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Relative Molar Response of lipophilic marine algal toxins in liquid chromatography electrospray ionization mass spectrometry

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Abstract :

Rationale

Accurate quantitative analysis of lipophilic toxins by liquid chromatography-mass spectrometry (LC-MS) requires calibration solution reference materials (RMs) for individual toxin analogs. Untargeted analysis is aimed at identifying a vast number of compounds and thus validation of fully quantitative untargeted methods is not feasible. However, a semi-quantitative approach allowing for profiling is still required and will be strengthened by knowledge of the relative molar response (RMR) of analogs in liquid chromatography-mass spectrometry (LC-MS) with electrospray ionization (ESI).

Methods

RMR factors were evaluated for toxins from the okadaic acid (OA/DTXs), yessotoxin (YTX), pectenotoxin (PTX), azaspiracid (AZA) and cyclic imine (CI) toxin groups, in both solvent standards and environmental sample extracts. Since compound ionization and fragmentation influences the MS response of toxins, RMRs were assessed under different chromatographic conditions (gradient, isocratic) and MS acquisition modes (SIM, SRM, All-ion, target MS/MS) on low and high resolution mass spectrometers.

Results

In general, RMRs were not significantly impacted by chromatographic conditions (isocratic vs gradient), with the exception of DTX1. MS acquisition modes had a more significant impact, with PnTX-G and SPX differing notably. For a given toxin group, response factors were generally in the range of 0.5 to 2. The cyclic imines were an exception.

Conclusions

Differences in RMRs between toxins of a same chemical base structure were not significant enough to indicate major issues for non-targeted semi-quantitative analysis, where there is limited or no availability of standards for many compounds, and where high degrees of accuracy are not required. Differences in RMRs should be considered when developing methods that use a standard of a single analogue to quantitate other toxins from the same group.

Keywords : Marine toxins, Mussel, Screening, high-resolution tandem mass spectrometry, SPATT, reference materials

INTRODUCTION

Shellfish such as mussels, oysters and clams can accumulate toxins produced by microalgae. The consumption of contaminated shellfish thus represents a significant potential threat to human health. Lipophilic marine toxins include okadaic acid (OA), dinophysistoxins (DTXs), yessotoxins (YTXs), azaspiracids (AZAs), pectenotoxins (PTXs) and cyclic imines such as spirolides (SPXs) and pinnatoxins (PnTXs) (Figure 1).

Harmful algal blooms (HAB) are complex to manage due to their intermittent nature. While the conditions that promote the occurrence and distribution of individual species are not yet fully understood[1], climate change and anthropogenic activities are suspected to be significant factors[2]. Rising seawater temperature and acidity, and the transfer of ship ballast waters are factors contributing to the dispersion of toxin producing microalgae[3, 4]. For this reason some toxins have been described as "emerging toxins"[5]. The emergence may be from the discovery of new toxin analogs or the appearance of known toxins in regions where they had not previously been described[5]. LC-MS is the reference method for the analysis of lipophilic toxins in shellfish[6]. Reference materials (RMs) are vital for calibration in LC-MS to ensure accurate analytical results [7]. Considerable efforts have been made to produce certified standards for most of the regulated lipophilic toxins[8, 9]. Focus has primarily been on producing standards for toxins that are specifically regulated or linked to poisoning events, with a reduced availability for related toxins (e.g. PTX1 or the hydroxylated YTXs) or emerging classes of compounds[10-20]. In the absence of RMs for specific toxins the typical approach is to use a standard for a toxin analog from the same group[21]. This practice is acceptable if relative molar responses (RMRs) between analogs have been assessed. Studies evaluating response factors for toxins have been limited, but a recent study highlighted that RMRs for AZAs were dependent both on chromatographic and MS acquisition parameters[22-

[24]. There is lack of knowledge concerning response factors for other regulated toxins including OA/DTXs, the PTXs, the YTXs and the cyclic imines. Assessment of RMR factors would increase confidence in methods that are used to screen for the presence of analogs of known groups of toxins and for emerging toxins.

To date the majority of LC-MS methods designed for the analysis of lipophilic toxins are targeted, looking for known toxins using low resolution MS[25-28]. The use of high resolution mass spectrometry (HR-MS) for the analysis of phycotoxins has evolved and has recently shown good suitability for both untargeted[29-32] and targeted approaches [33-36] .

A significant strength of HR-MS is the ability to retrospectively analyze data in an untargeted fashion, enabling screening for a large number of toxins. However, using an untargeted approach in a semi-quantitative fashion is impeded by the limited availability of standards. Hence, knowledge on RMRs between analogs from the same toxin family is valuable.

In this study, RMRs were evaluated for toxin analogs from the OA/DTXs, YTXs, PTXs, AZAs and cyclic imines (CI) families, under different chromatographic and MS conditions. These evaluations were carried out on different LC-MS systems including low and high resolution mass systems.

EXPERIMENTAL

Chemicals and Reagents

Calibration solution CRMs were from the National Research Council Canada (Measurement Science and Standards, Halifax, NS, Canada): AZA1, -2, and -3, PTX2, OA, DTX1 and -2, YTX and homo-YTX, 13-desMe-SPX-C and PnTX-G. A blank mussel tissue reference material (CRM-Zero-Mus) and an in-house PTX11 calibration solution were also provided.

HPLC grade methanol, acetonitrile and formic acid (98%) were obtained from Sigma Aldrich (Steinheim, Germany) and Caledon (Georgetown, ON, Canada). Ammonium formate was acquired from Fluka (St. Louis, MI, USA). Milli-Q water was obtained in-house to 18MΩ/cm quality, using a Milli-Q integral 3 system (Merck Millipore, Guyancourt, France). For HR-MS acetonitrile and high purity water were obtained from Fisher Scientific (Illkirch, France).

Sample Preparation

Solid phase adsorption toxin tracking (SPATT) devices containing 300 mg of HP-20 resin were deployed over a 1-week period at Villefranche-sur-Mer bay (France). Extracts of these passive samplers were prepared and extracted with 15 ml of MeOH as described previously[37]. CRM-Zero-Mus was extracted using a protocol adapted from McCarron, et al. [38] by extracting 2 g portions with 5.5 mL volumes of MeOH (× 4). The supernatants were combined and brought to 25 mL into a volumetric flask.

Calibration solution CRMs of each toxin were used to prepare stock mixtures containing toxins from the same group at approximate equimolar concentrations. Working solutions with final concentrations were then obtained by diluting the stock solutions, using methanol, SPATT extracts, or mussel extracts. Final concentrations were: OA/DTXs mix: 0.73 nM OA, 0.77 nM DTX1 and 0.88 nM DTX2; AZA mix: 0.056 nM AZA1, 0.059 nM AZA2 and

0.054 nM AZA3; YTX mix: 0.22 nM YTX and 0.21 nM homo-YTX; PTX mix: 0.19 nM PTX2 and 0.17 nM PTX11; CI mix: 13-desMe-SPX-C and PnTX-G at 43 nM.

A separate mixed solution containing OA at 162 nM, DTX1 at 167 nM and DTX2 at 176 nM was prepared for experiments examining ESI source conditions.

Liquid Chromatography-Mass Spectrometry

Method 1

Experiments were performed using an Agilent 1200 LC system connected to a hydrid triple quadrupole-linear ion trap API4000 QTRAP mass spectrometer (Sciex, Concord, ON, Canada), equipped with a TurboV® electrospray ionization source. Chromatographic separation was realized on a Synergi MaxRP C_{12} (50 × 2 mm; 3 µm) (Phenomenex Inc., Torrance, CA, USA) column using a mobile phase composed of water/acetonitrile (62:38 v/v) containing 5 mM ammonium acetate at a flow rate of 0.3 mL/min and a column temperature of 20 °C[39]. Injection volumes were 5 µL.

The collision and source parameters, in positive ionization mode, were: 20 psi curtain gas, 4500 V ion spray (IS) voltage, 275 °C aux gas temperature, 50 psi pressure nebulizer and auxiliary gases, level 4 (pressure arbitrary units) collision-activated dissociation. Full scan spectra were acquired using a Q1 scan range of *m/z* 700-900, with source declustering potentials (DPs) of 20, 40 and 60 V. Selected ion monitoring (SIM) was conducted using DPs of 20 and 60 V, monitoring *m/z* 769.5, 787.5, 805.5, 822.5, 827.5, 843.5, 849.5 and 863.5 for OA and DTX2, and *m/z* 783.5, 801.5, 819.5, 836.5, 841.5, 857.5, 863.5, 877.5 for DTX1. In negative ion mode the equivalent source conditions were applied except for the IS (-4500 V), acquiring full-scan spectra with a Q1 range of *m/z* 700-900, with SIM using DPs of 20 and 60 V, monitoring *m/z* 803.5 for OA and DTX2, and *m/z* 817.5 for DTX1.

Method 2

Experiments were performed using an LC system (UFLC XR Nexera, Shimadzu, Japan) connected to a hydrid triple quadrupole-linear ion trap API4000 QTRAP mass spectrometer (Sciex, Concord, ON, Canada), equipped with a TurboV® electrospray ionization source. Chromatographic separation was realized on a Phenomenex Kinetex XB-C_{18} column (100 × 2.1 mm, 2.6 µm) maintained at 40 °C, using a mobile phase composed of (A) water and (B) 95% acetonitrile, both containing 2 mM ammonium formate and 50 mM formic acid. The elution gradient ran from 5 to 50 % B over 3.5 min, to 100% B over the next 5 min and was held for 2.5 min before re-equilibration. For isocratic runs the mobile phase was set to 60% B (7 min run) for all toxins except the cyclic imines, which were analyzed with 40% B during 7 min. All methods were operated at 0.4 mL/min with an injection volume of 3 µL.

The API4000 QTrap mass spectrometer was operated in SIM mode or in selected reaction monitoring (SRM) mode. Positive and negative acquisition experiments were established using the following source settings: curtain gas at 30 psi, ion spray at 5500 V (ESI^+) and -4500 V (ESI^-), Turbogas temperature of 550 °C, gas 1 and 2 at 40 and 50 psi, respectively, and entrance potential of 10 V.

Method 3

Analyses were performed using a UHPLC 1290 Infinity LC (Agilent Technologies) coupled to a 6540 UHD high-resolution quadrupole time-of-flight hybrid mass spectrometer (Q-TOF; Agilent Technologies) equipped with a Dual Jet Stream® ESI source. Chromatographic separation was carried out using the conditions described for *Method 2*. The instrument was operated in "All-ion" MS/MS mode and targeted MS/MS mode with a mass resolution of 25000-40000 Full Width at Half Maximum (FWHM). The "All-ion" MS/MS mode acquired a full scan acquisition over *m/*z 100 to 1700, with a collision energy of 40 eV and an

acquisition rate of 2 spectra/s per experiment (1 full scan and 1 MS/MS). Targeted MS/MS experiments were over a scan range *m/z* 50 - 1700, with a MS scan rate of 10 spectra/s and a MS/MS scan rate of 3 spectra/s. Three different CEs (20, 40 and 60 eV) were applied to the precursor ions.

The source temperature was 205°C with drying and sheath gas flow-rates of 5 L/min and 12 L/min, respectively. The sheath gas temperature was 355°C and the capillary and nozzle voltages were 3500 V and 500 V, respectively. Calibration was carried out continuously over the entire run time using reference masses *m/z* 121.0509 (purine) and *m/z* 922.0099 (hexakis phosphazine) and data were analyzed using MassHunter software (Agilent Technologies). A summary of acquisition parameters is displayed in Table 1.

Quantitative and Statistical Analyses

Mixed toxin solutions for AZAs, OA/DTXs, YTXs, PTXs, and CIs were separately injected three times per day on three different days (methods 2 and 3). RMR factors were determined as the ratio of the response of a compound to that of a reference compound. In this study, the reference compounds used were: AZA1 for AZA2 and -3, OA for DTX1 and -2, YTX for homo-YTX, PTX2 for PTX11 and 13-desMe-SPX-C for PnTX-G. RMRs were calculated using:

$$RMRi = \frac{Ai * Cj}{Aj * Ci}$$

where RMR*i* is the relative molar response of the investigated compound (*i*) related to the reference analogue; A*i* is the peak area of the investigated compound (*i*); A*j* is the peak area of the reference analogue; C*i* is the molar concentration of the investigated compound (*i*), and C*j* is the molar concentration of the reference compound.

Statistical evaluations were carried out using SigmaPlot 12.5. Comparison of response factors between different chromatographic, acquisition and mass spectrometry conditions was carried

out using the two-way repeated measures analysis of variance (ANOVA) or the t-test. Differences were considered significant at $p < 0.05$. As part of ANOVA tests, the normality and homoscedasticity of data was verified using Shapiro-Wilk and equal variance tests, respectively.

RESULTS and DISCUSSION

The ion chosen for quantitation in SIM mode, or for fragmentation in SRM mode, is an important consideration when evaluating response factors and can be significantly influenced by ESI source conditions. An experiment was conducted using a mixture of OA, DTX1 and DTX2 (*Method 1*). Full scans (Q1) were run in positive and negative mode to determine the distribution of charge for each toxin, i.e. which ion clusters show significant abundance. In positive ionization mode, a variety of adducts or water losses, resulting from in-source fragmentation, were observed for OA, DTX2 and DTX1 (Figure 2 and Table S1). The abundance of ion species was not the same for OA, DTX1 and DTX2. In negative ionization mode, the deprotonated molecular ion was the only characteristic ion observed.

The distribution of charge across ion clusters was affected by source conditions, in particular the DP setting. Response factors for DTX1 and DTX2 (relative to OA) were assessed at DPs of 20 eV and 60 eV (Figure 3). In positive mode differences in RMRs for DTX1 and DTX2 to OA were not statistically significantly different for several adducts (sodium, ammonium, and potassium). However, reliance on such adducts is not ideal for quantitative purposes. On the contrary, differences in RMRs between DP 20 and DP 60 for the molecular ion of DTX2 were statistically significant ($p < 0.05$). The same is true for the ions resulting from one water loss (for DTX1) and from two water loss (for DTX1 and DTX2). The Q1 scans also demonstrated the presence of multiple in-source water losses that varied by up to more than a factor of 2. DP is used to break up ion-clusters, including the stripping of residual water-molecules from the mobile phase; therefore possible water losses from polyether and hydroxylated toxin molecules must be evaluated and considered.

In negative mode (ESI^-) it appeared that DP had less influence on the RMRs for DTX1 and DTX2. Response factors were close to 1 for DTX1 and DTX2, irrespective of the DP value. Analysis of OA and DTXs in ESI^+ yielded more fragment ions, thus resulting in a lower

detection abundance for quantifying ions ($[M+H]^+$), compared to ESI^- for which only the deprotonated ion was observed, at a higher abundance. Some earlier LC-MS methods analyzed OA and DTXs in positive ionization mode[25, 40]. However, these toxins are now more frequently monitored in negative ionization mode[26, 28, 39] meaning that RMRs should not be a major issue for OA/DTXs under similar conditions. However, this example demonstrates that source parameters such as DP settings and temperatures should be considered when developing methods for toxins for which standards are not available. All other experiments conducted as part of this work focused on analysis of OA and DTXs in negative ion mode only.

Data from experiments conducted using the QTrap (*Method 2*) shows that differences in RMRs were generally in the range of 0.5 and 2, with the exception of PnTX-G in SRM mode (Figure 4). This shows that for screening purposes possible variations in response factors may not be hugely significant (i.e. orders of magnitude) and use of a representative toxin standard should provide reasonable semi-quantitative data. Using two-way repeated measures ANOVA, an overall comparison of all relative molar responses (Supporting information Table S2) obtained with *Methods 2 and 3* was carried out: isocratic *vs* gradient results were compared on one side and acquisition mode results on the other side. RMRs for specific toxins were not significantly different between gradient and isocratic conditions for most toxins, except for DTX1 and PnTX-G in SRM and SIM mode, and for AZA2 in SIM mode (Figure 4). The composition of the mobile phase in gradient elution can have a significant impact on ionization and therefore on response factors[41]. RMRs were generally close to 1 for gradient separations, when the retention time of the investigated compound was within 0.5 min of the reference compound. This was the case for homo-YTX compared to YTX (0.03 min RT difference) and DTX2 compared to OA (0.27 min RT difference) (Figure 5). The observed differences in RMR for OA and DTX1 are attributable to different ionization

yields as a result of different solvent compositions (ΔB% ~ 10 % in gradient mode) when the analyte arrives at the ESI source, rather than due to differences in MS acquisition parameters (Figure 4 and 5). In addition, the difference between RMR in isocratic and gradient chromatographic conditions was statistically significant ($p<0.05$) in both SRM and SIM acquisition mode. These results indicate that mobile phase composition can influence the ionization and therefore yield different relative responses (this was also the case for OA and DTX1 using *Method 3*; see Supporting Information Table S2).

RMRs for PnTX-G deviated most significantly, in particular in SRM mode (RMR >2 in some cases). The PnTX-G RMRs were determined relative to 13-desMe-SPX-C. Although PnTX-G and 13-desMe-SPX-C possess structural similarities as cyclic imines, the notable structural differences unsurprisingly had a significant impact on the RMRs. Differences in relative responses in SIM mode were low compared to SRM, but still significant in some cases showing that differences in source ionization of the cyclic imines is also a factor of variation.

RMR factors on the 6540 QToF (*Method 3*) were significantly different for DTX1, AZA2, and PnTX-G for mussel extract and AZA2 and PnTX-G for SPATT matrix (Figure 6 and Table S2). Hence, the additional difference in RMRs for AZA2 might be attributed to slightly different source design affecting ionization yields for SPATT and mussel matrices.

The sample matrix used did not seem to significantly impact the RMRs, with an exception for PnTX-G (Figures 4 and 6). However, as shown in a previous study[42], the nature of the matrix can have a significant bearing on ionization efficiency (Figure S1). Matrix effects can also be dependent on the chromatographic conditions.

From the results presented here it is clear that the acquisition mode (SIM, SRM) can significantly influence the relative response of toxins from the same group. On the API4000 QTrap (*Method 2*), this was particularly significant for PnTX-G, DTX1 and PTX11 (Figure 4). RMRs obtained in SRM acquisition mode deviated more from an equimolar response

than those acquired in SIM mode. While SIM mode is likely to be only affected by differences in ionization efficiency[43], SRM mode is additionally affected by efficiency in production of fragment ions. Therefore, larger differences observed in SRM mode are understandable when considering differential fragmentation as a result of collision-induced-dissociation. On the QToF (*Method 3*), RMRs obtained in All-ion acquisition mode were not significantly different (p>0.05, t-test) from those obtained in target MS/MS mode for all toxins, except for PnTX-G (Table S2). As PnTX-G does not have the same base structure as the reference compound, it is not surprising that the fragmentation efficiency is not quantitatively comparable; and this difference was consistent between low and high resolution mass spectrometry.

Thus, differences between low and high resolution mass spectrometry appear relatively insignificant and the selectivity benefits of HR-MS can be fully utilized for screening of unknowns. It is particularly encouraging that the 'All-ion' acquisition mode was minimally affected by differences in RMRs since this mode provides both full-scan and fragmentation HR-MS information on the compounds observed.

CONCLUSIONS

In the quantitative analysis of marine biotoxins by LC-MS many factors play a role in accuracy of results. The absence of standards for some marine biotoxins, in particular for 'emerging' classes of toxins, means that frequently a related analogue is used for quantitation and equal RMRs are assumed. RMR factors were evaluated for analogues belonging to several lipophilic toxin groups. Chromatographic conditions and MS acquisition parameters were investigated on different LC-MS systems. RMRs generally varied by a factor of 0.5 to 2 between toxins from the same group under different LC and MS conditions. A significant exception was PnTX-G which was evaluated relative to 13-desMe-SPX-C. Although both these toxins are related cyclic imines, their structural differences resulted in significantly deviated response factors due to differences in ionization and fragmentation. The results for the AZA group were consistent with those obtained by other authors [24, 44]. Interestingly, RMRs were in general impacted less by chromatography conditions (*e.g.* gradient or isocratic) than by MS acquisition parameters. It was also shown that RMRs in SPATT matrix were similar to those in standard solutions, illustrating a benefit of passive samplers for non-targeted screening of marine biotoxins.

LC-MS is an extremely powerful and important method for algal toxin detection and quantitation. Toxin standards are available for most regulated analogs, and it is important that they are used when available to ensure accuracy of results. In cases where it is not possible to obtain certified calibrants for specific toxins possible differences in RMRs should be considered when using related toxin analog standard to generate information on toxin occurrence. The approach presented here and the knowledge of RMR ranges within toxin groups is of value when considering semi-quantitative screening of toxins using untargeted approaches such at high resolution mass spectrometry.

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Table 1: MS acquisition parameters for QTof (method 3) and QTrap (method 2) experiments.

			QToF		QTrap			
Toxin	**Ionization mode**	**Ion**	**Mass (*m/z*)**	**Fragmentor (V)**	**SIM (*m/z*)**	**SRM transitions (*m/z*)**	**DP (V)**	**CE (eV)**
OA	ESI^-	$[M-H]^-$	803.4587	180	803.4	803.4/255.1*	-170	-62
						803.4/113.1		-92
DTX2	ESI^-	$[M-H]^-$	803.4587	180	803.4	803.4/255.1*	-170	-62
						803.4/113.1		-92
DTX1	ESI^-	$[M-H]^-$	817.4744	180	817.5	817.5/255.1*	-170	-68
						817.5/113.1		-92
YTX	ESI^-	$[M-H]^-$	1141.4717	180	1141.4	1141.4/1061.6*	-120	-48
						1141.4/855.6		-98
Homo-YTX	ESI^-	$[M-H]^-$	1155.4874	180	1155.6	1155.5/1075.6*	-120	-48
						1155.5/869.4		-98
AZA 1	ESI^+	$[M+H]^+$	842.5049	200	842.6	842.6/654.4*	116	69
						842.6/672.3		69
AZA 2	ESI^+	$[M+H]^+$	856.5206	200	856.6	856.6/654.4*	116	69
						856.6/672.4		69
AZA 3	ESI^+	$[M+H]^+$	828.4893	200	828.6	828.6/640.4*	116	69
						828.6/658.4		69
13-desMe-SPX-C	ESI^+	$[M+H]^+$	692.4521	200	692.6	692.6/164.2*	121	71
						692.6/444.3		53
PnTX-G	ESI^+	$[M+H]^+$	694.4677	200	694.4	694.4/164.1*	141	75
						694.4/458.3		55
PTX2	ESI^+	$[M+NH_4]^+$	876.5104	200	876.6	876.6/823.6*	91	31
						876.6/805.6		37
PTX11	ESI^+	$[M+NH_4]^+$	892.5053	200	892.6	892.6/821.5*	91	37
						892.5/839.5		31

(*): transition used for quantification

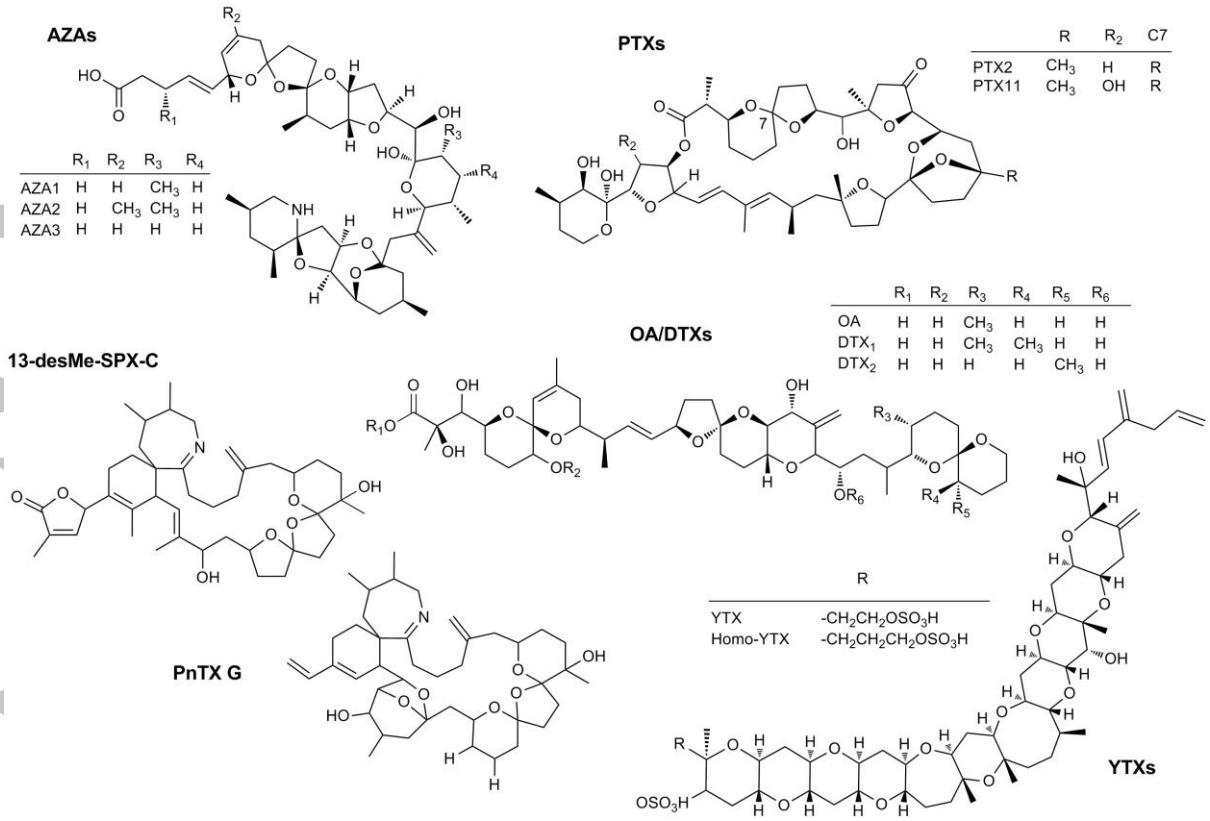

Figure 1: Structures of investigated toxins: AZA1-3; OA, DTX1 and -2; YTX and homo-YTX; PTX2 and 11; PnTX-G; and 13-desMe-C.

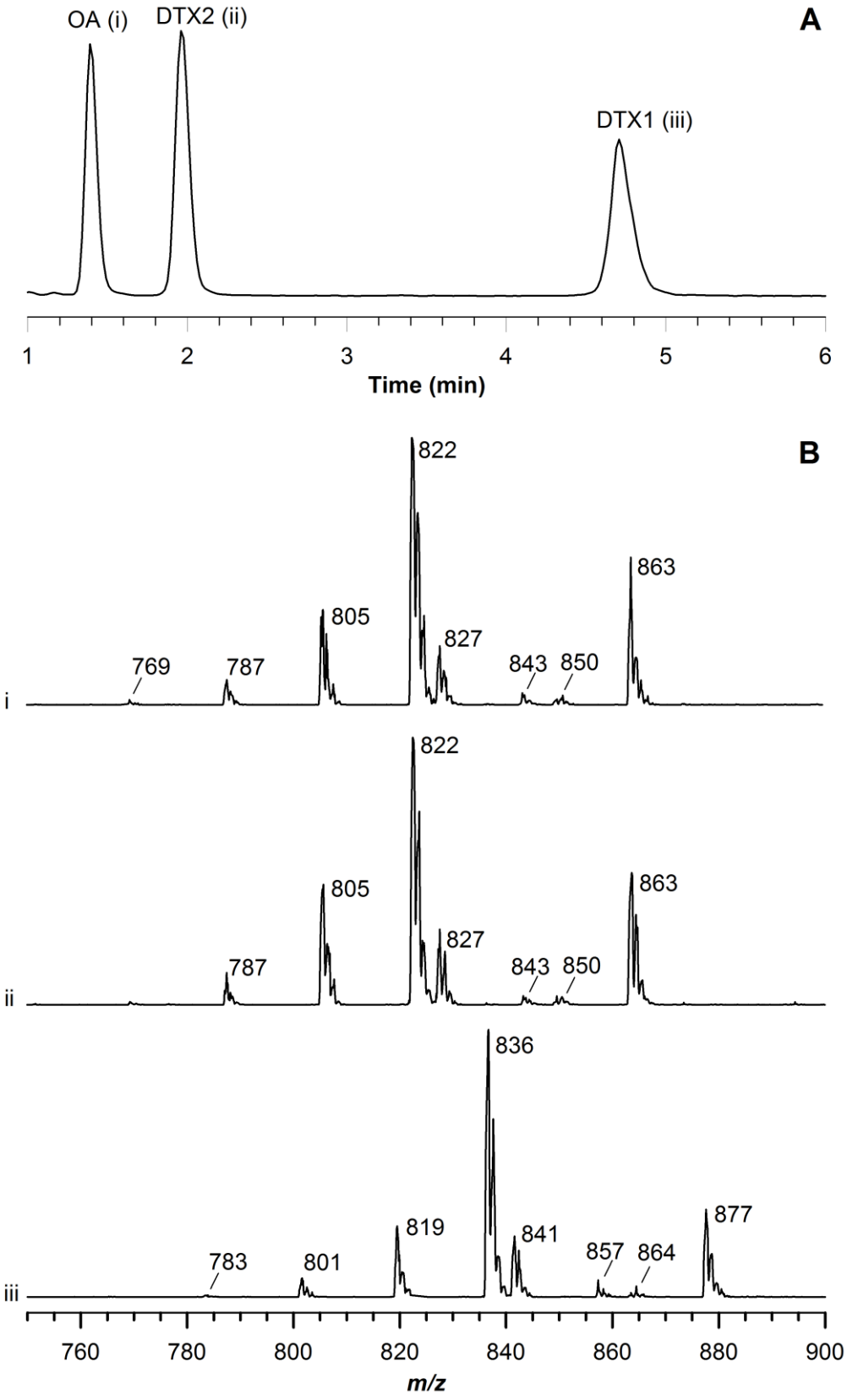

Figure 2: Positive ion mode total ion count (TIC) from LC-MS analysis (Method 1) of a mixture of OA, DTX1 and DTX2 (A), and extracted Q1 traces for the individual toxins (B).

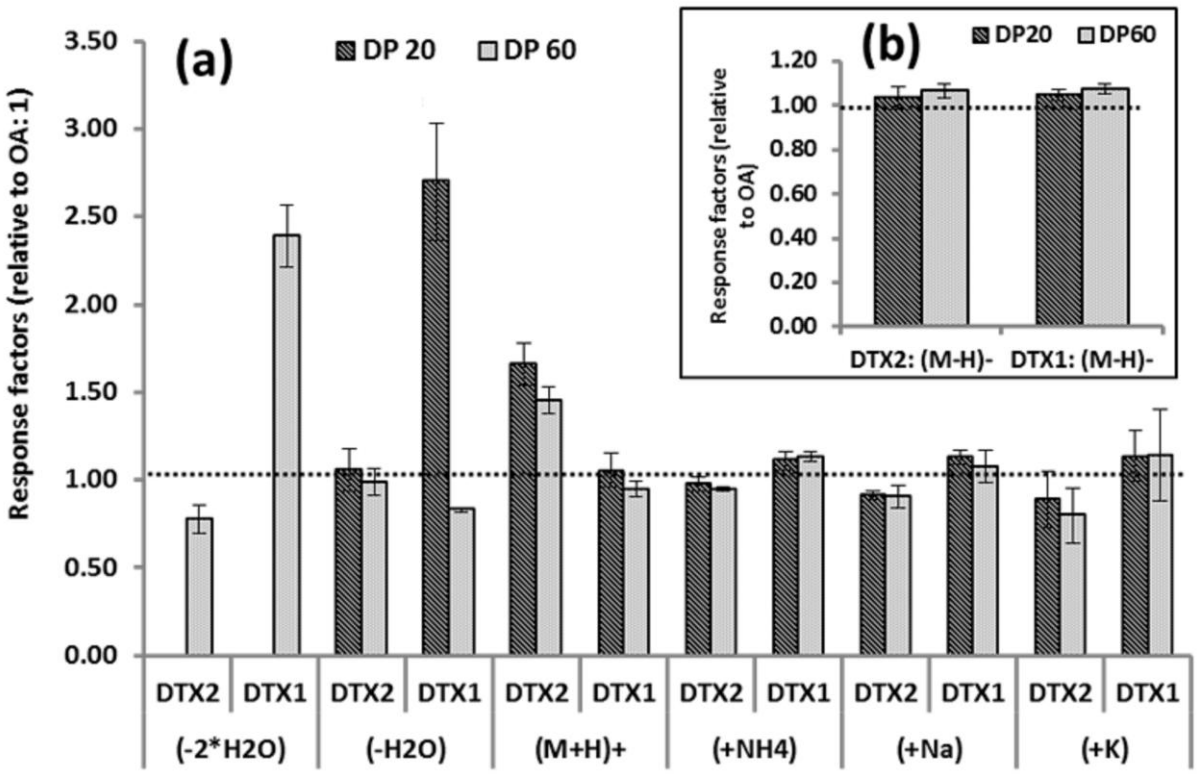

Figure 3: RMR factors ± SD (n=5) for different ions in quantitation of DTX1 and DTX2 relative to OA (Method 1), at DPs of 20 and 60, in (a) positive and (b) negative ionization modes.

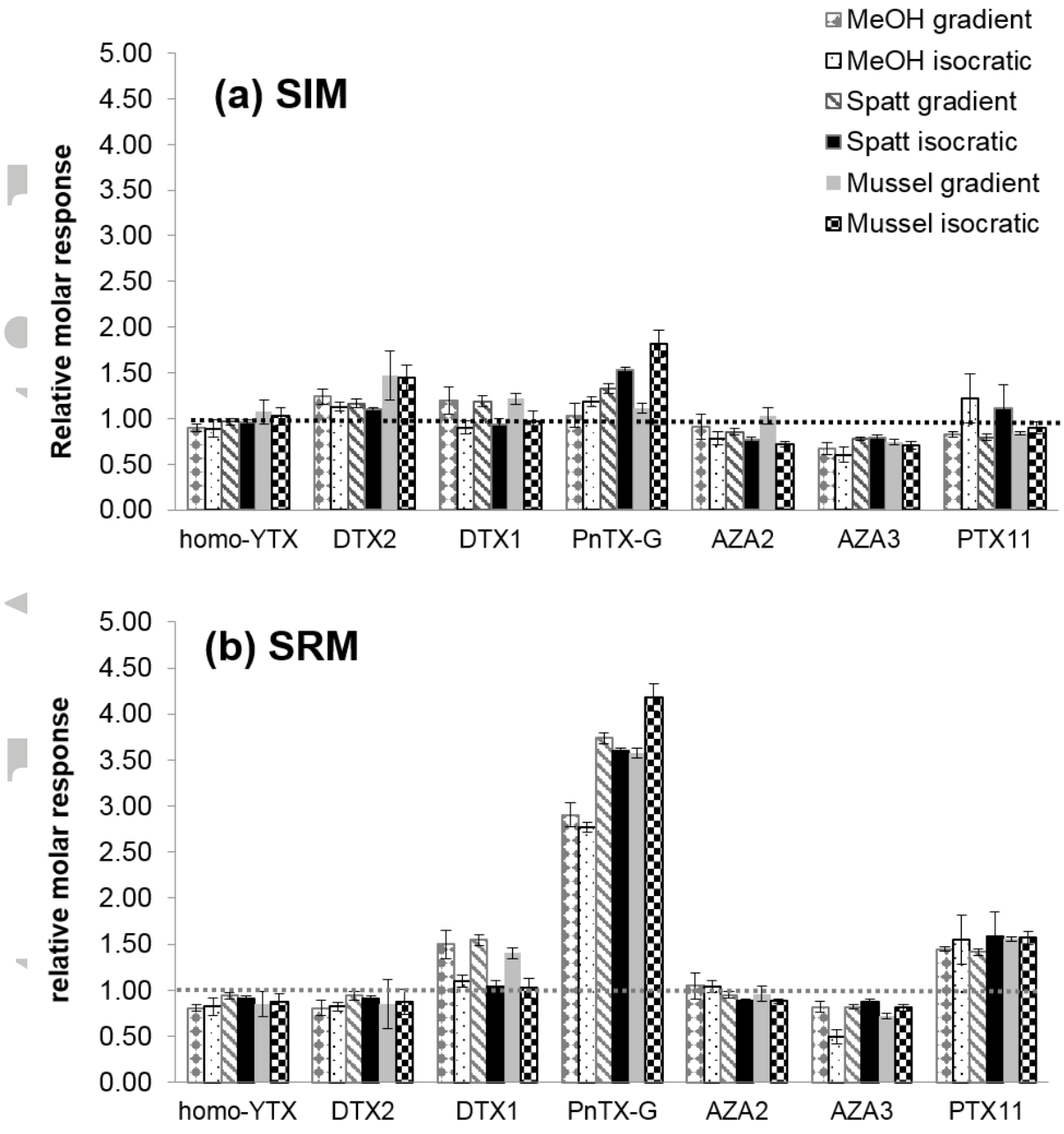

Figure 4: Comparison of RMR factors (± SD; n=9) (Method 2) for methanol, SPATT and mussel extracts, in isocratic and gradient conditions with (a) SIM acquisition mode and (b) SRM acquisition mode.

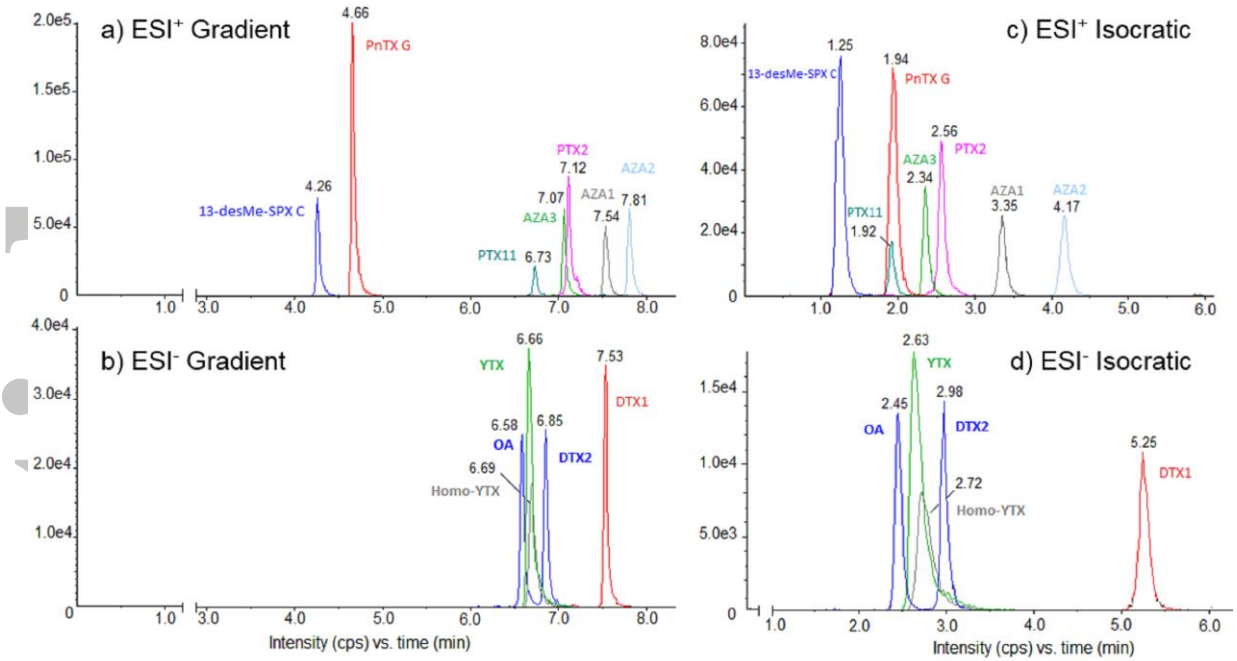

Figure 5: Reconstructed chromatograms with average (n=9) retention times (min) for OA/DTXs, YTXs, AZAs, CIs and PTXs under gradient (ESI+ (a); ESI- (b)) and isocratic conditions (ESI+ (c); ESI- (d)) using Method 2.

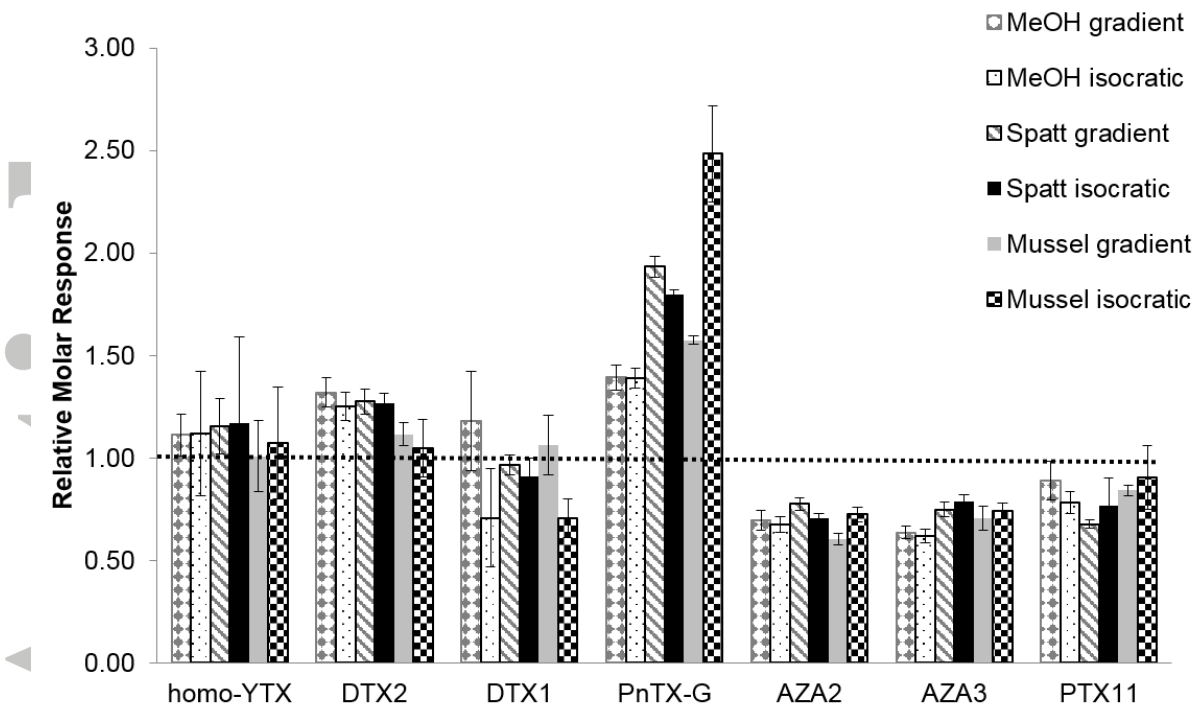

Figure 6: Comparison of response factors (RMRs ± SD; n=9) for homo-YTX, DTX1 and 2, PnTX-G, AZA2 and 3 and PTX11, in isocratic and gradient conditions (Method 3) in All-ion acquisition mode.