Development, Comparison, and Validation Using ELISAs for the Determination of Domoic Acid in California Sea Lion Body Fluids

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Mortalities of California sea lions (Zalophus californianus) attributed to the neurotoxin domoic acid (DA) produced by the diatom Pseudo-nitzschia have occurred repeatedly along the U.S. west coast since the late 1990s. Quantifying the amount of DA in these animals and correlating this information with the presence of DA in phytoplankton and the local food web has become a research focus for many scientists. However, differences in materials, equipment, technical capability, budgets, and objectives of the various groups and/or agencies involved in this work have influenced the DA quantification platforms used. The goal of the present study was to compare the performance of two commercially available ELISAs for the determination of DA in a spectrum of California sea lion body fluids and to compare the results with LC/MS of the same samples. The results indicated differences among these approaches, presumably owing to matrix effects (particularly urine) and antibody reactivities. This information implies that care should be taken in attempting to compare datasets generated using different analytical platforms and interpreting the results of published studies.

The diatom Pseudo-nitzschia has been known as a common member of the phytoplankton community in California since the early 1990s (1–3); however, the capability of Pseudo-nitzschia to produce the neurotoxin domoic acid (DA) and the threat that toxin can pose for human and wildlife health was not identified until the end of the century. DA can be bioaccumulated in organisms feeding directly on toxic Pseudo-nitzschia cells such as zooplankton (4, 5), shellfish (6–8), and planktivorous fish (9–13), which in turn serve as vectors transporting the toxin to higher trophic levels of the food web. The first outbreak of human illness occurred in 1987 on Prince Edward Island, Canada, when over 100 people were sickened and three died after consuming DA-contaminated blue mussels (6, 8, 14). DA has not caused any widespread human illnesses since the initial Canadian outbreak, a situation that is most likely a consequence of the extensive coastal monitoring programs for DA implemented by health departments worldwide since 1987. Conversely, DA continues to be the cause of marine bird (13, 15, 16) and marine mammal (5, 17–20) mortality events in areas where Pseudo-nitzschia occurs, and these mortalities are often the first sign of an emerging DA event in a given area.

The marine mammal predominantly associated with DA mortality events on the U.S. west coast is the California sea lion (CSL: Zalophus californianus), undoubtedly due to its large population and overlapping distribution with Pseudo-nitzschia in coastal waters (17, 20–22). Two types of DA exposure are currently identified in CSLs: acute DA toxicity that occurs when a CSL is exposed to a single high dose of DA, and chronic DA toxicity that occurs when a CSL is repeatedly exposed to sublethal concentrations of DA (23, 24). Presumably, these different types of DA exposure influence the magnitude of DA concentrations present in the body fluids of stranded animals at the time of rescue. Females comprise the majority of CSL strandings, and DA exposure not only threatens their health but also the health of a fetus they may be carrying (25–27). The impact of DA on CSLs following rehabilitation can be seen in alterations of CSL behavior, movement, dive pattern, and survival (23, 28).

Identifying strong positive correlations between the presence of DA-producing Pseudo-nitzschia and CSL strandings is impeded by several factors: the type of exposure (acute or chronic), the amount of time between exposure and stranding and rescue, the health of the animal admitted to the rehabilitation center, the vector responsible for the exposure, the amount of DA produced...
Table 1. Summary of DA concentrations and analysis methods reported in the literature for several pinniped species

<table>
<thead>
<tr>
<th>Species</th>
<th>Amniotic fluid, ng/mL</th>
<th>Feces, µg/g</th>
<th>Gastric fluid, ng/mL</th>
<th>Serum, ng/mL</th>
<th>Urine, ng/mL</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>California sea lion (Zalophus californianus)</td>
<td>2.5–152.0</td>
<td>1.3–182.0</td>
<td>1.31–182.0 µg/mL</td>
<td>170.0–200.0</td>
<td>30.0–3720.0</td>
<td>HPLC</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>4.0–34.0</td>
<td>0.5–15.0</td>
<td>10.0–82.02</td>
<td>7.0–261.0</td>
<td>LC</td>
<td>RBA</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>3.0–9.3</td>
<td>0.3–44.0</td>
<td>1.4–96.8</td>
<td>2.0–17.6</td>
<td>LC/MS/MS</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.2–96.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harbor seal (Phoca vitulina)</td>
<td>10.0</td>
<td>0.002–0.063</td>
<td>8.0–10.0</td>
<td>2.0–16.0</td>
<td>BS ELISA</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>Northern fur seal (Callorhinus ursinus)</td>
<td>20.0 ng/g</td>
<td>0.002–18.6</td>
<td>2–288 ng/g</td>
<td>1.0–2784.0 ng/g</td>
<td>BS ELISA</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.53–2.80</td>
<td>190.0–13661.0 ng/g</td>
<td>310.0–13661.0 ng/g</td>
<td>10.0–13661.0 ng/g</td>
<td>LC</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.44–54.73</td>
<td>811.0–826.0</td>
<td>371.0–5630.0 ng/g</td>
<td>512–12693 ng/g</td>
<td>LC/MS/MS</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td></td>
<td>46.47</td>
<td></td>
<td></td>
<td></td>
<td>RBA</td>
<td>34</td>
<td></td>
</tr>
</tbody>
</table>

* RBA = Receptor-binding assay.

by *Pseudo-nitzschia*, and the abundance of *Pseudo-nitzschia* cells producing DA. Unlike dinoflagellates that can form conspicuous blooms capable of altering the color of the water, *Pseudo-nitzschia* does not typically reach cell abundances large enough to be visibly noticeable. Moreover, coastal monitoring programs for *Pseudo-nitzschia* and DA are routinely established at surface water stations accessible from the shore, where the presence of DA in the phytoplankton and/or shellfish is most likely to overlap with human activities. Yet, marine animals can be in contact with *Pseudo-nitzschia* blooms present offshore and/or in thin layers (29–31), and the strandings of these animals often are the first sign of an emerging DA event prior to the *Pseudo-nitzschia* cells being physically transported to shore and subsequently seen by a coastal monitoring program.

The DA concentrations reported for fluids and solids collected from stranded pinnipeds vary in range, fluid type, collection, and DA quantification protocols (Table 1). Multiple platforms exist for the measurement of DA, including mouse bioassay, LC, LC/MS, receptor-binding assay (RBA), and ELISA. Each method has its own array of advantages and potential shortcomings, and laboratories generally choose the methodology that will best suit their needs in terms of cost, technical sophistication, and research goals. For example, commercially available ELISAs generally offer lower cost alternatives to analytical chemical approaches (i.e., LC, LC/MS) that require a significant investment in equipment and technical expertise. Several studies have implemented the rapid and lower cost methodologies (i.e., RBA and ELISA) as a prescreening tool prior to analysis by chemical methods (24–26).

The primary objective of the present study was to compare the performance of several of the methodologies available for quantification of DA in CSL body fluids, thereby enabling some degree of extrapolation across existing datasets and to provide context for comparing new results to previously published studies. This objective was met through validating a protocol adapted for the measurement of DA by ELISA in sea lion body fluids that minimized the sample volume required and reduced sample handling procedures; comparing the performance of two commercially available ELISAs, the monoclonal antibody ELISA manufactured by Mercury Science, Inc. (MeS; Durham, NC) and the polyclonal antibody ELISA manufactured by Biosense Laboratories (BS; Bergen, Norway) using the modified protocol; and comparing the results from ELISA with a well-established analytical method utilizing LC/MS. The CSL body fluids used in the validation study include amniotic fluid (AF), cerebral spinal fluid (CSF), serum, and urine.

Experimental

**DA Quantification Methods**

Three method platforms for the analysis of DA (two commercially available ELISAs and LC/MS) were compared for their ability to accurately measure DA in CSL body fluids and to provide information on how to compare data collected using these different platforms. Each fluid type selected (AF, CSF, serum, and urine) has unique properties that may cause interferences for ELISA or LC/MS methodologies, warranting examination of each fluid individually. The MeS ELISA is a monoclonal antibody assay developed by the National Oceanographic and Atmospheric Association Centers for Coastal Ocean Science, National Ocean Service, the Northwest Fisheries Science Center with Mercury Science, Inc. It has been validated for the analysis of DA in shellfish tissues and in dissolved and particulate phytoplankton samples (35, 36). The BS ELISA is a polyclonal antibody-based assay developed by Biosense Laboratories; it has been validated by both single and interlaboratory studies for the analysis of DA in shellfish tissues (37, 38) and for the analysis of DA concentrations present in rat serum and brain samples (39). An Agilent (Santa Clara, CA) 6130 LC/MS system operated in positive electrospray ionization mode with an Agilent Zorbax Rapid Resolution column and selected ion monitoring of DA.
(312 amu) was used for LC/MS analysis generally following the method of Wang et al. (40). Quantification was based on peak area and an external standard curve using National Research Council Canada CRM-DA-f standards. Peaks were confirmed based on the presence of daughter fragments at 266 and 248 amu. Because the objective of the study was to compare methods and matrices, the unknown samples were run blind and not corrected for matrix effects using standard addition or an internal standard.

**Sample Collection and Selection for Validation Study**

Samples of AF, CSF, serum, and urine were obtained from stranded CSLs treated by the Pacific Marine Mammal Center (PMMC; Laguna, CA) during 2007 and 2009. Following collection, samples were stored at −20°C at the PMMC, transported frozen to the University of Southern California (USC; Los Angeles, CA), and once again stored at −20°C until analysis via ELISA. Samples collected in 2007 were initially analyzed by the BS ELISA, and samples collected in 2009 were initially analyzed by the MeS ELISA, as described previously in a spike and recovery study conducted during the fall of 2009 that utilized simultaneous analysis by all three platforms. Samples collected during 2009 that yielded measurable DA concentrations using the MS ELISA and contained sufficient remaining volume to be analyzed simultaneously on all three platforms were also stored and included in the fall 2009 study to allow for comparison of naturally DA positive fluid samples across all three platforms. These latter samples also allowed determination of any DA degradation that may have occurred in the samples during storage.

**Modified ELISA Protocol**

A modified ELISA protocol was developed in 2009 with the primary goal of minimizing the required sample volume and reducing sample preparation. We reasoned that a methanol extraction step typically used in the extraction of DA from solid matrices (i.e., phytoplankton cells, shellfish tissues) might not be necessary for fluid samples as the DA would already be in the dissolved form. Fluid samples were vortexed for 1 min and diluted 1:25 with the sample buffer provided by the respective ELISA manufacturer, and the diluted sample was briefly vortexed immediately prior to pipetting onto the ELISA plate. The expected LOD for each ELISA platform was calculated from the plate sensitivity reported by the manufacturer and adjusted for the 1:25 minimum dilution. The BS ELISA is reported to have a 0.01 ng/mL plate sensitivity; therefore, the LOD for a sample diluted 1:25 is expected to be 0.25 ng/mL. The MeS ELISA is reported to have a 0.1 ng/mL plate sensitivity, and the LOD expected is 2.5 ng/mL.

**Method Comparison Study**

Analyses of CSL body fluids using the modified protocol for both ELISAs and LC/MS were performed within 1 week in October 2009 at the University of California Santa Cruz in order to minimize degradation of DA that may occur in samples over long storage periods. Samples selected for the spike and recovery portion of the comparison study were specifically determined to be below the LOD of the respective ELISA platform during their initial receipt and analysis in 2007 or 2009. A portion of the samples measured in the spring of 2009 that had quantifiable concentrations of DA using the MeS ELISA and the modified protocol were reanalyzed simultaneously on all three platforms. The study was designed to allow the following comparisons: performance of the modified ELISA protocol on the determination of DA concentrations in AF, CSF, serum and urine samples spiked with known concentrations of DA; comparison of the results obtained with the modified protocol by the MeS and BS ELISAs; comparison of results obtained from SPE cleaned spiked samples analyzed by LC/MS and the MeS ELISA; and comparison of LC/MS results on SPE cleaned samples to MeS and BS ELISA results without SPE cleanup (Figure 1).

**Preparation of Standards**

The DA standard used to spike CSL fluid samples and to prepare standard curves for LC/MS analysis was obtained from the National Research Council, Canada (CRM-DA-f, Ottawa, Ontario). Two standard curves were prepared for the LC/MS analysis, one in Milli-Q water and the second in LC/MS grade 50% methanol. A subset of the Milli-Q water standards was SPE cleaned up (see section below) prior to analysis by LC/MS in order to quantify the amount of DA lost during the cleanup procedure. The LC/MS standard curves were made through serial dilution with final concentrations of 1, 2, 5, 20, 50, 100, 250, and 500 ng/mL. A standard of 1000 ng/mL was prepared in Milli-Q water to use in the spiking of AF, CSF, serum and urine samples to the following concentrations: 12.5, 15, 18, 21.5, 26, 31, 37, 44.5, 53.5, 64, 77, 92.5, and 110 ng/mL. A matrix-free 1 ng/mL Milli-Q water standard was used for assessing ELISA platform performance during the study.

**LC/MS Sample Handling Procedure**

The procedure used for LC/MS analysis was modified from a procedure previously used for seawater and phytoplankton samples, described in Wang et al. (40). The same samples analyzed via ELISA methods were also analyzed by LC/MS but were cleaned up using Bond Elut SPE columns with LRC-C18 resin (Varian, Inc., now Agilent Technologies). The columns were conditioned prior to the addition of sample by vacuum filtering 10 mL of 100% methanol followed by 10 mL of LC/MS grade water (Fisher Scientific, Pittsburgh, PA). Samples were acidified with 0.5 mL formic acid–methanol–water (2 + 5 + 93, v/v/v), and 4 mL 5% formic acid was added prior to introduction onto the SPE column. Samples were pipetted into the column, 4 mL of 1.5% formic acid was added to the sample followed by vacuum
Figure 2. DA concentrations measured using different analytical platforms and protocols in samples of AF spiked with DA standard. The dotted lines in all graphs show the expected DA concentration based on the amount of DA added to each sample. Error bars represent SDs of triplicate replicates. (A) Results from all platforms, MeS non-SPE cleaned up sample results represented by black circles and the solid black line, the MeS SPE cleaned up sample results represented by white circles and the long dashed and dotted line, the BS ELISA non-SPE cleaned up sample results represented by gray circles and the gray line, and the LC/MS results represented by black triangles and the long dashed line; (B) DA concentrations measured using the BS ELISA (non-SPE cleaned up samples) plotted with the concentrations obtained using the MeS ELISA (non-SPE cleaned up samples); (C) DA concentrations obtained using the MeS ELISA (SPE cleaned up samples) plotted with the LC/MS results; (D) DA concentration obtained using the MeS ELISA (non-SPE cleaned up samples) represented by the black circles and black line and the DA concentration results obtained using the BS ELISA (non-SPE cleaned up samples) represented by gray circles and the gray line are plotted versus the LC/MS (SPE cleaned up samples) results.

filtration, and the extract was discarded. The final extraction step used vacuum filtration of 3 mL of 50% methanol onto the column, and the resulting extract was collected for analysis. The method LOD of 0.48 ng/mL was determined using seven spiked Milli-Q water samples with SPE cleanup.

Analysis of Spike and Recovery of DA Results

The quality of the fluid samples collected by rehabilitation centers can be impacted by multiple factors, as discussed above, and fluid samples from a number of individual animals were used in the spike and recovery portion of the study to account for this variability. The samples were divided into two portions; one portion of each sample remained unspiked and the other portion was spiked with DA standard at known concentrations (Figure 1). The unspiked portions were run on each platform, with non-SPE cleaned samples analyzed using the MeS and BS ELISAs and SPE-cleaned up samples analyzed by MeS ELISA and LC/MS. The remaining spiked portions of each sample were vortexed for 1 min and then analyzed as noted in the previous sections detailing the modified ELISA protocol and LC/MS sample handling procedure.

ELISA samples were run in triplicate and spread across two plates to account for any inter- or intra-plate variability that may have arisen during commercial fabrication. DA concentrations for each ELISA sample were determined from the average results of duplicate wells and calculated using Excel spreadsheets provided by the respective manufacturers. BS ELISA results were quantified using an Excel Macro that uses a four-parameter logistics curve-fitting model to produce a standard curve for each plate, and sample concentrations were determined by extrapolating from the standard curve. MeS ELISA results were calculated using an Excel spreadsheet that used a ratio between the maximal absorbance signal (a control containing no DA) and the absorbance signal of the sample in conjunction with constants for the midpoint and slope of a standard curve determined by the manufacturer when the method was developed. Both quantification spreadsheets automatically identified samples outside the working range of the ELISA (i.e., too dilute or too concentrated) and calculated coefficients of variation (CVs) for the duplicate sample wells. Samples with CVs greater than 15% were eliminated as recommended by the manufacturers because the high amount of variation makes it difficult to identify which of the duplicate wells can be used to calculate an accurate DA concentration.

The measured DA concentrations determined by the respective platforms were plotted against the known DA concentration spiked into each sample. The ELISA and LC/MS samples were treated blindly, with no correction of ELISA or LC/MS results for the known DA concentrations spiked into the samples. This was done to more appropriately mimic the manner in which CSL body fluid samples with an unknown DA concentration might be handled in different laboratories. The slopes and R² values of the linear regressions plotted for each platform were recorded, and the slopes were statistically compared. The linear regressions were carried out with SigmaPlot (v. 11.0.0, Systat Software, Inc., San Jose, CA). A Student’s t-test was computed as the difference between the two slopes being compared and divided by the SE of the difference between the slopes. The comparison of slopes using the Student’s t-test has been identified as reliable for determining agreement between two methods and agreement to the ideal equality line with a slope of 1 (41, 42). Deming regressions were performed to test for linear relationships between platform results using the XLSTAT Macro for Microsoft Excel (v. 2012, Addinsoft SARL, New York, NY).

Results and Discussion

Spike and Recovery of DA in CSL AF Samples

The DA concentrations measured by each method for the spiked CSL AF samples were plotted versus the known DA concentrations added to each sample, and linear regressions were performed to determine the R² and slope (Figure 2A; Table 2). The highest R² value of 0.94 was obtained for the MeS ELISA results using non-SPE cleaned up samples, indicating good linearity across the range of DA concentrations examined. The slope obtained for the MeS non-SPE cleaned up sample regression line was 0.67, lower than and statistically different from the ideal slope of 1 (t0.05,2,8 > 2.31). The BS ELISA results using non-SPE cleaned up samples also showed good linearity across the range of DA concentrations as indicated by an R² of 0.90. The slope of the regression line was 1.4, higher than and statistically different.
Table 2. \( R^2 \) and slope values obtained from linear regression lines plotted for each method platform and body fluid versus the known spike concentration of DA

<table>
<thead>
<tr>
<th>Body fluid</th>
<th>MeS ELISA Non-SPE cleaned up</th>
<th>MeS ELISA SPE cleaned up</th>
<th>BS ELISA Non-SPE cleaned up</th>
<th>BS ELISA SPE cleaned up</th>
<th>LC/MS Non-SPE cleaned up</th>
<th>LC/MS SPE cleaned up</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( R^2 )</td>
<td>Slope</td>
<td>( R^2 )</td>
<td>Slope</td>
<td>( R^2 )</td>
<td>Slope</td>
</tr>
<tr>
<td>Amnionic fluid</td>
<td>0.94</td>
<td>0.67</td>
<td>0.88</td>
<td>0.29</td>
<td>0.89</td>
<td>1.42</td>
</tr>
<tr>
<td>Cerebral spinal fluid</td>
<td>0.82</td>
<td>0.78</td>
<td>0.56</td>
<td>0.11</td>
<td>0.92</td>
<td>1.52</td>
</tr>
<tr>
<td>Serum</td>
<td>0.85</td>
<td>0.38</td>
<td>0.86</td>
<td>0.20</td>
<td>0.94</td>
<td>0.73</td>
</tr>
<tr>
<td>Urine</td>
<td>0.55</td>
<td>0.38</td>
<td>0.84</td>
<td>0.24</td>
<td>0.36</td>
<td>0.71</td>
</tr>
</tbody>
</table>

from the ideal slope of 1 \( (t_{0.05,2,10} > 2.23) \). The results from the SPE cleaned up samples analyzed on the MeS ELISA had a high \( R^2 \) (0.88), but the slope of the linear regression line was 0.29, much lower than and statistically different from the ideal slope of 1 \( (t_{0.05,2,10} > 2.23) \). The slope of 0.87 obtained for the LC/MS measured SPE cleaned up samples was not statistically distinguishable from the ideal slope of 1 \( (t_{0.05,2,10} < 2.37) \), and the \( R^2 \) value of 0.75 indicated a relatively good linearity across the range of DA concentrations tested.

DA concentrations quantified by the BS and MeS ELISAs were the result of averaged duplicate wells with CVs less than the manufacturer-recommended 15%, with each sample run in triplicate. The CVs calculated for AF samples using the BS ELISA were commonly higher than the MeS ELISA, and as a consequence, more BS measured results were rejected than MeS results. The BS ELISA results on the non-SPE cleaned up sample spiked AF samples had an average CV of 12%, and 10 sample results were discarded because of CVs > 15%. The MeS ELISA results had an average CV of 5% for the non-SPE cleaned up sample spiked AF samples and 4% with SPE cleanup, and only one sample replicate yielded a CV > 15%

Comparison of the slopes calculated using each analytical approach with the AF spiked samples revealed that the slope obtained using the MeS ELISA on non-SPE cleaned up samples (0.67) was not statistically different from the slope of the LC/MS regression line (0.87; \( t_{0.05,2,15} < 2.13 \)), while the slopes obtained for the remaining platforms were statistically different from one another (Figure 2A). The latter result was not unexpected given the basic dissimilarities in sample preparation and analytical methods. Results obtained for the non-SPE cleaned up samples measured by the MeS ELISA were plotted versus the BS ELISA results, and an \( R^2 \) and slope were determined (Figure 2B). The \( R^2 \) of 0.89 indicated good linearity between the results of the two ELISA methods, and a Deming regression analysis was performed to confirm a linear relationship. The computed \( P \)-value of 0.95 for the Deming regression was greater than \( \alpha = 0.05 \) and supported the null hypothesis that the relationship between the MeS and BS ELISA on non-SPE cleaned up sample AF results was linear. The slope of the Deming regression was 2.4, most likely influenced by the MeS ELISA slightly underestimating DA concentrations at the higher levels investigated and the BS ELISA overestimating DA concentrations at the higher levels.

The results for the SPE cleaned up AF samples measured by LC/MS and the MeS ELISA were plotted versus one another to compare samples that were handled in the same manner but analyzed by these two different methods (Figure 2C). The \( R^2 \) of 0.73 demonstrated relatively good linearity between the two methods, further confirmed through a Deming regression with computed \( P \)-value of 0.34, greater than \( \alpha = 0.05 \), supporting the null hypothesis that the relationship was linear. The low slope of 0.27 for the regression line indicated significant underestimation of DA concentrations of SPE cleaned up AF samples by the MeS ELISA. Underestimation of DA concentrations in the SPE cleaned up AF samples was also evidenced by the slope of 0.20 obtained when these values were plotted versus the known spike concentrations (Figure 2A). SPE cleanup produced an extract comprising 50% methanol, which may have an inhibitory effect on ELISA antibodies and therefore cause underestimation of DA concentrations. The low slope values obtained when plotting the MeS results for SPE cleaned up samples of AF versus the spiked DA concentration (Figure 2A) and the low slope obtained in a Deming regression performed versus LC/MS results (Figure 2C) on the same samples indicate that SPE cleanup may be contraindicated for analysis of AF samples on an ELISA platform.

The results obtained by both ELISAs with non-SPE cleaned up AF samples were plotted versus the LC/MS results on the SPE cleaned up AF samples (Figure 2D). The \( R^2 \) values for the regression lines of the MeS and BS ELISA results were 0.60 and 0.56, respectively, indicating a moderate linearity present between the ELISA and LC/MS measured values. Deming regressions of the MeS and BS ELISA results versus the LC/MS results produced \( P \)-values of 0.34 in each regression and supported the null hypothesis that the MeS and BS ELISA results on non-SPE cleaned up AF samples have a linear relationship with the LC/MS SPE cleaned up sample results.

**Spike and Recovery of DA in CSL CSF Samples**

DA concentrations measured by each method for the spiked CSL CSF samples were plotted versus the known DA concentrations, and linear regression was performed to determine the \( R^2 \) and slope values (Figure 3A; Table 2). The highest \( R^2 \) value of 0.92 was tabulated for the BS ELISA results of non-SPE cleaned up CSF samples, indicating good linearity across the range of DA concentrations measured. The slope of the BS ELISA regression line was 1.5, greater and statistically different than the ideal slope of 1 \( (t_{0.05,2,10} > 2.23) \). The BS ELISA overestimated DA concentrations in CSF samples, similar to the result obtained for the AF samples analyzed by the BS ELISA discussed above. The slope of the regression line of MeS ELISA results on non-SPE cleaned up CSF samples was 0.78 and not statistically different than the ideal slope of 1 or the slope of the LC/MS regression line \( (0.70; t_{0.05,2,18} < 2.10, \) and \( t_{0.05,2,8} < 2.31, \) respectively). The \( R^2 \) of 0.82 for the non-SPE cleaned up CSF samples analyzed using the MeS ELISA indicated a relatively good linearity across the range
of DA concentrations tested. The LC/MS regression line had a relatively poor $R^2$ value (0.48), but the slope of the regression line was not statistically different than the ideal slope of 1 (0.70; $t_{0.05,2}=2.45$). The low $R^2$ value may have been a consequence of matrix influence on the ability of the LC/MS to quantify DA concentrations, or DA lost in the SPE-cleanup procedure. This could be addressed in the future by including standard additions for the unknown samples, or by using the same matrix for the standard curve. The SPE cleaned up CSF samples that were analyzed on the MeS ELISA platform exhibited relatively good linearity with an $R^2$ value of 0.55, but the slope of the regression line was relatively flat (0.11) and was statistically different from the ideal slope of 1 ($t_{0.05,2}=2.45$). Whether this result was a consequence of the impact of 50% methanol extract on the ELISA or the SPE cleanup procedure itself is unknown. However, these results verified previous findings that the SPE cleanup step was contraindicated for ELISA.

The CVs calculated for non-SPE cleaned up CSF samples were higher using the BS ELISA than the MeS ELISA, similar to the results obtained with the analysis of the AF samples discussed above. Non-SPE cleaned up sample and SPE cleaned up samples measured by the MeS ELISA both had average CVs of 4%, and no results were discarded for CVs > 15%. In contrast, the non-SPE cleaned up samples analyzed by the BS ELISA had an average CV of 14%, with 20 results having CVs > 15% (and were therefore discarded).

The slopes of the regression lines obtained for the non-SPE cleaned up samples analyzed by the MeS and BS ELISAs when plotted versus the known DA concentration were statistically different ($t_{0.05,18}=2.13$; Figure 3A). The absolute values obtained for the two methods slightly overestimated (BS) or underestimated (MeS) the DA concentrations relative to the known amounts of DA spiked into these samples. When the results for each ELISA were plotted against each other, the $R^2$ of 0.92 indicated good linearity across the range of DA concentrations measured by each platform, but the BS ELISA yielded values that were consistently higher than values obtained using the MeS ELISA (Figure 3B). A Deming regression confirmed the null hypothesis of a linear relationship between the MeS and BS ELISA results with the computed $P$-value of 0.082 ($\alpha = 0.05$).

Results for SPE cleaned up CSF samples analyzed by the MeS ELISA and LC/MS were plotted versus one another, and an $R^2$ and slope value were calculated (Figure 3C). Although samples were handled in an identical manner prior to analysis, the SPE cleanup only appeared to impact the results of the MeS ELISA analysis, presumably due to the effect of the 50% methanol extract produced by SPE cleanup. The impact on toxin detection capabilities of SPE cleaned up CSF samples analyzed by the MeS ELISA was reflected in the slope value of 0 and confirmed the finding that SPE was contraindicated for the analysis of DA samples by ELISA. A Deming regression computed a $P$-value of 0.51 ($\alpha = 0.05$) and supported the null hypothesis that the relationship between the LC/MS and MeS ELISA results was linear, in spite of the flat slope and a low $R^2$ value of 0.15.

The results of non-SPE cleaned up CSF samples using both ELISAs were plotted versus the LC/MS results of the SPE cleaned up CSF samples, and Deming regressions were performed to test for linear relationships (Figure 3D). $R^2$ values of 0.59 and 0.54 were obtained for the BS and MeS ELISA results of non-SPE cleaned up CSF samples, respectively. The moderate linearity inferred from the $R^2$ value for the BS ELISA results was confirmed with a Deming regression with a computed $P$-value of 0.70 ($\alpha = 0.05$) supporting the null hypothesis of a linear relationship. The Deming regression computed a $P$-value of 0.99 ($\alpha = 0.05$) to support the null hypothesis that the non-SPE cleaned up sample MeS ELISA results had a linear relationship with the SPE cleaned up sample LC/MS results.

**Spike and Recovery of DA in CSL Serum Samples**

The DA concentrations measured by each method for spiked CSL serum samples were plotted versus the known DA concentrations, and linear regression was performed to determine the $R^2$ and slope (Figure 4A; Table 2). The regression line slope of DA concentrations in SPE cleaned up serum samples analyzed using LC/MS was 1.1 and not statistically different from the ideal slope of 1 ($t_{0.05,2}=2.45$; $R^2$ value was 0.99). The absolute values obtained by LC/MS were all slightly less than the expected (spiked) values, indicating a slight loss of DA, presumably during the SPE cleanup, or possibly suppression of ionization due to matrix effects; again, this could be corrected in the future with the use of a matrix-specific standard curve or with internal spikes of known concentrations. The BS ELISA results on non-SPE cleaned
up serum samples returned the second highest $R^2$ values with a
0.94 and a slope of 0.73 that was not statistically different than the
ideal slope of 1 ($t_{0.05,3} < 3.18$). The absolute toxin concentrations
for the BS ELISA method were all very similar to the expected
(spiked) concentrations of DA, but the results may be misleading
because the number of samples plotted was reduced considerably
by the elimination of sample results yielding poor CV values.

The CVs calculated for the BS ELISA averaged 18%, with
23 results discarded (CVs > 15%). The frequency of high CV values
obtained with the BS ELISA may have been influenced by the color
developed by the serum samples analyzed. Serum is blood plasma
with the fibrinogens removed and should not contain any red or
white blood cells. The removal of red and white blood cells is
affected by the efficiency of the centrifugation and the quality of
the serum sample collected from the animal. We speculate that
the presence of red blood cells (or material from lysed cells) in
a serum sample may interfere with the determination of DA
concentrations by ELISA methods. However, this effect was not observed for the MeS ELISA analysis of either
the non-SPE or SPE cleaned up serum samples. The non-SPE and
SPE cleaned up serum CVs for the MeS ELISA averaged 4%,
and no results were discarded for high CVs.

The $R^2$ value determined for the MeS ELISA for non-SPE
cleaned up serum samples was 0.85, and the SPE cleaned up
samples gave an $R^2$ value of 0.86. The $R^2$ values indicate good
linearity across the range of DA concentrations measured;
however, all the DA concentrations analyzed by using the MeS
ELISA platform were less than the expected values, and the
slopes of 0.38 and 0.29, respectively, were statistically different
than the ideal slope of $1$ ($t_{0.05,2} > 2.45, t_{0.05,2,4} > 2.78$; Figure 4A).
The $R^2$ of 0.08 and slope of 0.35 obtained when plotting the
results of the non-SPE cleaned up serum samples measured
using both ELISAs indicated a lack of agreement between the
two platforms (Figure 4B). However, the number of samples
included in the comparison plot was small (four) because many
of the samples measured using the BS ELISA were discarded due
to CVs > 15%. Deming regression analysis computed a $P$-value
of 0.76 ($\alpha = 0.05$), supporting the null hypothesis that there
is a linear relationship between the non-SPE cleaned up serum
samples analyzed by the MeS and BS ELISAs.

The regression for the SPE cleaned up serum samples measured
by LC/MS and the MeS ELISA yielded an $R^2$ of 0.89, indicating
a good linearity between the measured DA concentrations
(Figure 4C). However, most of the MeS ELISA values were less
than the values obtained using LC/MS, particularly for samples
with high concentrations of DA added, as indicated by the slope of
the regression line (0.27). Deming regression analysis computed a
$P$-value of 0.98 ($\alpha = 0.05$), supporting the null hypothesis of
a linear relationship between SPE cleaned up serum samples
analyzed by the MeS ELISA and LC/MS.

The results obtained from non-SPE cleaned up serum samples
using the two ELISA platforms were plotted versus the LC/MS
results (Figure 4D). The $R^2$ for the BS ELISA analysis was 0.92,
indicating good linearity between the measured LC/MS and BS
ELISA measured DA concentrations. However, relatively few
samples were compared because many of the BS ELISA results
were discarded due to high CVs, and those samples that were
compared were all higher than values obtained by LC/MS. The $R^2$
value for the MeS ELISA results was 0.29, indicating poor
linearity between DA concentrations in spiked serum samples
measured by LC/MS and the MeS ELISA. Deming regressions
computed $P$-values of 0.76 ($\alpha = 0.05$) for the MeS and BS
ELISA non-SPE cleaned up sample serum results in comparison
to the SPE cleaned up sample LC/MS results. The null hypothesis
was supported in both instances, with the non-SPE cleaned up
sample MeS ELISA results having a linear relationship with the
LC/MS SPE cleaned up sample results, and the non-SPE cleaned up
sample BS ELISA results having a linear relationship with the
LC/MS SPE cleaned up sample results.

**Spike and Recovery of DA in CSL Urine Samples**

The DA concentrations measured by each method for the
spiked CSL urine samples were plotted versus the known
DA concentrations, and linear regressions were performed
to determine the $R^2$ and slope values (Figure 5A; Table 2). The
highest $R^2$ (0.84) was obtained for the values determined using
the MeS ELISA for SPE cleaned up urine samples. However,
the slope of 0.24 was lower than and statistically different from
the ideal slope of 1, and all absolute values were considerably
less than values anticipated from the DA concentrations added
to the samples. The non-SPE cleaned up samples analyzed by
the MeS and BS ELISAs were 0.55 and 0.36, respectively, but
the absolute values obtained were generally more similar to the expected (spiked) concentrations of DA. Therefore, the SPE cleanup improved the precision of replicate samples analyzed by the MeS ELISA, but the results underestimated the known DA concentrations, especially at the higher concentrations of DA used in the spiked samples. The $R^2$ value of 0.19 obtained for the LC/MS measured results was the lowest $R^2$ value not only in the analysis of the urine samples but in all CSL body fluids tested by LC/MS. Similarly, the $R^2$ values for the MeS and BS ELISA results on non-SPE cleaned up sample urine samples were the lowest of all body fluids tested by these platforms.

The poor performance of the urine analysis by the three platforms may be attributable to the high salt content of CSL urine, which is 2.5 times more concentrated than seawater and 7-8 times more concentrated than their blood. The concentrated urine produced by these animals is a mechanism for ridding their bodies of excess salt and reducing freshwater loss. It is possible that the salt inhibits antibody performance in the ELISA platforms, affects the loss of DA from samples during the SPE cleanup procedure, and/or impacts the efficiency of ion formation during LC/MS analysis. SPE cleanup is recommended for the analysis of DA concentrations in seawater by LC/MS, as the salt presence impacts MS signal stability (40).

Direct comparisons of the non-SPE cleaned up urine samples analyzed by MeS and BS ELISAs ($R^2 = 0.08$; Figure 5B), the SPE cleaned up samples analyzed by MeS ELISA and LC/MS ($R^2 = 0.14$; Figure 5C), and the non-SPE samples analyzed by the MeS and BS ELISAs to results of LC/MS ($R^2 = 0.05, 0.06$, respectively; Figure 5D) all produced low $R^2$ values. These results reflected the poor relationships identified between each of the methods and the expected DA concentrations spiked into urine samples (Figure 5A; Table 2). Further, a large number of samples were discarded from the ELISA results due to high CVs. The non-SPE cleaned up samples analyzed using the MeS ELISA had an average CV of 6%, but five sample results were discarded for exceeding 15%. The CVs calculated for the SPE cleaned up urine samples measured using the MeS ELISA averaged 3%, and no results were discarded. The BS ELISA analysis of non-SPE cleaned up samples averaged 14%, with 24 sample results having CVs of greater than 15%.

**Analysis of DA in CSL Body Fluids from Natural Samples**

The modified DA protocol described in this study for the analysis of DA in CSL body fluids was adopted at the USC laboratory in 2009. Samples collected from stranded CSLs in Orange County, California, by the PMMC were analyzed within 1 month of sample receipt using the modified protocol of the MeS ELISA. Samples with DA concentrations thought to be significant enough to survive storage at $-20^\circ$C until the planned validation study were set aside to be reanalyzed by the three platforms simultaneously in the fall of 2009. DA degradation in particular phytoplankton samples has been shown to be highly variable (43) and can potentially influence successful quantification of DA concentrations in CSL bodily fluids after long-term storage. AF samples collected in the spring of 2009 were below the LOD of the MeS ELISA and therefore no naturally positive AF samples were available for analysis.

Available fluid samples from 11 different animals were analyzed simultaneously using the four methods (Table 3). Samples from eight animals were stored at $-20^\circ$C for 6 months, samples from two animals were stored for 8 months, and samples from one animal were stored for 9 months. The amount of DA degradation observed in individual samples analyzed by the MeS ELISA using the modified protocol was highly variable. There was a 49% decrease in the DA concentration measured in the urine sample stored for 9 months, an average decrease 40% in DA concentrations for urine samples stored for 8 months, and an average 43% decrease in DA concentrations for urine samples stored for 6 months, for an overall average loss of 44%. The individual decreases in DA concentrations measured by the MeS ELISA in urine samples ranged from 4 to 72%, and in two instances the DA concentrations measured were higher than the original DA concentrations measured from fresh samples. Four serum samples were stored for 6 months, and losses of DA ranged from 3 to 64% when measured by MeS ELISA. Two of the four samples decreased in DA concentration during storage to levels below the LOD of the MeS ELISA. One of the CSF samples stored for 6 months decreased 54% in DA concentration as determined by MeS ELISA analysis, and the other decreased to below the LOD of the MeS ELISA. It is recommended that body
### Table 3. Measurements of DA concentrations naturally present in CSL body fluids across the three platforms

<table>
<thead>
<tr>
<th>Animal</th>
<th>Cerebral spinal fluid, ng/mL</th>
<th>Serum, ng/mL</th>
<th>Urine, ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MeS non-SPE (orig)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>MeS non-SPE&lt;sup&gt;b&lt;/sup&gt;</td>
<td>BS non-SPE&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Z-09-01-23-014</td>
<td>547.7 ± 281.9 ± 57.6</td>
<td>335.3 ± 56.2</td>
<td>117.6 ± 25.4</td>
</tr>
<tr>
<td>Z-09-02-25-032</td>
<td>74.6 ± 34.1 ± 5.0</td>
<td>55.6 ± 1.3</td>
<td>bd</td>
</tr>
<tr>
<td>Z-09-02-25-033</td>
<td>49.8 ± 54.5 ± 9.5</td>
<td>54.5 ± 9.5</td>
<td>23.8 ± 2.0</td>
</tr>
<tr>
<td>Z-09-04-19-058</td>
<td>10.3 ± 3.4 ± 0.9</td>
<td>bd</td>
<td>bd</td>
</tr>
<tr>
<td>Z-09-04-24-065</td>
<td>9.4 ± 6.2</td>
<td>bd</td>
<td>bd</td>
</tr>
</tbody>
</table>

<sup>a</sup> The original DA concentration determined by MeS ELISA with the modified DA analysis protocol within 1 month of receipt of samples at the USC.

<sup>b</sup> Values reported for MeS non-SPE, BS non-SPE, and MeS SPE analysis during the validation study in fall 2009 are averages of triplicate replicates.

<sup>c</sup> bd = Below detection.
fluid samples be analyzed for DA content promptly following receipt, as DA degradation in samples during storage was found to be highly variable.

The impact of SPE cleanup of naturally positive samples destined for ELISA analysis could be directly quantified in the simultaneous analysis of the stored samples (Table 3). The SPE cleaned up urine samples achieved 51% lower DA concentrations than the non-SPE cleaned up samples analyzed by the MeS ELISA (range of 91 to 92% lower). The SPE cleaned up urine samples analyzed by the MeS ELISA were consistently lower than the LC/MS measured DA concentrations for the same sample. The MeS ELISA SPE cleaned up samples averaged 67% of the LC/MS measured concentrations (range of 47 to 95% lower). These results are consistent with the results obtained from the spike and recovery portion of this validation study in that SPE cleanup did not improve DI analysis of CSL body fluids using ELISA methods. The 50% methanol extract obtained from the SPE cleanup appeared to inhibit antibody performance, leading to underestimation of the DA concentrations.

Conclusions

The purpose of this study was to provide a comparison of DA concentrations reported in CSL body fluids across methods, laboratories, and datasets for a spectrum of matrixes. The amount, quality, and type of body fluid available for DA analysis from an individual animal are variable and highly dependent on the health status of the animal when brought into rehabilitation facilities. In addition, animal suffering from chronic DA toxicity could be sickened at lower concentrations of DA in their body fluids than animals afflicted with acute DA toxicity. Accurate determination of the toxin concentration is useful for assessing these different conditions and relating animal body burdens to the occurrence of toxin in local planktonic environments. The platform and methodology used by a given laboratory will be determined by the materials, equipment, cost, and technical ability, as well as the specific research goals of the project. The availability of multiple platforms to a single laboratory is a reality, and therefore the ability to extrapolate results across platforms is essential.

The modified protocol described in the reported study for the analysis of CSL body fluids by an ELISA platform was validated as a protocol for analysis of DA concentrations. SPE cleanup was shown to be contraindicated for the removal of matrix effects stemming from the different body fluid matrixes because the 50% methanol extract was more inhibitive to antibody performance than the individual fluid types. False positives were not observed using either ELISA platform, regardless of sample type, using a 1:25 minimal dilution without a methanol extraction step. Overall, the BS ELISA yielded accurate DA concentrations or slightly underestimated DA concentrations, possibly due to the multiple epitopes targeted by the polyclonal antibodies. Whether a slightly higher dilution of body fluid samples would have overcome this inhibition was not tested. Overestimation of DA concentrations using the BS ELISA to analyze body fluids was also noted in previous studies comparing BS ELISA measured results of DA in rat serum and brain samples, relative to LC/MS measurements (39). The MeS ELISA slightly underestimated DA concentrations in general, possibly due to greater sensitivity of the monoclonal antibody to the body fluid matrixes relative to the polyclonal antibodies of the BS ELISA. However, the BS ELISA results were less reproducible than MeS ELISA results, with the BS ELISA values yielding more high SDs and CVs. A total of 77 samples analyzed by the BS ELISA were discarded due to high CVs, while only six samples analyzed by the MeS ELISA were discarded. High CVs can be a consequence of operator error during analysis (i.e., pipet error, use of an unhomogenized sample, or procedural error), matrix impacts on antibody performance, and/or a manufacturing error. It is unknown if matrix effects were solely responsible for the higher CVs observed in the BS ELISA analysis.

The linear relationship between the spiked DA concentrations and those measured by the BS and MeS ELISAs for the AF, CSF, and serum samples, as well as the fact that both assays generally correlated well with each other, may allow for matrix corrected assays to be developed. This would involve applying a correction factor for each assay that adjusts the average ELISA values to match those obtained by LC/MS. Testing of additional samples could then be used to confirm whether the relationship holds across samples from different animals. If so, both assays may be used to rapidly obtain reasonable estimates of DA concentrations in AF, CSF, and serum samples.

The accurate correlation of DA present in phytoplankton samples with unusual marine mammal mortality events in a given area relies upon the positive identification of DA presence in body fluids and the magnitude of the DA present. The ELISA and LC/MS methods described in this paper have been shown to be capable of meeting these goals, without introducing a significant risk of false positives. The platforms demonstrated relatively good agreement (high R² values) with known DA concentrations added to CSL body fluid samples, and the linearity observed when platform results were directly compared verified that the magnitude of DA concentrations measured by each platform were comparable. Urine was identified as a complicated matrix, most likely due to the high salt content, and further investigation is needed to determine if additional sample preparation procedures can reduce the interference observed in the urine results of the present study. It is therefore recommended that DA concentrations for urine samples be an average of multiple replicates, regardless of the platform being used.

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