NRC·CNRC

NRC Publications Archive
Archives des publications du CNRC

Non-target analysis and stability assessment of reference materials using liquid chromatography–high-resolution mass spectrometry

Wright, Elliott J.; Beach, Daniel G.; McCarron, Pearse

This publication could be one of several versions: author's original, accepted manuscript or the publisher's version. / La version de cette publication peut être l'une des suivantes : la version prépublication de l'auteur, la version acceptée du manuscrit ou la version de l'éditeur.
For the publisher's version, please access the DOI link below./ Pour consulter la version de l'éditeur, utilisez le lien DOI ci-dessous.

Publisher's version / Version de l'éditeur:
https://doi.org/10.1016/j.aca.2022.339622
Analytica Chimica Acta, 1201, C, pp. 1-9, 2022-02-17

NRC Publications Archive Record / Notice des Archives des publications du CNRC :
https://nrc-publications.canada.ca/eng/view/object/?id=dda5ba29-9b72-4494-bfe0-3b54aec6913e
https://publications-cnrc.canada.ca/fra/voir/objet/?id=dda5ba29-9b72-4494-bfe0-3b54aec6913e

Access and use of this website and the material on it are subject to the Terms and Conditions set forth at
https://nrc-publications.canada.ca/eng/copyright
READ THESE TERMS AND CONDITIONS CAREFULLY BEFORE USING THIS WEBSITE.

L'accès à ce site Web et l'utilisation de son contenu sont assujettis aux conditions présentées dans le site
https://publications-cnrc.canada.ca/fra/droits
LISEZ CES CONDITIONS ATTENTIVEMENT AVANT D'UTILISER CE SITE WEB.

Questions? Contact the NRC Publications Archive team at PublicationsArchive-ArchivesPublications@nrc-cnrc.gc.ca. If you wish to email the authors directly, please see the first page of the publication for their contact information.

Vous avez des questions? Nous pouvons vous aider. Pour communiquer directement avec un auteur, consultez la première page de la revue dans laquelle son article a été publié afin de trouver ses coordonnées. Si vous n'arrivez pas à les repérer, communiquez avec nous à PublicationsArchive-ArchivesPublications@nrc-cnrc.gc.ca.

National Research Council Canada | Conseil national de recherches Canada

Canada

Non-Target Analysis and Stability Assessment of Reference Materials Using Liquid Chromatography–High-Resolution Mass Spectrometry

Elliott J. Wright, Daniel G. Beach*, Pearse McCarron

Biotoxin Metrology, National Research Council of Canada, 1411 Oxford Street, Halifax, Nova Scotia, B3H 3Z1, Canada

*corresponding author:
Tel +1 902-426-8274
Fax +1 902-426-5426;
Email: Daniel.Beach@nrc-cnrc.gc.ca

Abstract

Development and characterization of biological and environmental matrix certified reference materials (CRMs) for organic analytes typically relies heavily on targeted analytical methods, such as liquid chromatography (LC) with triple-quadrupole mass spectrometry detection. LC with high-resolution mass spectrometry (LC–HRMS) can also provide high quality data for both targeted and non-targeted analytes, with the potential for retrospective data analysis. Here, we demonstrate the utility of non-target analysis (NTA) using LC–HRMS for profiling and stability assessment of a mussel tissue matrix CRM certified for several classes of marine algal toxins (CRM-FDMT1). First, the NTA method was developed using data-dependent MS/MS acquisition and commercial metabolomics software for data processing. Of 128 toxin analogues previously reported in CRM-FDMT1, 125 were detected by LC-HRMS, with 97 triggered for MS/MS by data dependant acquisition. Automated data processing detected 119 of these compounds and 109 were retained after automated filtering of results for putative toxin analogues. Those analogues not detected were low abundance ions, or poorly resolved isomers. The method was then used to demonstrate new strategies for CRM stability assessment considering the stability of certified analytes, related toxin analogues, and unrelated matrix compounds. Several analogues from each toxin class in CRM-FDMT1 as well as other unrelated matrix compounds were observed to be significantly less stable than the certified toxins. Using this method, no instability was measured for any compounds at conditions ≤4 °C, providing a greater degree of confidence in CRM stability than could be achieved using conventional targeted approaches to stability assessment targeting only the certified analytes. The NTA method and stability assessment approach presented are applicable to future CRM development with other matrices and organic analyte classes.

Keywords

Metrology, Phycotoxin, Algal Toxin, Stability, Data-Dependent Analysis, Compound Discoverer

1 Introduction

Matrix certified reference materials (CRMs) for organic chemical analytes are required for development and validation of analytical methods across many life science disciplines and demand for these materials continues to grow as the broad scope of analytical testing evolves [1,2]. Considerable resources are required to produce matrix CRMs, including experiments for characterization, homogeneity and stability testing, which require high quality analytical methods. Targeted methods such as LC with triple-quadrupole tandem mass spectrometry using selected reaction monitoring (SRM) or spectroscopic detection are most often used during the various stages of matrix CRM development to achieve the high selectivity and repeatability required for metrological quantitation of trace analytes in complex matrices [3-6].

Liquid chromatography with high resolution mass spectrometry (LC–HRMS) enables selective detection of a broad range of ionizable species in a single run, with data sets amenable to retrospective analysis [7]. Detailed comparisons of quantitative performance of LC–HRMS and low resolution SRM have been reported [8,9], indicating that full-scan LC–HRMS provides quantitative data of equivalent quality for all but lowest abundance ions. Different modes of LC–HRMS data acquisition are available that combine full-scan and tandem mass spectrometry, such as data-dependant acquisition (DDA), data-independent acquisition (DIA), and related approaches [10]. These techniques can be used to confirm and quantitate the presence of targeted analytes, while providing data on many additional compounds present in samples being analysed without prior knowledge of their identity.

A significant challenge to the use of LC–HRMS is handling and interpreting the large data sets generated. While data analysis for known or expected compounds can be relatively straightforward, analysis in a less targeted fashion (e.g. identification of previously unknown

compounds of interest) is more challenging. To address this, commercially available, open-source, or in-house data processing software tools have been developed to automate many steps of data analysis and aid in data interpretation [11-16]. These software applications are used for peak detection and simplifying full-scan data into a list of detected compounds with associated information (e.g. predicted molecular formula, retention time, intensity, isotopes and adducts detected). Data analysis functions used to detect and identify compounds of interest may include automated database searching, finding compounds yielding diagnostic product ions in MS/MS spectra [17,18], or chemometric assessment [19]. Due to the complexity and scope of non-targeted LC–HRMS data sets, it can be a significant challenge to optimize and validate acquisition and processing methods.

Several CRMs have been produced for algal biotoxins in mussel tissues matrices, which have been developed primarily using targeted LC–MS/MS methods [5,6,20]. Methods using LC–HRMS for algal biotoxins and other analytes in shellfish matrices have been reported for screening and routine quantitation [17,21-24], but to date LC–HRMS has not been used extensively during CRM development. Recently, a freeze-dried mussel tissue CRM (CRM-FDMT1) containing certified analytes from several marine algal toxin classes (Fig. 1) including azaspiracids (AZAs), okadaic acid group toxins (OA/DTXs), pectenotoxins (PTXs), yessotoxins (YTXs) and cyclic imines was profiled using a variety of HRMS acquisition modes with laborious manual data interpretation, revealing over 128 toxin analogues [25].

Here, CRM-FDMT1 was used to develop and evaluate an LC–HRMS/MS non-target analysis (NTA) approach for toxin profiling using commercial metabolomics software. Method performance was assessed by comparing the number of toxin analogues detected in comparison to the previously reported manual profiling results [25]. The NTA methodology was then applied to

stability study samples to explore the concept of non-targeted LC–HRMS stability testing, evaluating data for certified toxins, related toxin analogues, and unrelated matrix compounds.

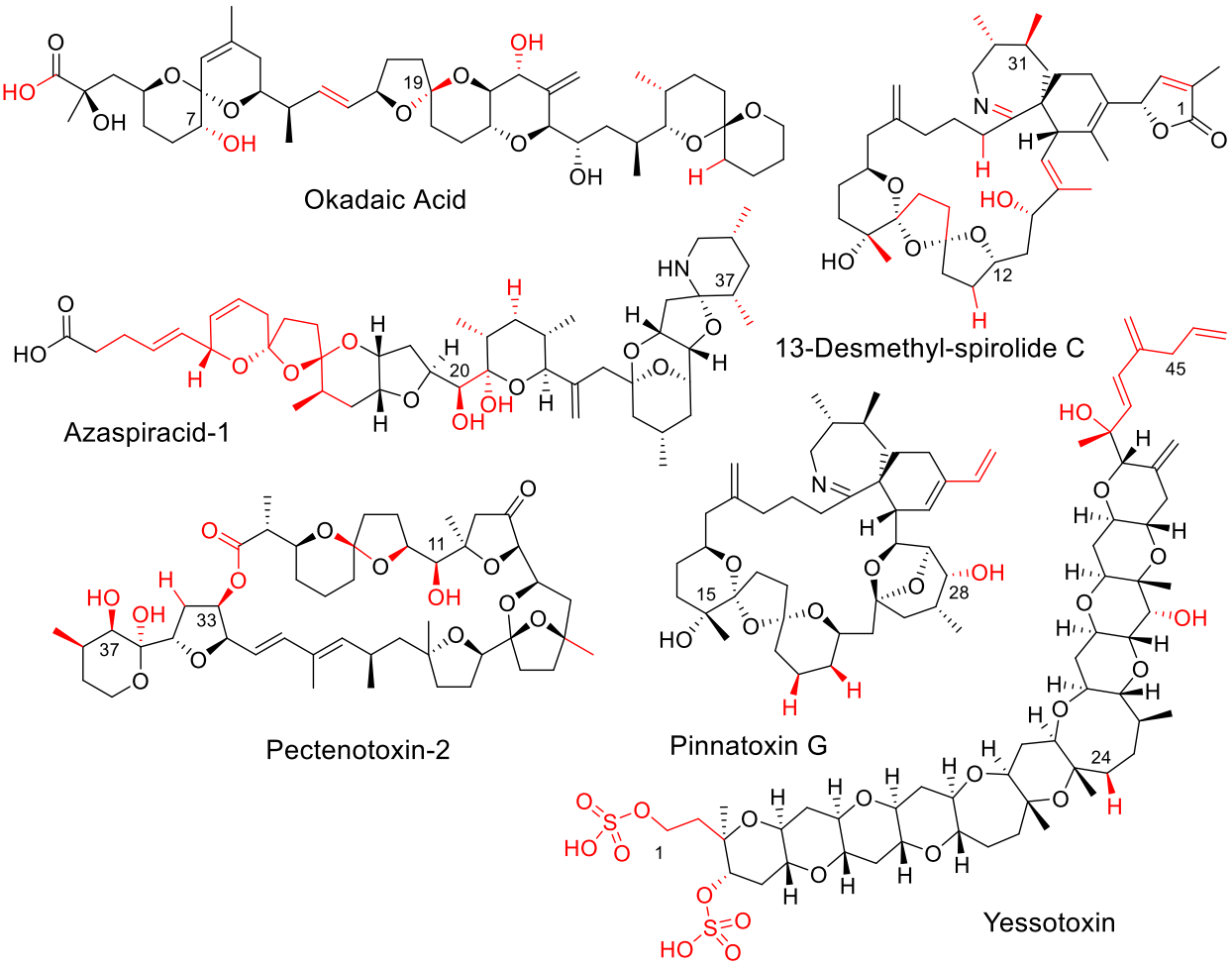

Fig. 1. Representative algal toxins from classes analyzed as part of this study, with some of the known sites of structural variation highlighted in red, including sites of reported conjugation, oxidation, reduction, substitution, or changes in stereochemistry, to demonstrate the diversity within each toxin class.

2 Materials and Methods

2.1 *Reagents*

Methanol and acetonitrile (Optima LC–MS) were obtained from Fisher Scientific (Whitby, ON, Canada). Formic acid (98 %) and ammonium formate (LC–MS grade) were from Honeywell–Fluka (Oakville, ON, Canada). Deionized water was obtained from a Milli-Q Reference A+ water purification system (Millipore Corp., Billerica, MA, USA). Algal toxin CRMs including CRM-FDMT1, CRM-AZA1, CRM-AZA2, CRM-AZA3, CRM-OA-b, CRM-DTX1, CRM-DTX2, CRM-PTX2, CRM-SPX1, CRM-PnTX-G, and CRM-YTX were obtained from the National Research Council (NRC, Halifax, NS, Canada).

2.2 *Sample Preparation*

For toxin profiling, a concentrated FDMT1 extract was prepared by solid phase extraction as previously described [25]. Briefly, 1 g of dry tissue was reconstituted with 5 mL water and extracted twice with 20 mL methanol. The extract was evaporated to 2.5 mL and then made up to 5 mL with 40 % methanol. This was loaded on a preconditioned 1 g Oasis HLB solid phase extraction cartridge (Waters, Mississauga, Canada), eluted with 10 mL 90 % methanol followed by 20 mL of 100 % methanol and evaporated under nitrogen at ambient temperature before being reconstituted in 1 mL methanol.

For stability assessment, a subsample (0.35 g) was taken from single unopened bottles of CRM-FDMT1 that had been stored at −20 °C since previous isochronous stability studies. Stability study conditions were −20, 4, 18, and 60 °C for 0, 2, 8, 16, and 30 days (short-term study) and −20, 4, and 18 °C for 0, 2, 3, 6, 9, and 12 months (long-term study), with samples held at –80 °C until analysis used as the reference condition [20]. Subsamples were extracted using a 4-step

methanol extraction (total volume 25 mL), as described previously [26]. A pooled quality control sample was prepared by mixing equal volumes of extract from the reference condition and the final time point of each test condition. All samples were filtered using 0.45 µm PVDF centrifugal filters (Merck Millipore, Oakville, Canada) prior to analysis.

2.3 LC–HRMS Analysis

LC separations were performed on an Agilent 1200 LC system (Agilent, Palo Alto, CA, USA), with an Agilent Poroshell 120 SB-C18 column (2.1 × 150 mm, 2.7 µm) held at 40 °C. A binary mobile phase of 50 mM formic acid and 2 mM ammonium formate in water (A) and 95 % acetonitrile (B) was used at a flow rate of 0.275 mL min^{-1} with a linear gradient from 5 % to 100 % B over 20 min and then held at 100 % B while the flow rate was increased linearly over 7 min to 0.6 mL min^{-1} and held for 11 min before returning to 0.275 mL min^{-1} over 1 min and re-equilibrating for 5 min. Mobile phase was diverted to waste for the first 6 min of the method and during re-equilibration.

The LC was coupled to a Q Exactive HF mass spectrometer with a heated electrospray ionization probe (Thermo Fisher Scientific, Waltham, MA, USA) using a spray voltage of 3.0 kV (ESI^+) or −2.7 kV (ESI^-), capillary temperature of 350 °C, sheath gas of 40, auxiliary gas of 15 (arbitrary units), auxiliary gas heat of 300 °C, and an S-Lens RF level of 90.

Profiling was performed using separate DDA methods for ESI^+ and ESI^-. Full-scan resolution was set to 60,000 with a mass range of *m*/*z* 450–1450, an automatic gain control (AGC) target of 3×10^6 and a maximum ion injection time (maxIT) of 200 ms. Product-ion spectra were acquired using the 15,000 resolution setting on the most abundant ions detected in each full scan spectrum (Top 10 in ESI^+ and Top 5 in ESI^-), with a 0.7 *m*/*z* precursor-ion isolation window and

apex triggering from 3–30 s with isotope exclusion turned on and dynamic exclusion for 3.5 s. In ESI^+ a 2×10^5 AGC target was used with a maxIT of 19 ms, a DDA intensity threshold of 1.1×10^5 and a collision energy spread using 35 and 60 eV. In ESI^- an AGC target of 2×10^5 was used with a maxIT of 100 ms, an intensity threshold of 8×10^4, and a normalized collision energy of −35 eV. Exclusion lists were prepared at the beginning of each sequence by exporting the most intense 50 ions in every 2 min interval across the chromatographic run of a 5 µL methanol injection and were used with a 3 ppm mass tolerance in all DDA runs.

Analysis of stability samples used a full-scan method with polarity switching at the 60,000 resolution setting with a mass range of *m*/*z* 200–1400, an AGC target of 3×10^6 and a maxIT of 200 ms. All stability samples were injected (1 µL) in triplicate using a randomized order, with QC samples injected at the beginning and end of the sequence, and approximately every 10 samples.

2.4 Data Processing

Compound Discoverer 3.0 (Thermo Scientific) was used to automate data processing. A schematic of the data analysis workflow (Fig. S1) and a detailed table of settings (Table S1) are given in the supporting information. Data processing for ESI^+ and ESI^- were performed separately. Thresholds for peak detection included a minimum of six data points per peak, a minimum of three detected isotopes and minimum intensities of 5×10^4 (ESI^+) or 2×10^3 (ESI^-) counts. Ion definitions specified for data reduction were based on expected adducts and in-source fragments of marine toxin classes (Table 1).

Table 1. Characteristic precursor and product ions from each class of lipophilic toxins represented in CRM-FDMT1 for use in data processing.

Toxin Class Precursor Ions	**Neutral Losses** Monoisotopic Mass (Da)	**Product Ion Formulae**	***m/z***	**Product Ion Formulae (cont'd)**	***m/z***
Azaspiracids	**$[M+H-nH_2O]^+$**	$C_9H_{16}NO^+$	154.1226	$C_{21}H_{32}NO_2^+$	330.2428
$[M+H]^+$	18.0106, 36.0211,	$C_{10}H_{16}NO^+$	166.1226	$C_{22}H_{34}NO_2^+$	344.2584
$[M+H-H_2O]^+$	54.0318, 72.0423	$C_{10}H_{18}NO^+$	168.1383	$C_{21}H_{34}NO_3^+$	348.2533
$[M+Na]^+$		$C_{14}H_{22}NO^+$	220.1696	$C_{22}H_{34}NO_3^+$	360.2533
$[M+K]^+$	**$[M+H-CO_2-nH_2O]^+$**	$C_{15}H_{22}NO_2^+$	248.1645	$C_{22}H_{36}NO_3^+$	362.2690
	43.9898, 62.0004,	$C_{16}H_{22}NO_2^+$	260.1645	$C_{22}H_{34}NO_5^+$	392.2431
	80.0110	$C_{16}H_{24}NO_2^+$	262.1802		
Spirolides & Pinnatoxins	**$[M+H-nH_2O]^+$**	$C_{10}H_{16}N^+$	150.1277	$C_{23}H_{38}NO_4^+$	392.2795
$[M+H]^+$	18.0106	$C_{11}H_{18}N^+$	164.1434	$C_{25}H_{40}NO_3^+$	402.3003
$[M+Na]^+$	36.0211	$C_{12}H_{19}N^+$	177.1512	$C_{26}H_{40}NO_4^+$	430.2952
	54.0318	$C_{11}H_{16}NO^+$	178.1223	$C_{26}H_{38}NO_5^+$	444.2745
	72.0423	$C_{11}H_{18}NO^+$	180.1383	$C_{27}H_{42}NO_4^+$	444.3108
		$C_{14}H_{24}N^+$	206.1903	$C_{27}H_{44}NO_4^+$	446.3265
		$C_{15}H_{26}N^+$	220.2060	$C_{28}H_{44}NO_4^+$	458.3265
		$C_{14}H_{24}NO^+$	222.1852	$C_{26}H_{42}NO_6^+$	464.3007
		$C_{15}H_{26}NO^+$	236.2009	$C_{27}H_{44}NO_6^+$	478.3163
Pectenotoxins	**$[M+H-NH_3-nH_2O]^+$**	$C_{11}H_{13}O_2^+$	177.0910	$C_{47}H_{67}O_{12}^+$	823.4627
$[M+NH_4]^+$	35.0371	$C_{11}H_{15}O_3^+$	195.1016		
$[M+Na]^+$	53.0477	$C_{11}H_{17}O_4^+$	213.1121	$C_8H_9O_2^-$	137.0608
$[M+K]^+$	71.0582	$C_{17}H_{21}O_2^+$	257.1536	$C_8H_{11}O_3^-$	155.0714
$[M+NH_4-SO_3]^+$	89.0688	$C_{17}H_{21}O_3^+$	273.1485	$C_{19}H_{27}O_7^-$	367.1762
$[M-H]^-$	107.0794	$C_{17}H_{23}O_3^+$	275.1642	$C_{29}H_{41}O_9S^-$	565.2477
		$C_{17}H_{23}O_4^+$	291.1591	$C_{47}H_{67}O_{13}^-$	839.4587
	$[M-C_{16}H_{32}O_2]^-$	$C_{29}H_{43}O_{10}^+$	551.2851	$C_{47}H_{69}O_{14}^-$	857.4693
	256.2402	$C_{29}H_{43}O_{11}^+$	567.2800	$C_{46}H_{67}O_{16}S^-$	907.4155
Okadaic Acid & Dinophysistoxins	**$[M-H-C_{16}H_{32}O_2]^-$**	$C_6H_9O_2^+$	169.1223	$C_{18}H_{29}O_3^-$	293.2122
	256.2402	$C_{14}H_{23}O_2^+$	223.1693	$C_{19}H_{31}O_3^-$	307.2279
$[M+H]^+$		$C_{14}H_{19}O_5^+$	267.1227	$C_{19}H_{29}O_4^-$	321.2071
$[M+H-nH_2O]^+$ [a]				$C_{20}H_{31}O_4^-$	335.2228
$[M+Na]^+$		$C_6H_9O_2^-$	113.0608	$C_{30}H_{41}O_9^-$	545.2756
$[2M+Na]^+$		$C_7H_9O_2^-$	125.0608	$C_{30}H_{43}O_{10}^-$	563.2862
$[M+K]^+$		$C_9H_{11}O_2^-$	151.0765	$C_{44}H_{66}O_{12}^-$	785.4482
$[2M+K]^+$		$C_{13}H_{19}O_4^-$	239.1289	$C_{45}H_{68}O_{12}^-$	799.4638
$[M-H]^-$		$C_{13}H_{19}O_5^-$	255.1238		
Yessotoxins	**$[M-H-SO_3]^-$**	$C_5H_9O_5S^-$	181.0171	$C_{42}H_{63}O_{17}S^-$	871.3791
$[M-H]^-$	79.9568	$C_8H_{13}O_6S^-$	237.0432	$C_{46}H_{69}O_{17}S^-$	925.4261
$[M-2H]^{2-}$		$C_{35}H_{53}O_{13}S^-$	713.3212	$C_{46}H_{69}O_{18}S^-$	941.4210
$[M-SO_3-H]^-$		$C_{35}H_{53}O_{14}S^-$	729.3162		
		$C_{42}H_{63}O_{16}S^-$	855.3842		

[a] n=1–3

195 Data analysis functions included mass-list and database searching as well as detection of
196 class-specific product ions. Mass-list searching was performed using a list of *m*/*z* values from
197 published lists [22,27] using a tolerance of ± 5 ppm. Product ion spectra were scored against the
198 Natural Toxins spectral library of collision-induced dissociation spectra from the mzCloud
199 database (www.mzcloud.org) and the detection of characteristic product ions from each toxin class
200 (Table 1) were tabulated in Compound Discoverer using the Compound Class Scoring node and a
201 custom neutral loss node (provided by Robert L. Ross and Patrick A. Limbach of the University
202 of Cincinnati). In order to limit the resulting compound tables to a list of potential toxin analogues,
203 results were filtered for compounds with molecular weight above 500 Da and having a compound
204 class coverage score > 0, a mass-list match, or an mzCloud match.
205 For stability study data analysis, QC samples were analyzed to determine the extent of
206 retention time variability, and verify consistent mass accuracy throughout the analysis. The results
207 table was filtered to display only compounds detected in 100 % of the quality control samples with
208 a relative standard deviation for peak area ≤30 %. Statistical analysis included generating ratios of
209 the average of peak areas from triplicate injections for each compound from each temperature
210 condition to that of the reference condition. Manual processing of stability sample data was done
211 using Xcalibur 4.0 to integrate peaks for certified analytes in CRM-FDMT1, and pinnatoxin G
212 (PnTX-G) using 5 ppm mass windows. Long-term stability uncertainty (u_{LTS}) was calculated for
213 PnTX-G using the approach reported previously for CRM-FDMT1 [20]. Profiling samples were
214 included in Compound Discoverer profiling as "identification only" to allow MS/MS spectra to be
215 appended to entries in the compound table without otherwise impacting the data otherwise.
216
217

3 Results and Discussion

Development of the overall LC–HRMS NTA approach first required optimization and verification of the data acquisition method before the automated data processing method could be developed. The performance of acquisition and processing methods were then critically evaluated against the complex profile of toxin analogues previously reported in CRM-FDMT1 to ensure peak detection and analysis were optimal in the data analysis workflow. The developed methodology was then applied to stability assessment of CRM-FDMT1 using all available data from both certified and non-certified toxin analogues, as well as matrix components.

3.1 *LC–HRMS/MS Data Acquisition Method Development*

The concentrated SPE sample used for CRM profiling contained toxin analogues ranging in polarity and concentration (low ng mL^{-1} to low µg mL^{-1}) and included several groups of isomers varying in chromatographic resolution. To improve the LC separation of this complex mixture, a long LC gradient with an extended isocratic hold at high organic was used, ensuring complete elution of the least polar analytes. The long analysis time served to reduce the number of co-eluting chemical species, which in turn decreased the demand for MS/MS coverage depth at any given time in the chromatogram.

The use of an SPE concentrated sample for profiling allowed high quality MS/MS spectra to be collected for toxin analogues that wouldn't be otherwise concentrated enough to afford informative spectra in non-concentrated samples, but could still be detected in full-scan. Using low abundance toxin ions to optimize instrument parameters for MS^2 acquisition allowed detection of characteristic product ions (Table 1) in as many toxin analogues as possible. During this optimization it was evident that negative MS/MS acquisition required a longer maxIT than positive

MS/MS to obtain sufficient spectral quality to yield characteristic product ions, hence a Top 5 negative polarity experiment was chosen with a 100 ms fill time, while the positive method used Top 10 acquisition with a much shorter, 19 ms, maxIT. Apex trigger and dynamic exclusion settings were optimized to minimize oversampling of abundant precursor ions in DDA, while avoiding long exclusion times detrimental to data acquisition for closely eluting isomers. An example of MS/MS triggering of closely eluting cyclic imines, a class that gave narrow chromatographic peaks, is shown in Fig 2.

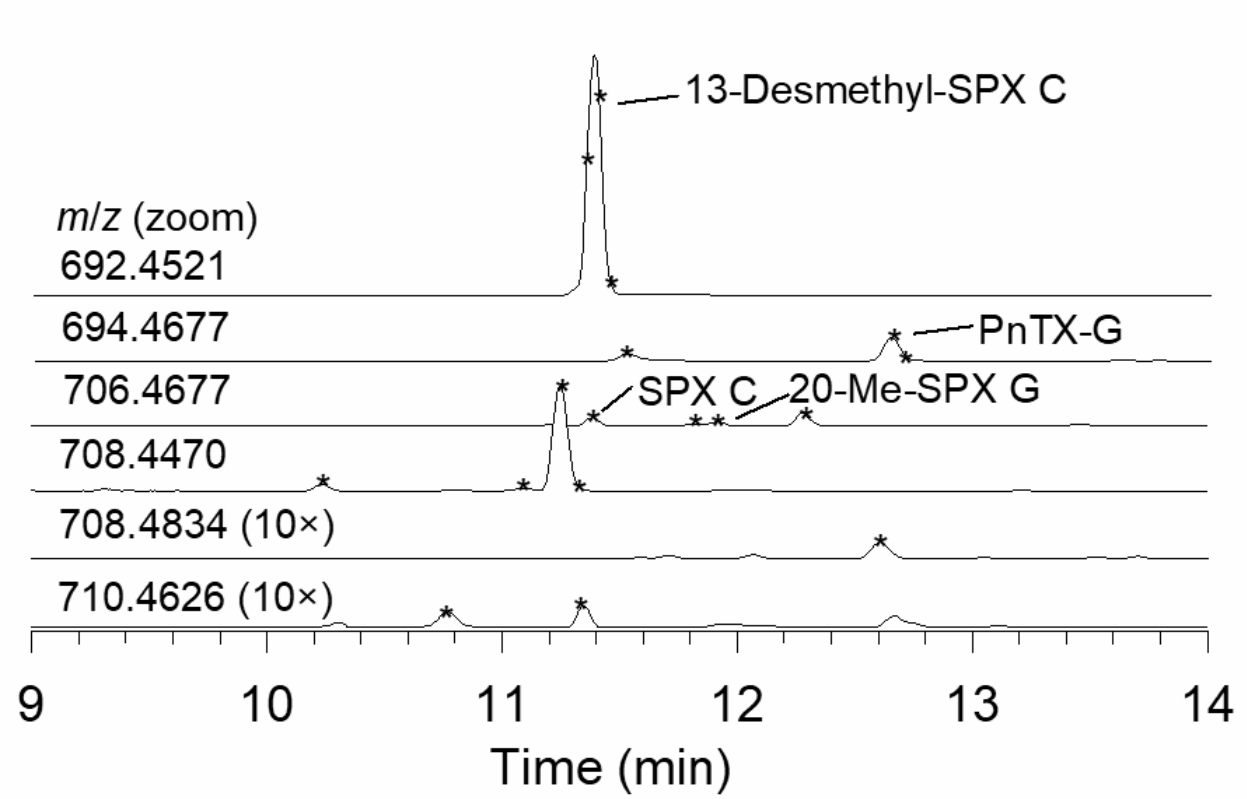

Fig. 2. Full-scan extracted ion chromatograms of selected cyclic imine analogues in SPE-concentrated CRM-FDMT1 extract indicating the retention times where MS/MS acquisition was triggered (*) for each given *m*/*z* using data-dependent acquisition. Chromatograms are scaled to the max intensity of *m*/*z* 692.4521 (6.95×10^7 counts per second)**.**

Performance of the full-scan/DDA acquisition method was evaluated for each compound reported in previous manual profiling results for CRM-FDMT1, using an identical sample preparation and similar chromatography [25]. Out of 128 confirmed toxin analogues in that study,

125 were detected in full scan, with three low-level compounds not detected due to small differences in isomer resolution between the chromatographic methods used. Of the compounds detected, 97 triggered MS/MS acquisition. As seen in Fig. 3, compounds not triggered for MS/MS were among the lowest intensity analogues, which were measured at peak intensities near, or below the DDA triggering thresholds set in the method. Effective acquisition of quality MS/MS spectra for these low-abundance analytes would require additional, dedicated MS/MS experiments with longer C-trap fill times than feasible for DDA and/or additional sample preconcentration. The results obtained using the developed DDA methods demonstrated success at acquiring comprehensive HRMS/MS data in an untargeted fashion for the complex reference material sample tested.

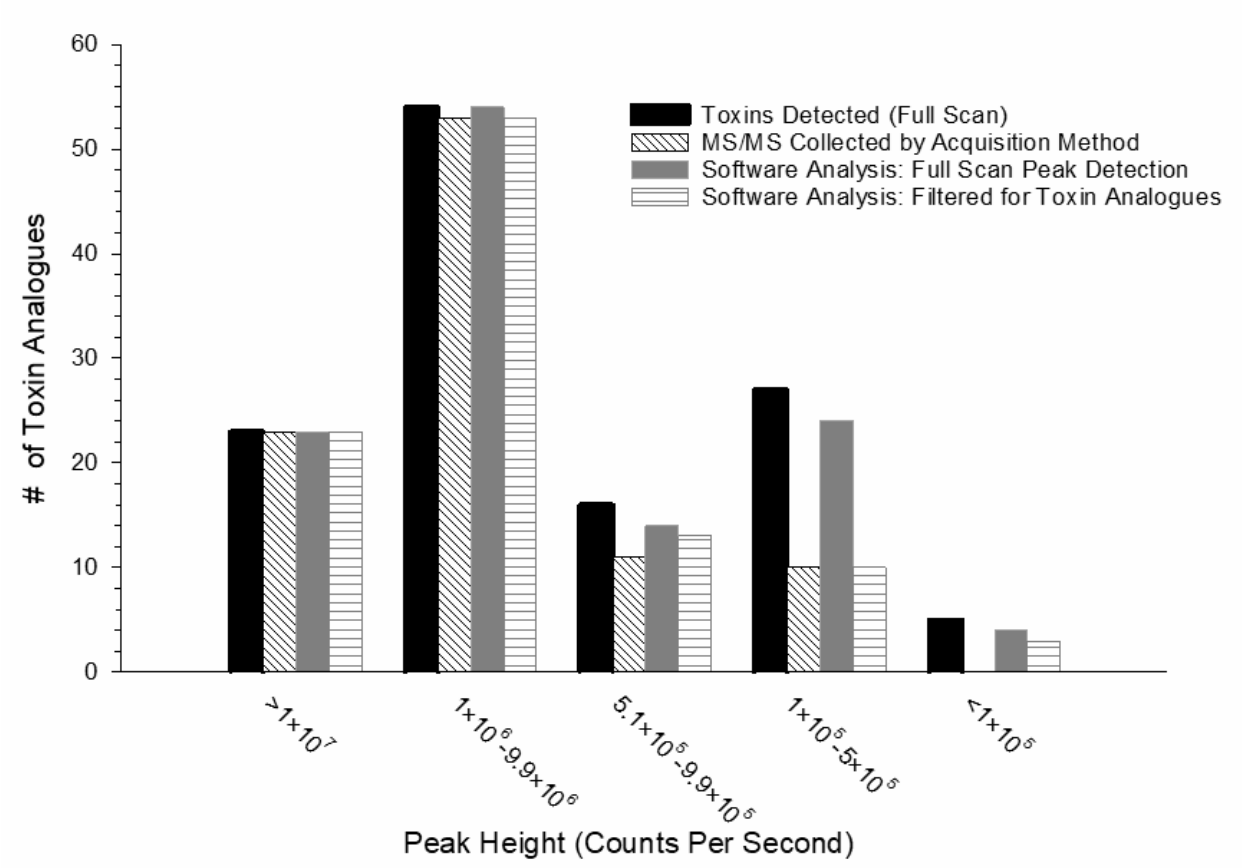

Fig. 3. Data acquisition and processing method performance across the range of full-scan ion intensities observed for toxin analogues in profiling of the SPE concentrated CRM-FDMT1

sample. Black bars represent the total number of putative toxin analogues detected from full scan data (solid) and those where MS/MS spectra were acquired (diagonal lines). Grey bars represent the number of putative toxins that were detected using Compound Discoverer (solid) and those remaining in the final results table after automated filtering (horizontal lines).

3.2 *Data Processing Method Development and Profiling Evaluation*

The aim when developing the automated data processing workflow was to use Compound Discoverer to reduce complex LC–HRMS/MS data into a list of potential toxin analogues detected, which could then be used as the starting point for data interpretation. Each compound entry in the Compound Discoverer results table (Fig. 4) includes the neutral monoisotopic accurate mass, retention time, full-scan spectra including adducts detected, peak area, predicted molecular formula, best available MS/MS spectra, mass list and database matching results, and the number of class-characteristic product ions and neutral loss masses detected.

Fig. 4. Screen capture of a Compound Discoverer results table sorted by PTX class coverage from ESI$^+$ profiling of CRM-FDMT1 showing the extracted ion chromatogram, and full scan HRMS spectrum for the selected compound, pectenotoxin-2 seco acid (PTX2sa).

One of the main challenges of processing method optimization was determining peak detection thresholds that gave an appropriate balance between detection of low-abundance toxin analogues while minimizing the number of artefactual entries in the compound table. The availability of a well characterized reference material was critical in optimizing the parameters for peak detection, which can be difficult to critically assess using either unknown or spiked samples. Initial parameters for peak detection were chosen based on retention time, peak width and intensity, number of full-scan data points across each peak, and the number of isotope peaks observed for known toxin analogues detected in full-scan. Optimization of peak detection parameters in Compound Discoverer was done using automated mass-list searching against a list of the known toxin analogues in CRM-FDMT1, which allowed for rapid assessment of the number of properly detected peaks in the resulting compound table. Using this approach, it was determined that the most effective peak detection threshold settings that gave the best balance of detection for low intensity toxins while minimizing artefactual entries in the compound table were the minimum number of full scan points across the chromatographic peak (6) and number of isotope peaks observed in full scan (3). By analyzing the rich isomer profile in CRM-FDMT1 it was observed that using a maximum width at half height as a peak detection parameter was detrimental to detection of unresolved isomers.

During optimization of the peak detection settings, it was observed that molecular formulae were often incorrectly assigned for compounds primarily observed as ammonium adducts in positive full-scan data. This was particularly relevant in this study for mass-list searching of PTXs, which primarily formed $[M+NH_4]^+$ adducts and for which little or no $[M+H]^+$ was detected. This could be addressed by including the formulae of both protonated and ammoniated PTXs as separate mass list entries, and by manually verifying the presence of the $[M+Na]^+$ adduct during

311 subsequent data interpretation. An example of PTX detection using the final method is shown in
312 Fig. 4 where an additional NH_3 was observed in the molecular formula for the compound in row
313 7, which is one of the lowest abundance isomers of the 16:0 acyl ester of pectenotoxin-2 seco acid
314 (PTX2sa), while isomers in rows 2–5 show the correct molecular formula although these were not
315 assigned a name due to their absence in the mass list.

316 Detection of suspected toxins was performed by searching for characteristic product ions
317 from each toxin class (Table 1). This approach is similar to that used previously with low resolution
318 precursor ion scanning and manual data processing to detect lipophilic algal toxins [28-30], and
319 more recently in an LC–HRMS/MS study detecting cyclic imines [17]. Here, "compound class
320 scoring" reported the percentage of product ions detected from the list of ions in each class. In
321 some instances, low specificity associated with using low-mass product ions resulted in matrix
322 compounds remaining in the filtered results table. It was therefore important to use several
323 characteristic product ions, allowing the results table to be sorted by class score for each toxin
324 class as an initial step when viewing the results table (Fig. 4). For the lipophilic toxins in this study,
325 characteristic neutral losses (Table 1) are among the most abundant product ions in MS/MS spectra
326 at the collision energies used, however, their specificity is typically low. Additional confirmation
327 was achieved by searching the mzCloud spectral library for natural toxins, containing 64
328 compounds, [31] 26 of which were algal toxins considered in this study. These gave match scores
329 based on comparison of database data with experimental spectra (Fig. S2), thereby providing
330 additional confirmation of known toxins with available standards.

331 In order to critically evaluate the performance of the data processing method in detecting
332 unknown toxin analogues, a mass list consisting only of lipophilic shellfish toxins previously
333 reported [22,27] prior to the characterization of CRM-FDMT1 was used for all subsequent method

evaluation. The entries in the "Name" column (Fig 4) are only populated for compounds with either mzCloud or mass list matches using this workflow. For compounds annotated based on mass-list entries, manual MS/MS interpretation and/or retention time matching with standards are required to further identify the compound, which was beyond the scope of this work.

Evaluation of the NTA method was carried out by comparing the entries in the final compound table to the results of previous exhaustive manual LC–HRMS/MS profiling of CRM-FDMT1 [25]. First, the results table was searched for the 125 compounds detected by the data acquisition method before and after results filtering, showing >95 % and 87 % of these compounds, respectively. Toxin analogues not detected as compounds from full-scan data exhibited either low overall ion abundance or poor chromatographic behaviour (e.g. noisy baseline, poor isomer resolution). Low-abundance compounds often did not have enough full-scan points across the peak or enough isotopic peaks to meet the minimum thresholds set (see Table S1). However, decreasing these thresholds to capture more low-abundance toxin analogues led to a disproportionate increase in artefactual entries in the results table, rendering it less useful. Toxin analogues detected as compounds but not retained after filtering the results table were typically low-abundance ions without sufficient precursor ion intensity to yield useful MS/MS spectra that were also not present on the mass list used.

Filtered results tables from ESI^+ and ESI^- contained 1424 and 833 compounds, respectively. Critical analysis of these combined entries revealed well over 300 compounds that were considered probable toxin analogues based on suitable retention times, toxin-like fragmentation, or likelihood as isomers of more abundant known toxin analogues. As can be seen from the summary of suspected toxin analogues (Table S2), many of these additional compounds were fatty acid acyl esters of known toxins, which are invariably detected in shellfish along with

their non-conjugated parent toxins. The presence of a complex acyl ester toxin profile was noted previously, although results for these ester variants were not individually tabulated [25]. Among the processed results from this automated approach were two new putative AZA analogues that had not been previously summarized in CRM-FDMT1 during manual profiling (Fig. S3 and S4). The AZA with an accurate mass of *m*/*z* 702.4589 observed in this study was overlooked in previous studies because of its partial co-elution with AZA1, which is present at much higher levels (Table S2). Examples such as these demonstrate the method's ability to selectively collect and summarize data for true unknowns among the targeted toxin classes.

The use of DDA with automated profiling in this study provided comprehensive analysis of individual chromatographic peaks at a level of detail that is prohibitively labour intensive to do manually. Extensive analogue profiling information can enhance the utility of biological and environmental CRMs and will also be of value for CRM users who are increasingly using more complex analytical methods in their own laboratories. In this work, the availability of CRM-FDMT1 as a tool to critically assess method performance in profiling a large number of toxin analogues across a broad range of retention time and ion abundances provides confidence in automated peak detection. In future use of the method, improvements in compound name annotation can be made using mass lists updated with a comprehensive list of toxin analogues including those identified here and in forthcoming studies.

3.3 *Application of NTA approach to CRM Stability Assessment*

Providing evidence of stability for certified analytes in a reference material matrix is a requirement for CRM certification and can have a significant impact on the overall uncertainty budgets associated with the certified values. Conservative uncertainty estimates frequently

adopted for stable analytes in matrix CRMs [32] are highly dependent on the magnitude of measurement uncertainty (e.g. repeatability). Before applying the developed NTA methodology to CRM stability assessment, the quality of LC–HRMS data collected was assessed for the certified toxins in CRM-FDMT1. Relative standard deviations calculated from the reference samples (n = 9) demonstrated repeatability ranging from 2–11 % for the certified analytes in CRM-FDMT1, which was equivalent to the range of repeatability observed by targeted SRM methods (1–11 %) [20,33], which aligns with previously reported LC–HRMS methods using similar instrumentation [34].

The use of LC–HRMS allows for the retrospective evaluation of new analytes of interest for certification from existing data. One such example in CRM-FDMT1 is PnTX-G, an algal toxin from the cyclic imine group, which now has a calibration solution CRM available [35]. Using the conservative uncertainty estimation approach previously employed for CRM-FDMT1, LC–HRMS data for PnTX-G was used to estimate a stability uncertainty of 3.1 %, which is reasonable in comparison to previously reported values for 13-desmethylspirolide-C (1.9 %) considering PnTX-G is present at a concentration approximately two orders of magnitude lower [36]. Although the data used to derive this stability uncertainty estimate showed no detectable instability, it would remain a significant component in the final combined uncertainty.

The methods developed can also be used to gain additional insight into CRM stability during storage and transport from the data acquired for many other compounds beyond just certified analytes. In the original CRM-FDMT1 stability studies, instability of certified analytes was only detected at 60 °C, showing AZA3 to be the most significantly degraded of the certified toxins [20]. Stability assessment of additional compounds was performed in Compound Discoverer without any additional manual processing. This could be done by normalizing peak

areas to the average peak area of control samples and analyzing this ratio for trends across the time series graphically (Fig. S5), or by analyzing the ratio of each compound's average peak area for a temperature condition to that of the reference condition.

Through sorting the result table by class score and using the approaches described above, several analogues from each toxin class were observed to be less stable than the certified analytes in CRM-FDMT1 at the 60 °C condition. By including DDA runs from profiling experiments in the stability data processing workflow, corresponding MS/MS spectra were appended to compound table entries from the stability study, enabling sorting by class score for individual toxin classes. Doing this for spirolides and pinnatoxins (Fig. S5), compounds observed to be forming or degrading at test conditions could quickly be compared to the stability trends for certified analytes (Fig. 5). Among the most significantly degrading toxin analogues were PTX2sa acyl esters, with the palmitoyl (16:0) ester eluting at 27.63 min decreasing in intensity by approximately 8-fold after 30 days. Late eluting isomers of AZA3 (25.20 min, Fig. 5) and 13-desMe-SPX C (12.88 min, Fig. S5), had large relative increases in peak areas over the course of the study, showing 2- and 3-fold increases, respectively, at 60 °C for 30 days. No instability of these compounds was detected at temperatures ≤18 °C.

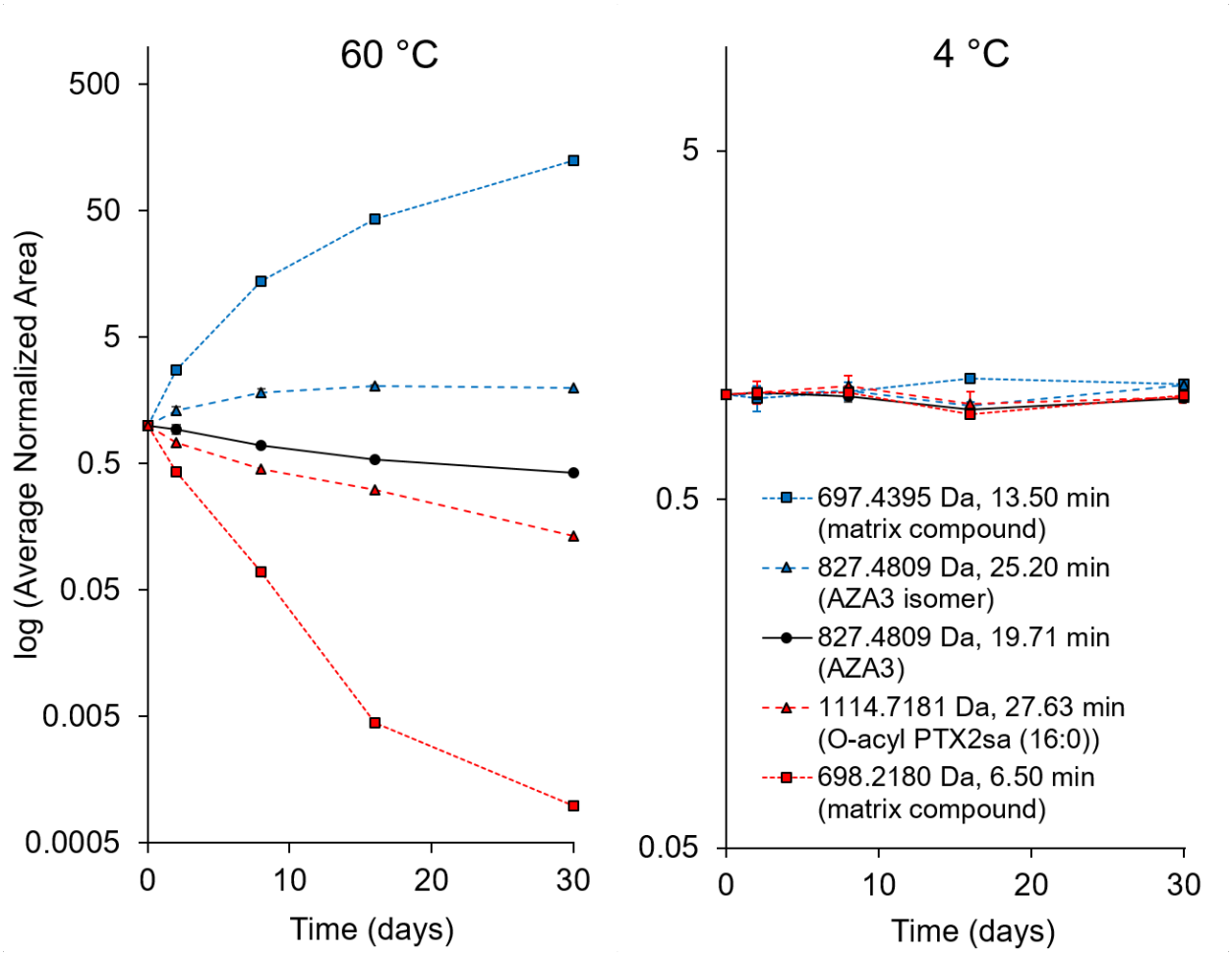

Fig 5. Short-term stability data for 60 °C (left) and 4 °C (right) conditions showing AZA3 (black circles), the most significantly forming (blue) and degrading (red) toxin analogues (triangles) and non-targeted matrix compounds (squares). Mean (n = 3) peak areas for compounds shown are the sum of all detected adducts from the neutral monoisotopic mass given at the specified retention time at each sample point and normalized to the mean area (n = 9) at the reference (−20 °C) condition. Error bars are ± the relative standard deviation about the mean results.

A non-targeted assessment of the stability of CRM FDMT1 was then carried out by looking for stability trends in the unfiltered results table containing all detected compounds. One of the largest increases observed in the control-normalized peak area ratio after 30 days at 60 °C was a compound with a monoisotopic neutral mass of 697.4395 Da eluting at 13.5 min with a ratio over 35-fold higher than any detected toxin analogue (Fig. 5). This indicated that compounds present in CRM-FDMT1 not previously targeted in stability studies are far more susceptible to degradation

than certified toxins, and the toxin analogues assessed in this study. Literature searching and MS/MS interpretation tentatively identified this compound as one of several degradation products of erythromycin A [37], an antibiotic added to CRM-FDMT1 during preparation of the CRM [38]. Over 190 compounds exhibited reduced stability compared to any of the toxin analogues in the short-term stability results table. It is also noteworthy that no compound instability could be detected at 4°C for the one year period of the long-term stability study.

While it is still necessary to characterize the stability of certified analytes, other compounds with reduced stability such as those detected in this study offer the potential to serve as early indicators of instability during ongoing stability monitoring of CRM products. Additionally, their instability relative to certified analogues could be considered when evaluating the need to assign uncertainty contributions for certified analogues from stability study data.

For CRM-FDMT1, the demonstrated use of non-targeted stability assessment shows that at the recommended storage conditions of −12 °C there is negligible risk of instability. The NTA approach to stability assessment developed in this study could also be used to assess the stability of matrix CRMs certified to be free of an analyte of interest, as well as a tool to study potential changes in commutability of a matrix CRM over time.

4 Conclusion

This study demonstrates the potential of incorporating NTA into matrix CRM development. The availability of CRM-FDMT1, with information on the presence of over 100 toxin analogues, provided a unique opportunity for development and evaluation of DDA and automated data processing methods using commercial metabolomics software. The methods presented were effective at profiling toxin analogues in CRM-FDMT1, providing a detailed summary of the data collected with significantly less effort than manual data analysis. Previously

unreported putative AZA compounds were detected, demonstrating the broader value of the non-targeted approach to profiling applications.

When applied to a set of stability study samples for CRM-FDMT1, non-targeted LC–HRMS methods provided high quality data on both certified and uncertified toxin analogues and a wide range of matrix components. These data were suitable for retrospective stability analysis that could inform certification of additional analytes without the need for new experimentation. The availability of relative stability data on uncertified toxin analogues and matrix components over an order of magnitude less stable than certified analytes offers a data-driven approach for evaluating overall CRM stability. In the future, this could include ongoing monitoring of selected stability markers along with certified analytes. The approaches presented here can be considered broadly applicable to CRMs for other analyte classes or matrices and could be particularly useful for negative control matrix CRMs.

5 Acknowledgements

Robert L. Ross (College of Medicine) and Patrick A. Limbach (Department of Chemistry) of the University of Cincinnati are gratefully acknowledged for providing the custom neutral loss node for use in our Compound Discoverer workflow. Tim Stratton (Thermo Scientific) is acknowledged for his efforts to include NRC CRMs into the mzCloud database for use in this project. Melanie MacArthur (NRC) is acknowledged for technical assistance. We would like to thank Christopher O. Miles (NRC) for careful internal review of this manuscript.

6 References

[1] S.A. Wise, M.M. Phillips, Evolution of reference materials for the determination of organic nutrients in food and dietary supplements—a critical review, Anal. Bioanal. Chem. 411 (2019) 97–127.
[2] S.A. Wise, What is novel about certified reference materials?, Anal. Bioanal. Chem. 410 (2018) 2045–2049.
[3] M. Yang, F. Liu, M. Wang, J. Zhou, R. Wei, R. Wang, L. Zhang, L. Jian, T. Wang, New matrix certified reference material for accurate measurement of ciprofloxacin residue in egg, Anal. Bioanal. Chem. 412 (2020) 635–645.
[4] S. Grimalt, S. Harbeck, P. Shegunova, J. Seghers, B. Sejerøe-Olsen, H. Emteborg, M. Dabrio, Development of a new cucumber reference material for pesticide residue analysis: feasibility study for material processing, homogeneity and stability assessment, Anal. Bioanal. Chem. 407 (2015) 3083–3091.
[5] P. McCarron, S.D. Giddings, K.L. Reeves, P. Hess, M.A. Quilliam, A mussel (*Mytilus edulis*) tissue certified reference material for the marine biotoxins azaspiracids, Anal. Bioanal. Chem. 407 (2015) 2985–96.
[6] P. McCarron, K.L. Reeves, S.D. Giddings, D.G. Beach, M.A. Quilliam, Development of certified reference materials for diarrhetic shellfish poisoning toxins, part 2: shellfish matrix materials, J. AOAC Int. 99 (2019) 1163–1172.
[7] J.B. Renaud, M.W. Sumarah, Data independent acquisition-digital archiving mass spectrometry: application to single kernel mycotoxin analysis of Fusarium graminearum infected maize, Anal. Bioanal. Chem. 408 (2016) 3083–3091.
[8] A. Kaufmann, High-resolution mass spectrometry for bioanalytical applications: Is this the new gold standard?, J. Mass Spectrom. 55 (2020) e4533.
[9] B. Grund, L. Marvin, B. Rochat, Quantitative performance of a quadrupole-orbitrap-MS in targeted LC–MS determinations of small molecules, J. Pharm. Biomed. Anal. 124 (2016) 48–56.
[10] J. Guo, T. Huan, Comparison of full-scan, data-dependent, and data-independent acquisition modes in liquid chromatography–mass spectrometry based untargeted metabolomics, Anal. Chem. 92 (2020) 8072–8080.
[11] G. Yan, H. Sun, W. Sun, L. Zhao, X. Meng, X. Wang, Rapid and global detection and characterization of *aconitum* alkaloids in Yin Chen Si Ni Tang, a traditional Chinese medical formula, by ultra performance liquid chromatography–high resolution mass spectrometry and automated data analysis, J. Pharm. Biomed. Anal. 53 (2010) 421–431.
[12] A.M. Knolhoff, T.R. Croley, Non-targeted screening approaches for contaminants and adulterants in food using liquid chromatography hyphenated to high resolution mass spectrometry, J. Chromatogr. A 1428 (2016) 86–96.
[13] Y. Verkh, M. Rozman, M. Petrovic, A non-targeted high-resolution mass spectrometry data analysis of dissolved organic matter in wastewater treatment, Chemosphere 200 (2018) 397–404.
[14] N. Jaitly, A. Mayampurath, K. Littlefield, J.N. Adkins, G.A. Anderson, R.D. Smith, Decon2LS: An open-source software package for automated processing and visualization of high resolution mass spectrometry data, BMC Bioinf. 10 (2009) 87.
[15] M.E. Dasenaki, S.K. Drakopoulou, R. Aalizadeh, N.S. Thomaidis, Targeted and untargeted metabolomics as an enhanced tool for the detection of pomegranate juice adulteration, Foods 8 (2019) 212.

[16] F. Fenaille, P. Barbier Saint-Hilaire, K. Rousseau, C. Junot, Data acquisition workflows in liquid chromatography coupled to high resolution mass spectrometry-based metabolomics: Where do we stand?, J. Chromatogr. A 1526 (2017) 1−12.
[17] F. Varriale, L. Tartaglione, S. Cinti, A. Milandri, S. Dall'Ara, A. Calfapietra, C. Dell'Aversano, Development of a data dependent acquisition-based approach for the identification of unknown fast-acting toxins and their ester metabolites, Talanta 224 (2021) 121842.
[18] J.P. Walsh, J.B. Renaud, S. Hoogstra, D.R. McMullin, A. Ibrahim, C.M. Visagie, J.B. Tanney, K.K.-C. Yeung, M.W. Sumarah, Diagnostic fragmentation filtering for the discovery of new chaetoglobosins and cytochalasins, Rapid Commun. Mass Spectrom. 33 (2019) 133−139.
[19] K. Purschke, M. Vosough, J. Leonhardt, M. Weber, T.C. Schmidt, Evaluation of nontarget long-term LC–HRMS time series data using multivariate statistical approaches, Anal. Chem. 92 (2020) 12273−12281.
[20] P. McCarron, H. Emteborg, S.D. Giddings, E. Wright, M.A. Quilliam, A mussel tissue certified reference material for multiple phycotoxins. Part 3: homogeneity and stability, Anal. Bioanal. Chem. 400 (2011) 847−858.
[21] P. Blay, J.P.M. Hui, J. Chang, J.E. Melanson, Screening for multiple classes of marine biotoxins by liquid chromatography-high-resolution mass spectrometry, Anal. Bioanal. Chem. 400 (2011) 577−585.
[22] A. Gerssen, P.P.J. Mulder, J. de Boer, Screening of lipophilic marine toxins in shellfish and algae: development of a library using liquid chromatography coupled to orbitrap mass spectrometry, Anal. Chim. Acta 685 (2011) 176−185.
[23] I. Dom, R. Biré, V. Hort, G. Lavison-Bompard, M. Nicolas, T. Guérin, Extended targeted and non-targeted strategies for the analysis of marine toxins in mussels and oysters by (LC-HRMS), Toxins 10 (2018) 375.
[24] Z. Zendong, P. McCarron, C. Herrenknecht, M. Sibat, Z. Amzil, R.B. Cole, P. Hess, High resolution mass spectrometry for quantitative analysis and untargeted screening of algal toxins in mussels and passive samplers, J. Chromatogr. A 1416 (2015) 10−21.
[25] E.J. Wright, P. McCarron, A mussel tissue certified reference material for multiple phycotoxins. Part 5: profiling by liquid chromatography–high-resolution mass spectrometry, Anal. Bioanal. Chem. 413 (2021) 2055-2069.
[26] P. McCarron, E. Wright, H. Emteborg, M.A. Quilliam, A mussel tissue certified reference material for multiple phycotoxins. Part 4: certification, Anal Bioanal Chem 409 (2017) 95−106.
[27] C.O. Miles, D.J. Stirling, Toxin mass list, 2017. https://www.researchgate.net/profile/Christopher-Miles-2. (Accessed October 10 2019).
[28] R. Rossi, C. Dell'Aversano, B. Krock, P. Ciminiello, I. Percopo, U. Tillmann, V. Soprano, A. Zingone, Mediterranean *Azadinium dexteroporum* (Dinophyceae) produces six novel azaspiracids and azaspiracid-35: a structural study by a multi-platform mass spectrometry approach, Anal. Bioanal. Chem. 409 (2017) 1121−1134.
[29] J. Aasen, S.L. Mackinnon, P. Leblanc, J.A. Walter, P. Hovgaard, T. Aune, M.A. Quilliam, Detection and identification of spirolides in Norwegian shellfish and plankton, Chem. Res. Toxicol. 18 (2005) 509−515.
[30] P. De La Iglesia, P. McCarron, J. Diogène, M.A. Quilliam, Discovery of gymnodimine fatty acid ester metabolites in shellfish using liquid chromatography/mass spectrometry, Rapid Commun. Mass Spectrom. 27 (2013) 643−653.

[31] T. Stratton, Delivering confidence for small molecule identification, 2018. https://assets.thermofisher.com/TFS-Assets/CMD/Reference-Materials/wp-65374-ms-mzcloud-libraries-wp65374-en.pdf. (Accessed February 27 2022).
[32] T.P.J. Linsinger, J. Pauwels, A. Lamberty, H.G. Schimmel, A.M.H. van der Veen, L. Siekmann, Estimating the uncertainty of stability for matrix CRMs, Fresenius' J. Anal. Chem. 370 (2001) 183–188.
[33] P. McCarron, E. Wright, M.A. Quilliam, Liquid chromatography/mass spectrometry of domoic acid and lipophilic shellfish toxins with selected reaction monitoring and optional confirmation by library searching of product ion spectra, J. AOAC Int. 97 (2014) 316–324.
[34] A. Domènech, N. Cortés-Francisco, O. Palacios, J.M. Franco, P. Riobó, J.J. Llerena, S. Vichi, J. Caixach, Determination of lipophilic marine toxins in mussels. Quantification and confirmation criteria using high resolution mass spectrometry, J. Chromatogr. A 1328 (2014) 16–25.
[35] National Research Council Canada, Certified reference materials. https://nrc.canada.ca/en/certifications-evaluations-standards/certified-reference-materials. (Accessed May 12 2021).
[36] E. Wright, K.L. Reeves, S.D. Giddings, P. McCarron, CRM-FDMT1, a freeze-dried mussel tissue certified reference material for multple marine toxins: technical report CRM-FDMT1-20070717, National Research Council Canada, Halifax, 2016.
[37] D.A. Volmer, J.P.M. Hui, Study of erythromycin A decomposition products in aqueous solution by solid-phase microextraction/liquid chromatography/tandem mass spectrometry, Rapid Commun. Mass Spectrom. 12 (1998) 123–129.
[38] P. McCarron, H. Emteborg, C. Nulty, T. Rundberget, J.I. Loader, K. Teipel, C.O. Miles, M.A. Quilliam, P. Hess, A mussel tissue certified reference material for multiple phycotoxins. Part 1: design and preparation, Anal. Bioanal. Chem. 400 (2011) 821–833.