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Selective Extraction and Purification of Azaspiracids from Blue Mussels (*Mytilus edulis*) using Boric Acid Gel

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Abstract

Azaspiracids belong to a family of more than 50 polyether toxins originating from marine dinoflagellates such as *Azadinium spinosum*. All of the azaspiracids reported thus far contain a 21,22-dihydroxy group. Boric acid gel can bind selectively to compounds containing *vic*-diols or α-hydroxycarboxylic acids via formation of reversible boronate complexes. Here we report use of the gel to selectively capture and release azaspiracids from extracts of blue mussels. Analysis of the extracts and fractions by LC–MS showed that this procedure resulted in an excellent clean-up of the azaspiracids in the extract. Analysis by ELISA and LC–MS indicated that most azaspiracid analogues were recovered in good yield by this procedure. The capacity of boric acid gel for azaspiracids was at least 50 μg/g, making this procedure suitable for use in the early stages of preparative purification of azaspiracids. In addition to its potential for concentration of dilute samples, the extensive clean-up provided by boric acid gel fractionation of azaspiracids in mussel samples almost eliminated matrix effects during subsequent LC–MS, and could be expected to reduce matrix effects during ELISA analysis. The method may therefore prove useful for quantitative analysis of azaspiracids as part of monitoring programs. Although LC-MS data showed that okadaic acid analogues also bound to the gel, this was much less efficient than for azaspiracids under the conditions used. The boric acid gel methodology is potentially applicable to other important groups of natural toxins containing diols, including ciguatoxins, palytoxins, pectenotoxins, tetrodotoxin, trichothecenes, and toxin glycosides.

Keywords: azaspiracid; boronate; purification; matrix effect; LC–MS; ELISA; diol

Introduction

Natural toxins, such as mycotoxins and algal toxins, generally occur at low levels in natural samples, often in difficult matrices such as grain products or seafood. Extracts from such samples typically contain relatively small amounts of the toxins in a complex mixture of much larger amounts of other compounds from the matrix. This not only makes analysis challenging, but also makes isolation of pure toxins a difficult and tedious process.

Azaspiracids (azaspiracids) are a large group of polyether toxins (Figure 1) responsible for human intoxications of shellfish consumers.[1, 2] Methods have been established for routine monitoring of many of the known azaspiracids in shellfish by LC-MS, with regulatory limits in the EU as well as some other areas.[2] Although such methods are selective and sensitive, detection of known azaspiracid analogues is not trivial and matrix effects are a significant issue in assay development and validation.[2-4] The supply of azaspiracids for use in standards, method development, and monitoring programs was limited by the difficulty of their purification from shellfish[5-8] until the recent development of improved isolation procedures and production of azaspiracid-1, **1** and azaspiracid-2, **2** in cultures,[9-11] although large-scale production of azaspiracids in culture and isolation of minor azaspiracid analogs is challenging. Furthermore, the availability of minor azaspiracids produced by metabolism in shellfish remains inadequate and their isolation difficult.[6, 7, 12, 13] Azaspiracids are required for use in method (HPLC, LC-MS, immunoassay and other methods) development and validation, as analytical standards, for toxicological assessment, and for biochemical studies aimed at elucidating the mechanism of action and possible development of lead compounds of potential pharmaceutical interest. Further improvements in azaspiracid isolation would therefore be very beneficial to researchers, analysts and regulators, and better knowledge and methodologies would improve the

degree of public protection from azaspiracid-associated food poisoning events while minimizing unnecessary disruptions to shellfish harvesting and production.

The most selective extraction methods should be based on the specific functional groups present in the toxin, and their three dimensional disposition. Specific binding proteins, such as antibodies or cellular receptors, are often excellent for this purpose in the form of affinity columns. However, the specificity of some antibodies can be too narrow unless they have been intentionally designed for broad specificity.[14] The range of suitable antibodies available is also relatively limited and, although immunoaffinity columns for azaspiracids have been reported,[14] the binding capacity and cost may make them unsuitable for preparative or routine analytical purposes. Molecularly imprinted polymers (MIPs) are also potentially highly specific, but again availability and "cross-reactivity" may not be sufficient for some analyte groups, and no MIPs for azaspiracids have been reported.

An alternative approach is the use of polymers containing reagents that reversibly bind to specific functional group in the target molecules. While such polymers should be much less specific than antibody- or MIP-based approaches, this can be an advantage if the intention is to capture a large family of structurally related compounds, such as azaspiracids. Provided the family of compounds contain the functional group targeted by the polymer, then all of them would be expected to bind to the polymer. The modest specificity of this approach is not expected to be a problem when more selective methods are used in subsequent analytical steps; for example, LC–MS or immunoassay if the aim is chemical analysis, or additional purification steps if the aim is isolation of toxin analogues. The advantages of this approach are well illustrated by the recent report of the selective extraction and concentration of the mycotoxin zearalenone from plant oils, prior to analysis by HPLC with fluorescence detection.[15] In this case, the method was based on

reversible reaction of a ketone group on zearalenone to form a hydrazone with a polymer-bound hydrazide, and resulted in a highly sensitive analytical method that was selective for zearalenone and zearalanone and did not detect closely related non-ketone metabolites such as zearalenols.

Most of the known natural azaspiracids contain a range of functional groups, including a cyclic secondary amine (at C-40), a carboxylic acid (at C-1), and two hydroxyl groups (at C-20 and C-21) (Figure 1). These are very common functional groups, and therefore not of themselves likely to be particularly helpful in the development of specific extraction methods. However, the two hydroxyl groups are vicinal to each other, a feature that has been exploited in a structurally diagnostic periodate cleavage reaction to identify partial structures of new azaspiracids.[12, 13, 16] Some 1,2- and 1,3-diols and α-hydroxycarboxylic acids are known to bind reversibly with boronates to form boronate esters (Figure 2),[17-19] and polymer-bound boronates are sometimes utilized in the isolation of carbohydrate derivatives,[20] catechols,[21, 22] other types of compounds containing *vic*-diols[23] or α-hydroxycarboxylic acids,[24] and in the analysis of α-hydroxyamines such as florfenicol amine residues in fish muscle.[25]

The primary aim of this study was to determine whether azaspiracids bind reversibly to boric acid gel (Figure 3), a polymer containing 3-aminophenylboronic acid coupled via an amide linkage, and, if so, to test whether this could provide a basis for methods to clean up azaspiracids from shellfish samples as a prelude to analysis or purification. A secondary aim was to evaluate the ability of the gel to bind to okadaic acid (OA) analogues (Figure 4), which contain an α-hydroxycarboxylic acid moiety, in the same shellfish samples.

Materials and Methods

Materials. Reservoirs, frits, and column caps were from Isolute SPE accessories (Teknolab, Ski, Norway). Boric acid gel was from Aldrich Chemical Co. (Steinheim, Germany, or St Louis, MO). All other inorganic chemicals and organic solvents were of reagent grade or better. The Bruckless methanolic mussel hepatopancreas extract was as described by Rehmann et al.[26] IRNO DSP/AZA LRM 05/2[27] is an in-house reference material (RM) produced according to the principles outlined by Hess et al.[28] from a mixture of blue mussels, mussel hepatopancreas, purified toxins and algal culture extracts, and contains an array of regulated marine algal toxins at levels (including **1**–**3** and **23**–**25** at 310, 70, 90, 340, 340, and 960 μg/kg, respectively) close to or below their EU regulatory limits, . Extraction of the IRNO DSP/AZA LRM was as described elsewhere.[27] RM-AZA123, and certified reference materials (CRMs) of **1**, **2**, azaspiracid-3, **3**, okadaic acid, **23**, dinophysistoxin-1, **24**, dinophysistoxin-2, **25**, and algal-toxin-free mussel tissue (CRM-Zero-Mus), were from the National Research Council, Halifax, Canada, RMs of azaspiracid-4–10, **4**–**10** were prepared as described by Kilcoyne et al.,[12] and azaspiracid-33, **21** was available from previous work.[9]

Preparation of boric acid gel columns. Preparation of the boric acid gel columns was based on Technical Information Bulletin no. AL-102 from Aldrich (Sigma-Aldrich, St. Louis, MO).[29] Columns of approximately 1 mL were prepared, by suspending 1.2 g boric acid gel in milli-Q water, adding it to SPE cartridges, and inserting the porous frit. The columns were then activated by running 0.5 M HCl (10 mL) through the columns, washing with water (4 × 10 mL), and finally with MeOH (6 mL) to equilibrate the columns with respect to the samples.

Fractionation procedure. (1) Extract from Bruckless mussel hepatopancreas (0.2 mL) was applied to a boric acid gel column and washed with MeOH (5.0 mL), with the load and wash collected together (total volume ca 5.2 mL). The column was then eluted with 0.1% formic

acid in MeOH (3 × 5.0 mL), with each aliquot of eluent collected separately. An aliquot of the Bruckless hepatopancreas extract (0.2 mL) was also made up to 5.0 mL with MeOH for comparison with the fractions from the gel column.

(2) Extract from the IRNO DSP/AZP LRM (5.0 mL) was applied to a boric acid gel column and washed with MeOH (5.0 mL), with the load and wash collected together (total volume ca 10.0 mL). The column was then eluted with 0.1% formic acid in MeOH (3 × 5.0 mL) as above.

(3) Extract from CRM-Zero-Mus (10.0 mL) was applied to a boric acid gel column, and the column washed with MeOH (5 mL) and eluted with 0.1% formic acid in MeOH (3 × 5.0 mL), as above. For the matrix effect evaluation, the first and second elution fractions were combined (total volume 10 mL) before spiking with **1**–**3**.

Matrix effect study. CRM-Zero-Mus tissue (2.08934 g) was transferred to a 50 mL centrifuge tube, weighed and extracted in four steps with 5.5 mL aliquots of MeOH, following a procedure described previously for azaspiracid reference materials.[30] A DVX 2500 multi tube vortex mixer (VWR, West Chester, PA) was used for the first step (2500 rpm, 2 min) prior to centrifuging (3000 *g*, 10 min) and decanting the liquid phase. The second and third extraction steps used an Omni prep homogenizer (Omni Int., Kennesaw, GA) (10,000 rpm, 3 min). Vortex mixing was used again for the final step. The extracts from all four steps were combined in a 25 mL volumetric flask and brought to volume with MeOH.

A stock solution of **1**–**3** was prepared using NRC-RM-AZA123, a calibration solution RM containing a mixture of **1**, **2** and **3** at 168, 125 and 116 ng/mL, respectively. A working solution was made by preparing a 2-fold serial dilution of this stock, which was subsequently used for preparation of a five-level dilution series in the unfractionated CRM-Zero-Mus extract and in the same extract after elution from a boric acid gel column (using the combined

first and second 5-mL eluates). Spiking was performed by mixing 50 µL aliquots of each dilution level with 200 µL aliquots of either MeOH, unfractionated CRM-Zero-Mus extract, or CRM-Zero-Mus extract following boric acid gel clean-up (first 10 mL eluted from the column). A Microlab diluter (Hamilton Company, Reno, NV) was used for preparing the dilutions. Concentrations of **1**–**3** in the spiked samples were 2.2–34, 1.6–25, and 1.5–23 ng/mL, respectively. Aliquots of the spiked extracts were passed through 0.45 µm regenerated cellulose filters (Millipore Corp, Billerica, MA) prior to analysis by LC-MS/MS (method D). Least-squares fitting of data to straight lines was performed using SigmaPlot version 12.5 (Systat Software Inc., San Jose, CA).

LC–MS analysis. Extracts and fractions were analyzed by one or more of the following LC–MS methods.

LC–MS (method A). Liquid chromatography was performed on a 100 × 2.1 mm i.d., 3.5 µm, Symmetry C18 column (Waters, Milford, MA) using a Surveyor MS Pump Plus and a Surveyor Auto Sampler Plus (Finnigan, Thermo Electron Corp., San Jose, CA) as described previously, eluted with a linear gradient (0.3 mL/min) of acetonitrile (A) and water (B) each containing 0.1% formic acid. The gradient was from 22–75% A over 10 min, to 95% A at 11 min (1 min hold), followed by a return to 22% A with a 3-min hold to equilibrate the column. The HPLC system was coupled to a Finnigan LTQ ion trap mass spectrometer (Finnigan Thermo Electron Corp.) operated in positive electrospray ionization mode (*m/z* 500–1600). The ion injection time was set to 100 ms with a total of three micro-scans. ESI parameters were a spray voltage of 6 kV, a capillary temperature of 375 °C, a sheath gas rate of 55 units N_2 (ca 550 mL/min) and an auxiliary gas rate of 5 units N_2 (ca 50 mL/min). Figures 5 and 6 show base-peak chromatograms in full scan mode (*m/z* 500–1000) obtained with this method, with the chromatograms in each Figure displayed with the same vertical scale (0–1.5 × 10^7 counts in Figure 5, 0–2 × 10^5 counts in Figure 6) after correction for the volume of each

fraction, such that the intensities of peaks in all chromatograms in each Figure are directly comparable.

LC–MS/MS (method B). Analysis was performed on an Acquity UPLC coupled to a Xevo G2-S QToF (Waters, Manchester, UK) operated in positive electrospray ionization MS^e mode, scanning from *m/z* 100−1200. Leucine encephalin was used as the reference compound. The cone voltage was 40 V, collision energy was 50 V, the cone and desolvation gas flows were set at 100 and 1000 L/h, respectively, and the source temperature was 120 °C. Linear gradient elution was used, with phase A consisting of water and phase B of 95% acetonitrile in water (both containing 2 mM ammonium formate and 50 mM formic acid). The column used was a 50 mm × 2.1 mm i.d., 1.7 µm, Acquity UPLC BEH C18 (Waters, Wexford, Ireland). The gradient was from 30–90% B over 5 min at 0.3 mL/min, held for 0.5 min, and returned to the initial conditions and held for 1 min to equilibrate the system. The injection volume was 2 µL and the column and sample temperatures were 25 °C and 6 °C, respectively.

LC–MS/MS (method C). For analysis of okadaic acid analogues, the same mobile phase, LC conditions and column were used as for analysis of the azaspiracids (method B), but with the mass spectrometer operating in negative ion mode. The gradient was from 5–50% B over 1 min at 0.3 mL/min, increased to 90% B over 1 min, held for 1 min and returned to the initial conditions and held for 1 min to equilibrate the system.

Peak areas were measured from extracted ion chromatograms at *m/z* of $[M+H]^+$ for each analyte (5 ppm window). Quantitation of **1**–**3**, and **23**–**25** were relative to CRMs of the relevant analytes, while **4**–**10** were quantitated relative to LRMs prepared by Kilcoyne et al.[12] and azaspiracid-17, **17** and azaspiracid-19, **19** were quantitated relative to the AZA1 CRM

LC–MS/MS (method D). LC-MS/MS analyses for **1**–**3** and **6** were performed with a method described previously for analysis of azaspiracids in mussel tissue reference materials,[30] using

a 1200 LC system (Agilent, Palo Alta, CA) connected to a Q-TRAP API4000 mass spectrometer (AB-Sciex, Concord, ON, Canada) equipped with a turbospray ionization source. A 50 × 2.1 mm i.d., 2.5 μm, Luna C18(2) HST column (Phenomenex, Torrence, CA) was eluted with a binary mobile phase of water (A) and acetonitrile–water (95:5) (B), each containing 5 mM ammonium acetate (pH 6.8). A gradient was run from 25–100% B over 5 min at 350 μL/min at 15 °C, with 5 μL injections. The MS was operated in positive selected reaction monitoring (SRM) mode with the following transitions: **1** (*m*/*z* 842.5 → 672.5 and 362.3); **2** (*m*/*z* 856.5 → 672.5 and 362.3); **3** (*m*/*z* 828.5 → 658.5 and 362.3), and; **6** (*m*/*z* 842 → 658.5 and 362.3). The collision energies for **1**–**3** were 65 and 75 eV for the transitions to *m*/*z* 672.5/658.5 and to *m*/*z* 362.3, respectively. The declustering potential (DP) was 50 eV, the source temperature was 350 °C, and the ion-spray voltage was 5500 V.

ELISA analysis. The concentration of azaspiracids in each extract was determined by indirect competitive enzyme-linked immunoasorbent assay (ELISA)[31] using antibodies produced against a synthetic azaspiracid-fragment.[14] All incubations were performed at room temperature. The standard of **1** in methanol (1 μg/mL) was diluted in PBST and sample-buffer (10% methanol in PBST), to give a methanol concentration of 10%. Serial three-fold dilutions of the standard of **1** were performed with sample buffer, giving 10 standards from 0.0279–550 ng/mL. Serial dilutions of standards and samples were performed in duplicate. Absorbances were measured at 450 nm using a plate reader.

Results and Discussion

Many extraction methods for analyzing shellfish for lipophilic algal toxins use either 80–100% MeOH.[32] Therefore the boric acid gel-manufacturer's recommendations were adapted to accommodate 100% MeOH, although in preliminary trials the gel columns appeared to

function equally well with 90% MeOH (data not shown). Initially, the columns were loaded with a suitable small volume of extract (under 1 mL), washed with 100% MeOH (5 mL), and then eluted with 100% MeOH containing a weak acid (3 × 5 mL).

After initial method development, the procedure was tested with a highly contaminated extract from hepatopancreas excised from cooked blue mussels obtained during a major azaspiracid contamination event at Bruckless, Ireland, in 2005.[26] Fractionation of the colored Bruckless extract on boric acid gel resulted in a yellow load/wash fraction, with the three subsequent elution fractions being nearly colorless. Analysis by LC–MS (method A) in full scan mode (*m*/*z* 500–1000) revealed that although the unfractionated Bruckless extract was highly enriched in azaspiracids, significant contaminating peaks were present in the chromatogram—especially from 7.5–8.6 min (Figure 5A). Although the column-wash contained small but significant amounts of azaspiracids, it contained virtually all of these non-azaspiracid contaminants (Figure 5B, Table 1). Elution with acidic MeOH produced fractions highly enriched in azaspiracids, and virtually free from contaminants detectable with the LC–MS method used for analysis (Figures 5C–E, Table 1). Most of the azaspiracids were present in the first 5 mL fraction collected (Figure 5C), but significant amounts were also present in the second 5 mL fraction (Figure 5D, Table 1).

Quantitative LC–MS/MS analysis (method B) for azaspiracids **1**–**10**, **17** and **18** showed generally good recoveries, and for most azaspiracids, ca 70–80% was eluted in the first 5 mL elution fraction, and 80–90% was eluted in the first 10 mL (Tables 1 and 2). Notable exceptions, however, were 23-hydroxyazaspiracids **5** and **10**, which appeared to bind much more tightly to the boric acid gel and were harder to elute (Table 2), and the results indicated that these compounds were incompletely eluted from the column even after elution with 15 mL (Table 1). This effect is attributable to a second 1,3-diol moiety formed by the axial 21- and 23-hydroxyl groups in the E-ring, or to coordination of the 23-OH to the trigonal boronate

ester formed with the nearby 21- and 22-hydroxy groups to form a tetrahedral boronate complex (Figure 2). It may be possible to improve recovery of these 23-hydroxyazaspiracids by changing the eluent composition, or by adding a competing diol (such as glycerol) to the eluent.

Analysis of the fractions by ELISA was used to provide an estimate of the total concentration of azaspiracids in the fractions, as the antibodies have previously been shown to recognize compounds containing the C-27–40 moiety present in most azaspiracids (Figure 1) regardless of the substitution pattern present at C1–C-26.[14, 31] Results by ELISA for total azaspiracids were 1.3–2.0-fold those obtained by LC-MS/MS for the sum of **1**–**10**, **17** and **18** (Table 3), similar to results previously obtained[31] for unfractionated extracts of mussels. These results suggest that boric acid gel fractionation may also function for minor and as-yet unidentified azaspiracids, which would be expected to be recognized by the antibodies, in addition to the azaspiracids that were quantitated by LC-MS/MS (**1**–**10**, **17** and **18**). That this may be the case is supported by the detection by LC–MS (method A) of **21** in the fractions (Figure 5).

Small amounts of the α-hydroxycarboxylic acid toxins **23** and **25** (Figure 4) were also present in the Bruckless extract. Although **23** and **25** were partially retained (both at ca 35%) by the boric acid gel column (Table 2), this effect was too weak to provide a practical extraction and clean-up method under the conditions developed for azaspiracids. Boric acid-catalyzed esterification of some α-hydroxycarboxylic acids has been reported in methanol,[33] however the high overall recovery (98.1%) of **23** and **25** in the load/wash and elution fractions (Table 1) precludes the possibility that these compounds were converted to their methyl esters on the column, and methyl esters of **23** and **25** were not detected in any of the fractions by LC–MS.

The same boric acid gel fractionation procedure was then tested on an extract from an RM representing whole mussels contaminated with much lower levels of azaspiracids and also

containing okadaic acid analogues. This type of sample is more typical of the sort of toxin levels likely to be found in real contaminated samples, and the matrix components are those from the whole shellfish, rather than from the hepatopancreas as in the Bruckless extract. Figure 6A shows the full scan chromatogram for this sample, which is characterized by small peaks of the main azaspiracids partially overlapped with small peaks from the sample and surrounded by much larger sample peaks. The column wash removed the bulk of the non-azaspiracid peaks, although once again a small proportion of the azaspiracids were also removed by the washing step (Figure 6B). Elution with acidic MeOH provided an extract in which azaspiracids were major components in the LC–MS chromatograms, with very few overlapping interferences remaining (Figure 6C). Examination of the individual peaks in the chromatograms gave similar results to those for the Bruckless sample (Table 1), with most of the azaspiracids eluted in the first 10 mL, although concentrations for minor azaspiracids were uncertain due to their low concentrations.

Significant matrix suppression (27–41%) was evident from the slopes of standard curves of **1**–**3** spiked into crude CRM-Zero-Mus extracts compared to MeOH (Figure 7). However, only minimal matrix effects (5–8%), close to the precision of the method, were observed for **1**–**3** spiked into CRM-Zero-Mus extract that had been subjected to boric acid gel clean-up (Figure 7). The degree of clean-up provided by the column was also evident in visual comparison of CRM-Zero-Mus extracts before and after boric acid gel clean-up.

The procedure was developed to achieve acceptable binding and release of azaspiracids using the sample matrix and the boric acid gel material available. Excellent results were obtained when loading 0.2 mL of the concentrated Bruckless hepatopancreas extract (Tables 1–3 and Figure 5) and with 5.0 mL of the much less concentrated IRNO LRM extract (Figure 6), suggesting that selective concentration of azaspiracids from larger volumes of very weak samples may be feasible. While Table 1 shows a binding capacity of at least 20 μg of total

azaspiracids, retention of 50 μg of azaspiracids per column was observed, as was reusability of the columns, during the initial method development (data not shown), indicating the potential of boric acid gel for preparative applications. The potential utility of boric acid gel fractionation for analytical applications was demonstrated by the greatly reduced matrix effects for **1**–**3** compared to those normally observed during LC-MS analysis of azaspiracids in mussels. This methodology may also be applicable to azaspiracids in samples from other sources, such as algal cultures and blooms, passive samplers, and derivatized azaspiracids. In addition to azaspiracids, many other groups of natural toxins contain functionalities with the potential to complex to boronates, and promising results have already been obtained using boric acid gel with tetrodotoxins (D. Beach, E. Kerrin, and C. O. Miles, unpublished results). In light of the results presented here, inclusion of boronic acid derivatives may prove advantageous in the preparation of MIPs designed for selective binding of appropriate diol-, α-hydroxyacid- and α-hydroxyamine-containing toxins, in a similar manner to that used to develop MIPs for carbohydrate-containing molecules.[34]

In summary, while further optimization is required, the preliminary results presented here indicate that use of boric acid gel in a column format has the potential to afford very good cleanup of azaspiracid-containing mussel extracts, removing many of the interfering peaks from LC–MS chromatograms as well as much of the color. Acceptable recoveries were obtained for all azaspiracids analyzed, apart from the 23-hydroxylated variants **5** and **10**, which appeared to be more strongly retained on the column. Application of boric acid gel has the potential to simplify analysis of azaspiracids, because it was shown to greatly reduce matrix effects in LC–MS analysis, it can be used to concentrate dilute samples, and it should make it easier to detect and identify novel azaspiracids that contain the 20,21-diol moiety. Boric acid gel may also be applicable to some of the numerous other natural toxins that contain potential boronate-binding diols, hydroxyamines and hydroxycarboxylic acids, such

as trichothecenes, tetrodotoxins, ciguatoxins, palytoxins, pectenotoxins, as well as many toxin glycosides.

Abbreviations Used

AZA, azaspiracid; CRM, certified reference material; MIP, molecularly imprinted polymer; OA, okadaic acid; RM, reference material.

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Supporting Information Description

Color photographs of fractions from the three boric acid gel columns, molecular model of a tetrahedral phenylboronate complex with a 22-desmethyl-23-hydroxyazaspiracid, tabulated amounts of azaspiracid analogues from the fractionated IRNO LRM extract, and graphs showing matrix effects for LC–MS analysis of **2** and 3 in spiked extracts of mussels with and without boric acid gel fractionation.

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Figure Captions

Figure 1. Structures of some of the azaspiracids reported in the literature,[2] with the C-20 stereochemistry as revised by Kenton et al.[35]

Figure 2. Equilibria between phenylboronic acid and a generic 1,2-diol in an aqueous solvent (modified from Scheme 1 of Bromba et al.[18]), with the trigonal form of the boronate on the left and the tetrahedral form on the right.

Figure 3. Reversible reaction between an azaspiracid (see Figure 1 for structures and atom numbering) and boric acid gel, a polymer containing amide-linked 3-aminophenyl boronic acid.

Figure 4. Structures of common okadaic acid analogues.[36, 37]

Figure 5. LC–MS analysis (method A) of: (A) Bruckless mussel hepatopancreas extract, 200 µL diluted to 5.0 mL; and fractions from boric acid gel-fractionation of the extract: (B) column load/wash fraction, 5.2 mL; (C–E) first, second and third elution fractions, 5.0 mL each.

Figure 6. LC–MS analysis (method A) of: (A) IRNO whole mussel extract (5.0 mL), and fractions from boric acid gel-fractionation of the extract; (B) column load/wash fraction, 10 mL), and; (C) first elution fraction, 5.0 mL). The second and third elution fractions are not shown, but results closely paralleled those shown in Figure 5.

Figure 7. Relative response of **1** (LC-MS/MS method D) in spiked samples of MeOH, an unfractionated extract of CRM-Zero-Mus, and the same extract after elution from a boric acid gel column. Each sample was spiked with a dilution series containing **1**–**3**.

Table 1. Amounts of Azaspiracids, **1**–**10**, **17** and **18** and Okadaic Acid Analogues, **23** and **24** Recovered after Boric Acid Gel Fractionation of the Bruckless Mussel Hepatopancreas Extract, as Determined by LC–MS/MS.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**17**	**18**	AZAs[a]	**23**	**25**	OAs[a]
	ng	ng	ng	ng	ng	ng	ng	ng	ng	ng	ng	ng	ng	ng	ng	ng
Applied	10466.1	2961.9	3151.2	1888.3	485.2	638.1	239.4	306.7	525.6	154.6	40.4	23.1	20880.7	302.5	57.5	360.0
Wash[b]	1061.8	384.3	426.4	160.3	3.0	145.3	17.8	36.2	79.6	0.0	13.0	8.6	2336.2	181.2	33.3	214.5
Elute 1	8350.9	2299.1	2639.3	1751.9	70.9	537.3	267.3	211.0	665.8	27.1	50.0	19.9	16890.6	117.3	17.3	134.5
Elute 2	1184.5	312.3	213.8	126.4	101.5	63.7	46.5	57.5	50.2	43.2	5.6	1.7	2206.8	3.3	0.3	3.5
Elute 3	185.5	41.0	24.3	14.1	87.2	8.3	7.6	37.1	5.2	36.8	0.5	0.1	447.7	0.3	0.3	0.5
% Recovery[c]	103.0	102.5	104.8	108.7	54.1	118.2	141.7	111.4	152.4	69.3	170.9	131.4	104.8	99.8	88.7	98.1
% Elute 1–3[d]	90.2	87.3	87.1	92.2	98.9	80.7	94.8	89.4	90.1	100.0	81.3	71.5	89.3	40.0	34.8	39.2

[a]AZAs = sum of **1**–**10**, **17** and **18**; OAs = sum of **23**–**25**.

[b]Includes the load and wash volumes together (total 5.2 mL).

[c]Total amount recovered in the Wash plus Elute 1–3 fractions, as a percentage of the amount applied.

[d]Total amount recovered in the Elute 1–3 fractions, as a percentage of the total amount in the Wash plus Elute 1–3 fractions.

Table 2. Total Recovered Amounts of Each Azaspiracid, **1**–**10**, **17** and **18** and Okadaic Acid Analogue, **23** and **25** Present in the Load/Wash and Elution fractions During Boric Acid Gel Fractionation of the Bruckless Mussel Hepatopancreas Extract, as Determined by LC–MS/MS.[a]

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**17**	**18**	AZAs[b]	**23**	**25**	OAs[a]
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Wash[c]	9.8	12.7	12.9	7.8	1.1	19.3	5.2	10.6	9.9	0.0	18.7	28.5	10.7	60.0	65.2	60.8
Elute 1	77.4	75.7	79.9	85.3	27.0	71.2	78.8	61.7	83.1	25.3	72.3	65.6	77.2	38.8	33.8	38.1
Elute 2	11.0	10.3	6.5	6.2	38.7	8.4	13.7	16.8	6.3	40.3	8.1	5.7	10.1	1.1	0.5	1.0
Elute 3	1.7	1.4	0.7	0.7	33.2	1.1	2.2	10.9	0.7	34.4	0.8	0.2	2.0	0.1	0.5	0.1

[a]This table indicates the *distribution* of each compound in the wash and three elute-fractions, not the overall recovery (which is presented in Table 1). Thus, each column adds to 100%, although recoveries relative to the amount loaded are not 100%.

[b]AZAs = sum of **1**–**10**, **17** and **18**; OAs = sum of **23**–**25**.

[c]Includes the load and wash volumes together (total 5.2 mL).

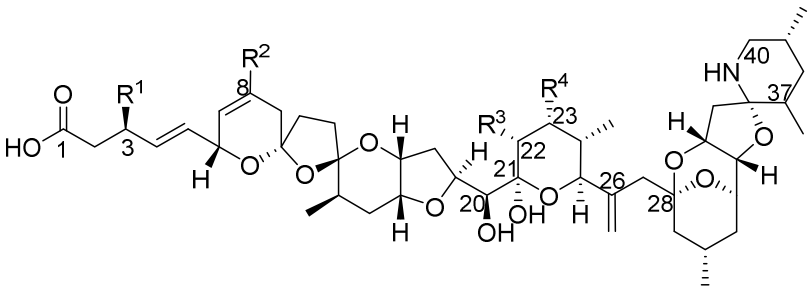
Table 3. Comparison of Total Azaspiracids as Measured by ELISA and LC–MS/MS in Fractions from the Boric Acid Gel Column Fractionation of the Bruckless Mussel Hepatopancreas Extract.

	ELISA	LC–MS[a]	Ratio
	µg	µg	
Applied	—	20.9[b]	—
Wash[c]	4.6	2.3	2.0
Elute 1	25.9	16.9	1.5
Elute 2	3.4	2.2	1.5
Elute 3	0.6	0.4	1.3

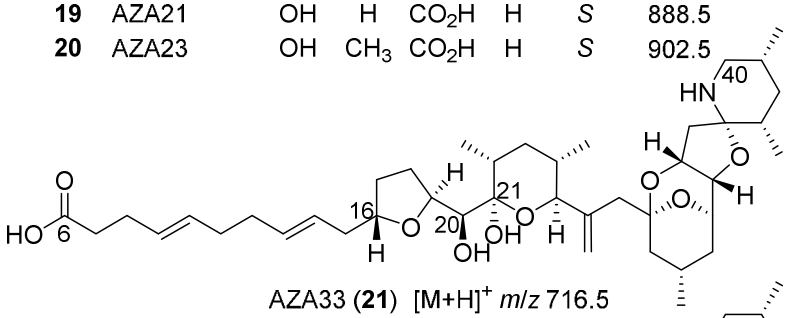
[a]Sum of **1**–**10**, **17** and **18** (from Table 1).

[b]The total azaspiracids detected in the wash and three elution fractions was 21.9 µg.

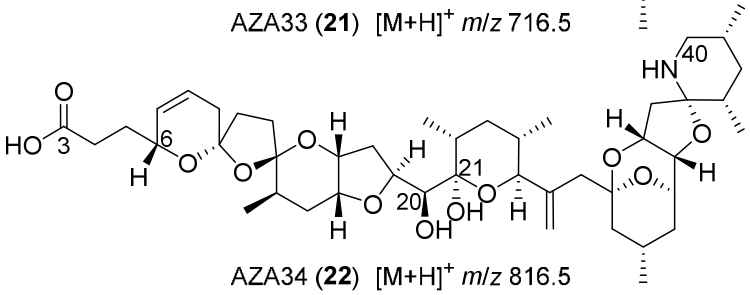
[c]Includes the load and wash volumes together (total 5.2 mL).



	Name	R^1	R^2	R^3	R^4	C-37	*m/z* $[M+H]^+$
1	AZA-1	H	H	CH_3	H	*S*	842.5
***epi*-1**	37-*epi*-AZA1	H	H	CH_3	H	*R*	842.5
2	AZA2	H	CH_3	CH_3	H	*S*	856.5
3	AZA3	H	H	H	H	*S*	828.5
4	AZA4	OH	H	H	H	*S*	844.5
5	AZA5	H	H	H	OH	*S*	844.5
6	AZA6	H	CH_3	H	H	*S*	842.5
7	AZA7	OH	H	CH_3	H	*S*	858.5
8	AZA8	H	H	CH_3	OH	*S*	858.5
9	AZA9	OH	CH_3	H	H	*S*	858.5
10	AZA10	H	CH_3	H	OH	*S*	858.5
11	AZA11	OH	CH_3	CH_3	H	*S*	872.5
12	AZA12	H	CH_3	CH_3	OH	*S*	872.5
13	AZA13	OH	H	H	OH	*S*	860.5
14	AZA14	OH	H	CH_3	OH	*S*	874.5
15	AZA15	OH	CH_3	H	OH	*S*	874.5
16	AZA16	OH	CH_3	CH_3	OH	*S*	888.5
17	AZA17	H	H	CO_2H	H	*S*	872.5
18	AZA19	H	CH_3	CO_2H	H	*S*	886.5
19	AZA21	OH	H	CO_2H	H	*S*	888.5
20	AZA23	OH	CH_3	CO_2H	H	*S*	902.5



AZA33 (**21**) $[M+H]^+$ *m/z* 716.5



AZA34 (**22**) $[M+H]^+$ *m/z* 816.5

Figure 1

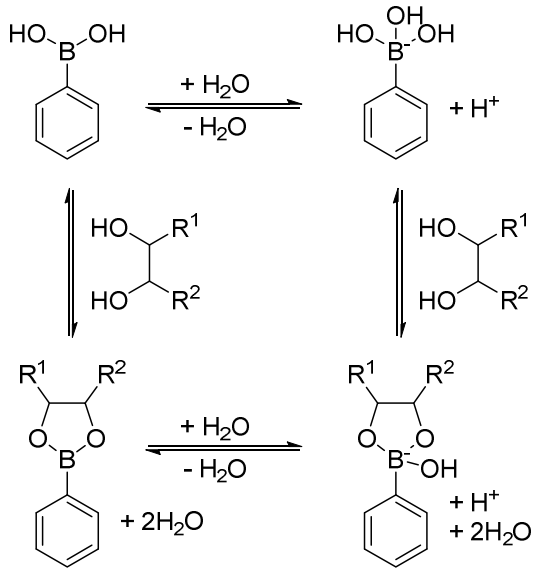

HO
B
OH
+ H2O
- H2O
OH
HO
B
OH
+ H+
HO
R1
HO
R2
HO
R1
HO
R2
R1
R2
O
O
B
+ H2O
- H2O
+ 2H2O
R1
R2
O
O
B
OH
+ H+
+ 2H2O

Figure 2

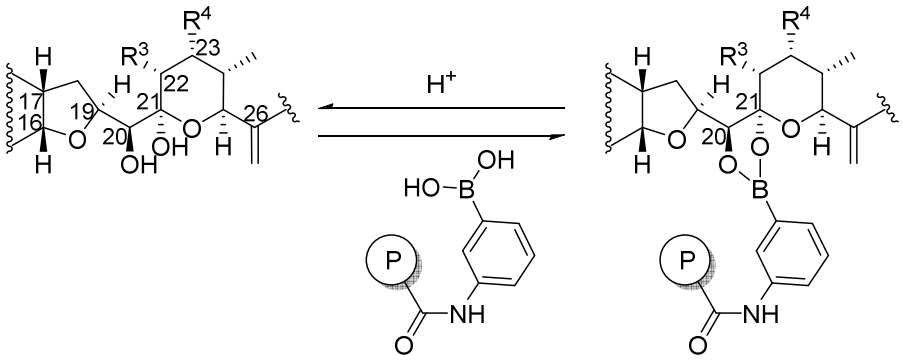
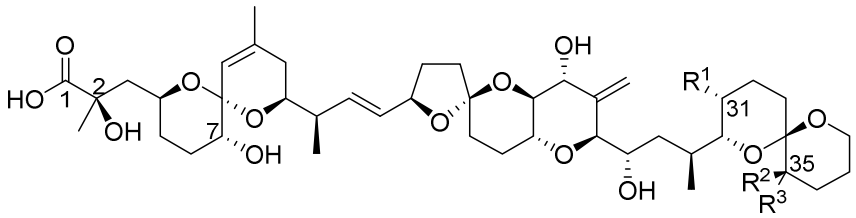


Figure 3



		R^1	R^2	R^3
23	Okadaic Acid	CH_3	H	H
24	Dinophysistoxin-1	CH_3	H	CH_3
25	Dinophysistoxin-2	H	CH_3	H

Figure 4

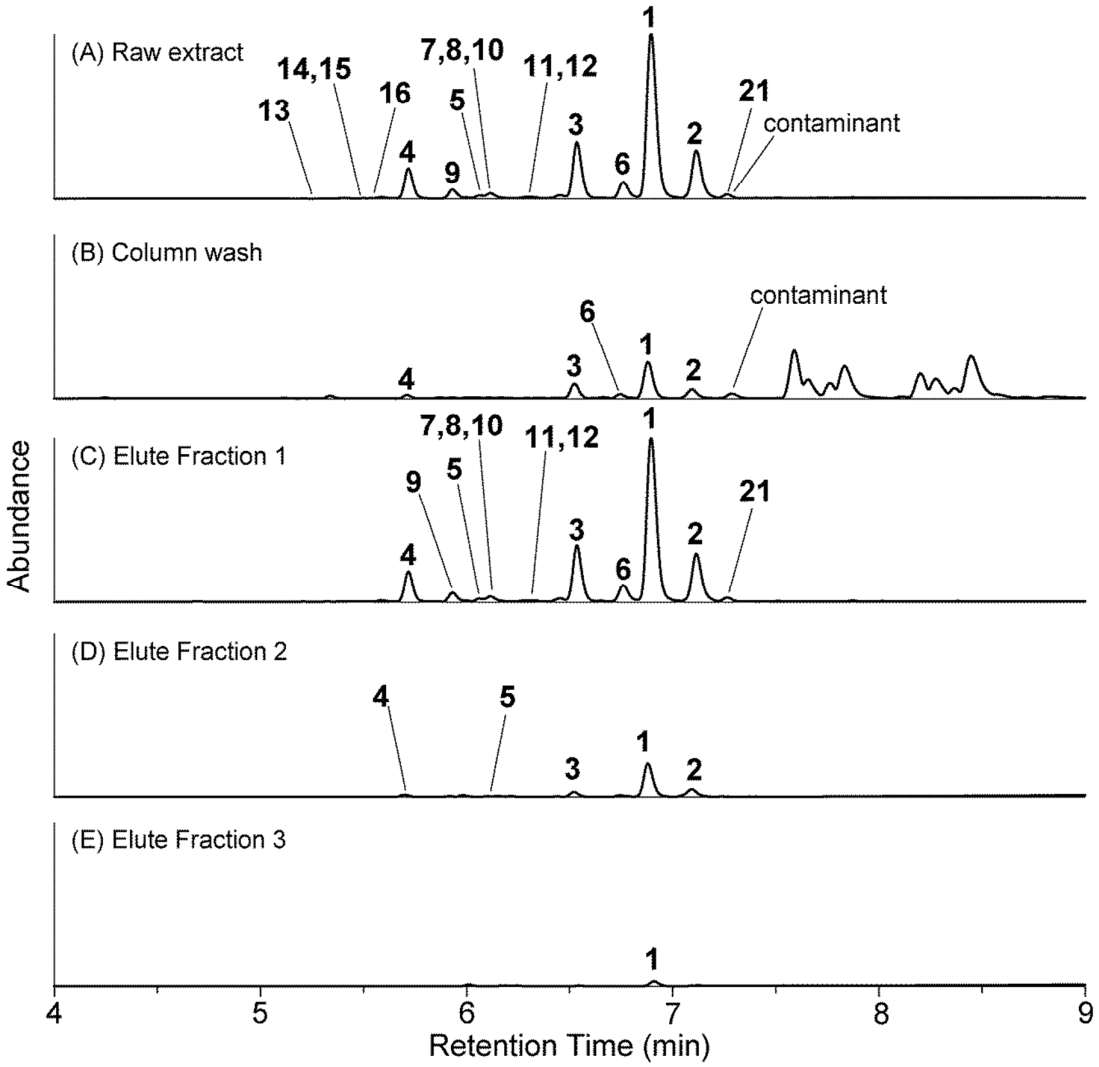

(A) Raw extract
13
14,15
16
4
9
7,8,10
5
11,12
3
6
1
2
21
contaminant
(B) Column wash
4
3
6
1
2
contaminant
(C) Elute Fraction 1
4
9
7,8,10
5
11,12
3
6
1
2
21
(D) Elute Fraction 2
4
5
3
1
2
(E) Elute Fraction 3
1
Abundance
4
5
6
7
8
9
Retention Time (min)

Figure 5

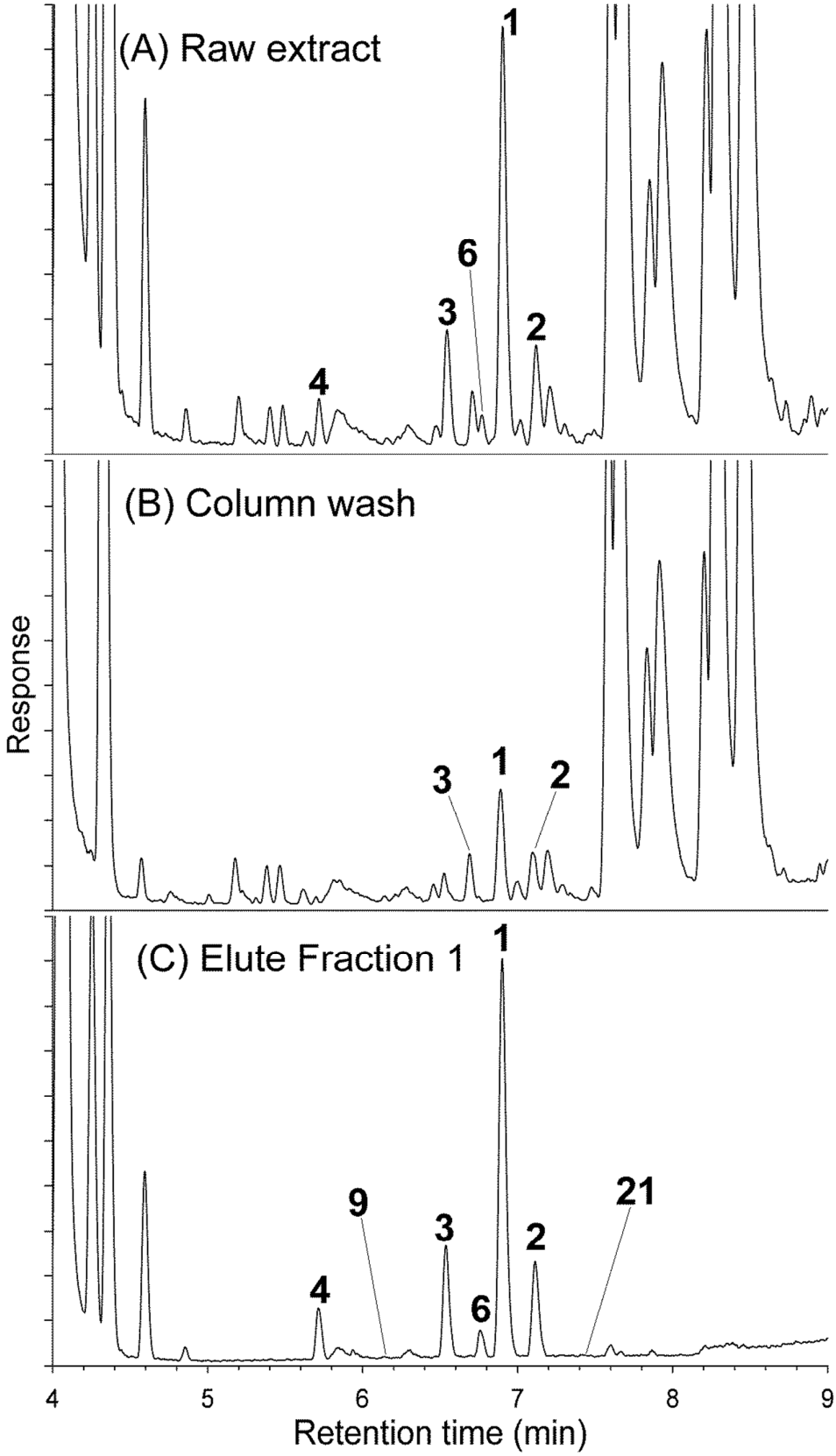

(A) Raw extract
1
6
3
2
4
(B) Column wash
3
1
2
Response
(C) Elute Fraction 1
1
9
21
3
2
4
6
4
5
6
7
8
9
Retention time (min)

Figure 6

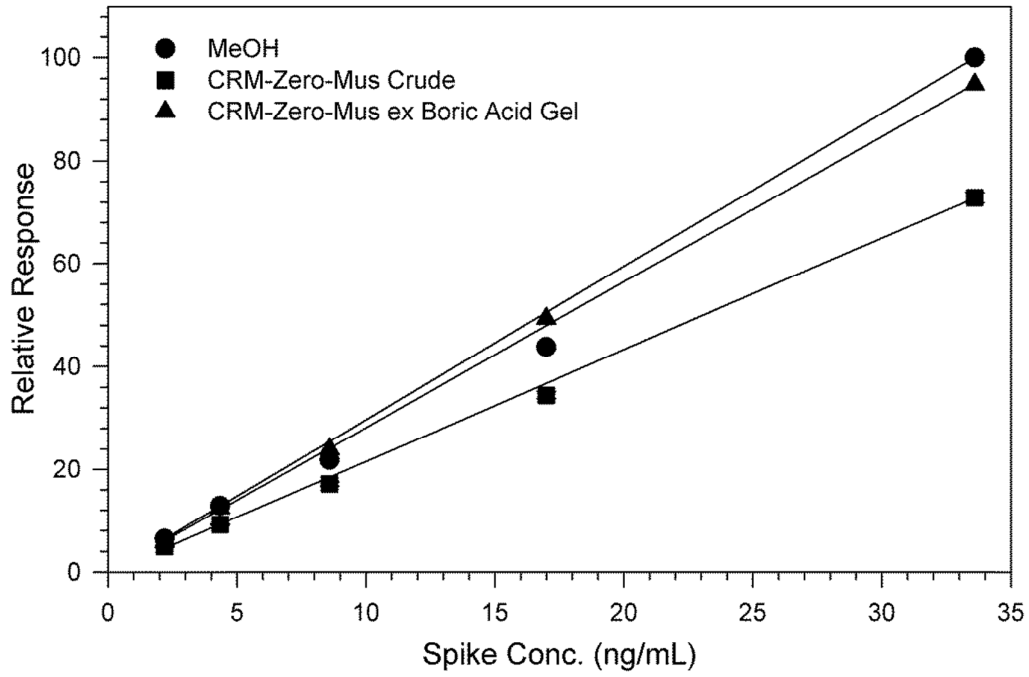

MeOH
CRM-Zero-Mus Crude
CRM-Zero-Mus ex Boric Acid Gel
Relative Response
Spike Conc. (ng/mL)
0
20
40
60
80
100
0
5
10
15
20
25
30
35

Figure 7

Table of Contents Graphic