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Comment on “Effect of Uncontrolled Factors in a Validated Liquid Chromatography–Tandem Mass Spectrometry Method Question Its Use as a Reference Method for Marine Toxins: Major Causes for Concern”

Patrick T. Holland,^{*,†} Paul McNabb,[†] and Michael A. Quilliam[‡]

[†]Cawthron Institute, 98 Halifax Street East, Private Bag 2, Nelson 7042, New Zealand

[‡]National Research Council Canada, Institute for Marine Biosciences, 1411 Oxford Street, Halifax, Nova Scotia, B3H 3Z1, Canada

ABSTRACT: This recent paper by Otero and co-workers presents some data from analysis of okadaic acid group toxins by liquid chromatography–tandem mass spectrometry (LC–MS/MS) using different instruments, operating parameters, and solvent conditions. They question the suitability of this tool for quantitative analysis. This paper reveals a lack of understanding of critical factors for the successful use of LC–MS methodology in general as well as some specific proficiency issues with the work reported on the three toxins. We show that there are problems with the conduct and reporting of the experiments, including possible injector carry-over and lack of quality assurance/quality control (QA/QC) controls. Therefore the specific conclusions they draw from their data are considered invalid.

Liquid chromatography–mass spectrometry (LC–MS) has developed into a powerful technology that is now widely used in many fields of organics trace analysis. In a recent paper, Otero et al.¹ have questioned the suitability of LC–MS as a reference method for protecting consumers from marine toxins. The LC–MS/MS multitoxin methodology used most commonly in various laboratories internationally, as well as by Otero et al., is based on methods developed in Canada.² Early validation studies demonstrated excellent performance and good interlaboratory reproducibility.³ There have been ongoing efforts to further validate methods, study the important factors for successful use of the technology, and provide training.^{4–7} The monitoring programs in many countries, including several National Reference Laboratories (NRLs) of Europe, are providing reliable data for toxins in shellfish based on LC–MS methods backed by rigorous quality assurance/quality control (QA/QC) procedures.

A fundamental assumption of Otero et al. apparently is that an LC–MS system should provide absolute responses in a similar way to high-pressure liquid chromatography–ultraviolet (HPLC–UV). The authors claim to have proved this is not the case for three toxins, and they have then concluded that the technique is unsuitable for routine testing. We show that there are severe problems with the conduct of the reported experiments, and therefore the specific conclusions they draw from their data are invalid. We also do not agree with their general conclusions which reveal a lack of understanding of critical factors for the successful use of LC–MS methodology. Furthermore, we believe their wording is unduly contentious in view of the immense international efforts in this area over the past decade. The credibility of their conclusions is very low, but a response is necessary because of ongoing implications for the control of marine biotoxins in seafood.

EXPERIMENTAL SECTION

Key details of their methodology are sparse and inadequate to allow replication of the experiments in another laboratory. While

Analytical Chemistry does have a tight page limit, the authors have skimmed on the experimental rather than on the sometimes controversial discussion. Specific areas where critical information is lacking include the following.

(1) Calibration. No calibration equation data is presented and it is unclear how any of the concentration data was generated. The authors only state that “Calibrations were linear over the range 10–500 ng/mL using 6 points for each standard and linear regression values were >0.999”. The calibration conditions for the data in Table 2 and Figure 1 are not clearly stated and therefore it is unclear what the % deviations refer to. In Results it vaguely states that “...each toxin type was first quantitated using its own calibration curve”. None of the data points in Table 2 match the standard concentrations, yet our meta-analysis (see below) indicates that instrument responses were generally linear. We must assume that some other, unspecified sets of instrument conditions were used for the calibrations and the standards may have been run at very different time periods to samples.

(2) Analyte concentrations. The concentrations of the three toxins used for calibrations and for Table 2 were much higher than are relevant to testing of shellfish. The concentrations tested to produce the data summarized in Figure 1 are not provided.

(3) Injection sequences. The time-frames and order of the injection sequences for standards and samples are crucial for the interpretation of the reported shifts in responses.

(4) Multiple reaction monitoring (MRM) parameters. The four sets of dwell and settling times used for the two instruments and the two key MRM experiments should have been provided. There is evidence that the lab did not verify that parameters used were appropriate for their instruments. In the case of the QTRAP, an early generation, entry-level instrument, the times for the 10 transition experiments were probably too short to allow proper settling of the mass analyzer.

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Table 1. Regression Equations for Expected Vs Found Concentrations from Table 2 of Otero et al. (Mean, $n = 18$)

toxin	slope $\pm$ RSD	R^2	intercept (range) ng/mL
OA	$0.998 \pm 7.7\%$	0.9970	7.7 (−17.4 to 21.4)
DTX1	$0.996 \pm 13\%$	0.9876	−9.6 (−38.3 to 11.9)
DTX2	$0.969 \pm 11\%$	0.9929	2.4 (−24.8 to 19.8)

Figure 1. Example Regression Plot of Data from Table 2 of Otero et al. (Okadaic Acid, Second Row)

DATA INTERPRETATION

The data provided in Table 2 allowed for some meta-analysis. We have obtained regression equations for each toxin by plotting the reported set of three found concentrations (ng/mL) versus expected (45, 160, 320 ng/mL) for the six experiments on two instruments under various solvent conditions (total of 18 plots per toxin). An example of one of these plots is provided in Figure 1, and Table 1 summarizes all the regression equations. The experiments using the 10 transition method 2 were excluded (problems with QTRAP, see point 4 above).

The mean slopes for these pseudocalibrations are within 3% of 1.00 with moderate RSDs and linearities are high, especially for OA and DTX2. This indicates that the instruments were generally holding the response factors (RFs) from their original calibrations, however they were performed, despite the various LC and sample solvents. However, the intercepts are large, variable, and generally positive for OA and DTX2 (which elute close to each other) but generally negative for DTX1 (which elutes later). Analyte signals from blank injections should be negligible in the highly selective tandem mass spectrometry (MS/MS) mode. Therefore the positive intercepts combined with highly linear responses for OA and DTX2 are strong evidence for an LC blank problem, possibly due to injector carryover. For DTX1, the more variable, generally negative intercepts plus the lower linearity may indicate that calibrations were more affected by carryover than the samples. Several alternative, more subtle explanations are possible for the nonzero intercepts but in any case point-concentration data will be directly affected.

The data in Otero et al., Figure 1 gives concentrations of one toxin calculated from the response of another. The authors

ascribe the large % deviations in found versus expected concentrations to differences in RRFs between the three toxins under the different solvent conditions used. As only a single concentration (not given) was used, the above meta-analysis is not possible. However, the serious blank problems deduced above can be presumed to also have been present when this point-concentration data was gathered, leading to erroneous conclusions. Also, the concentration of OA from the DTX1 calibration should be the exact inverse of DTX1 from the OA calibration for mixtures of the toxins run under the same set of conditions. This is often not the case. There is good agreement in Figure 1 for the standards diluted in two different solvents. All the data for adjacent pairs of methanol/30% acetonitrile treatments in each toxin/calibrant quadrant show nearly identical patterns across the three mobile phase treatments, which is contrary to the authors' conclusion: "A major source of results variability will be therefore the change of solvent in a given laboratory".

DISCUSSION AND CONCLUSIONS

The authors claim that there were large shifts in instrument responses when different solvents were used. However, they have chosen to present their data as % deviations in concentration from some calibrated ideal. These types of point comparisons can lead to erroneous conclusions, especially if there is a significant blank. The full calibration functions, i.e., the slopes (response factor), intercepts, and linearity, are the preferred parameters to enable insights for quantitative performance of instruments operated under various conditions.

The large nonzero intercepts for the regressions of their expected/found data are indicative of a serious problem directly affecting the reported point-concentration data. It seems likely that instrument blanks were large and variable (no blank data was reported). Okadaic acid group toxins are relatively lipophilic and also have a carboxyl group capable of ion exchange. Thus, extreme care is needed with the injection parameters, wash solvents, and sample sequences, especially when working with high toxin concentrations. Our estimates for the intercepts (OA + DTX1 + DTX2) are up to 3-fold higher than the regulatory limit of 16 ng/mL for a typical 1:10 shellfish extract. If the different solvents were affecting the MS responses, e.g., by signal enhancement/suppression, then the slopes should change but not the intercepts, which should be zero (in the absence of significant carryover). The reasonably uniform RFs calculated above imply that relative response factors (RRFs) between toxins were also constant, contradicting another of the authors' conclusions.

RRFs between compounds by LC–MS/MS depend upon effects of molecular structure and mobile phase composition on electrospray ion yield and structure effects on collision-induced fragmentation. However, RRFs should not change once a particular method is established. The conclusion that RRFs for the structurally very similar okadaic acid group can vary widely is not supported by McNabb et al.³ with a mussel certified reference material (CRM) (OA, DTX1) or recent work at NRCC with new CRMs (Quilliam, unpublished data). RRFs for DTX1 and DTX2 to OA differed by less than 20% from 1.0 and certainly the 200% claimed by Otero et al. is completely unsustainable. QUASIMEME⁸ interlab sample studies (rounds 53, 55, 57, 61; 13 laboratories using LC–MS) gave interlab RSDs that were similar for DTX1, DTX2, and OA (calibrant) indicating that RRFs for these three toxins were not a major variable. The issue of extrapolating RFs to toxins where no CRMs are available is a complex one,⁹ and

Otero et al. make no attempt to cover relevant points. It is obviously preferable to calibrate every key toxin using a CRM. In the case of the okadaic acid group, CRMs for all three toxins are now available from two sources (NRCC and L.Cifga) so this point has become a nonissue.

The use of LC–MS and other instrumental test methods in regulatory laboratories must be accompanied by rigorous quality control procedures for each sample batch. Data for test samples is accepted or rejected on the basis of set criteria for the calibration and QA/QC samples. It is fundamental when attempting to measure subtle effects, such as those being studied here, that similar checks are done to ensure instruments are stable and accurate in all their functions. There is evidence that some basic QC measures were not followed by these authors.

In addition to these serious technical deficiencies, the authors make a statement that is incorrect: page 5910, line 58 "... hence the legal toxic value is increased several-fold with the chemical approach...". The EC legislation prescribes that total OA + DTX1 + 160 µg/kg, the level toxic in the i.p. mouse bioassay (MBA). Mice respond very sensitively i.p. to some other toxins such as yessotoxin and gymnodimine. These toxins are relatively nontoxic orally and therefore have much higher or no set limits in shellfish. False positives (overestimation of toxin levels) are a major disadvantage of MBA and, in addition to ethical concerns, have led to its removal from seafood regulatory testing. The authors' implicit call for a return to MBA is not supported by their data.

Otero et al. have reported a number of observations and attempted to discredit LC–MS as a technique for quantitative analysis but have made no real effort to understand the reasons for the purported problems. Their wide-ranging conclusions relied solely on ill-defined comparisons of point-concentration data that are probably affected by sample carryover.

AUTHOR INFORMATION

Corresponding Author

*E-mail: patrick.holland@cawthron.org.nz.

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