Intra-laboratory validation of a fast and sensitive UHPLC-MS/MS method with fast polarity switching for the analysis of lipophilic shellfish toxins

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Introduction

Recent changes in the European legislation require the establishment of LC-MS/MS methodologies for the analysis of lipophilic shellfish toxins. Due to the structures and the physico-chemical properties of the compounds, acquisition in positive and negative electrospray ionization (ESI) is required to gain the best sensitivity for all compounds.

An UHPLC-MS/MS method has been developed for the analysis of 14 marine toxins including 13 lipophilic toxins regulated by Commission Regulation (EC) No 853/2004. In this study we show the results of the in-house validation for an UHPLC-MS/MS method using acidic mobile phase conditions. The method includes 14 lipophilic marine toxins which have been acquired with Dynamic MRM enabled for fast polarity switching. For the OA group compounds static MRM and dynamic MRM as well as different precursor species have been compared for sensitivity, repeatability and robustness of the method. For several samples new triggered MRM acquisition mode has been applied for the confirmation of new toxin analogues.

Conclusions

- An UHPLC-MS/MS method has been developed for the analysis of 14 lipophilic marine toxins and offers the option to further increase the number of toxins due to the use of dynamic MRM with fast polarity switching. Due to the UHPLC separation analysis time is significantly reduced compared to methods previously validated for routine operation.
- The use of dynamic MRM improved the reproducibility of the method which resulted in improved correlation coefficients for the matrix matched calibrations.
- For most analyzed toxins matrix effects in the electrospray ionization has been observed. Whereas signal enhancement has been observed for the OA group compounds, all other toxins were suppressed by the matrix. The use of matrix matched standards fully corrected for both, signal enhancement and signal suppression.
- Good accuracy and reproducibility has been observed during a 3-day intra-laboratory validation study for CRM-DSP-MUSb and FDMT, respectively.
- Triggered MRM (tMRM) has been shown to be a valuable tool for unequivocal confirmation of toxins in complex matrices.

Results and Discussion

Figure 1 shows the chromatogram of a contaminated mussel extract analyzed with the UHPLC-MS/MS method using dynamic MRM (dMRM) enabled for fast polarity switching. OA group compounds were acquired in positive and negative ionization, YTX group compounds were acquired in negative ionization, and all other compounds were acquired in positive ionization. Peak heights have been normalized to 100 % for each chromatogram.

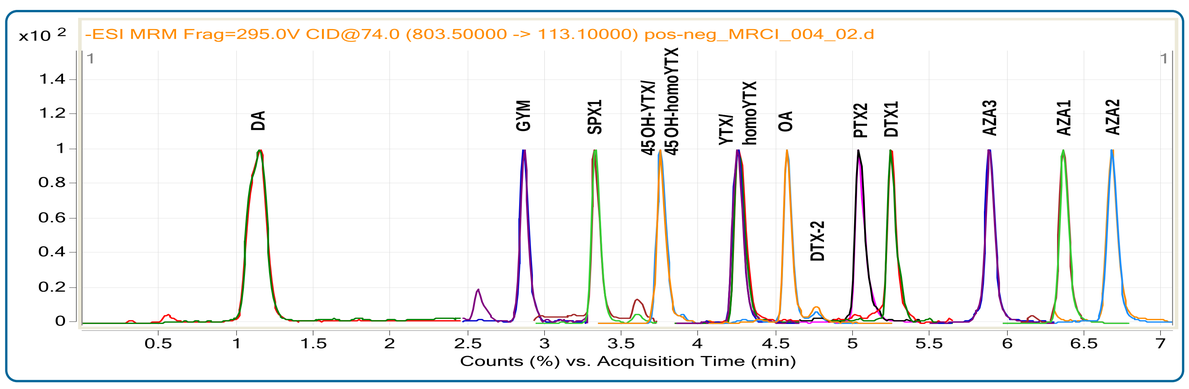


Figure 3: UHPLC-MS/MS chromatogram of a contaminated mussel extract analyzed with dMRM using fast polarity switching. Peak heights have been normalized for each chromatogram.

Comparative studies were carried out for the OA group toxins using static and dynamic MRM as well as for the different ion species measured in positive and negative mode. Figure 2 shows the calibration curves for OA for matrix matched standards for the negative mode (A) and for positive mode using [M+Na+]+ as the precursor species. Similar limit of detections (LODs) have been observed when using static MRM and dynamic MRM, however, when using dMRM reproducibility has been improved resulting in better correlation coefficients for the calibration curves. Sensitivities for the OA group compounds under acidic conditions have been slightly better when using positive ionization.

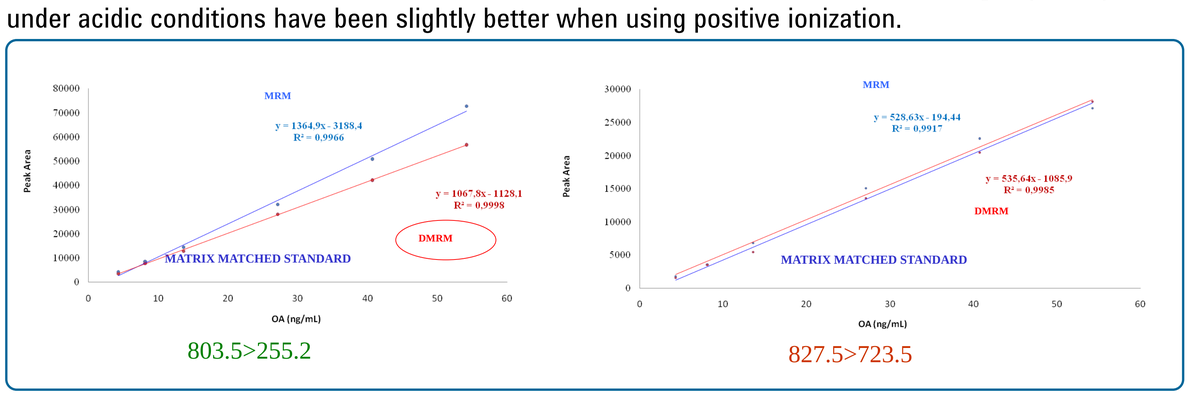


Figure 2: Calibration curve obtained for OA acquired with Static and Dynamic MRM with fast polarity switching in negative (A) and positive ESI (B).

Table 1: Theoretical LODs for OA group using positive and negative polarity using DMRM acquiring mode

OA positive	MeOH	0.23 ng/ml	**OA negative**	MeOH	0.43 ng/ml
	MMS	0.12 ng/ml		MMS	0.16 ng/ml
DTX2 positive	MeOH	0.06 ng/ml	**DTX2 negative**	MeOH	0.38 ng/ml
	MMS	0.08 ng/ml		MMS	0.01 ng/ml
DTX1 positive	MeOH	0.22 ng/ml	**DTX1 negative**	MeOH	0.5 ng/ml
	MMS	0.10 ng/ml		MMS	0.21 ng/ml

Matrix effects have been evaluated by comparing calibration curves prepared in methanol and blank mussel tissue (MUS-zero). Figure 3 shows the calibration curves for OA and AZA1 in methanol and in matrix matched standards. OA showed an enhancement of the signal in matrix matched standards while ion suppression in MMS was observed for the rest of lipophilic toxins. This also explains the lower LODs for the OA group toxins in matrix matched standards shown in table 1.

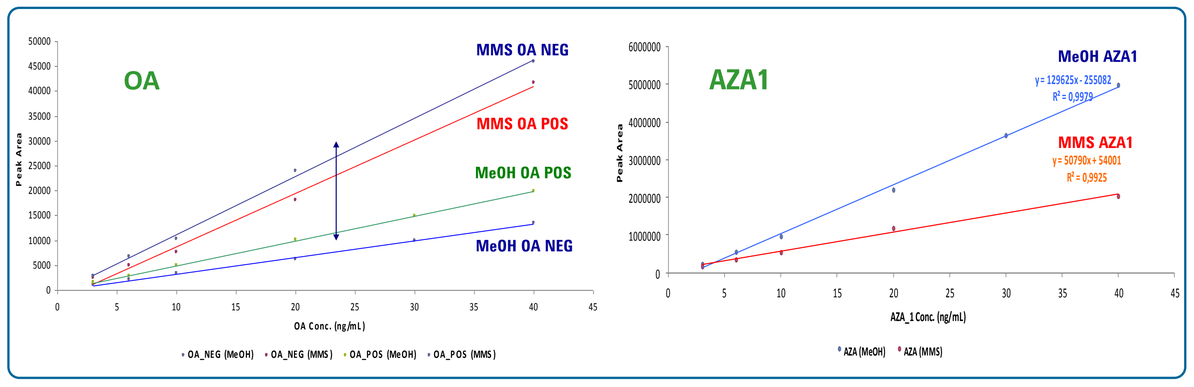
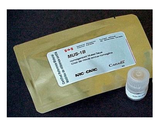


Figure 3 : Calibration curves for OA and AZA1 in methanol and matrix matched standard (MMS) (calibration range 3-40 ng/mL).

The accuracy of the method was evaluated using the certified reference material CRM-DSP-MUSb which has been measured within a 3-day intra-laboratory validation study. Table 2 shows the apparent recoveries for the OA group toxins obtained for positive and negative ionization. The recoveries have been within 97 to 118% of the certified values with good reproducibility. Within the 3-day intra-laboratory validation study a freeze dried mussel tissue sample has been measured with 6 replicates. Table 3 shows the reproducibility for all compounds included in the sample. The RSD values obtained were below 10% for all compounds with slightly higher variation for the OA group compounds when acquired in positive mode.

Table 2: Accuracy CRM-DSP-MUSb (n=6)



	DTX1^{-}	DTX1 $^{+}$	OA^{-}	OA $^{+}$
Rec (%)	103	97.9	108.9	118.1
RSD (%)	3.7	4.7	4.5	6.7

Table 3: RSDs for all lipophilic toxins included in the freeze-dried mussel tissue (FDMT) within the 3-day intra-laboratory validation.

	OA+	DTX1+	DTX2 +	OA -	DTX1 -	DTX2 -	YTX	PTX2	AZA1	AZA2	AZA3	SPX1
RSD (%)	6,8	15,3	4,6	2,7	2,3	10,0	2,4	2,5	0,9	3,8	5,2	1,4

Matrix interferences are a major challenge when analyzing shellfish for lipophilic toxins. Figure 4 shows the chromatogram of the FDMT for PTX2. In addition to the PTX2 peak (yellow) there is a second signal just 0.5 min separated with both, quantifier and qualifier present at right ion ratio. When using tMRM up to 8 additional transitions are triggered to acquire a comprehensive spectrum which can be searched against a user editable library. The comparison of the acquired spectra (top) with the library entry for PTX2 (bottom) is also shown in figure 4. Although the reference library match score is 77.9, it does not represent a perfect match for PTX2. However, due to the presence of all major fragment ions it well may be that this compound belong to the PTX family.

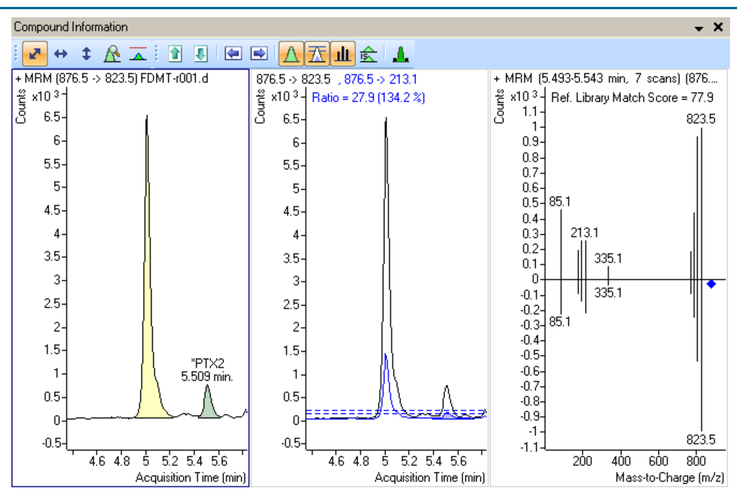


Figure 4: Additional information provided from tMRM library for PTX2 in FDMT. In addition to chromatograms for quantifier and qualifier traces a comparative spectrum for PTX2 in the mussel extract on top with the library entry for PTX2 below is shown.

Experimental

- **Sample preparation:**

  2 g shellfish tissue homogenate were extracted with 9 mL methanol by vortexing for 3 min. After centrifuging for 10 min @ 2000 g the supernatant was transferred to a 20 ml volumetric flask and the remaining tissue pellet was extracted again. The supernatant of the second extraction was combined with the first extract and the volumetric flask was filled up to the mark with methanol. The extract was injected as is or was hydrolyzed with 2.5 M NaOH @76°C for 40 minutes prior to injection to determine the total content of OA group toxins (EU-Harmonised-SOP-LIPO-LCMSMS_Version4)

- **UHPLC-MS/MS parameters:**

  – Agilent 1290 Infinity UHPLC system consisting of a G4220A Binary Pump, G4226A High performance autosampler, and G1316C Thermostated column compartment

  – Agilent G6460AA QQQ system (FW A.00.06.25); MassHunter Workstation B.04.01

  – Agilent ZORBAX RRHD SB-C8, 2.1 x 50 mm, 1.8 µm @ 40°C; Mobile Phase: (A) 2 mM ammonium formate + 50 mM formic acid and (B) 2 mM ammonium formate+ 50 mM formic acid in 95% acetonitrile, flow rate 0.4 ml/min; 0.5 min isocratic at 12% B, linear gradient to 50% B in 2.5 min, linear gradient to 90% B in 3.5 min, 1.5 min isocratic at 90% B, linear gradient to 12% B in 0.1 min. Total run-time 8 min.

  – Electrospray ionization, positive and negative MRM mode with two primary mass transitions per compound, Vcap ± 3 kV, Drying Gas 8 l/min @ 200°C, Nebulizer 45 psi, Sheath Gas 11 l/min @ 400°C, unit resolution

References

EU Commission Regulation (EC) No 853/2004; Official J. Eur. Union L226(2004)22
EU Harmonized SOP LIPO LCMSM Version 4 (http://www.aesan.msps.es/en/CRLMB/web/procedimientos_crlmb/crlmb_standard_operating_procedures.shtml; Accessed: 22-7-2011)

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