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ELISA Methods for Domoic Acid Quantification in Multiple Marine Mammal Species and Sample Matrices

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ELISA Methods for Domoic Acid Quantification in Multiple Marine Mammal Species and Sample Matrices

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Executive Summary

Over the past several years, considerable effort has been invested in developing sensitive methods for detecting algal toxins, specifically using enzyme-linked immunosorbent assay (ELISA) technologies. Previously, detection of algal toxins, such as domoic acid (DA), required expensive analytical equipment and highly trained personnel to perform high-performance liquid chromatography (HPLC) or liquid chromatography tandem mass spectrometry (LC/MS/MS) methodologies. While excellent methods exist for DA detection using these technologies for seafood safety programs, these methods lacked the sensitivity needed for researchers investigating the impacts of harmful algal bloom toxins in wildlife. The advent of ELISA kits for DA quantification provides a useful tool for diagnostic studies in natural marine mammal populations, but requires careful testing in new sample matrices before being employed. This technical memorandum provides protocols for DA quantification by ELISA in multiple marine mammal species and sample matrices and outlines consistent reporting criteria for use by the larger scientific community.

Acknowledgments

The authors especially thank Frances Gulland of The Marine Mammal Center for her guidance and mentorship through all of our work. We also thank the many folks involved in sample collection, storage, and delivery—which requires considerable effort without which this project would not have been possible—including Liz Wheeler, The Marine Mammal Center; Kathy Burek Huntington, Alaska Veterinary Pathology Services; members of the Alaska Marine Mammal Stranding Network; Verena Gill, U.S. Fish and Wildlife; Lori Quakenbush, Alaska Department of Fish and Game; and Kerri Danil, Southwest Fisheries Science Center. We appreciate the thoughtful comments and critiques of this manuscript from reviewers including Northwest Fisheries Science Center reviewers Nick Adams, Keri Baugh, Walt Dickhoff, and Michelle McClure.

Introduction

The problem of domoic acid (DA) poisoning in marine mammals has been well documented and tracked since it was first reported in the late 1990s in California sea lions (*Zolophus californianus*) from the central California coast (Lefebvre et al. 1999, Scholin et al. 2000, Gulland et al. 2002). Cases of DA poisoning as large outbreaks, clusters of several animals, or isolated cases have consistently been reported since that time (Bejarano et al. 2008). Primary clinical signs of DA neuroexcitotoxicity consist of seizures and are regularly used for preliminary diagnosis, often with follow-up by histopathological examination for characteristic brain lesions and measurements of toxin content in various matrices such as feces, urine, and blood serum. DA detection and quantification previously relied heavily on high-performance liquid chromatography (HPLC) or liquid chromatography tandem mass spectrometry (LC/MS/MS), which both require specialized equipment or expertise to perform (Quilliam et al. 1989, 1995, Hess et al. 2001, Tor et al. 2003, Leandro et al. 2010). Now, with the advent of commercially available enzyme-linked immunosorbent assay (ELISA)–based kits for DA quantification, any lab equipped with a plate reader has the capacity to detect and quantify DA.

Commercially available ELISA kits are excellent tools that allow for quick, convenient measurements of algal toxins in a large number of samples. They were originally developed for use with shellfish tissue; the details of sample preparation and dilution are well described in the product literature and instructional booklets for this particular matrix. However, these kits have not been thoroughly validated by the manufacturers for use with marine mammal sample matrices. Unlike shellfish samples, marine mammal samples come in many different matrix types, including feces, urine, serum, stomach contents, bile, and other bodily fluids. It should not come as any surprise that a fecal sample and a urine or serum sample are quite different in terms of matrix effects. "Matrix effects" refers to the interaction of the sample material itself with the ELISA reagents and the potential for components other than the target DA to result in a change in the absorbance signal, which will be interpreted as an incorrect DA concentration. For this reason, every new sample matrix needs to be tested to determine appropriate minimum dilutions to avoid these effects. Put simply, false positives can easily be obtained if samples are not adequately diluted.

Due to the issues related to matrix differences and the multiple techniques available for toxin detection, there is a need for a uniform protocol for processing and analyzing marine mammal samples for DA quantification. These protocols must include validation for each type of matrix and a minimum dilution level requirement. When reporting DA values in wildlife samples, the method, minimum dilution, and sample quantification limit should be reported along with the DA values. Knowledge of the analytical technique, minimum dilution, and sample quantification limit is important because different laboratories may use different methods for DA measurement (e.g., ELISA, HPLC, LC/MS/MS) and the sensitivity of these methods varies depending on the type of analysis used and the sample matrix (see Table 4 in Lefebvre and Robertson 2010 for a comparison of commonly reported DA detection methods). This

information must be made clear to those who are interpreting data from toxin analyses for wildlife health assessments. It should also be understood that the quantification limit of the instrument or kit is not the quantification limit for DA within the sample material itself (sample quantification limit). Sample quantification limits will usually be higher than that of the instrument or method due to dilutions needed for extraction, cleanup, and prevention of matrix effects.

The difference between sample and instrument quantification limits highlighted above explains why some samples can yield a positive result from ELISA, but not when analyzed by HPLC or LC/MS/MS. This can lead to confusion and uncertainty over results that are relayed to researchers, veterinarians, or other wildlife health professionals who receive the data. Further complications can arise when looking at past data analyzed via methods with higher minimum sample quantification limits. This is an increasing problem, as researchers are starting to report the "first" observation of DA in a region or species. Often these "firsts" are due to lack of previous sampling or the advent of more sensitive analytical methods.

The goal of this technical memorandum is to provide detailed extraction and quantification methods for DA in a variety of marine mammal samples from several representative species, including pinnipeds, cetaceans, and mustelids. Here we provide matrix-specific recommendations for the use of a commercially available ELISA kit for feces, blood, urine, bile, aqueous humor, and stomach contents. In addition to providing minimum dilutions for ELISA, a comparison of measurements of DA from selected species and matrix types using Biosense ELISA (Biosense Laboratories, Bergen, Norway) and LC/MS/MS is included.

Methods

Sample Collection

California Sea Lions

Samples of frozen California sea lion feces, urine, serum, bile, aqueous humor, stomach contents, and milk were provided from material at The Marine Mammal Center, Sausalito, California. Samples were collected from animals that stranded along the central California coast.

Common Dolphins

Samples of frozen dolphin (Delphinidae) feces, urine, and stomach contents were provided from material collected by the National Marine Fisheries Service's Southwest Fisheries Science Center, La Jolla, California. These animals were collected from the waters off the southern California coast in the San Diego area.

Northern Sea Otters

Samples of frozen northern sea otter (*Enhydra lutris*) urine were obtained from animals collected by U.S. Fish and Wildlife agents in areas along the shores of the Gulf of Alaska. Frozen urine and blood samples were also provided by U.S. Geological Survey agents from animals found on coastal beaches of Washington State.

Ice Seals

Samples of frozen stomach and intestinal contents from subsistence-harvested ice seal species—ribbon (*Histriophoca fasciata*), ringed (*Pusa hispida*), spotted (*Phoca largha*), and bearded (*Erignathus barbatus*)—were collected from seven Alaskan villages along the shores of the Bering and Chukchi seas.

Other Cetacean and Pinniped Species

Fecal, stomach content, or urine samples from other species such as beluga whales (*Delphinapterus leucas*), humpback whales (*Megaptera novaeangliae*), Steller sea lions (*Eumetopias jubatus*), harbor seals (*Phoca vitulina*), and northern fur seals (*Callorhinus ursinus*) were collected from stranded/dead animals by stranding network members from organizations in Alaska, Washington, and California.

Sample Handling

All samples were placed in plastic screw-cap tubes or Whirl-Pak baggies (Nasco, Salida, California) and frozen at or below −20°C as soon after collection as possible. Samples were kept

frozen until shipped to the Northwest Fisheries Science Center for analysis. Frozen samples were shipped on dry ice overnight from the collecting agency and stored frozen at −20°C until analysis.

Sample Extraction

All samples were thawed at room temperature. Depending on the amount of sample available, 1 to 4 g of sample was weighed out into a 15 mL polypropylene screw-cap tube (BD Falcon, BD Biosciences, San Jose, California). The initial extraction step was carried out by adding 50% aqueous methanol to the sample in a 1 in 4 wt/wt ratio (1 part sample, 3 parts 50% MeOH) and thoroughly vortexing the sample.

Fecal, Stomach Content, and Intestinal Content Samples

Samples were homogenized for at least 60 seconds using an Omni ES homogenizer (Omni International, Kennesaw, Georgia). The homogenized sample was then spun at 10,000 × g in a Sorvall RC 5C Plus centrifuge (Thermo Fisher Scientific Inc., Waltham, Massachusetts) for 20 minutes at 4°C. The supernatant was then filtered through a 0.22 μm membrane microcentrifuge tube filter (EMD Millipore, Billerica, Massachusetts) and spun in a desktop microcentrifuge for 10 minutes at 12,000 × g. Samples were stored at 4°C until analysis by ELISA or LC/MS/MS.

Urine, Blood Serum, Milk, Bile, and Aqueous Humor Samples

Samples were sonicated with a Branson Sonifier 450 (Branson Ultrasonics Corp., Danbury, Connecticut) at 50% pulse for 45 seconds at a setting of 5. Samples were then centrifuged at 10,000 × g in a Sorvall for 20 minutes at 4°C. The supernatant was then filtered through a 25 mm diameter, 0.45 μm pore size syringe filter (Pall Corp., Port Washington, New York). Samples were stored at 4°C until analysis by ELISA or LC/MS/MS.

DA Detection/quantification by Biosense ELISA

Matrix Effects Testing

Matrix effects testing was performed using material from animals known to be negative for DA. A dilution curve was made of the methanol extracts at the following dilutions: 1:1, 1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, and 1:5120. From this curve, the minimum dilution necessary to avoid matrix effects was determined for each species and sample matrix by plotting the absorbance (A) at each dilution with respect to the maximum absorbance possible (A_{max}). Ideally, A/A_{max} will approach a value of 1 at some dilution level. The slope of the curve is steeper at the lower end of the dilution series, but levels out as samples are diluted enough to overcome matrix effects. A value of 1 indicates that A (the absorbance of the DA-negative matrix control sample) is equal to that of A_{max} (the absorbance value in the DA-negative control). The dilution which corresponds to this flattening of the curve of A/A_{max} vs. dilution is the minimum dilution needed to avoid matrix effects.

The methanol in the sample was not a contributing factor in terms of matrix effects. The sample dilution buffer used with the Biosense kit is 10% methanol in PBS-Tween (PBS-T). All sample dilutions listed above resulted in a methanol content less than 10%, with the exception of the 1:1 (undiluted) extract, which had a methanol content of 37.5%.

Measurement of DA

Biosense ELISA measurements of DA were performed as described in the instruction protocol supplied by the manufacturer. Samples were diluted with sample buffer (10% methanol in PBS-T) as described above. In addition to samples, each plate included a 10-point standard curve, an A_{max} control (maximum binding of anti-DA-HRP conjugate, yielding maximum absorbance) and an A_{min} control (background absorbance of TMB peroxidase substrate, no anti-DA-HRP conjugate, yielding minimum absorbance). All samples, standards, A_{max}, and controls were run in duplicate. Precoated plates were soaked in washing buffer (PBS-T) for 10 minutes, then buffer was removed and sample was added to duplicate wells. Anti-DA-HRP conjugate was added to each well (except blank) and the plate was sealed and incubated for 1 hour in the dark at room temperature. Liquid was then removed and the plate washed four times with washing buffer (PBS-T) in a BioTek ELx50 plate washer (BioTek, Winooski, Vermont). TMB peroxidase substrate was added and the plate was incubated in the dark at room temperature for 15 minutes. The reaction was stopped with 0.3 molar H_2SO_4 and absorbance at 450 nm was read after 2 minutes on a VERSAmax microplate reader (Molecular Devices LLC, Sunnyvale, California).

Quantification of DA

A four-parameter logistic curve fit model is used by the software included with the Biosense ELISA kit to generate a DA standard calibration curve. Based on a working range determined by the I_{20} and I_{80} values from the calibration curve, DA values in samples were tagged as "within range," "too dilute," or "too concentrated." DA values from samples tagged "within range" were accepted. Values tagged as "too dilute" were given a value of "below quantification limit." Samples tagged "too concentrated" were diluted and rerun until obtaining a "within range" value.

DA Detection/quantification by LC/MS/MS

A Waters Agilent Micromass Quatromicro triple quadrupole electrospray tandem mass spectrometer was used for all samples. Separation was carried out using an Acquity UPLC BEH C-18 1.7 μm particle size, 2.1 x 100 mm column (Waters Corp., Milford, Massachusetts). Solvent A was water with 50 mM formic acid and Solvent B was 95% acetonitrile with 50 mM formic acid. The run consisted of a 3 minute gradient from 95% to 85% Solvent A, followed by a 0.5 minute hold at 85% Solvent A, followed by a 1.5 minute gradient return to 95% Solvent A. Injection volume was 20 μL and the flow rate was 0.8 mL/minute. Column oven temperature was 40°C. The retention time for DA was 1.4 minutes. LC/MS/MS data was obtained in the positive ionization electrospray ionization (ESI+) mode. The following settings were used: capillary voltage 2.6 kV, cone voltage 30 V, extractor voltage 8 V, and RF lens voltage 3 V. Source temperature was 125°C and desolvation temperature was 390°C. Confirmation was carried out with three ion products: *m/z* 266, *m/z* 248, and *m/z* 166.

Quantification was performed using the product ion of *m/z* 266. A standard curve was run for each set of samples. The lower detection limit, defined as 3 × background, was 5 ng/mL.

Results

Biosense ELISA

Matrix effects curves were performed for feces, urine, serum, bile, milk, stomach contents, and aqueous humor from DA-negative California sea lions (Figure 1). From these curves, minimum dilutions of 1:100 (feces), 1:10 (urine) 1:10 (serum), 1:100 (bile), 1:50 (milk), 1:50 (stomach contents), and 1:10 (aqueous humor) for the methanol extracts were determined to be necessary in order to avoid matrix effects. With the original 1 in 4 dilution performed during the extraction step, this translates into a total dilution of 1:400 (feces), 1:40 (urine), 1:40 (serum), 1:400 (bile), 1:200 (milk), 1:200 (stomach contents) and 1:40 (aqueous humor) in terms of the original sample material. The results from more than 90 ELISA assays, representing 3,240 samples, have generated working ranges varying between a mean minimum DA concentration of 9.8 (±2.9) pg/mL and a mean maximum DA concentration of 192 (±34.9) pg/mL. Based on these assays, we have established a standard minimum quantification limit for diluted extracts of 10 pg/mL, revising upwards in rare cases when the minimum I_{20} for a calibration curve is above a value corresponding to 10 pg/mL. When minimum dilutions to avoid matrix effects are taken into account, this means a minimum quantification level of 4 ng/g (or mL) for feces and bile, 2 ng/g (or mL) for milk and stomach contents, and 0.4 ng/g (or mL) for urine, serum, and aqueous humor in the original sample material.

Matrix effects for California sea lions were explored in the greatest number of sample matrices, due to access to large numbers of samples from which DA-negative material was available. Other species are not as well represented in our sampling and hence do not have as comprehensive matrix testing from such a wide range of sample types. Fecal material has been available for most other species and matrix effects tests have been run for common dolphins, beluga whales, humpback whales, Steller sea lions, bearded seals, ringed seals, ribbon seals, spotted seals, and northern sea otters (Figure 2). The curves vary with species, but level off in the extract dilution range of 1:100. There may be slight variation, but a minimum dilution of 1:100 for fecal extracts is appropriate for all species.

LC/MS/MS

A large number of DA-positive samples were available from California sea lions and were used for a comparison of Biosense ELISA and LC/MS/MS methods in fecal, urine, and serum matrices. Measurements of DA concentration by the ELISA and LC/MS/MS methods were in strong agreement (Figure 3). The Pearson product moment correlation was 0.989 ($P < 0.001$), indicating a tight correlation between DA quantification performed using the two measurement methods. The two methods were also in close agreement in terms of actual concentrations, with the slope of the linear regression being 0.858 ($r^2 = 0.981$).

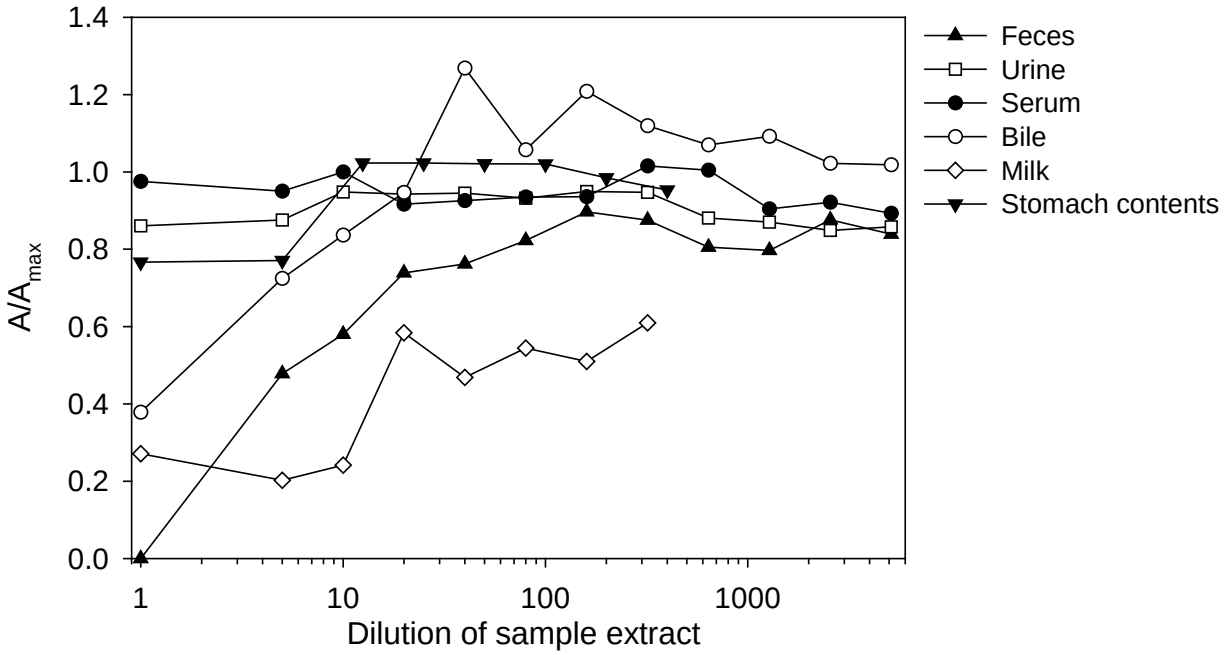

Figure 1. Matrix curves for Biosense ELISA from California sea lion feces, urine, serum, bile, milk, and stomach contents. Matrix effects are eliminated with the extract dilution indicated by the flattening curve.

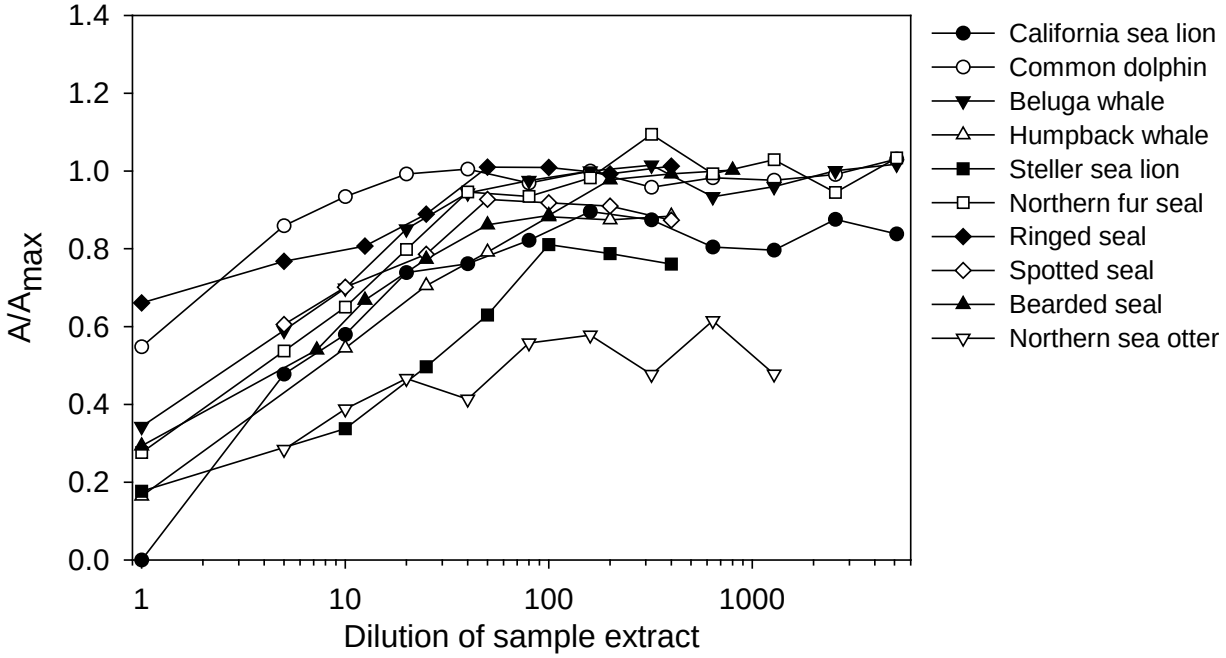

Figure 2. Fecal matrix effects curves from Biosense ELISA for several marine mammal species.

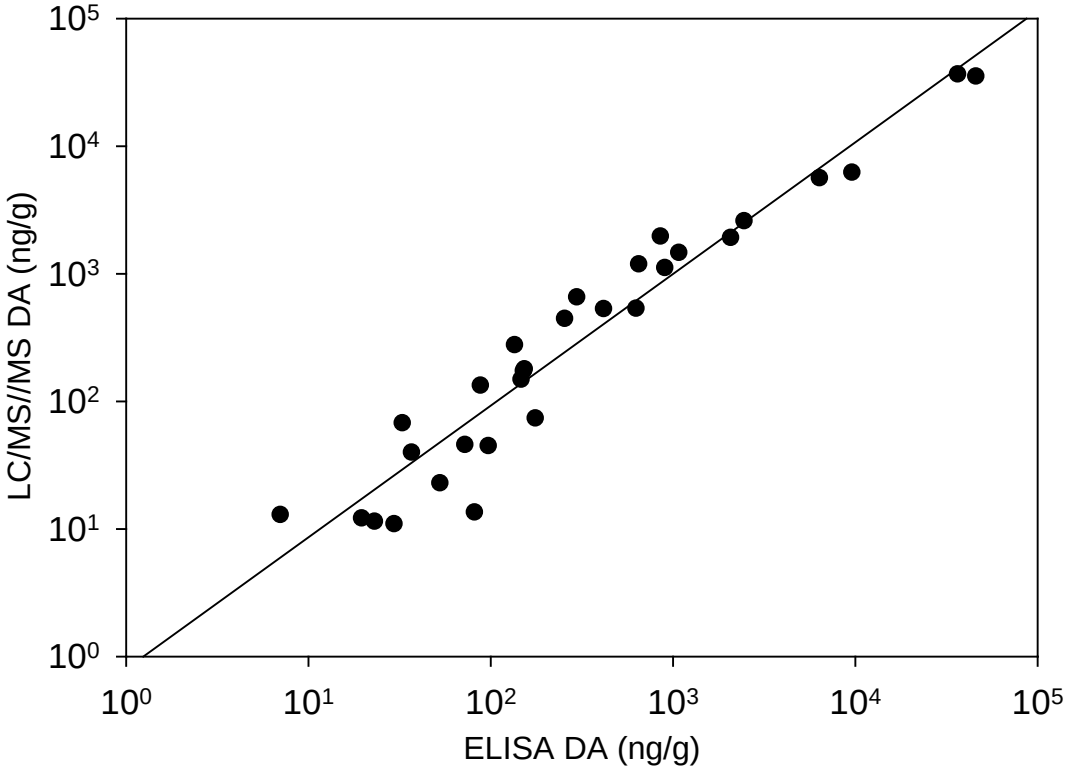

Figure 3. Comparison of DA as measured by Biosense ELISA and LC/MS/MS for selected California sea lion fecal, urine, and serum samples.

A DA standard at 5 ng/mL (5 ppb) was quantifiable (defined as >3 × background), while the standard at 1 ng/mL (1 ppb) was detectable, but not 3 × above background. The actual sample detection limits were higher in California sea lion sample material. Sample extracts were run undiluted for LC/MS/MS, but the extraction protocol itself involves a 1:4 dilution; so to be reliability quantifiable, the samples must have a DA concentration of approximately 20 ng/g in the original sample material, but the actual concentration needed for detection will be higher depending on the matrix effects. The quantification limit of the LC/MS/MS method (5 ng/g) is considerably higher than for the ELISA method (10 pg/g), which can lead to confusion due to positive ELISA results and negative LC/MS/MS findings for the same sample. Table 1 lists several examples of positive results on ELISA that are below the detection limit on LC/MS/MS; hence these would appear negative for DA if the LC/MS/MS method were used, but positive if the ELISA method were employed.

All (18) fecal samples with DA concentrations above 80 ng/g (as determined by Biosense ELISA) were confirmed by the LC/MS/MS method (Table 1). Three fecal samples ranging from 20 to 37 ng/g (as determined by Biosense ELISA) were also confirmed by LC/MS/MS, but 51 fecal samples with DA concentrations between 4 and 80 ng/g (as determined by Biosense ELISA) could not be accurately quantified by LC/MS/MS (>3 × background). This range reflects the high variability in matrix effects in fecal samples, which vary widely due to prey type and animal condition.

Detection by LC/MS/MS was more sensitive in the urine samples. Urine samples with DA concentrations above 29 ng/g (as determined by Biosense ELISA) were reliably quantifiable. Twenty-four samples with DA concentrations between 0.4 and 29 ng/g were not quantifiable by LC/MS/MS. The matrix effect from urine is much lower than from feces, making the quantification limit for urine samples closer to the expected limit of approximately 28 ng/g based on detection of clean DA standards.

Only 2 serum samples out of 25 measured in the study were quantifiable by LC/MS/MS. One of these was the highest DA positive serum sample as measured by ELISA at 72.2 ng/g and the other had a DA concentration of 7.0 ng/g. Twenty-three other serum samples between 0.4 and 7 ng/g were not quantifiable by LC/MS/MS.

LC/MS/MS performed on other species including sea otters and common dolphins has yielded similar agreement with ELISA results: 138.2 ng/g, 129.8 ng/g, and 34.7 ng/g for LC/MS/MS vs. 149.9 ng/g, 141.6 ng/g, and 42.5 ng/g, respectively in feces from common dolphins collected off San Diego, California.

Table 1. Comparison of DA values measured with the ELISA method and LC/MS/MS in California sea lion feces, urine, and serum. Asterisk (*) indicates not quantifiable (>3 × background).

DA ng/g feces		**DA ng/g urine**		**DA ng/g serum**	
ELISA	LC/MS/MS	ELISA	LC/MS/MS	ELISA	LC/MS/MS
45,800	35,486	2,446	2,607	72	46
36,386	36,864	296	659	7	13
9,560	6,245	254	447	2.7	*
6,342	5,665	153	180	1.8	*
2,067	1,925	135	278	—	—
1,075	1,470	97	45	—	—
899	1,118	88	134	—	—
852	1,974	52	23	—	—
648	1,196	33	68	—	—
626	537	29	11	—	—
415	533	26	*	—	—
226	*	24	*	—	—
175	74	18	*	—	—
151	174	—	—	—	—
147	149	—	—	—	—
123	*	—	—	—	—
108	*	—	—	—	—
81	14	—	—	—	—
37	40	—	—	—	—
23	12	—	—	—	—
20	12	—	—	—	—

Discussion

The ELISA method is becoming more and more widespread as a technique for measuring DA in natural samples, and as the use of prepackaged kits broadens, there should be consistency in reporting results obtained from these kits. This method is quick and efficient—allowing processing of multiple samples in a short period of time—but with this convenience comes the potential danger of employing the method without regard for issues such as matrix effects. This is important for sample types that were not the original intended target of these kits, such as marine mammal material. These samples require different treatment from the shellfish samples for which the kits were designed and validated by the manufacturer, especially when it comes to minimum sample dilution.

This report describes in detail the minimum dilutions needed for analysis of DA by Biosense ELISA for a wide range of California sea lion sample types and, to a lesser degree, sample types in several other species. Based on these results, it is clear that the variation between samples matrices is greater than that between species, though some interspecies differences are apparent, especially in "messier" matrices such as stomach contents, likely reflecting differences in diet. For this reason, we recommend the following general dilutions for sample extracts when matrix curves are not yet available: feces 1:100, urine 1:10, serum 1:10, aqueous humor 1:10, stomach contents 1:50, milk 1:50, and bile 1:100 (Table 2).

It is also important to realize that the detection limit of the Biosense ELISA kit, cited in the kit directions as 2 pg/g, is not the detection limit for DA in marine mammal matrices. Based on more than 3,000 sample runs, we have assigned a "practical" quantification limit of the kit at 10 pg/g. Using this as the default quantification limit in the diluted sample extracts run on the

Table 2. Minimum dilution of sample extracts and minimum detection limits in sample material for selected species and sample types. Asterisk (*) indicates matrix type not tested for this species.

	Matrix type			
Species	**Feces**	**Urine**	**Stomach contents**	**Serum**
California sea lion	100/4	10/0.4	50/2	10/0.4
Harbor seal	100/4	10/0.4	25/1	*
Ice seal spp.	100/4	10/0.4	50/2	*
Northern fur seal	100/4	10/0.4	25/1	10/0.4
Common dolphin	100/4	10/0.4	50/2	*
Sea otter	100/4	10/0.4	25/1	10/0.4
Steller sea lion	100/4	10/0.4	50/2	*
Humpback whale	100/4	*	50/2	10/0.4
Beluga whale	100/4	10/0.4	50/2	*

ELISA plate, and the minimum dilutions cited, the quantification limits in the original sample material are much higher; for example, a fecal sample is diluted 1:4 during extraction, then an additional 1:100 to avoid matrix affects, yielding a total dilution of 1:400. A quantification limit of 10 pg/g for this diluted sample translates to a 4,000 pg/g or 4 ng/g quantification limit in the original sample material. This sample quantification limit is still over two orders of magnitude lower than quantification limits reported for HPLC analyses and over one order lower than that for LC/MS/MS, the more commonly used methods historically.

The differences in DA quantification limits between ELISA, LC/MS/MS, and HPLC continue to be a source of confusion. The ELISA is the most sensitive method and, as its use increases, questions about meaningful toxin levels in relation to toxicological impacts in wildlife become more important. For example, many reports of "first" records of DA in a certain area or species as quantified by ELISA may not be firsts at all—especially when the only method previously applied in the area or species has been HPLC. It is important to report the previous detection method and the limits of detection of that method when declaring these first reports. It is also important to establish baseline levels of DA presence in each species of interest before attributing the detection of the toxin to disease, death, or any type of toxicological impact in wildlife populations.

Conclusions

The Biosense ELISA is a robust, quick, and efficient tool for measuring DA in marine mammal samples when the proper dilutions are used and reported with the results. Biosense ELISA results agree well with results from LC/MS/MS analysis and are far more sensitive and specific than from HPLC. By using the extraction and dilution guidelines outlined here, reports of DA in marine mammal sample material can be standardized and used for comparison purposes across temporal and spatial ranges. This will become increasingly important as the method gains users and changing environmental factors such as global climate change potentially contribute to expansion in range or duration of DA events.

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Appendix A: Domoic Acid Sample Collection and Storage Protocol

For detection of domoic acid (DA), the most reliable sample matrix (type) is feces (Table A-1). Urine, stomach contents, blood (serum), and other fluids (milk, amniotic, etc.) are also useful, but DA clears relatively quickly from blood, so serum samples must be taken from relatively recent strandings/deaths.

Collect material as soon as possible. Heat (above 40°C) and light degrade DA. Collect samples in plastic tubes or Whirl-Pak baggies (Nasco, Salida, California). If liquid chromatography tandem mass spectrometry analysis will be used, it is best to avoid any polystyrene products, (polyethylene is acceptable). At least 1 g (or mL for fluids) is needed for analysis, but 4 g (or mL) are recommended. Freeze samples as soon as possible and avoid thawing/refreezing.

Table A-1. DA concentration in two sample matrices (feces and urine) collected simultaneously from the same animal. Note the DA concentration in the fecal sample is almost always significantly higher than in the urine sample.

CSL no.	DA ng/g feces	DA ng/mL urine
6615	45,800.0	87.6
6858	9,560.0	135.0
6857	1,612.2	253.7
7181	648.0	17.5
6695	150.8	29.4
6921	43.0	5.8
6986	36.7	4.9
6887	20.9	0.7
7160	11.3	0.9
6836	10.1	1.1
6958	8.3	0.7
7157	5.9	0.6
7021	4.3	1.1
6958	3.9	0.7
7157	3.8	0.6
6884	3.6	0.6
6882	1.4	1.9
6874	1.2	0.3
6911	1.2	0.3
6727	1.0	0.1
7013	0.7	0.7
6704	0.7	1.0

Appendix B: Domoic Acid Extraction Protocols

Feces

1. Thaw sample.
2. Weigh out 4 g into a 50 mL plastic tube.
3. Dilute 1:4 with 50% methanol (4 g of feces, 12 mL of 50% methanol).
4. Homogenize using a tissue homogenizer until the sample is well ground—usually at least 60 seconds (keep samples on ice, since the homogenization generates heat).
5. Transfer homogenized sample into a centrifuge tube.
6. Centrifuge at 10,000 × g for 20 minutes at 4°C.
7. Transfer supernatant to a glass or plastic tube.
8. Filter supernatant using a microcentrifuge spin column. (Note: syringe filters can be used, but the fecal samples are often quite thick and require much patience. A prefiltration step using GF/C filters is often helpful.)
9. Depending on the volume, samples can be stored in glass screw-top vials or plastic tubes.
10. Store sample at 4°C (refrigerator). Do not freeze. Samples will remain stable for weeks, but it is best to analyze them as soon after extraction as possible.

Urine

1. Thaw sample.
2. Transfer 2 mL to a 15 mL plastic tube.
3. Dilute 1:4 with 50% methanol (2 mL of urine, 6 mL of 50% methanol).
4. Sonicate at 50% for 45–60 seconds (keep samples on ice).
5. Transfer sonicated sample to a centrifuge tube.
6. Centrifuge at 10,000 × g for 20 minutes at 4°C.
7. Transfer supernatant to a glass or plastic tube.
8. Filter supernatant using a 0.45 µm syringe filter.
9. Depending on the volume, samples can be stored in glass screw-top vials or plastic tubes.
10. Store sample at 4°C (refrigerator). Do not freeze. Samples will remain stable for weeks, but it is best to analyze them as soon after extraction as possible.

Serum

Follow procedure for urine.

Stomach Contents

Follow procedure for feces.

Other Body Fluids (Milk, Amniotic Fluid, Peritoneal Fluid, Etc.)

Follow procedure for urine.

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