

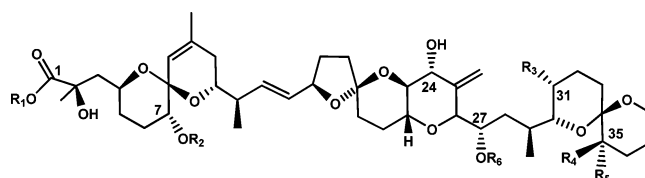
Development and Single-Laboratory Validation of a Pseudofunctional Biosensor Immunoassay for the Detection of the Okadaic Acid Group of Toxins

Linda D. Stewart,^{*,†} Philipp Hess,^{‡,§} Lisa Connolly,[†] and Christopher T. Elliott[†]

Institute of Agri-Food and Land Use, Queen's University of Belfast, Stranmillis Road, Belfast, Northern Ireland BT9 5AG, and Marine Institute, Oranmore, County Galway, Ireland

A rapid analytical optical biosensor-based immunoassay was developed and validated for the detection of okadaic acid (OA) and its structurally related toxins from shellfish matrix. The assay utilizes a monoclonal antibody which binds to the OA group of toxins in order of their toxicities, resulting in a pseudofunctional assay. Single-laboratory validation of the assay for quantitative detection of OA determined that it has an action limit of 120 $\mu\text{g/kg}$, a limit of detection of 31 $\mu\text{g/kg}$, and a working range of 31–174 $\mu\text{g/kg}$. The midpoint on the standard matrix calibration curve is 80 $\mu\text{g/kg}$, half the current regulatory limit. Inter- and intra-assay studies of negative mussel samples spiked with various OA concentrations produced average coefficient of variation (CV) and standard deviation (SD) values of 7.9 and 10.1, respectively. The assay was also validated to confirm the ability to accurately codetect and quantify dinophysistoxin-1 (DTX-1), DTX-2, and DTX-3 from shellfish matrix. Alkaline hydrolysis was not required for the detection of DTX-3 from matrix. Excellent correlations with the data generated by the biosensor method and liquid chromatography/tandem mass spectrometry (LC/MS/MS) were obtained using a certified reference material ($R^2 = 0.99$), laboratory reference material, and naturally contaminated mussel samples ($R^2 = 0.97$). This new procedure could be used as a rapid screening procedure replacing animal-based tests for DSP toxins.

Okadaic acid (OA) and its structurally related toxins, dinophysistoxin-1 (DTX-1) and DTX-2, are lipophilic marine biotoxins produced by phytoplankton and accumulated in and concentrated by shellfish.^{1,2} DTX-3 is a collective name given to the 7-*O*-acyl derivatives of these compounds which have not been detected in plankton but are thought to be produced by biotransformations



	R1	R2	R3	R4	R5	R6
OA	H	H	CH3	H	H	H
DTX1	H	H	CH3	CH3	H	H
DTX2	H	H	H	H	CH3	H
DTX3	H	acyl	as parent	as parent	as parent	H
Diol-ester	diol	H/acyl	as parent	as parent	as parent	H
DTX4	Diol-ester	H	as parent	as parent	as parent	H
DTX5	Diol-ester	H	as parent	as parent	as parent	H
27-O-acyl	H	H	CH3	H	H	acyl

Figure 1. Chemical structures of okadaic acid and dinophysistoxins.

of the parent toxins in the hepatopancreas of shellfish.^{3–7} The chemical structures of the three main toxins of the OA group differ in substituents and stereochemistry at carbon atoms 31, 34, and 35 of the skeleton⁸ (Figure 1). They are collectively called the diarrhetic shellfish poisoning (DSP) toxins⁹ because when shellfish contaminated by these toxins are consumed by humans the result is gastrointestinal disruption including nausea, vomiting, and diarrhea.^{10,11} The toxins exert their toxic effects through inhibition of protein phosphatases, PP1 and PP2A,^{12,13} and they have also been implicated in promoting tumor production.^{14,15}

Sporadic outbreaks of DSP toxins have been recorded in many countries throughout the world although the specific causative

* To whom correspondence should be addressed. Phone: +44 28 9097 6562. Fax: +44 28 9097 6513. E-mail: l.stewart@qub.ac.uk.

[†] Queen's University of Belfast.

[‡] Marine Institute.

[§] Current address: Ifremer, Centre de Nantes, Department of Environment, Microbiology and Phycotoxins, Rue de l'Île d'Yeu, Nantes, France.

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toxin varies in each occurrence and usually includes complex mixtures of toxins. OA is found worldwide but is reported to be the dominant OA-group toxin in Europe,¹⁶ whereas outbreaks of DSP in Japan have mainly been associated with DTX-1.¹⁷ DTX-2 has been reported in shellfish from Spain, Portugal, and Norway,¹⁸ and in Ireland there are reports of higher levels of DTX-2 than OA.¹⁹ Toxic episodes of DTX-3 have been reported in Norway, Chile, Portugal, and Spain.^{20–23} These toxins are a growing food safety problem and pose a major challenge to shellfish industries worldwide as producing countries are required to monitor for their presence in seafood. Improved monitoring programs can reduce the economic cost for the shellfish industry and lower the risk to the consumer. Under EU legislation²⁴ the mouse bioassay (MBA)²⁵ is the current reference method and as such is the most widely used method of analysis for these toxins. This method, however, is unethical and has severe limitations, including low sensitivity, is nonspecific and is unable to distinguish between toxins from different groups.^{26,27} In addition sample clean up is required to reduce interference by free fatty acids,²⁸ and the ester derivatives of OA/DTX can be lost in this process.²⁹ A further problem is that other lipophilic marine biotoxins are coextracted with OA, and of these, yessotoxins are known to interfere with MBA, producing false positive results.³⁰ A more effective and efficient analytical detection method is required. Alternative methods are permitted under European Legislation provided they have undergone protocol validation.³¹ Major limitations to any new technique for DSP toxins include the low availability and consequential high cost of the toxins and their analytical standards, and an added complication with OA and its structurally related toxins is that they have differing toxicity equivalence factors (TEFs). The TEFs for OA and DTX-1 have been established as 1; however,

the relative toxicity of DTX-2 is 0.6.¹⁸ The relative toxicity of DTX-3 is determined by the parent OA analogue (OA, DTX-1, or DTX-2),^{32,33} which is thought to be released in vivo when the esters are hydrolyzed in the human gastric system.^{21,32,33} It has been recommended that when substituting the MBA for lipophilic marine toxins in shellfish with analytical procedures relative toxicities of all analogues that contribute to health risk must be factored in.¹⁸ Although many alternative techniques have been described, they have various shortcomings ranging from long sample preparation techniques, low sensitivity and specificity, and the need for highly trained personnel.³⁴ Most of these procedures also require alkaline or enzymatic hydrolysis as part of the sample preparation procedure to facilitate detection of the DSP esters.³⁵ With the added complication of differing TEFs, alternative analytical techniques must be able to identify and quantify each toxin in order to calculate total toxicity; for example, if DTX-2 is detected its lower TEF requires that the values obtained by nonfunctional analytical methods of detection must be adjusted, by multiplying by a factor of 0.6, to provide an OA equivalence value to determine the level of contamination, and if the parent analogue of any contaminating DTX-3 is DTX-2 the analytical result for DTX-3 has also to be multiplied by the same factor. Alternative analytical replacement methods must be rapid, robust, cost-effective, specific, and sensitive and be able to factor in the relative toxicities of all OA analogues that contribute to consumer health risk.

Antibody-based immunoassay procedures offer the opportunity of rapid, low-cost, and high-throughput screening of samples and allow for the possibility of adaptation for on-site detection of toxins in shellfish extracts and water samples. However, the basis of antibody detection is structural recognition and not toxicity, so in many cases the ultimate usefulness of an antibody will lie in its specificity and cross-reactivity profile. Many antibody-based immunoassays for the detection of OA have been described using various detection formats, and several antibody-based kits are available commercially for the detection of OA including ERFA biotech, DSP-Check (Sceti, Japan), Rougier Bio-Tech ELISA, and E.F.2 (CER, Belgium). Although the limit of detection of these assays can be very low, issues exist regarding the cross-reactivity profile of the antibodies employed in them. Only the E.F.2 kit provides cross-reactivity data claiming to have 50% cross-reactivity to DTX-1 and DTX-2. The monoclonal antibody (mAb) employed in the DSP-Check cross-reacts with DTX-1 at a level comparable to OA, and it requires alkaline hydrolysis prior to ELISA for detection of DTX-3. However, the wells of this ELISA kit are coated with OA resulting in the kit being expensive to buy.^{34,36} Currently, there are no reports of any immuno-based kits which can detect DTX-3 without the need for hydrolysis. To date the most promising

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alternative methods for the lipophilic toxins are suggested to be based on liquid chromatography/tandem mass spectrometry (LC/MS/MS)¹⁸ with direct identification of the OA-group parent toxins followed by hydrolysis for detection of DTX-3. LC/MS/MS methods, however, require trained, experienced personnel and entail conversion of concentrations into toxic equivalent values using TEFs. Ideally, LC/MS/MS-based methods are calibrated using all toxin analogues as calibration standards.

Surface plasmon resonance (SPR)-based biosensor technology has been proven to be a highly reliable tool for detection of agri-food contaminants in many different applications.^{37–46} This label-free, automated analysis technique combines the high affinity of biochemical interactions with low limits of detection producing rapid and reliable results. Minimal amounts of analyte are required to produce the chip surfaces, and these surfaces can be reused many times without loss of activity. The development of a biosensor-based inhibition immunoassay for the detection of OA from shellfish matrixes has been previously reported.⁴⁷ However, as is the case with all other immunoassays described for this toxin, the antibody used in the assay could only accurately detect OA and not the important DTX toxins. The challenge was therefore to produce a binding molecule for use with this technology which could detect all members of the OA group of toxins that contributed to major contamination episodes. Monoclonal antibodies (mAbs) to OA were produced and characterized concurrently for binding to DTX toxins, during screening of the fusion. With the use of this approach a unique mAb was identified which was highly sensitive, having a midpoint on a standard buffer OA calibration curve of 4.8 ng/mL. Standard buffer calibration curves using DTX-1 and DTX-2 determined that this mAb cross-reacted with DTX-1 and DTX-2 with midpoints of 4.9 and 8.0 ng/mL, respectively, and from this the percentage cross-reactivity relative to OA was determined as 100% for DTX-1 and 60% for DTX-2. This cross-reactivity profile reflected exactly the intrinsic toxic potency of this group of toxins and preliminary matrix studies determined that the antibody was sensitive enough for detection of OA in shellfish matrix well below the regulatory limit of 160 µg/kg OA equivalents and could also codetect DTX-1 and DTX-2 with a similar cross-reactivity profile as in buffer. This mAb was therefore considered an excellent candidate for full assay development.⁴⁸ Here we report the development and single-laboratory validation

of a biosensor-based analytical immunoassay for the rapid detection of the OA group of toxins from shellfish matrix using this exceptional mAb.

MATERIALS AND METHODS

Source of Reagents and Apparatus. An optical SPR biosensor system (Biacore Q), Biacore Q control and evaluation software, CM5 sensor chip (research grade), HBS-EP buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% polysorbate 20 (v/v), pH 7.4) and 1 M ethanolamine hydrochloride were supplied from Biacore AB (Uppsala, Sweden). All organic solvents (Analar grade) were obtained from Sigma-Aldrich Chemical Co. (Dorset, U.K.). The MABTrap kit was from Amersham Biosciences AB (Uppsala, Sweden), and the vivaspin centrifugal concentrators tubes were from Sagittarius Stedim Biotech GmbH (Germany). The Millex-HV 0.45 µm filters were from Millipore (Ireland). OA was acquired from LC Laboratories (U.S.A.), and the CRM-OA-b and CRM-DSP-Mus-b were purchased from NRC (Canada). At the time of the study, no certified calibrants were available for DTX-1, DTX-2, or DTX-3. The DTX-1 used for the prevalidation studies was a gift from Biosense Laboratories (Norway), and the DTX-1 used in the validation study was obtained from the NRC (Canada). The DTX-2 and the laboratory reference materials (LRMs) for analysis were a gift from the Marine Institute, Galway (Ireland). The DTX-2 was part of the same purification lot as the one used by Aune et al.¹⁸ NMR analysis of the lot showed no major contamination of the pure compound, and LC/MS analysis showed 800 µg of DTX-2 present. DTX-3 was a gift from CRLMB, Vigo, (Spain) which had been originally obtained from Dr. Takeshi Yasumoto and was part of the same lot used in the study by Villar-Gonzalez et al.³² Naturally contaminated mussel samples were a gift from the Norwegian School of Veterinary Science, Department of Food Safety and Infection Biology, (Norway), and negative shellfish were a gift from the National Reference Laboratory for Marine Biotoxins (U.K.), Agri-Food and Biosense Institute, (N. Ireland).

Safety. Okadaic acid and its analogues cause diarrhea, vomiting, and nausea; therefore, when using DSP standard solutions and DSP-contaminated mussels, special care should be taken. Gloves should be worn at all times. Appropriate disposal methods should also be utilized.

ASSAY DEVELOPMENT

OA Immobilization onto a CM5 Sensor Chip Surface. The method described previously⁴⁷ was used to immobilize the OA onto the surface of a CM5 chip surface. Briefly, the carboxymethylated surface of a CM5 sensor chip was equilibrated to room temperature, and an EDC/NHS mixture (50 µL, 1:1; v/v) was incubated on the chip surface for 30 min. The excess solution was removed, and freshly prepared 1 M ethylene diamine (pH 8.5) (50 µL) was added to the NHS-ester chip surface and incubated for 1 h. The surface of the chip was washed with HBS-EP, buffer and the free NHS-ester groups were blocked with 1 M ethanolamine hydrochloride (pH 8.5) (50 µL) for 20 min. EDC (54.0 mg) and NHS (24.0 mg) were dissolved in 10 mM NaOAc buffer (pH 4.5) (1.0 mL). An aliquot of this mixture (10 µL) was added to OA (50 µg), which was previously dissolved in DMSO (10 µL) and 10 mM NaOAc buffer (pH 4.5) (30 µL). OA–NHS derivative (50 µL) was then added to the amine chip surface and

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incubated for 4 h. The excess solution was removed, and the chip surface was washed with deionized water, dried under a gentle stream of nitrogen gas, and stored desiccated (4 °C) when not in use.

Antibody Preparation. The crude supernatant of a mAb produced previously⁴⁸ was concentrated by centrifugation using vivaspin sample concentrator tubes, purified using a MAbTrap kit following the instructions supplied, and stored frozen in 1 mg/mL aliquots. During assay development studies the antibody was diluted 1/70 in antibody storage buffer and stored in the refrigerator. Under these conditions the antibody was found to be stable for the duration of the study. Just prior to use, the antibody was further diluted 1/4 in HBS-EP buffer resulting in a final working dilution of 1/280.

Sample Preparation—Standard Calibration Curve. A 1 g portion of known negative mussel tissue homogenates (i.e., from shellfish tested negative for DSP by MBA) was weighed into glass bottles. Aqueous acetonitrile (90%) (10 mL) was added, and the sample vortexed for 5 s prior to roller mixing (30 min). After centrifugation (3500 rpm, 10 min at 10 °C) an aliquot of each supernatant (1 mL) was transferred into a glass tube and evaporated to dryness (45 °C) under a gentle stream of nitrogen. The residue was reconstituted in HBS-EP (950 μ L), and appropriate standard concentration (50 μ L) added. Working standards (200, 150, 100, 50, 0 μ g/kg) for matrix calibration curves were made by dilutions in HBS-EP buffer from a stock standard solution (10 μ g/mL in methanol). The samples were vortexed for 20 s and filtered through a 0.45 μ m Millex-HV filter prior to analysis.

Fortified Sample Preparation. The protocol used was as described in the previous section except after weighing out the homogenized negative mussel, this was spiked with the appropriate standard concentration (50 μ L) (200, 160, or 80 μ g/kg) and vortexed before the aqueous acetonitrile (90%) (10 mL) was added. The residue obtained after drying down was then reconstituted in HBS-EP (1 mL) buffer before being vortexed and filtered as before.

Naturally Contaminated Sample Preparation. The protocol used was as described above for blank material. A 1 g portion of the naturally contaminated mussel sample was used, and the residue obtained after drying down was reconstituted in 1 mL HBS-EP buffer before being vortexed, filtered, and analyzed.

Certified Reference Material (CRM-DSP-Mus-b) Preparation. The CRM-DSP-Mus-b has a certified concentration for OA and DTX-1 determined by LC/MS/MS. CRM-DSP-Mus-b (0.1 g) was added to blank mussel tissue (0.9 g), and the sample was vortexed. The extraction protocol described above for naturally contaminated samples was followed. After filtering the sample was diluted in negative matrix extract to expected concentrations of 200, 160, and 80 μ g/kg before being assayed.

Analytical Biosensor Analysis. The diluted shellfish extracts were mixed 1:1 with OA mAb (1/280 dilution). Each sample (50 μ L) was injected over the sensor chip surface at a flow rate of 25 μ L/min. Report points were recorded before and after each injection, and the chip surface was regenerated with 25 μ L injection (flow rate 25 μ L/min) of 220 mM sodium hydroxide with 30% acetonitrile.

Cross-Reactivity and Specificity. Antibody cross-reactivity in sample matrix was determined using blank mussel samples

which were extracted as described previously for blank material and spiked with a range of working toxin calibration standards (200, 150, 100, 50, 0 μ g/kg) of DTX-1 or DTX-2 to produce standard calibration curves for each. The midpoint concentration (IC_{50}) of each calibration curve was determined, and the cross-reactivity calculated as a percentage relative to OA. The specificity of the antibody in matrix was determined by spiking shellfish extracts with working standards (250 μ g/kg) of azaspiracid, gymnodimine, yessotoxin, and pectenotoxin and calculating the percentage inhibition.

Assay Validation. The limit of detection (LOD) of the assay was calculated from the mean of the measured response units (RU) of different negative samples ($n = 20$) minus 3 times the standard deviation (SD) of the mean (mean – 3SD) to take into account the uncertainties associated with assay measurements.⁴⁷ Assay precision was defined by determining intra-assay (within run) and interassay (between run) variation expressed as coefficient of variation (CV) (SD/mean $\times$ 100). To establish the action limit for the procedure, negative mussel samples ($n = 30$) were spiked with 160 μ g/kg OA, analyzed, and the action limit established by again subtracting $3 \times$ SD from the mean value. The intra-assay and interassay variation were determined by evaluating mussel replicates spiked at 80, 160, and 200 μ g/kg ($n = 10$ at each concentration) on 3 separate days on three different biosensor instruments (total $n = 30$ at each concentration). The assay was also validated for the detection of DTX-1, DTX-2, and DTX-3. Intra-assay validation of DTX-1 and DTX-2 detection was achieved by analysis of mussel replicates ($n = 6$) spiked at 80, 160, and 200 μ g/kg, and due to lack of standard, DTX-3 validation was only assessed with samples ($n = 5$) spiked at 160 μ g/kg. To determine the accuracy of the data certified reference material (CRM-DSP-Mus-b), naturally contaminated mussel samples and laboratory reference material (LRM) were assayed.

RESULTS AND DISCUSSION

Cross-Reactivity and Specificity. The nonextracted matrix calibration curves using each of the OA toxin analogues showed that in matrix the cross-reactivity of the antibody had changed slightly, the antibody was now 90% cross-reactive with DTX-1 and 80% cross-reactive with DTX-2. Changes in antibody cross-reactivity are not unusual as matrix has been found in the past to interfere with cross-reactivity.^{41,42} Specificity studies determined that the antibody did not cross-react with gymnodimine, yessotoxin, pectenotoxin, or azaspiracid. This was an important finding as many of these toxins can be coextracted with the OA family of toxins from contaminated shellfish and yessotoxin can interfere with the results of the MBA. The assay was also validated for detection of DTX-1, DTX-2, and DTX-3, and the results, shown in Table 1, confirmed that the assay could accurately detect them all. Problems were initially encountered when developing the assay for detection of DTX-3. First, in the buffer system, the antibody was found not to bind to DTX-3; however, in matrix, the combination of the heat and the solvent in the extraction procedure, although not thought to be enough to cause hydrolysis of the acyl side chains, appears to sufficiently change the conformation of the DTX-3 molecule to allow the mAb to bind to this toxin. Second, because of its highly lipophilic moiety, DTX-3 was found to bind to the plastic filters being used, and a range of

Table 1. Biosensor Analysis Results for Inter- and Intra-Assay Precision Studies of the OA Group of Toxins

no. of samples	OA [$\mu\text{g/kg}$]			DTX-1 [$\mu\text{g/kg}$]			DTX-2 [$\mu\text{g/kg}$]			DTX-3 [$\mu\text{g/kg}$]
	$n = 30$			$n = 6$			$n = 6$			$n = 5$
spiked concentration [$\mu\text{g/kg}$]	80	160	200	80	160	200	80	160	200	160
mean of biosensor results [$\mu\text{g/kg}$]	59	147	180	55	130	160	47	91	120	120
SD	5.0	9.1	16.2	4.9	11.5	12.1	7.8	13.6	14.8	16.7
CV	8.5	6.2	5.0	8.9	8.5	8.3	16.6	14.9	7.4	13.9

filters had to be tested to find one to which did not exhibit the binding phenomena.

Assay Precision and Accuracy Studies. A matrix calibration curve for OA was produced using uncontaminated mussel extracts samples spiked with OA standard concentrations from 0 to 200 $\mu\text{g/kg}$. On the basis of this standard curve the assay produced a midpoint (IC_{50}) of 80 $\mu\text{g/kg}$. Ideally, in an analytical assay, the midpoint of the standard curve should be close to the regulatory limit as this is the most sensitive part of the assay. Adjusting various parameters of the biosensor assay could have increased this midpoint to make it closer to the current regulatory limit (160 $\mu\text{g/kg}$); however, phycotoxin regulatory limits within the EU are currently under review and there is the possibility, because of the association of OA and tumor promotion, of the DSP toxin limit being reduced. The dynamic range of the assay was calculated from the IC_{10} and the IC_{90} of the curve⁴⁵ and was found to be 31–174 $\mu\text{g/kg}$.

The LOD of the assay was determined as the mean relative response (333RU) of negative samples ($n = 20$) minus 3 times the SD (75.9), and the value obtained (257.1RU) read from the standard matrix calibration curve as 31 $\mu\text{g/kg}$. The action limit was calculated as the concentration corresponding to the average instrument response for 30 negative mussel samples fortified with 160 $\mu\text{g/kg}$ OA. The mean value obtained was 147 ± 9 $\mu\text{g/kg}$. The mean minus 3 times the SD was taken as the action limit of the procedure, i.e., 120 $\mu\text{g/kg}$. Under these assay conditions a CV of 6.2 was achieved. Intra-assay ($n = 10$) and interassay ($n = 3$) studies of negative mussel samples spiked with OA concentrations of 80, 160, and 200 $\mu\text{g/kg}$ (10 samples/day, three different days on three different instruments) produced CV and SD values of 8.5, 6.2, 9.0 and 5.0, 9.1 and 16.2, respectively (Table 1).

There was good apparent recovery of DTX-1 (77%) during the procedure, and the results at each concentration had a CV of below 9 and SDs of 12 or less. The results for DTX-2 showed apparent recoveries of approximately 60% from the matrix, so no adjustments were needed to be made to take into account the lower TEF of this toxin. Lack of availability of DTX-3 toxin permitted validation studies to be performed at one concentration only (160 $\mu\text{g/kg}$). The concentration of DTX-3 was determined by taking into account the differences between the molecular weight of the DTX-3 molecule and that of its parent compound in order to calculate how much parent compound should be there.³² The results generated confirmed that the assay was capable of detecting DTX-3 present in shellfish matrix without the need for hydrolysis and with a 75% recovery rate. This is a major advantage over all of the other assays described in the literature for detection of the OA group of toxins (apart from the MBA) as samples must be analyzed following hydrolysis, to determine the total amount of OA equivalents present.

Table 2. Comparison of Expected Results for CRM-DSP-Mus-b with Biosensor Analysis Results ($R^2 = 0.99$, $y = 1.25x$)

expected toxin concentration [$\mu\text{g/kg}$]	200	160	80
biosensor result [$\mu\text{g/kg}$]	249	202	104
percentage overestimation by biosensor	125%	126%	130%

ASSAY VALIDATION

Further validation of the biosensor assay was performed by analyzing a certified reference material (CRM-DSP-Mus-b), laboratory reference material (LRM), and a number of naturally contaminated shellfish samples.

CRM-DSP-Mus-b Analysis. The results for biosensor analysis of CRM-DSP-Mus-b showed an excellent correlation with the results expected by LC/MS/MS; there was, however, a slight but consistent overestimation of the amount of toxin in the sample, ($R^2 = 0.99$, $y = 1.25x$) (Table 2). The chromatogram of LC/MS/MS analysis of the CRM material supplied when purchased shows the presence of several isomers of OA and DTX-1. It is highly possible that the antibody employed in the biosensor assay recognized all of these analogues and thus accounted for the consistent small overestimation of toxin in the CRM-DSP-Mus-b material. Another possible contributing factor may be that the dilutions of this material were made based on the presence of 10.1 mg/kg OA and 1.3 mg/kg DTX-1. The certified concentrations of toxin in the sample are more accurately 10.1 ± 0.8 mg/kg for OA and 1.3 ± 0.2 mg/kg for DTX-1, so the sample may contain up to 9% more toxins. As the overestimation is slightly more than this 9% the most likely explanation is a combination of antibody recognition of the additional, as yet undetermined, DTX isomers known to be present in the sample and the possible extra toxins it may contain.

Naturally Contaminated Mussel Samples. The LC/MS/MS results for the naturally contaminated mussel samples showed that these samples contained a mixture of the analogues, isomers, and esters of OA and that the total levels of toxin were close to (above and below) the current regulatory limit for OA (160 $\mu\text{g/kg}$ OA equivalents). The results from biosensor analysis showed good correlation with the LC/MS/MS results ($R^2 = 0.97$, $y = 0.86x$) (Table 3).

LC/MS/MS analysis provides a quantitative determination of the amount of each toxin present in the sample which must then be adjusted depending on the TEF of the individual toxin. Each sample is hydrolyzed prior to determination of total OA-equivalents present. The action limit of the LC/MS/MS assay is the regulatory limit (160 $\mu\text{g/kg}$ OA equivalents), and its limit of quantification is 20 $\mu\text{g/kg}$ OA equiv. The biosensor assay, however, provides a quantitative result for the total amount of toxin present in the sample without the need for hydrolysis, and because of the unique cross-reactivity profile of the antibody no additional calculations

Table 3. Individual and Total Toxin Composition of Naturally Contaminated Mussel Samples from Norway as Determined by LC/MS/MS and Corresponding Biosensor Analysis Results

sample no.	OA	OA-acyl	DTX-1	DTX-1 acyl	DTX-2	DTX-2 acyl	total DTX-3	total DSP toxins by LC/MS/MS [$\mu\text{g/kg}$] ^a	biosensor result [$\mu\text{g/kg}$] ^a
OA [$\mu\text{g/kg}$] as the Major Toxin Present									
1	42	141	0	6	0	0	147	189 (pos)	234 (pos)
2	6	167	0	7	0	0	174	180 (pos)	177 (pos)
3	12	129	0	10	0	0	139	151 (neg)	105 (neg)
4	0	149	0	0	0	0	149	149 (neg)	144 (pos)
DTX-1 [$\mu\text{g/kg}$] as the Major Toxin Present									
5	0	10	38	81	0	2	92	130 (neg)	156 (pos)
6	0	13	69	99	0	0	112	181 (pos)	133 (pos)
DTX-2 [$\mu\text{g/kg}$] as the Major Toxin Present									
7	15	23	0	0	37	0	23	61 (neg)	71 (neg)
OA and DTX-1 [$\mu\text{g/kg}$] as the Major Toxins Present									
8	40	24	112	0	0	0	24	176 (pos)	186 (pos)
9 ^b	22	96	26	33	0	0	128	176 (pos)	129 (pos)
10	0	101	30	45	0	0	147	176 (pos)	120 (pos)
Blank [$\mu\text{g/kg}$]									
11	0	0	0	0	0	0	0	0 (neg)	8 (neg)
12	0	0	0	0	0	0	0	0 (neg)	69 (neg)

^a pos = positive sample, neg = negative sample according to the action limit of the respective technique. ^b Negative by mouse bioassay.

Table 4. Individual and Total Toxin Comparison of Laboratory Reference Material (LRM) as Determined by LC/MS/MS and Corresponding Biosensor Analysis Results

sample no.	OA [$\mu\text{g/kg}$]	OA-acyl [$\mu\text{g/kg}$]	DTX-1 [$\mu\text{g/kg}$]	DTX-1 acyl [$\mu\text{g/kg}$]	DTX-2 [$\mu\text{g/kg}$]	DTX-2 acyl [$\mu\text{g/kg}$]	total DTX-3 [$\mu\text{g/kg}$]	total DSP by LC/MS/MS [$\mu\text{g/kg}$]	biosensor result [$\mu\text{g/kg}$]
1	80	270	90	20	108	168	458	740	890
2	260	1810	0	0	0	0	1810	2070	1690
3	160	160	380	120	384	84	364	1290	1100

are required to take into account the individual TEFs of each toxin. The extraction procedure is quick and easy, and the assay is fully automated and does not require trained personnel to perform it. The result obtained is the result reported. The action limit of the biosensor assay was determined to be 120 $\mu\text{g/kg}$, and it has a working range of 31–174 $\mu\text{g/kg}$. LC/MS/MS analysis detected six samples that would have been considered positive relative to the regulatory limit and six negative samples. The biosensor assay detected eight positive samples and four negative samples. Although no false negative results were reported by the biosensor assay two samples were determined as positive which the LC/MS/MS analysis determined as negative. An examination of these individual results obtained by the two different procedures, however, showed that they were very similar: sample 4, LC/MS/MS result 149 $\mu\text{g/kg}$, biosensor result 144 $\mu\text{g/kg}$; sample 5, LC/MS/MS result 130 $\mu\text{g/kg}$, biosensor result 156 $\mu\text{g/kg}$. The major difference between the data generated lies in what is considered the action limit of the individual assays. It could be argued that in the interest of public safety the biosensor assay gave the more accurate results.

In the Norwegian School of Veterinary Science shellfish samples are analyzed routinely using an accredited LC/MS/MS method, although if requested, samples are analyzed using the MBA. MBA analysis was requested on one of the naturally contaminated samples (no. 9) and was found to be negative, but LC/MS/MS analysis determined that the sample was positive. Biosensor analysis of this same sample also produced a positive result. This positive sample would have been missed in a MBA screening program and reported as a false negative, and so again

it can be argued that in terms of public safety the biosensor assay outperformed the MBA. The lack of detection capability of the MBA for OA at concentrations close to the regulatory limit has also been independently recognized during a recent evaluation of the current regulatory methods in the EU.⁴⁹

Laboratory Reference Materials. The laboratory reference materials (LRMs) materials are naturally contaminated shellfish tissues which have been heat-treated, homogenized, and characterized for homogeneity and stability.⁵⁰ The Marine Institute, Galway is accredited for its in-house validated LC/MS/MS method which has been used for the characterization of these materials. The composition and concentrations of the individual OA toxins present in the LRM samples had been predetermined by LC/MS/MS analysis, but the biosensor-based measurements of toxin concentrations present in the samples were performed as a blind trial. All of the results obtained by the biosensor procedure for undiluted LRM material were determined to be above the calculated upper limit of the biosensor assay (174 $\mu\text{g/kg}$), so a series of dilutions of each extract in negative matrix extract were performed. The dilution which gave a result closest to the midpoint of the calibration curve (80 $\mu\text{g/kg}$) was used to calculate the final concentration (Table 4). Again there was good correlation between the results obtained by biosensor and LC/MS/MS.

All of the naturally contaminated samples used in the validation study contained a wide range of the OA group of toxins including acyl esters of OA, DTX-1, and DTX-2. On the basis of the

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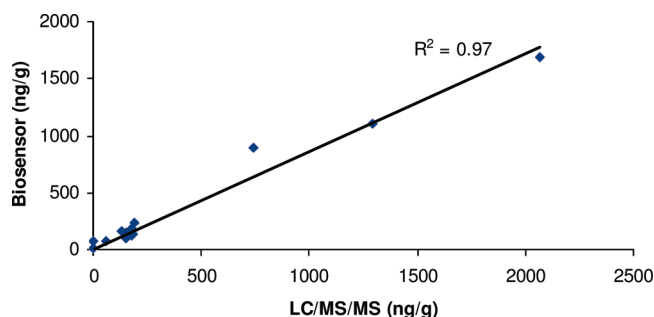


Figure 2. Comparison of biosensor and LC/MS/MS results of all naturally contaminated samples (LRMs and Norwegian samples).

quantitative data generated in the study it can be assumed that the biosensor assay was able to detect all of these derivatives as well as the parent compounds and provide an accurate quantitative determination of the total amount of toxin present without the need for hydrolysis or postanalytical calculations to take account of the lower TEF of DTX-2. The correlation coefficient between LC/MS/MS and biosensor analysis for all results obtained for the naturally contaminated samples was determined to be 0.97 ($y = 0.86x$) (Figure 2) suggesting that the antibody is recognizing the parent compound of DTX-3, and not the derivative, thus providing an accurate determination of OA equivalents present in the sample.

The requirements for a biosensor assay to be a feasible replacement for the MBA is dependent on multiple factors. The cost per analysis is an important consideration. In relation to this, one must consider the equipment and assay components. The SPR equipment used in the present study, a Biacore Q, has been shown to be an extremely useful tool in contaminant analysis. However, the cost of this apparatus is continually being flagged up as a difficulty in introducing the technology to a wider number of industrial and regulatory users. Several new SPR platforms, with a much lower cost basis, are now becoming commercially available, and it will be interesting to determine if they can generate equally as reliable data as the Biacore equipment. In relation to the assay, the ability to reuse the sensor chip surface is fundamental to the cost per analysis calculations. The surface chemistry used in the present study produced a highly stable chip surface using 50 μg of OA, resulting in a high coverage of toxin that allowed the binding of the antibody to be measured over 1000 cycles without any decrease in performance. This data strongly suggests the surface chemistry employed in the present study is an ideal candidate for future production of commercial assay kits,

and as the antibody employed is monoclonal there is an infinite supply of it.

CONCLUSION

In this study a unique mAb to OA and the DTX toxins was used to develop and validate a pseudofunctional biosensor-based analytical immunoassay which was competent in the quantitative detection of low levels of OA, its analogues, isomers, and esters in complicated matrixes. The nature of the assay allowed for a high sample throughput capability using minimal amount of toxin, and the unique cross-reactivity profile of the mAb employed conferred the ability to quantify the total amount of toxin based on differing TEFs with the additional benefit of not requiring hydrolysis for OA ester detection. The current mammalian assays used as the official reference methods for the detection of the OA group of toxins are insensitive, unethical, and are not capable of detecting OA-group toxins below the present EU regulatory level of 160 μg OA equivalents/kg shellfish meat. The biosensor assay development, inter- and intra-assay validation studies, and the investigations of naturally contaminated samples confirmed the suitability of this assay as a potential candidate to replace the MBA for routine monitoring purposes. With no cross-reactivity detected to any of the other lipophilic toxins the biosensor-based assay could be used for preliminary screening the toxicity of shellfish samples for the presence of the OA group of toxins, and if the sample was considered suspicious, complementary analytical techniques could then be used in parallel in order to provide a full individual toxin determination and quantification. The quantitative results provided by the biosensor assay provide invaluable risk management information for the shellfish industry. The assay can determine if the toxin levels found in a sample are low, close to, or above the maximum permitted level allowing regulatory authorities and shellfish producers to use this data to make judgments relating to sampling levels and harvesting.

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