and DTX1 (2.55 ± 0.20 µg/g) were purchased from Laboratorio Cifga S.A. (Spain). From the above certified reference material (CRM), series of standard solutions were prepared. Five concentration levels (10, 20, 50, 100, and 200 ppb) of AZA, DTX, OA, and pectenotoxin certified reference material (PTX CRM) solution were added into an uncontaminated *Pseudocardium sachalinense* shellfish extract as calibration solutions. Five concentration levels (1, 2.5, 5, 10, and 20 ppm) of domoic acid certified reference material (DA CRM) solution were added into an uncontaminated *Pseudocardium sachalinense* shellfish extract as calibration solutions. Five concentration levels (50, 75, 100, 250, 500 ppb) of yessotoxin certified reference material (YTX CRM) solution were added into an uncontaminated *Pseudocardium sachalinense* shellfish extract as calibration solutions.

All the shellfish samples came from self-controls that industries carried out before selling their products and were collected by the Bureau of Standards, Metrology and Inspection (Taiwan).

Instruments. HPLC analysis was performed on a system of Agilent Technologies 1200 series HPLC. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) data were acquired on AB SCIEX QTRAP 5500 (Applied Biosystems, Canada) triple quadrupole tandem mass spectrometer. Chromatography was carried out on an Agilent Poroshell EC-C18 column (Agilent, Germany). Hettich Mikro 120 (Hettich, Germany) was employed as centrifuge. The vortex mixer was Vortex Mixer VM-1000 (Yihder Co., Ltd., Canada).

Sample preparation. The sample of shellfish flesh was homogenized by using a blender. Methanol extraction was performed in duplicate by weighing 2 g of homogenized sample into a 50-ml plastic centrifuge tube to which 4 ml of 80% methanol/water (80/20, v/v) was added. The extracts were vortexed for 5 min and centrifuged at 4000 rpm for 5 min. The two supernatant extracts were combined and defatted twice with 5 ml *n*-hexane. After shaking for 5 min, take the bottom layer and blow dry with nitrogen to 3 ml as a sample solution for SPE cleanup.

The sample solution was loaded on an Oasis HLB SPE cartridge (60 mg, 3 ml) which was previously conditioned with 3 ml methanol and equilibrated with 3 ml water. The cartridges were washed by 3 ml water and 3 ml 10% methanol and eluted by 5 ml methanol. Collect the eluent, evaporate to dry with nitrogen at 45°C. Reconstitute the residue with 1 ml methanol and filter through 0.2 µm filter. After the SPE cleanup, the sample solution was ready for LC-MS/MS analysis.

Chromatography and mass spectrometric analysis. The shellfish toxins were separated on a 3.0 × 50 mm, 2.7 µm particle Agilent Poroshell EC-C18 column (Agilent Technologies, USA). The mobile phase consisted of water containing 2 mM ammonium acetate and 50 mM formic acid in channel A, and acetonitrile containing 2 mM ammonium acetate and 50 mM formic acid in channel B. The mobile phase flow rate was set at 1.0 ml/min and injection volume as 10 µl. The gradient elution was programmed to start with 30% B, increased to 90% B over 8 min, held for 4 min, decreased to 30% B over 1 min and held for 2 min to equilibrate the system before the next injection. Mass spectrum analysis was carried out using an electrospray ionization (ESI) method on AB SCIEX QTRAP 5500 (Applied Biosystems, Canada) triple quadrupole tandem mass spectrometer. The mass operation parameters were set as follows: curtain gas, 10 psi; collision gas, medium; ion spray voltage (*IS*), 5500 V; temperature (TEM), 400°C; ion source gas 1 (GS1), 50 psi; ion source gas 2 (GS2), 50 psi; dwell time,

https://doi.org/10.17221/125/2018-CJFS

0.1 sec; scan type, multiple reaction monitoring mode (MRM). For each toxin two transitions were monitored using the settings in brackets: DA 312.5.5 > 294.2 (DP: 70.3 V, CE: 22.0 eV) and 312.5 > 266.5 (DP: 70.3 V, CE: 26.0 eV), PTX2 876.7 > 823.8 (DP: 137.7 V, CE: 36.9 eV) and 876.5 > 551.8 (DP: 137.7 V, CE: 37.0 eV), AZA1 842.7 > 824.6 (DP: 110 V, CE: 43.0 eV) and 842.7 > 672.7 (DP: 110 V, CE: 69.0 eV) for the positive mode and OA 803.5 > 563.2 (DP: –157.0 V, CE: –56.6 eV) and 803.5 > 255.5 (DP: –157.0 V, CE: –68.2 eV), YTX 1141.4 > 1061.6 (DP: –119.0 V, CE: –48.6 eV) and 1141.4 > 855.4 (DP: –119.0 V, CE: –105.0 eV), DTX1 817.6 > 255.2 (DP: –220.0 V, CE: –69.5 eV) and 817.6 > 113.1 (DP: –220.0 V, CE: –94.4 eV) for the negative mode. The quantifier ion transition for DA was 312.5 > 266.5, PTX2 876.7 > 823.8, AZA1 842.7 > 824.6, OA 803.5 > 255.5, YTX 1141.4 > 1061.6, DTX1 817.6 > 255.2, respectively. The qualifier ion transition for DA was 312.5.5 > 294.2, PTX2 876.5 > 551.8, AZA1 842.7 > 672.7, OA 803.5 > 563.2, YTX 1141.4 > 855.4, DTX1 817.6 > 113.1, respectively. The structures of these mass fragments are reviewed in detail by Suzuki (2018). The total chromatogram for the shellfish toxins in this study is shown in Figure 1. The observed MRM chromatograms for AZA1, DA, DTX1, OA, PTX2 and YTX are shown in Figure 2A–F.

Method validation. According to Commission Decision No. 2002/657/EC regulation, the confirmatory analysis of food contaminants must meet the qualitative and quantitative performance criteria. The validation scheme in the present study was adopted from Verdon *et al.* (2007a). Three triplicates, at each of the five spiking levels (10, 20, 50, 100, and 200 ppb) into uncontaminated shellfish samples were analysed for AZA, DTX, OA, and PTX. Three triplicates, at each of the five spiking levels (1, 2.5, 5, 10, and 20 ppm) into uncontaminated shellfish samples were analysed for DA. Three triplicates, at each of the five spiking levels (50, 75, 100, 250, and 500 ppb) into uncontaminated shellfish samples were analysed for YTX. Calibration curves of each toxin for three triplicates were obtained for three days to calculate the CC_α and CC_β levels of the shellfish toxins. Qualitative performance was evaluated through retention time, identification point, and ion ratio on three separate days. The quantitative parameters were assessed through linearity, accuracy and precision, decision limit CC_α and detection capability CC_β. The decision limit CC_α and detection capability CC_β were calculated according to ISO Standard 11843 (Verdon *et al.* 2007b).

RESULTS AND DISCUSSION

Validation of qualitative performance. The qualitative performance was validated by retention time, identification point (*IP*), and ion ratio. The relative deviations of the chromatographic retention times for all the toxins were within ± 2.5% tolerance of 657/2002/EC. Two MRM transitions were monitored for each of the toxins as described in the chromatographic analysis section, so four IPs were earned and

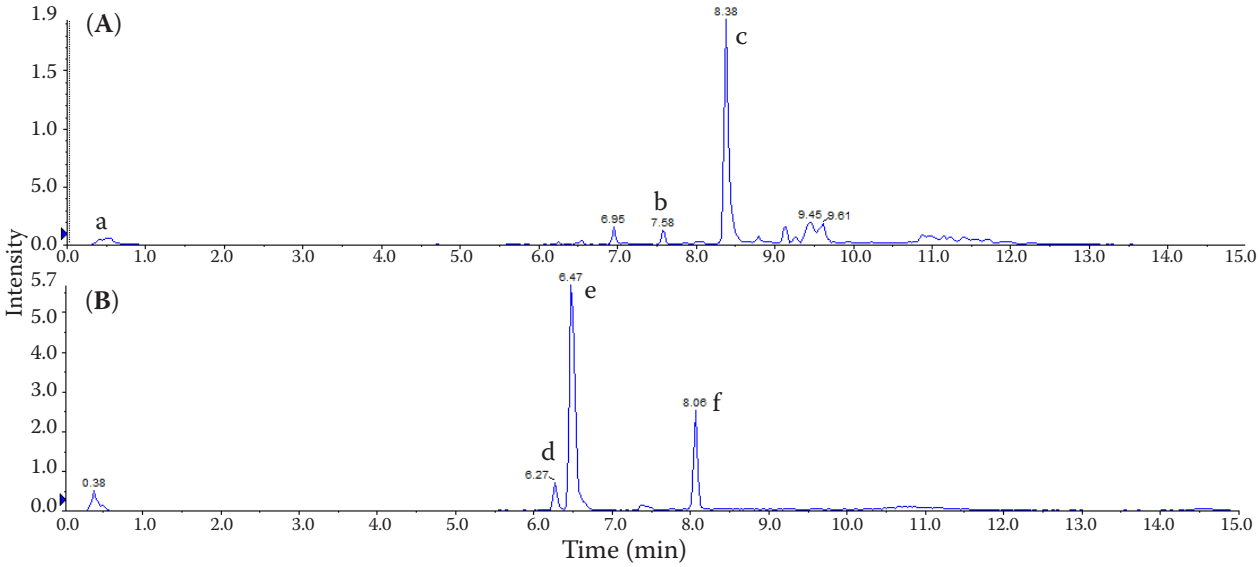

Figure 1. Total ion chromatograms (TICs) of toxin reference materials: positive ionization (**A**); negative ionization (**B**)
a – domoic acid (*DA*) 500 (ng/ml); b – pectenotoxin-2 (PTX2) (10 ng/ml); c – azaspiracid-1 (AZA1) (10 ng/ml); d – okadic acid (*OA)* (10 ng/ml); e – yessotoxin (YTX) (50 ng/ml); f – dinophysistoxin-1 (DTX1) (10 ng/ml)

https://doi.org/10.17221/125/2018-CJFS

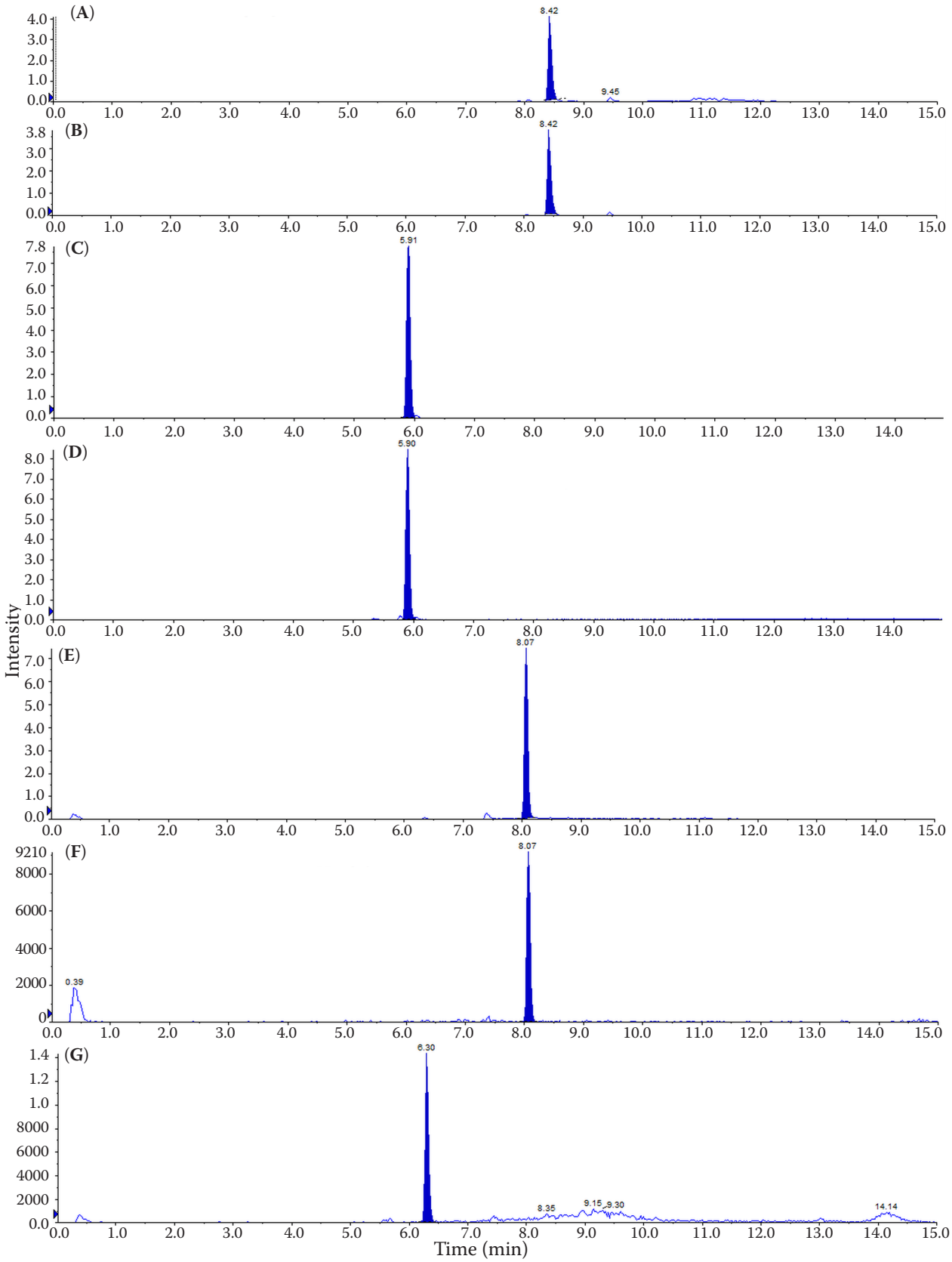

Figure 2. MRM chromatograms of shellfish toxins: AZA1 quantitative (**A**) and qualitative ion (**B**); DA quantitative (**C**) and qualitative ion(**D**); DTX1 quantitative (**E**) and qualitative ion (**F**); OA quantitative (**G**) and qualitative ion (**H**); PTX2 quantitative (**I**) and qualitative ion (**J**); YTX quantitative (**K**) and qualitative ion (**L**); *For abbreviations see Figure 1

https://doi.org/10.17221/125/2018-CJFS

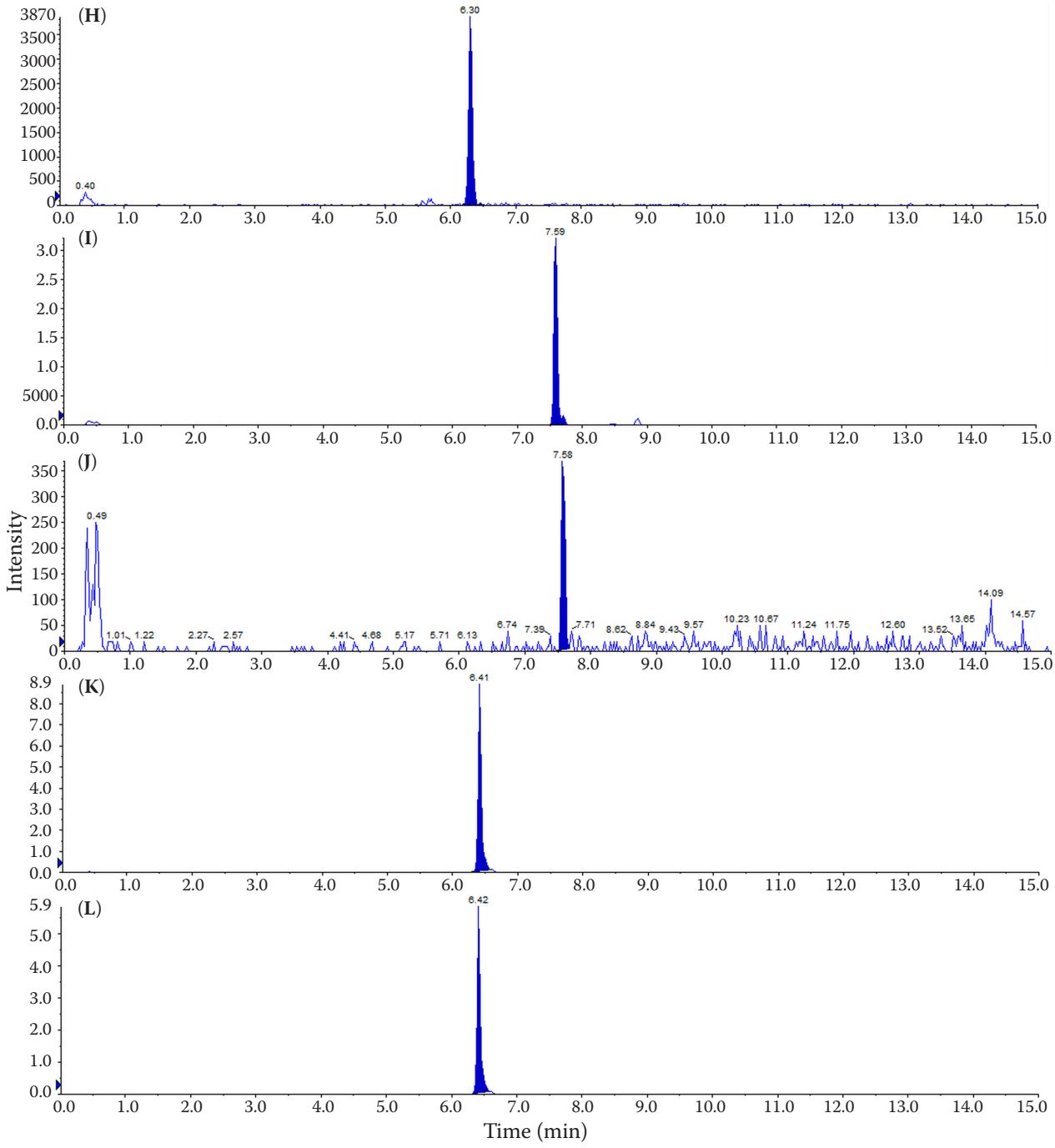

Figure 2. To be continued

none of the toxins shared the same transitions. The ion ratio deviations observed for AZA, DA, DTX, OA, PTX and YTX were all within the 657/2002/EC requirements for ion ratio.

Linearity of calibration curve. The linearity of the chromatographic response was evaluated by calibration curves in triplicate for three days using 5 calibration concentration points (10, 20, 50, 100, and 200 ppb) for AZA, DTX, *OA*, and PTX; 5 calibration concentration points (1, 2.5, 5, 10, and 20 ppm) in triplicate for three days for *DA*; and 5 calibration concentration points (50, 75, 100, 250, and 500 ppb) in triplicate for three days for YTX. Linear regression analysis was carried out by plotting the peak area of the shellfish toxin versus the shellfish toxin concentrations. Linear ranges, slopes, intercepts, and correlation coefficients are listed in Table 1. Good linearity was obtained as all correlation coefficients were ≥ 0.990.

https://doi.org/10.17221/125/2018-CJFS

Precision and accuracy. The precision and accuracy were checked by spiking 20 ppb AZA, 2500 ppb *DA*, 20 ppb DTX, 20 ppb *OA*, 20 ppb PTX, and 75 ppb YTX in a blank shellfish matrix in three replicates for three days. The extraction and cleanup steps were performed according to the procedure described above. The method precision and accuracy values are listed in Table 2. For azaspiracid-1 (AZA1), domoic acid (*DA*), dinophysistoxin-1 (DTX1), okadaic acid (*OA*), pectenotoxin-2 (PTX2), and yessotoxin (YTX) toxins the recovery rate was 99.4, 92.7, 114.1, 90.2, 115.2 and 87.8%, respectively. The recovery rate was between 80 and 120%, which was like in the previous study by Braña-Magdalena (2014). The procedure for the calculation of intra-day RSD and inter-day RSD was adopted from Kaloudis (2016) and listed in the supplement file. The intra-day RSD was between 0.93 and 8.43% for all six shellfish toxins. The inter-day RSD was less than 5% for AZA, DTX, PTX, YTX, 7.85 % for *DA*, and 12.95% for *OA*.

Table 1. Linear range, slope, intercept, and correlation coefficients of calibration curves

	Linear range (µg/kg)	Slope	Intercept	Correlation coefficient
AZA1	10–200	103 021	– 617 419	0.998
DA	1000–20 000	987	634 068	0.996
DTX1	10–200	12 030	– 34 551	0.998
OA	10–200	2588	1070	0.999
PTX2	10–200	6265	–24 169	0.998
YTX	50–500	3164	58 816	0.998

AZA1 – azaspiracid-1; DA – domoic acid; DTX1 – dinophysistoxin-1; OA – okadaic acid; PTX2 – pectenotoxin-2; YTX – yessotoxin

Table 2. Method recovery rate, intra-day RSD and inter-day RSD (n = 3)

	Spiked level	Recovery	Intra-day RSD	Inter-day RSD
	(ppb)		(%)	
AZA1	20	99.4	0.93	2.4
DA	2500	92.7	8.43	7.85
DTX1	20	114.1	1.50	2.97
OA	20	90.2	3.10	12.95
PTX2	20	115.2	2.81	2.97
YTX	75	87.8	4.02	3.55

*For abbreviations see Table 1

Table 3. Decision limit (CC_α), detection capability (CC_β), EU regulation and ESFA suggested limits (ppb)

Toxin	CC_α	CC_β	EU regulation[a]	EFSA 2009[b]
AZA	13.6	14.8	160	30
DA	1883	2051	20 000	4500
DTX	12.3	13.4	160	45
OA	8.0	8.7	160	45
PTX	12.1	13.2	160	120
YTX	36.9	40.1	1000	3750

*For abbreviations see Table 1; [a]Gerssen *et al*. 2010; [b]Paredes *et al*. 2011

Decision limit (CC_α) and detection capability (CC_β). The decision limit (CC_α), detection capability (CC_β), current EU regulation limit and European Food Safety Authority (EFSA) suggested regulation limit (Gerssen *et al*. 2010; Paredes *et al*. 2011) are listed in Table 3. For all the toxins, the decision limits (CC_α) are all less than one tenth of the current EU regulation level.

Analyses of real shellfish samples. A total of 20 shellfish samples supplied by the Bureau of Standards, Metrology and Inspection (Taiwan) from local industries that carried out self-controls were analysed by the developed LC-MS/MS method. Most of the samples were not contaminated with shellfish toxins. One *Placopecten magellanicus* was contaminated with 2.2 ppb PTX, one *Patinopecten yessoensis* was contaminated with 1.8 ppb PTX, 213 ppb YTX and 9.0 ppb DTX, and one *Ruditapes philippimarum* with 1.7 ppb PTX and 1.9 ppb OA. The detected toxin levels are all under the EU regulated limits. The results demonstrated the capability of the present LC-MS method for multiple shellfish toxin analysis.

CONCLUSIONS

The monitoring of six EU regulated shellfish toxins with one single run by fast polarity switching LC-MS/MS with acidic mobile phase is demonstrated to be acceptable and is validated according to Commission Decision 2002/657/EC. For azaspiracid-1 (AZA1), domoic acid (*DA*), dinophysistoxin-1 (DTX1), okadaic acid (*OA*), pectenotoxin-2 (PTX2), and yessotoxin (YTX) toxins the recovery rate was 99.4, 92.7, 114.1, 90.2, 115.2 and 87.8%, respectively. The intra-day RSD was less than 5% for all the shellfish toxins except for 8.43% for *DA*. The inter-day RSD was less than 5% for AZA, DTX, PTX, YTX, 7.85% *DA*, and 14.63% for *OA*. The decision limit (CC_α) and detection capability (CC_β) for AZA were 13.6 and 14.8 ppb; for *DA* they were 1883 and 2051 ppb; for

https://doi.org/10.17221/125/2018-CJFS

DTX 12.3 and 13.4 ppb; for *OA* 8.0 and 8.7 ppb; for PTX 12.1 and 13.2 ppb; for YTX 36.9 and 40.1 ppb.

The present LC-MS/MS method could not only meet the future ESFA requirement but also it could analyse six regulated shellfish toxins in a single run. In the future, further work should be done to reduce human labour in sample preparation and increase the sample throughput of the present method.

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