ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	**ASP ELISA kit for determination of Domoic acid**
Name of the Method Developer	**Biosense Laboratories AS**
Developer Contact Information	**Hans Kleivdal** **Biosense Laboratories AS** **HIB-ThorMohlensgate 55** **N-5008 Bergen** **Norway**

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.	Y	The method is a rapid, simple, and reliable method for domoic acid analysis in shellfish for the industry sector. The method is easy to set up and therefore accessible for small test facilities close to the site of operation.
2. What is the intended purpose of the method?	Y	The method is developed and validated for the determination of domoic acid in mussels, oysters and scallops. However, the method has been validated for several other matrices at the SLV level.
3. Is there an acknowledged need for this method in the NSSP?	Y	The supply of rapid, simple, reliable and easily accessible method alternatives to demanding liquid chromatography methods, will enable the shellfish industry to employ local test facilities close to the site of operation. This will again enable preventive countermeasures and immediate response to elevated DA levels locally, significantly reducing the risk of exposing the consumers to contaminated shellfish.
4. What type of method? i.e. chemical, molecular, culture, etc.	Y	Direct competitive Enzyme-linked Immunosorbent Assay.
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	ASP ELISA kit for determination of Domoic acid
Method Scope	Y	For the determination of domoic acid in shellfish.
References	Y	1) Biosense Laboratories AS (2009) *A31300401 - ASP ELISA user manual.* Biosense Laboratories AS, Bergen, Norway (www.biosense.com) 2) Garthwaite, I., Ross, K.M., Miles, C.O., Hansen, R.P., Foster, D., Wilkins, A.L. & Towers, N. (1998) Polyclonal antibodies to domoic acid and their use in immunoassay for domic acid in sea water and shellfish. *Nat. Toxins* **6**, 93-104. 3) Kleivdal, H, Kristiansen, SI, Nilsen, MV, Briggs, L. (2007) Single-Laboratory Validation of the Biosense

		Direct Competitive Enzyme-Linked Immunosorbent Assay (ELISA) for Determination of Domid Acid Toxins in Shellfish. *J. AOAC Intl.* **90**; 1000-1027. 4) Kleivdal, H, Kristiansen, SI, Nilsen, MV, Goksoyr, A, Briggs, L, McNabb, P, Holland, P. (2007) Determination of Domoic acid Toxins in Shellfish by Biosense ASP ELISA – A Direct Competitive Enzyme-linked Immunosorbent Assay: Collaborative Study. *J. AOAC Intl.* **90**; 1011-1027. 5) AOAC OMA 2006.02, AOAC International Official Methods of Analysis. AOAC International, Gaitersburg, MD, USA. 6) Commission regulation (EC) No 1244/2007 of 24 October 2007 amending Regulation (EC) No 2074/2005 as regards implementing measures for certain products of animal origin intended for human consumption and laying down specific rules on official controls for the inspection of meat (Official Journal of the European Union L281/12 of 25 October 2007).
Principle	Y	The domoic acid is extracted from the mollusc tissue by the extracting shellfish flesh homogenate with 50% methanol. The methanol extract containing the domoic acid is diluted with buffer before it is loaded on the microplate wells for analysis. The ASP ELISA assay is in a direct competition format, where free DA in the sample competes with DA-conjugated protein coated on plastic wells for binding to anti-DA antibodies free in the solution. The polyclonal ovine anti-DA antibodies are conjugated to horseradish peroxidase (HRP). Sample diluted in buffer is incubated in the wells with the anti-DA-antibody-HRP conjugate. After washing, the amount of conjugate remaining bound to the well is measured by incubation with a substrate that gives a blue product upon reaction with the HRP enzyme. Addition of acid stops the reaction and changes the product colour from blue to yellow. The colour intensity is measured spectrophotometrically on a plate-reader at 450 nm, and is inversely proportional to the concentration of DA in the sample solution. The assay is calibrated using dilutions of a DA calibration solution supplied with the kit.
Any Proprietary Aspects	Y	Unique antibody conjugate.
Equipment Required	Y	Specified in the user manual for ASP ELISA A31300401, page 7.
Reagents Required	Y	Most reagents are supplied with the kit, while a few additional reagents are required. All is specified in the user manual for ASP ELISA A31300401, page 7.
Sample Collection, Preservation and Storage Requirements	Y	Sample preparation procedure is specified in the user manual for ASP ELISA A31300401, page 10. The ASP ELISA kit storage requirements are specified in the user manual for ASP ELISA A31300401, page 10. The ASP ELISA kit is stable for 24 months after production when stored at 4˚C.
Safety Requirements	Y	Safety precautions are specified in the user manual for ASP ELISA A31300401, page 6.
Clear and Easy to Follow Step-by-Step Procedure	Y	Specified in the user manual for ASP ELISA A31300401, as a detailed procedure and as a Quick Guide supplied with the ASP ELISA kit.
Quality Control Steps Specific for this Method	Y	Quality assurance measures are specified in the user manual for ASP ELISA A31300401, page 16.
C. Validation Criteria		
1. Accuracy / Trueness	Y	The closeness of agreement between the ASP ELISA test results and the spike value using Certified

		Reference Materials provided by NRC Canada, was determined both in the SLV study (r2 at 0.999, slope at 1.070) and the collaborative study (r2 at 0.992, slope at 1.015), as specified in the respective publications.
2. Measurement Uncertainty	N	This was not a requirement for the AOAC at the time of validation.
3. Precision Characteristics (repeatability and reproducibility)	Y	The repeatability precision was determined (mean 9.3%) in the SLV study, as specified in the respective publication. In the Collaborative study, the within-lab repeatability precision (mean 14.8%) and the inter-lab reproducibility (mean 22.7%) were determined, as specified in the respective publication.
4. Recovery	Y	The ASP ELISA test recovery was calculated based on the analysis of several matrix samples spiked with Certified Reference Materials provided by NRC Canada, in both the SLV study (mean 102.2%) and the collaborative study (104%), as specified in the respective publications.
5. Specificity	Y	The antibody specificity was determined by Garthwaite and coworkers (1998).
6. Working and Linear Ranges	Y	The calibration curve range of the assay is approximately 10-260 pg/mL, with a dynamic working range for DA toxins in shellfish that is linear from 0.01 to at least 250 mg/kg, as specified in the SLV study.
7. Limit of Detection	Y	The shellfish LOD is 0.0033 mg/kg as specified in the SLV study.
8. Limit of Quantitation / Sensitivity	Y	The shellfish LOD is 0.010 mg/kg as specified in the SLV study.
9. Ruggedness	Y	A ruggedness study is described in the SLV study.
10. Matrix Effects	Y	The selectivity has been well documented wrt structural analogues, and the matrix effects have been studied wrt several shellfish matrices, *Pseudonitzchia sp.* cultures and mammalian body fluids, as specified in the SLV study.
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	Y	The ASP ELISA test comparability to liquid chromatography-based methods has been described in both the SLV study and the Collaborative study.
D. Other Information		
1. Cost of the Method	Y	$500 for 36 duplicate sample results ($13.9 pr sample).
2. Special Technical Skills Required to Perform the Method	Y	Some technical skills are required, but familiarity with laboratory setting is adequate. User courses and instructional DVD is available for specific training.
3. Special Equipment Required and Associated Cost	Y	A microwell Strip reader and manual pipettes are required. The estimated cost is about $1,800.
4. Abbreviations and Acronyms Defined	Y	ELISA: Enzyme-linked Immunosorbent Assay DA: Domoic Acid ASP: Amnesic Shellfish Poisoning
5. Details of Turn Around Times (time involved to complete the method)	Y	The sample preparation will depend on the number of samples, but 10 samples can be prepared in 30 minutes. 36 prepared samples can be analyzed in approximately 2 hours.
6. Provide Brief Overview of the Quality Systems Used in the Lab	Y	The quality policy contains a) manufacturing quality system, b) documentation control system, c) written master batch record including master formula, labeling and manufacturing SOPs, d) individual batch records which are maintained and kept through product expiration, e) product performance testing requirements which are conducted on each batch, f) unique lot numbers for each batch which are traceable from raw materials through finished products, g) a stability program, h) a quality audit function, and i) a mechanism for disposition of non-conforming materials.

Submitters Signature  	Date: 6th October 2009
Submission of Validation Data and Draft Method to Committee	Date:
Reviewing Members	Date:
Accepted	Date:
Recommendations for Further Work	Date:
Comments:	

DEFINITIONS

1. **Accuracy/Trueness** - Closeness of agreement between a test result and the accepted reference value.
2. **Analyte/measurand** - The specific organism or chemical substance sought or determined in a sample.
3. **Blank** - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. **Comparability** - The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. **Fit for purpose** - The analytical method is appropriate to the purpose for which the results are likely to be used.
6. **HORRAT value** - HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. **Limit of Detection** - the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. **Limit of Quantitation/Sensitivity** - the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. **Linear Range** - the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. **Measurement Uncertainty** - A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. **Matrix** - The component or substrate of a test sample.
12. **Method Validation** - The process of verifying that a method is fit for purpose.[1]
13. **Precision** - the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. **Repeatability** - the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. **Reproducibility** - the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. **Quality System** - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision-making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. **Recovery** - The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. **Ruggedness** - the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. **Specificity** - the ability of a method to measure only what it is intended to measure.[1]
18. **Working Range** - the range of analyte or measurand concentration over which the method is applied.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
3. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.
4. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

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RESEARCH ARTICLE

Polyclonal Antibodies to Domoic Acid, and Their Use in Immunoassays for Domoic Acid in Sea Water and Shellfish

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ABSTRACT Ovine antibodies raised against conjugates linked through the secondary amino group of domoic acid (**1**) were used, together with activated-ester-derived conjugates of domoic acid (DA) as the plate coater, to develop a robust indirect competitive enzyme-linked immunosorbent assay (cELISA) for DA in shellfish and seawater. The ELISA was used to analyze shellfish samples for DA, and was compatible with several extraction procedures. The ELISA had a detection limit below 0.01 ng ml^{-1}, a limit of quantitation (LOQ) of 0.15 ng ml^{-1} and a working range of 0.15–15 ng ml^{-1} DA. The LOQ is equivalent to 38 ng g^{-1} DA in shellfish flesh, assuming a 250-fold dilution during extraction. This is more than 500 times lower than the maximum permitted level (20 μg g^{-1} flesh). The ELISA is designed for use alongside regulatory analyses, and, following formal validation, should be available for pre-screening of regulatory shellfish flesh samples. The ELISA was also shown to be appropriate for analysis of DA in algal cultures and in samples of seawater, and thus has the potential to provide early warning of developing algal blooms.

Key words: domoic acid; ASP; immunoassay; ELISA; shellfish; antibodies

INTRODUCTION

The occurrence of the potent neurotoxin domoic acid (DA) (**1**) (Figure 1) in shellfish was first recognized following human consumption of contaminated mussels from Prince Edward Island, Canada (Wright *et al.*, 1989). The source of the toxin was shown to be *Pseudo-nitzschia pungens* forma *multiseries*. DA is produced by a number of marine algae, including microalgae of the genus *Pseudo-nitzschia*, and is accumulated by shellfish filter-feeding during *Pseudo-nitzschia* blooms. Ingestion of contaminated shellfish causes amnesic shellfish poisoning (ASP), and has caused the death of both animal and human consumers in severe cases (Wright *et al.*, 1989; Bates *et al.*, 1987). To protect consumers from ASP, most countries have set a regulatory limit for DA in shellfish of 20 μg g^{-1} (20 ppm) as determined by the method of Iverson *et al.* (1994).

The incidence of *Pseudo-nitzschia* blooms is increasing world-wide, and toxic blooms have been reported in many localities (Ravn, 1995). The genus was first recorded in New Zealand (NZ) waters nearly 40 years ago (Rhodes *et al.*, 1996), and DA was first identified in NZ in shellfish harvested during 1993 (Rhodes *et al.*, 1996). NZ regulatory authorities conduct an extensive shellfish sampling and analysis program to protect consumers and to satisfy overseas markets that NZ produce is safe and conforms to the regulatory limits. Shellfish are now routinely tested for DA in NZ under the Marine Biotoxin Monitoring Programme, which tests

Abbreviations: DA domoic acid, KA kainic acid, PBS phosphate-buffered saline, ELISA enzyme-linked immunosorbent assay, cELISA competitive ELISA, ASP amnesic shellfish poisoning, DSP diarrhetic shellfish poisoning, NSP neurotoxic shellfish poisoning, PSP paralytic shellfish poisoning, LOQ limit of quantitation, I_{50} concentration giving 50% maximum inhibition, HPLC high performance liquid chromatography, NMR nuclear magnetic resonance spectroscopy, TLC thin layer chromatography, EDAC 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide, NHS *N*-hydroxysuccinimide, OVA ovalbumin, HRP horseradish peroxidase, BSA bovine serum albumin, FET fetuin, THY thyroglobulin, TMB 3,3',5,5'-tetramethylbenzidine, DACS-1 DA analytical standard.

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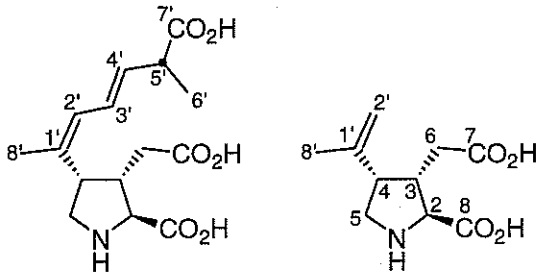

Domoic acid (**1**) Kainic acid (**2**)

Figure 1. Structures of domoic (**1**) and kainic (**2**) acids. The atom numbering system of Walter *et al.* (1994) for **1** is applied to **2** and its derivatives to facilitate comparison of NMR data.

samples from both commercial and recreational harvest sites from around our 5650 km coastline.

To compound the problem, NZ has experienced toxicity attributable to all four regulated algal toxin classes-ASP, DSP, PSP, and NSP. All shellfish exported must be tested for each class of toxin, which places a heavy workload on the accredited laboratories and is extremely expensive to the industry. We are working with the shellfish industry and the Ministry of Agriculture and Fisheries (MAF) to establish a pre-screening procedure to minimize the number of mouse bioassays and expensive HPLC analyses that must be performed (Towers *et al.*, 1997). The ELISA technique, being quick, cheap and easy to perform, is ideal for such a program, so long as robust assays of sufficient sensitivity are available. The development of an ELISA for DA, and its validation for analysis of shellfish, is a key step in the establishment of the pre-screening procedure.

Analysis for DA is currently performed by HPLC (Lawrence *et al.*, 1989). Other assays available for DA include a modification of the PSP mouse bioassay capable of detecting DA at concentrations over 40 μg g^{-1}, and a receptor binding assay (Van Dolah *et al.*, 1994) with a limit of quantitation (LOQ) of 0.1 ng ml^{-1}.

Several groups have developed immunological assays for DA (Newsome *et al.*, 1991; Osada *et al.*, 1995), but only Smith and Kitts (1995) have reported using such an assay on shellfish flesh. They found the ELISA results to be highly correlated with results obtained by HPLC, but they were generally approximately 9% higher, perhaps because the ELISA recognized the presence of DA isomers not determined by HPLC. Unfortunately, this assay relies on the very limited resource of antiserum from a single mouse.

This paper describes the generation of novel hapten chemistries for DA and kainic acid (KA) (Figure 1), the production of antibodies against DA, and establishment of an assay for ASP toxins suitable for use in a shellfish pre-screening procedure. We also report the effect of extraction solvents upon the assay system, in preparation for the development of a universal extraction system that will be suitable for ASP, DSP, PSP, and NSP toxins. A preliminary account of this work has been presented elsewhere (Garthwaite *et al.*, 1997).

MATERIALS AND METHODS

General

(±)-7-Octene-1,2-diol and $NaBH_3CN$ were from Aldrich, USA. Domoic acid dihydrate (DA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), kainic acid monohydrate (KA), L-glutamic acid, L-glutamine, formimino-L-glutamic acid, *N*-hydroxysuccinimide (NHS), ovalbumin (OVA), Freund's complete adjuvant, Freund's incomplete adjuvant, and horseradish peroxidase (HRP) were from Sigma, USA. Bovine serum albumin (BSA) and fetuin (FET) were from Life Technologies, NZ. Anti-sheep secondary antibody was from Silenus Laboratories, Australia. Thyroglobulin (THY) and 3,3′,5,5′-tetramethylbenzidine (TMB) were from Boehringer Mannheim GmbH, Germany. f/2 Medium was prepared according to the method of Guillard (1975). The analytical standard of DA (DACS-1) was from NRC, Canada. Tween-20 and solvents were of 'Analar' grade from BDH, NZ. Except where indicated, NMR spectra were obtained from D_2O solutions at 300 K with either a Bruker AC-300 or Bruker DRX-400 instrument. Chemical shifts in D_2O are reported relative to methanol (i.e. CH_3OD) where δ (^1H) = 3.52 ppm and δ (^{13}C) = 49.1 ppm, respectively. Chemical shifts in $CDCl_3$ are reported relative to internal $CHCl_3$ (δ ^1H 7.26) and $CDCl_3$ (δ ^{13}C 77.1) TLC was performed on silica gel (Merck art. 5554), and compounds were visualized with ninhydrin (0.2% in ethanol) and heat or $KMnO_4$ (5% in H_2O). Flash chromatography (Still *et al.*, 1978) was performed on silica gel (Merck art. 9385). Ozonolysis was performed with a Fischer Ozon-generator 502 running at 50 l^{-1} h and set at 50 scale units. Reactions were performed at ambient temperature (*c.* 20°C) unless otherwise specified. Yields of kainic and domoic acid derivatives were calculated by assuming these to be in the form of the monohydrates and dihydrates, respectively. Phosphate buffer (0.1 M, pH 7.4) was used to prepare phosphate buffered saline (PBS) (0.01 M phosphate, 0.15 M NaCl, pH 7.4). GC-MS was performed on a DB1 capillary column (30 m) with helium as the carrier gas and temperature programmed from 40°C (5 min hold) to 240°C (10 min hold) at 5°C min^{-1}. Phosphate–citrate buffer (pH 6.5) was prepared by adding sodium phosphate (0.1 M) to citric acid (0.1 M). Protein conjugates were freed of contaminating hapten by repeated concentration from PBS in a centrifugal ultrafiltration apparatus (30 kDa cutoff, Filtron, USA), then freeze-dried and stored at −20°C under vacuum until required. Animal manipulations were approved by the Ruakura

Animal Ethics Committee, established under the Animal Protection (code of ethical conduct) Regulations Act, 1987.

*Kainic acid (**2**)*

NMR δ^1H 5.24 (s, H-2_A′), 4.96 (s, H-2_B′), 4.30 (d, J = 3.3 Hz, H-2), 3.83 (dd, J = 11.4, 7.3 Hz, H-5β), 3.63 (t, J = 11.4 Hz, H-5α), 3.29 (m, H-3), 3.23 (m, H-4), 2.68 (dd, J = 16.7, 6.3 Hz, H-6_A), 2.58 (dd, J = 16.7, 8.1 Hz, H-6_B), 1.96 (s, H-8′); δ^{13}C 22.3 (C-8′), 33.4 (C-6), 40.9 (C-3), 46.0 (C-4), 46.7 (C-5), 65.9 (C-2), 113.8 (C-2′), 140.2 (C-1′), 173.4 (C-8), 176.3 (C-7).

*N-(5-Hydroxypentyl)kainic acid (**5**)*

$NaBH_3CN$ (30.0 mg, 480 μmol) in methanol (300 μl) was added to kainic acid (**2**) monohydrate (10.1 mg, 43.6 μmol) and glutaraldehyde (350 μl of 25% solution in water, 930 μmol). After 24 h, TLC (EtOH-HOAc, 24:1) revealed the reaction to be complete, and the product was purified by flash chromatography (EtOH-water, 24:1) to give a quantitative yield of *N*-(5-hydroxypentyl)kainic acid (**5**). NMR δ^1H 5.21 (s, H-2_A′), 4.90 (s, H-2_B′), 4.08 (d, J = 3.1 Hz, H-2), 4.04 (dd, J = 11.1, 6.3 Hz, H-5β), 3.79 (t, J = 6.5 Hz, H-5″), 3.58 (t, J = 11.1 Hz, H-5α), 3.48 (dd, J = 8.8, 7.2 Hz, H-1″), 3.18 (m, H-3, H-4), 2.52 (dd, J = 15.2, 6.3 Hz, H-6_A), 2.40 (dd, J = 15.2, 7.2 Hz, H-6_B), 1.95 (s, H-8′), 1.92 (m, H-2″), 1.77 (m, H-4″), 1.62 (m, H-3″); NMR δ^{13}C 22.3 (C-8′), 22.4 (C-3″), 25.3 (C-2″), 31.0 (C-4″), 37.6 (C-6), 42.1 (C-3), 46.0 (C-4), 55.8 (C-5), 56.5 (C-1″), 61.6 (C-5″), 74.0 (C-2), 113.6 (C-2′), 140.0 (C-1′), 173.5 (C-8), 179.9 (C-7). HR-FAB-MS *m/z* 299 (21%), 298.1686 (100, M-H$^-$. Calc. for $C_{15}H_{24}NO_5$, 298.1654). The reaction was repeated with phosphate buffer (pH 6.0) as solvent and 1 equivalent of $NaBH_3CN$ in an attempt to obtain the desired aldehyde, but ^{1}H NMR revealed a *c.* 1:1 mixture of the alcohol and the aldehyde derivatives.

*N-(2,3-Dihydroxypropyl)kainic acid (**8**)*

To a suspension of **2** (11.0 mg, 47.6 μmol) in methanol (500 μl) and water (200 μl) was added DL-glyceraldehyde (23 mg, 260 μmol) and a solution of $NaBH_3CN$ (3.2 mg, 52 μmol) in CH_3OH (108 μl). The suspension dissolved after stirring for 2 h, and at 24 h TLC showed absence of **2.** The product was purified by flash chromatography (2-propanol–water, 4:1) to give *N*-(2,3-dihydroxypropyl)kainic acid (**8**) (13 mg, 89%). NMR (resonances for each diasterisomer are separated by a slash) δ^1H 5.21 (s, H-2_A′), 4.92 (s, H-2_B′), 4.26 (m, H-2″)/4.18 (m, H-2″), 4.20 (d, J = 3.1 Hz, H-2)/4.16 (d, J = 2.3 Hz, H-2), 4.12 (bdd, J = 11, 6 Hz, H-5_A)/4.08 (bdd, J = 11, 6 Hz, H-5_A), 3.83 (dd, J = 12.0, 4.5 Hz, H-3_A″)/3.82 (dd, J = 12.0, 4.6 Hz, H-3_A″), 3.77 (dd, J = 12.0, 5.3 Hz, H-3_B″), 3.66 (m, H-5_B)/3.64 (m, H-5_B), 3.63 (m, H-1_A″)/3.61 (m, H-1_A″), 3.55 (m, H-1_B″)/3.53 (m, H-1_B″), 3.20 (m, H-3, H-4), 2.51 (dd, J = 15.3, 6.4 Hz, H-6_A), 2.41 (m, H-6_B), 1.95 (s, H-8′); δ^{13}C 22.32/22.28 (C-8′), 37.4 (C-6), 42.2/41.9 (C-3), 46.02/45.95 (C-4), 57.2/55.4 (C-5), 59.0/58.3 (C-1″), 63.6/63.5 (C-3″), 67.5 (C-2″), 75.1/74.5 (C-2), 113.6/113.4 (C-2′), 140.0 (C-1′), 173.4 (detected in HMBC spectrum only, ±0.5 ppm, C-8), 180.0 (C-7). HR-FAB-MS *m/z* 287 (14%), 286.1296 (100, M-H$^-$, Calc. for $C_{13}H_{20}NO_6$, 286.1291).

*Attempted oxidation of N-(2,3-dihydroxypropyl)kainic acid (**8**)*

To *N*-(2,3-dihydroxypropyl)kainic acid (4.70 mg, 15.4 μmol) in D_2O (*c.* 0.5 ml) was added sodium metaperiodiate in portions (0.4, 0.3, 0.3, 0.3, and 0.3 equiv.) from a stock solution (70 mg ml^{-1} in D_2O). Monitoring by ^{1}H NMR showed disappearance of starting material and appearance of two products. One of the products was identical by ^{1}H NMR to **2,** and the second gave rise to resonances at δ 8.64 (s, weak) and 5.58 (t, J = 5.4 Hz). TLC (2-propanol–water, 4:1) also revealed disappearance of starting material and that the product co-eluted with, and gave the same color reaction with ninhydrin, as authentic kainic acid (**2**). The product was isolated from the reaction by flash chromatography (2-propanol–water, 4:1) and gave the correct ^{1}H NMR resonances for **2,** with no detectable contaminants present.

*2-Methoxy-7-(hydroxymethyl)oxepane (**9**)*

Ozone was bubbled through a solution of (±)-7-octene-1,2-diol (1.00 g, 6.93 mmol) in methanol (190 ml, −78 °C). The reaction was stopped after 35 min, when TLC (dichloromethane–methanol, 24:1) indicated that the octene had been consumed. The product was concentrated in vacuo and purified by flash chromatography (dichloromethane–methanol, 24:1) to give (±)-2-methoxy-7-(hydroxymethyl)oxepane (**9**) (996 mg, 90 %) as a colorless oil. NMR indicated **9** to be a *c.* 1:1 mixture of diastereoisomers in $CDCl_3$. NMR ($CDCl_3$; where resolved, pairs of resonances for the diastereoisomers are separated by a "/") δ^1H 10.68 (bs, OH), 4.734 (dd, J = 6.2, 5.5 Hz, H-2)/4.728 (dd, J = 6.3, 5.4 Hz, H-2), 3.72 (m, H-7), 3.655/3.653 (dd, J = 11.0, 3.1 Hz, H-8_A), 3.49 (s, OCH_3), 3.43 (dd, J = 11.1, 7.5 Hz, H-8_B), 1.66, 1.45 (both m, H-3-H-6); δ^{13}C 108.7 (d, C-2), 72.20/72.14 (d, C-7), 66.83/66.80 (t, C-8), 55.91/55.88 (q, OCH_3), 32.9/32.8, 31.2/31.1, 25.3/25.2, 24.6/25.5 (all t, C-3-C-6). With $CDCl_3$-C_5D_5N (2:1) as solvent, peaks attributable to diastereoisomers were not observed for **9**: NMR ($CDCl_3$-C_5D_5N, 2:1) δ^1H 4.83 (t, J = 5.8 Hz, H-2), 3.80 (m, H-7), 3.70 (dd, J = 10.9, 3.7 Hz, H-8_A), 3.58, (dd, J = 10.9, 7.2 Hz, H-8_B), 3.51 (s, OCH_3), 1.81 (m, H-6_A), 1.72 (m, H-6_B), 1.56 (H-4_A), 1.53 (m, H-3), 1.47 (m, H-5), 1.44 (m, H-4_B); δ^{13}C 107.7 (d, C-2), 71.2 (d, C-7), 65.9 (t, C-8), 54.9 (q, OCH_3), 32.6 (t, C-3), 31.1 (t, C-6), 24.8 (t, C-4), 24.2 (t, C-5). GC-MS R_t 28.1 min, *m/z* 145 (16%, M-CH_3^+), 113 (100). EI-DCI *m/z* 145.0862 (13%, M-CH_3^+.

Calc. for $C_7H_{13}O_3$, 145.0865). Upon standing for 1 yr, **9** became contaminated with a compound identified by GC-MS and NMR as (±)-methyl 6,7-dihydroxyheptanoate. NMR ($CDCl_3$) δ^1H 3.69 (m, H-6), 3.65 (s, OCH_3), 3.61 (dd, J = 11.1, 3.0 Hz, H-7_A), 3.42 (dd, J = 11.1, 7.6 Hz, H-7_B), 2.31 (t, J = 7.4 Hz, H-2), 1.65 (m, H-3), 1.45 (m, H-5), 1.44 (m, H-4); δ^{13}C 174.4 (s, C-1), 72.0 (d, C-6), 66.7 (t, C-7), 51.6 (q, OCH_3), 33.9 (t, C-2), 32.6 (t, C-5), 25.1 (t, C-4), 24.8 (t, C-3). GC-MS R_t 15.4 min, *m/z* 87 (33%), 69 (100).

*N-(6,7-Dihydroxyheptyl)kainic acid (**3**)*

To kainic acid (**2**) monohydrate (41.2 mg, 178 µmol) and acetal-**9** (56.2 mg, 351 µmol) in methanol (500 µl) was added $NaBH_3CN$ (18 mg, 290 µmol) in methanol (0.6 ml). After 24 h, TLC showed loss of kainic acid and a new product, which was purified by flash chromatography (2-propanol-water, 4:1) to give *N*-(6,7-dihydroxyheptyl)kainic acid (**3**) as a white solid (62 mg, 96%). NMR δ^1H 5.21 (s, H-2_A'), 4.92 (s, H-2_B'), 4.08 (d, J = 3.1 Hz, H-2), 4.04 (dd, J = 11.0, 6.2 Hz, H-5β), 3.88 (m, H-6''), 3.77 (dd, J = 11.6, 3.8 Hz, H-7_A''), 3.65 (dd, J = 11.6, 6.8 Hz, H-7_B''), 3.58 (t, J = 11.0 Hz, H-5α), 3.47 (m, H-1''), 3.19 (m, H-3, H-4), 2.52 (dd, J = 15.2, 6.6 Hz, H-6_A), 2.40 (dd, J = 15.2, 7.4 Hz, H-6_B), 1.95 (s, H-8'), 1.92 (m, H-2''), 1.68 (m, H-5_A''), 1.62 (m, H-4''), 1.60 (m, H-3''), 1.58 (m, H-5_B''); δ^{13}C 22.4 (C-8'), 24.5 (C-4''), 25.5 (C-2''), 25.9 (C-3''), 32.2 (C-5''), 37.6 (C-6), 42.2 (C-3), 46.0 (C-4), 55.8 (C-5), 56.7 (C-1''), 65.8 (C-7''), 72.0 (C-6''), 74.0 (C-2), 113.7 (C-2'), 140.0 (C-1'), 173.6 (C-8), 180.0 (C-7). HR-FAB-MS *m/z* 343 (23%), 342.1925 (100, M-H$^-$. Calc. for $C_{17}H_{28}NO_6$, 342.1917).

*N-(6-Oxohexyl)kainic acid (**4**)*

To a solution of *N*-(6,7-dihydroxyheptyl)kainic acid (**3**) (19.3 mg, 53.4 µmol) in water (1 ml) was added sodium metaperiodiate (20 mg, 94 µmol) in methanol (200 µl). The reaction was stopped after 24 h and the product purified by flash chromatography (2-propanol–water, 4:1) to give a white solid (17.5 mg, 99%). ^{1}H and ^{13}C NMR indicated the product to exist in solution as a *c.* 1:1 mixture of the desired aldehyde (**4**) and its cyclic hemiacetal ester derivative. Tentative assignments are given below, and where identifiable, NMR resonances attributable to **4** and its hemiacetal derivative are designated by the subscripts I and II, respectively, and are separated by a "/". NMR δ^1H 9.87 (t, J = 1.7 Hz, H-6_I''/5.22 (m, H-6_{II}''), 5.22 (s, H-2_A'), 4.92 (s, H-2_B'), 4.08 (d, J = 3.1 Hz, H-2), 4.04 (dd, J = 11.4, 6.5 Hz, H-5_A), 3.59 (t, J = 11.4 Hz, H-5_B), 3.48 (m, H-1''), 3.20 (m, H-3, H-4), 2.76 (td, J = 7.2, 1.7 Hz, H-5_I''), 2.53 (dd, J = 15.3, 6.7 Hz, H-6_A), 2.41 (dd, J = 15.3, 7.3 Hz, H-6_B), 1.95 (s, H-8'), 1.92 (m, H-2''), 1.83 (m, H-4''), 1.8 (m, H-5_{II}'') 1.60 (m, H-3''); δ^{13}C 209.0 (d, C-6_I'')/91.3 (d, C-6_{II}''), 179.9 (s, C-7), 173.56/173.53 (s, C-8), 140.0 (s, C-1'), 113.6 (t, C-2'), 74.0 (d, C-2), 56.5/56.4 (t, C-1''), 55.8 (t, C-5), 45.9 (d, C-4), 43.1/36.9 (t, C-5''), 42.1 (d, C-3), 37.6 (t, C-6), 25.6/25.41, 25.46/25.3, and 23.7/21.0 (t, C-2'', C-3'', and C-4''), 22.3 (q, C-8').

Conjugation of N-(6-oxohexyl)kainic acid to protein

BSA (10.20 mg), OVA (9.95 mg), and THY (10.63 mg) were each dissolved in phosphate–citrate buffer (1 ml, pH 6.53). Aldehyde-**4** (1.95 mg, 5.93 µmol) was dissolved in water (300 µl), and 100 µl of this solution was added to each protein solution. After 24 h, $NaBH_3CN$ (1.6 mg, 25 µmol) in water (100 µl) was added to each reaction mixture, which was then allowed to stand at room temperature for 150 min before ultrafiltration.

*Domoic acid (**1**)*

^{1}H NMR δ 6.53 (dd, J = 14.9, 11.0 Hz, H-3'), 6.31 (d, J = 11.0 Hz, H-2'), 5.96 (dd, J = 14.9, 7.9 Hz, H-4'), 4.16 (d, J = 8.1 Hz, H-2), 4.01 (ddd, J = 7.9, 7.7, 7.4 Hz, H-4), 3.89 (dd, J = 12.2, 7.9 Hz, H-5β), 3.67 (dd, J = 12.2, 7.4 Hz, H-5α), 3.47 (dq, J = 7.9, 7.0 Hz, H-5'), 3.23 (dddd, J = 9.0, 8.1, 7.7, 5.8 Hz, H-3), 2.94 (dd, J = 16.8, 5.8 Hz, H-6_A), 2.68 (dd, J = 16.8, 9.0 Hz, H-6_B), 1.98 (s, H-8'), 1.45 (d, J = 7.0 Hz, H-6'); δ^{13}C 16.7 (C-6'), 21.7 (C-8'), 33.5 (C-6), 40.8 (C-4), 42.7 (C-3), 43.0 (C-5'), 47.1 (C-5), 65.0 (C-2), 126.8 (C-3'), 130.9 (C-2'), 131.9 (C-1'), 133.2 (C-4'), 173.1 (C-8), 175.6 (C-7), 180.0 (C-7').

*N-(6,7-Dihydroxyheptyl)domoic acid (**6**)*

To domoic acid (**1**) (4.2 mg, 12 µmol) in water (1 ml) was added acetal-**9** (4.0 mg, 25 µmol) in methanol (200 µl). $NaBH_3CN$ (1.5 mg, 24 µmol) in water (50 µl) was added and the reaction mix was stirred for 48 h, when TLC (2-propanol–water, 4:1) indicated that reaction was complete. The product was purified by flash chromatography (2-propanol–water, 4:1) to give **6** (7.0 mg, 120%) as a colorless solid. NMR δ^1H 6.45 (dd, J = 14.7, 10.9 Hz, H-3'), 6.28 (d, J = 10.9 Hz, H-2'), 5.93 (dd, J = 14.7, 8.1 Hz, H-4'), 4.06 (dd, J = 11.1, 7.3 Hz, H-5β), 3.98 (d, J = 6.7 Hz, H-2), 3.89 (m, H-4, H-6''), 3.78 (dd, J = 11.8, 3.8 Hz, H-7_A''), 3.65 (dd, J = 11.8, 6.8 Hz, H-7_B''), 3.56 (t, J = 11.1 Hz, H-5α), 3.47 (dd, J = 7.6, 9.0 Hz, H-1''), 3.24 (dq, J = 8.1, 7.1 Hz, H-5'), 3.15 (ddt, J = 7.3, 7, 6.7 Hz, H-3), 2.56 (d, J = 7.3 Hz, H-6_A), 1.97 (s, H-8'), 1.93 (m, H-2''), 1.65 (m, H-4''), 1.60 (m, H-3''), 1.58 (m, H-5''), 1.37 (d, J = 7.1 Hz, H-6'); δ^{13}C 18.0 (C-6'), 22.1 (C-8'), 24.5 (C-4''), 25.4 (C-2''), 25.9 (C-3''), 32.2 (C-5''), 37.0 (C-6), 40.5 (C-4), 43.9 (C-3), 46.8 (C-5'), 55.4 (C-5), 56.0 (C-1''), 65.7 (C-7''), 71.9 (C-6''), 73.4 (C-2), 125.4 (C-3'), 130.4 (C-1'), 131.0 (C-2'), 136.4 (C-4'), 173.5 (C-8), 179.5 (C-7), 184.6 (C-7'). The chemical shifts reported for H-3'', H-4'', and H-5'' were centres of multiplets identified in a low resolution HMQC spectrum (300 MHz), and the assignments for C-3'' and C-4'' were by analogy with those established for diol-**3.** HR-FAB-

MS *m/z* 442 (16%), 441 (29), 440.2276 (100, M-H$^-$. Calc. for $C_{22}H_{34}NO_8$, 440.2284).

N-(6-Oxohexyl)domoic acid (7)

Sodium metaperiodiate (1 equiv.) in D_2O (20 µl) was added to the diol-**6** (6.9 mg, *c.* 10 µmol) in D_2O (*c.* 0.5 ml) and the reaction was monitored by ^1H NMR spectroscopy. Complete reaction, demonstrated by loss of the diol resonances at δ 3.65 and 3.78 (H-7″) and 3.89 (H-6″) and the appearance of new resonances at δ 9.86 (H-6″) and 2.75 (H-5″), occurred within 15 min. As with aldehyde-**4** (above), ^1H and ^{13}C NMR suggested the product to exist in solution as a *c.* 1:1 mixture of the desired aldehyde (**7**) and its cyclic hemiacetal ester derivative. Tentative assignments are given below, and where identifiable, NMR resonances attributable to **7** and its hemiacetal derivative are designated by the subscripts I and II, respectively, and are separated by a "/". NMR included δ^1H 9.86 (bs, H-6$_I$″)/5.21 (t, J = 5.4 Hz, H-6$_{II}$″), 6.45 (dd, J = 14.6, 11.2 Hz, H-3′), 6.27 (d, J = 11.2 Hz, H-2′), 5.92 (dd, J = 14.6, 8.4 Hz, H-4′), 4.07 (dd, J = 11.1, 7.3 Hz, H-5β), 3.99 (d, J = 6.6 Hz, H-2), 3.87 (ddd, J = 11.1, 7.3, 7 Hz, H-4), 3.56 (t, J = 11.1 Hz, H-5α), 3.47 (m, H-1″), 3.24 (dq, J = 8.4, 7.0 Hz, H-5′), 3.14 (ddt, J = 7.3, 7, 6.6 Hz, H-3), 2.75 (bt, J = 6.5 Hz, H-5$_I$″), 2.56 (d, J = 7.3 Hz, H-6), 1.96 (s, H-8′), 1.92 (m), 1.82 (m), 1.60 (m), 1.37 (d, J = 7.0 Hz, H-6′); $\delta^{13}C$ 209.0 (C-6$_I$″)/91.3 (C-6$_{II}$″), 184.5, 179.4, 173.5, 136.4, 131.0, 130.4, 125.4, 82.1, 73.4, 55.4, 46.8, 43.9, 43.1, 40.4, 36.9, 25.7, 25.5, 25.4, 25.3, 23.7, 22.0, 21.0, 18.0.

Conjugation of N-(6-Oxohexyl)domoic acid to protein

BSA (19.28 mg), OVA (20.76 mg), THY (20.99 mg), and FET (10.12 mg) were each dissolved in phosphate–citrate buffer (1 ml, pH 6.5). The solution of aldehyde-**7** in D_2O (above) was lyophilized, the residue dissolved in phosphate buffer (800 µl), and 200 µl of this solution was added to each protein solution. The protein solutions were held at room temperature for 3 h, then $NaBH_3CN$ (3.9 mg) in buffer (200 µl) was added to each solution and the reaction was allowed to proceed for 3 h.

Carboxy-linked Domoic Acid

In a modification of the method of Newsome *et al.* (1991), **1** (3.0 mg, 8.6 µmol) was reacted with NHS (5.6 mg, 49 µmol) and EDAC (2.0 mg, 10 µmol) in DMSO (400 µl), and 100 µl of the resulting activated ester solution was added to each of BSA (10.7 mg), OVA (10.4 mg), THY (11.0), or HRP (8.0 mg) in $NaHCO_3$ (1.0 ml, 0.01 M), and the reaction solution was allowed to stand at 4 °C overnight before ultrafiltration.

Immunization

Sheep (5 each) were immunized with DA-carboxy-linked hapten coupled to THY or OVA. Sheep (3 each) were immunized with DA-*N*-linked hapten coupled to THY, FET or OVA. Immunogen for primary immunization was prepared as a 1:1 emulsion of conjugate dissolved in PBS (1 mg ml^{-1}) and Freund's complete adjuvant. Secondary and subsequent immunizations were administered as emulsions in incomplete adjuvant at 4-weekly intervals. Immunogen was administered by intramuscular injection (2 sites per animal, 250 µl per site). Test bleeds (10 ml) were taken 14 days after the third and subsequent immunizations, and analyzed for activity by ELISA. A serum pool was obtained from sheep once antisera of sufficient titer were identified.

ELISA

ELISA plates (NUNC Immunoplate I, Denmark) were coated with DA-carboxy-linked BSA conjugate in 0.05 M sodium bicarbonate buffer pH 9.6 (50 µl, 2.0 µg^{-1}ml), DA-N-linked BSA conjugate (2.5 µg^{-1}ml), or KA-N-linked BSA conjugate (2.5 µg^{-1}ml), overnight at 20 °C. After washing with PBS, additional binding sites were blocked by incubation with BSA (1 % w/v, 300 µl, 1 h, 20–25 °C). Plates were washed once with PBS and used immediately or stored at 4 °C for up to 7 days. For assay, sample or standard (50 µl) was added to the wells together with antiserum at the appropriate dilution (50 µl, e.g. 1/3000). After incubation at 20–25 °C for 2 h, the wells were washed twice with PBS + 0.05 % Tween 20 (PBST) and twice with PBS. Anti-sheep secondary antibody (100 µl, 1/3000–1/5000) was then added to the wells and incubated for 2 h, wells were aspirated, washed twice with PBST and twice with PBS. TMB substrate solution, prepared by adding 100 µl TMB stock (10 mg^{-1}ml in DMSO) to sodium acetate buffer (10 ml, 0.1 M, pH 5.5 containing 0.005 % H_2O_2), was then added and incubated for 15 min. The reaction was stopped by addition of H_2SO_4 (50 µl, 2 M), and absorbance at 450 nm determined with a microplate spectrophotometer (Biorad 3550, Biorad, USA). Standards and samples were prepared for ELISA by dilution in the following diluents: (1) methanol in PBS to a maximum methanol concentration of 10 % (v/v); (2) ethanol in PBS to a maximum ethanol concentration of 10 % (v/v); (3) AOAC acid extract (50 g shellfish homogenate was boiled for 5 min with an equal volume of 0.5 M HCl, diluted to 100 ml with 0.5 M HCl) neutralized with NaOH then diluted with PBS (AOAC 1997); (4) f/2 culture medium. All samples were analyzed in at least duplicates, and over a range of dilutions.

Assay Characterization

The optimum concentration of each assay reagent was determined empirically by checkerboard titration. Anti-

body titers were determined by incubation of antiserum dilutions with PBS-methanol (9:1) as sample. Titer was calculated as the dilution giving 50% maximum binding. Assay standard curves were calculated using the Logit transform, Microplate Manager software (Biorad, USA). The assay working range was defined as the linear portion between 20 and 80% of maximum absorbance. Cross-reactivity of the assay against structural analogs was calculated from the concentration of analog giving 50% inhibition (I_{50}) of binding to the protein-DA solid phase, expressed relative to the I_{50} for free DA.

Sample Extraction – Shellfish Flesh

Shucked and drained shellfish were homogenized for 1–2 min in an MC-3 Waring blender. Solvent (5 ml g^{-1} shellfish tissue) was added and the material again homogenized for 2 min. For spike recovery experiments, DA was added to the homogenized flesh, and the shellfish blended for a further 1–2 min before addition of solvent. The volume of solvent recovered after centrifugation was accurately determined, and this value used in calculations of spike recovery, assuming 100% extraction into the solvent.

The following protocols were investigated for their ability to recover DA from shellfish without introducing matrix effects into the assay: (1) AOAC hot acid extraction; (2) aqueous methanol extraction (50%, 70%, 80% and 90% methanol (v/v)); (3) aqueous ethanol extraction (70%, 80% and 90% ethanol (v/v)). Green lipped mussel (*Perna canaliculus*), scallop (*Pecten novaezelandiae*), and Bluff (*Tiostera lutaria*) and Pacific (*Crasostrea gigas*) oysters were analyzed.

Sample Extraction – Algal Culture and Field Samples

Algal cultures were analyzed by ELISA after dilution into PBS, with and without disruption by immersion in an ultrasonic bath (Branson, UK). Samples were diluted to a minimum of 1:4. Field samples (15 ml) were filter-concentrated and resuspended in 0.5 ml PBS. Net-tow samples were also resuspended in PBS, the volume depending on the weight of cells collected. The samples were further processed by ultrasonic disruption, and diluted for assay. In all analyses, media or similarly processed seawater blanks were employed as negative controls.

RESULTS AND DISCUSSION

Previous immunoassays for DA (Newsome *et al.*, 1991; Osada *et al.*, 1995; Smith and Kitts, 1995) were based on antibodies raised against DA (**1**) coupled, via its carboxyl groups, to proteins. DA contains 3 carboxyl groups and is thus capable of multivalent coupling. To avoid possible complications arising from this, we sought a coupling methodology which would give rise to a univalent hapten and which would result in conjugates in which the DA side chains (C-1′–C-8′, C-6–C-7, and C-8) would be available for recognition by the immune system. We therefore developed a method for coupling DA via its amino group.

The coupling methodology was developed using the readily-available kainic acid (KA) (**2**) as a model system. Reductive alkylation of 2 with excess glutaraldehyde and $NaBH_3CN$ unexpectedly gave the corresponding amino-alcohol (**5**) in good yield, rather than the desired aminoaldehyde. Attempts to generate the aminoaldehyde by controlling the pH and restricting the amount of $NaBH_3CN$ were only partially successful. In an attempt to circumvent the over-reduction of aminoaldehyde by $NaBH_3CN$, **2** was reductively alkylated with D,L-glyceraldehyde to give diol-**8**, in the expectation that oxidation of **8** with periodate would generate the corresponding amino-linked acetaldehyde derivative of KA. However, TLC and NMR indicated that oxidation of diol-**8** was accompanied by scission of the bond between the diol moiety and the amino group, regenerating **2**.

It was anticipated that aminodiol oxidation would proceed smoothly to the aldehyde if the diol moiety was separated from the amino group by more than one methylene unit. A suitable precursor was conveniently prepared by ozonolysis of (±)-7-octene-1,2-diol. The resulting dihydroxyaldehyde was isolated in high yield, and NMR spectoscopy showed it to exist as cyclic acetal-**9**. Reductive alkylation of **2** with **9** proceeded smoothly to give diol-**3** in high yield. The oxidation of **3** with periodate was monitored by ^1H NMR spectroscopy, which indicated smooth conversion to an aldehyde (**4**) that appeared to be in equilibrium with a form in which the formyl group (C-6″) cyclized with the neighbouring carboxyl group (C-8) to form a hemiacetal ester. This mixture was coupled by addition to the desired protein, and the resulting imine-linked conjugate was stabilized by reduction with $NaBH_3CN$ to the corresponding amine-linked conjugate. The procedure was applied to DA (**1**) to provide diol-**6** and aldehyde-**7**, and to couple aldehyde-**7** to proteins to generate immunogens.

The location of the introduced groups in compounds **3**–**8** (Figure 2) were established from analysis of one- and two-dimensional NMR spectral data. For example, the ^1H and ^{13}C NMR chemical shifts of the kainic acid heptanediol adduct (**3**) were correlated in inverse-mode HMQC and HMBC experiments which identified ^1J, and $2J$ and ^3J, connectivities, respectively. HMBC correlations between H-2 (4.08 ppm) and the resonance at 56.7 ppm (C-1″), and between H-1″ (3.47 ppm) and resonances at 74.0 ppm (C-2), 55.8 ppm (C-5), and 25.5–25.9 ppm (either or both of C-2″ and C-3″) indicated that

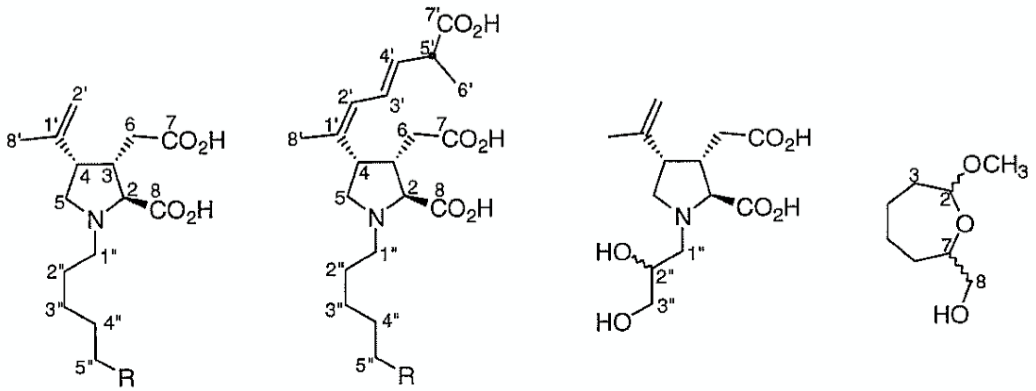

Figure 2. Derivatives of domoic (**6, 7**) and kainic (**3–5, 8**) acids used in this study, and linker acetal-**9** used to produce haptens **3** and **6.**

the heptanediol side chain of **3** was attached to the nitrogen atom, as did the presence in the ROESY spectrum of correlations betwen H-1″ (3.47 ppm) and resonances at 4.08 ppm (H-2), 3.58 ppm (H-5α), 1.92 ppm (H-2″), and 1.60 ppm (H-3″). The proton and carbon resonances of the heptanediol side chain of **3** were elucidated in like manner from a combination of COSY, HMQC, and HMBC spectral data. COSY correlations between the H-1″ (3.47 ppm) and H-2″ (1.92 ppm) enabled the C-2″ (25.5 ppm) signal to be distinguished from the C-3″ (25.9 ppm) signal via ^{13}C–^{1}H correlations observed in the HMQC spectrum of **3**, while the C-4″ (24.5 ppm) and C-5″ (32.2 ppm) resonances were identified via HMBC correlations observed for the H-6″ and H-7″ resonances. ^{1}H and ^{13}C NMR assignments obtained for **1–8** in this study closely paralleled those determined by Walter *et al.* (1994) for several analogs of **2.**

Assay Development and Characterization

Animals were immunized with antigen and were bled 10–14 days after each boost. Sera were harvested, and assessed for titer by indirect ELISA. Antibodies of sufficient titer for use in ELISA were obtained following the third immunization.

Titers of all sera were compared on both *N*-linked and carboxy-linked-DA plate coaters at this stage. In general, antisera gave a higher response on the corresponding plate coater (e.g. *N*-linked immunogen on *N*-linked coater). Both immunization chemistries elicited a specific response, and 8 animals of high (>4000 with either *N*- or carboxy-linked plate coater) titer were identified (Table 1). Specific immune responses varied greatly among animals. No single immunogen elicited a uniformly better response than any other, although the OVA-carboxy-linked-DA immunogen generally raised a higher titer than the THY-carboxy-linked-DA immunogen. FET-*N*-linked-DA immunogens raised consistently higher titers than OVA and THY-*N*-linked immunogen, although it should be noted that the apparent titers for FET immunized animals are over-estimated as there is a small contribution due to non-specific binding between common epitopes of FET and BSA. Titers of OVA and THY-*N*-linked-DA immunized animals were generally similar, although a single THY-*N*-linked-DA sheep showed high titer binding to both plate coaters.

Sera from the 8 selected sheep were analyzed for their ability to bind KA–plate coating protein conjugates, and their utility for DA ELISA analysis further assessed. Competition ELISAs were set up under limiting antibody conditions, using sera at dilutions giving 50% maximum absorbance (see Table 1). They were assessed for assay sensitivity, cross-reactivity and working range using both DA plate coating conjugates. In all cases, cELISAs employing carboxy-linked-DA plate coater gave higher sensitivity for DA than those employing *N*-linked plate coater, with a 5–20-fold difference in sensitivity between the conjugates, although the former sera gave steeper assay slopes resulting in a narrower assay working range (Table 2). Sera raised against the *N*-linked immunogen generated assays of higher sensitivity than those raised against the carboxy-linked immunogen (Table 2). In specificity studies, the antibody titers were low when assessed over BSA-KA plate coater, serum 7012 being the only one showing appreciable binding (Table 3). No cross-reactivity was observed with either KA or L-glutamine at concentrations below 50 $\mu g\,ml^{-1}$ when assessed on *N*-linked-DA-BSA coated plates. No cross-reactivity was detected for L-glutamine on carboxy-linked BSA-coated plates, although cross-reactivity was

Table 1. Midpoint titers, determined as 1/antiserum dilution giving 50% maximum absorbance, for each antiserum on both carboxy-linked and *N*-linked plate coater[a]

Sheep number	Immunogen	Antibody titer (1/antibody dilution giving 50% max. absorbance)	
		N-linked plate coater	carboxy-plate coater
4129	THY-carboxy-DA	1000	3500
4292	THY-carboxy-DA	1500	2500
4523	THY-carboxy-DA	2000	2500
4621	THY-carboxy-DA	2000	2000
4748	THY-carboxy-DA	1000	4000
4047	*OVA-carboxy-DA*	*5000*	*10000*
4441	*OVA-carboxy-DA*	*2000*	*10000*
4551	*OVA-carboxy-DA*	*6000*	*20000*
4691	*OVA-carboxy-DA*	*1500*	*4000*
4799	OVA-carboxy-DA	300	500
7014	*FET-N-linked-DA*	*9000*	*2300*
7035	*FET-N-linked-DA*	*6000*	*2500*
7046	*FET-N-linked-DA*	*6000*	*3500*
7012	*THY-N-linked-DA*	*10000*	*3000*
7072	THY-*N*-linked-DA	1500	850
7090	THY-*N*-linked-DA	1700	1000
7028	OVA-*N*-linked-DA	3000	1500
7036	OVA-*N*-linked-DA	3000	1500
7079	OVA-*N*-linked-DA	750	300

[a] Italicized entries correspond to antisera of highest titer, selected for further study.

detected for KA with a number of sera in cELISA employing carboxy-linked-DA conjugates as plate coater (Table 3). Cross-reactivity was highest for sera 4441 (0.6%), 4691, and 4047 (both *c.* 0.3%). Despite showing the highest titer binding to BSA-KA, serum 7012 showed less than 0.005% cross-reactivity with free KA. Sera from sheep 4551 (OVA-carboxy-linked-DA) and 7012 (THY-*N*-linked-DA) were chosen for further study of their potential to provide a sensitive assay with little or no interference. These two sera also appeared to provide the most robust assays (data not shown). Following reagent titration, cELISA conditions were optimized using BSA-carboxy-linked-DA conjugate as plate coater, and working ranges were established (Figure 4). Employing the conservative cut-off values of 20% and 80% maximum absorbance, the assay employing serum 4551 had an LOQ of 2.5 ng ml^{-1}, and a working range of 2.5–125 ng ml^{-1}. The assay using serum 7012 gave an LOQ of 0.15 ng ml^{-1}, and working range of 0.15–15 ng ml^{-1}.

The specificity of the two cELISAs was determined for all readily available structural analogs, and for a number of compounds which are known to interfere with DA receptor binding assays (Van Dolah *et al.*, 1994). The assays were found to be extremely specific for DA, with no cross-reactivity to L-glutamic acid, L-glutamine, formimino-L-glutamic acid, proline or γ-aminobutyric

Table 2. DA cELISA sensitivity for eight selected sera, employing BSA *N*-linked-DA and carboxy-linked-DA plate coaters

Serum	DA-*N*-linked-BSA		DA-carboxy-linked BSA	
	Working range[a] (ng ml^{-1})	I_{50} (ng ml^{-1})	Working range[a] (ng ml^{-1})	I_{50} (ng ml^{-1})
4047	> 10 000–20	1000	295–3.2	31
4441	> 10 000–20	1000	96–1.4	12
4551	> 10 000–3	300	125–2.5	17
4691	> 10 000–10	1200	275–4.4	35
7014	> 10 000–20	1000	85–1.4	11
7035	> 10 000–10	700	28–0.3	3
7046	> 10 000–0.2	100	12–0.2	1
7012	> 10 000–1	50	15–0.15	1

[a] Defined as the concentrations giving 20–80% maximum absorbance.

Table 3. Specificity of the eight selected sera. Antibody titer for binding to BSA-KA conjugates, working range, and I_{50} (ng ml^{-1}) for competition of KA (**2**) and L-glutamine over DA plate coaters

Sheep	Binding to BSA-KA (titer)	Cross-reactivity				
		DA-*N*-linked-BSA		DA-carboxy-linked-BSA		
		KA competition	Glutamine competition	KA competition (working range)	KA (I_{50})	Glutamine competition
4047	100	n.d.[a]	n.d.	>10 000–5	10 000	n.d.
4441	100	n.d.	n.d.	>10 000–5	2 000	n.d.
4551	200	n.d.	n.d.	n.d.	>50 000	n.d.
4691	150	n.d.	n.d.	>10 000–5	10 000	n.d.
7014	900	n.d.	n.d.	>10 000–100	>10 000	n.d.
7035	600	n.d.	n.d.	>10 000–100	>10 000	n.d.
7046	800	n.d.	n.d.	>10 000–100	>10 000	n.d.
7012	2000	n.d.	n.d.	>100 000–250	20 000	n.d.

[a] n.d., not detected, i.e. no competition at 50 μg ml^{-1}.

acid (GABA). Cross-reactivity with KA was less than 0.005% for serum 7012, and no KA cross-reactivity was observed with serum 4551.

Analysis of Shellfish Extracts–Matrix Effects

Shellfish extracts were prepared using a range of solvents. Under most extraction protocols, clarification of the extract by centrifugation followed by dilution was sufficient to allow direct application of samples to the assay. To ascertain the minimum processing of extract that permitted analysis, the dilution required to avoid matrix effects was determined. Standard curves were established with the diluted extracts, and matrix effects were deemed to be eliminated when the curves over the working range coincided with the standard dilution curve obtained from PBS or PBS–ethanol (9:1).

AOAC Acid Extract

Dilution 1/50, or neutralization with NaOH followed by dilution 1/20, removed matrix effects for extracts of green lipped mussel (*Perna canaliculus*), and oyster (*Tiostrea lutaria* and *Crasostrea gigas*). AOAC extracts of scallop (*Pecten novaezealandiae*) were not tested.

Alcoholic Extracts

Extracts of shellfish prepared in aqueous methanol and ethanol were suitable for direct application to the assay after dilution with PBS-ethanol (9:1). In all cases, dilution of the extract by only 1/50 was sufficient to avoid matrix effects in the assay. Aqueous ethanolic extracts (70–90%) were chosen for further study of toxin recovery rates. An example of the matrix effect titration for greenshell mussels extracted with 90% ethanol is shown in Figure 5.

Analysis of Shellfish Extracts–Extraction Efficiency

Spiked tissues, prepared by adding DACS-1 standard at the regulatory level (20 μg^{-1} g) and at half the regulatory level (10 μg^{-1} g) to mussel homogenates, were extracted with 70, 80 or 90% aqueous ethanol (5 ml^{-1} g). Extracts were prepared and analyzed in duplicate on four separate

Table 4. Effect of extraction solvent (ethanol) concentration on spike recovery of DA (**1**) from mussel tissues spiked at 20 μg^{-1}g (the regulatory limit) and 10 μg^{-1}g

Ethanol (%)	Spike level (ng ml^{-1})	Recovery (%)[a]
90	20	58.1 ± 5.6, 50.4 ± 5.7, 67.7 ± 3.9, 71.7 ± 14.1
90	10	50.8 ± 1.9, 51.0 ± 19, 70.8 ± 8.6, 76.2 ± 0.4
80	20	69.5 ± 11.9, 92.6 ± 1.6, 94.3 ± 2.8, 87.2 ± 9.5
80	10	79.9 ± 0.4, 110.5 ± 2.9, 87.9 ± 5.2, 93.3 ± 8.1
70	20	72.4 ± 1.6, 75.1 ± 12.7, 96.4 ± 3.3, 81.1 ± 4.9
70	10	77.1 ± 0.1, 110.9 ± 5.9, 102.6 ± 5.9, 101.6 ± 4.1

[a] Results are means of extracts, analyzed in duplicate, at three dilutions, and re-assayed on four separate occasions (days 1, 27, 30, 34).

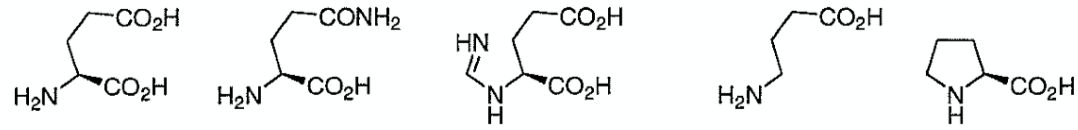

Figure 3. Structural analogs of domoic acid (**1**) used in this study.

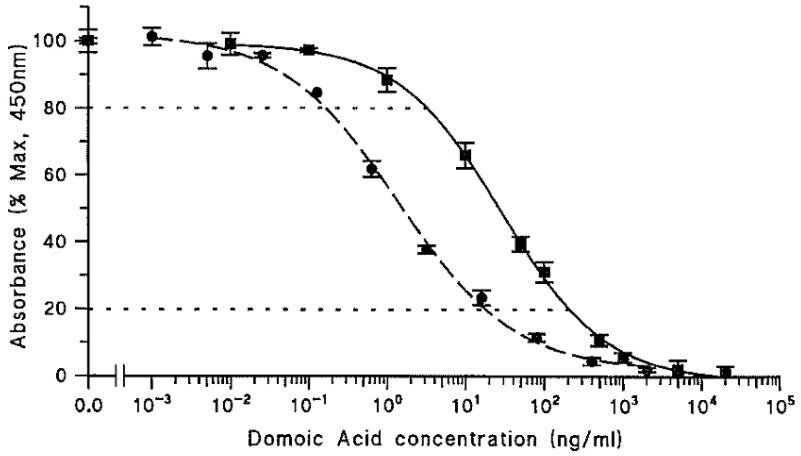

Figure 4. Domoic acid cELISA standard curves. Serum 4551 (■) and Serum 7012 (●).

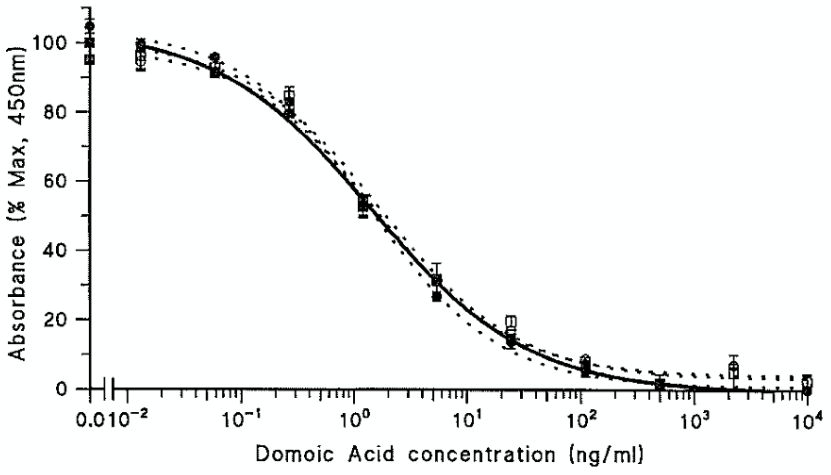

Figure 5. Matrix effects were eliminated by dilution of the extract. The standard curve in mussel (*Perna canaliculus*) extract (90% ethanol) at 1/50 (□) and 1/100 (○) dilutions coincided with standard curves in PBS (●) and PBS-ethanol (9:1) (■). Solid line shows standard curve in PBS-ethanol (9:1). Dotted lines show logit-transforms for the other matrices.

occasions (day 1, 27, 30, and 34). Samples were analyzed at 1/50, 1/100, and 1/200 dilutions. Data for samples analyzed on day 1 were processed at 1/200 dilution. In general, the 1/100 dilution was closest to the center of the standard curve and gave the lowest variance. The 1/100 dilution data points were used for calculation of recovery on days 27, 30 and 34. Higher recovery of DA was achieved with lower percentages of alcohol in the solvent (Table 4).

The reproducibility of toxin recovery may be improved

by increasing the solvent to tissue ratio used in extraction. In future work we plan to investigate this possibility. Extracts of samples spiked at the regulatory limit required dilution of 1/100 or more for analysis, demonstrating the feasibility of this approach. The 'single solvent extraction' ELISA system that we propose to employ for analysis of shellfish for ASP, DSP, PSP, and NSP toxins may, however, impose constraints on this approach. For assay of these toxins a low solvent to flesh ratio may be required to avoid the introduction of an expensive concentration step.

Analysis of Algal Cultures and Field Samples

A number of methods for the extraction of toxin from algal cultures and field samples were investigated. The sensitivity of the DA ELISA allowed the detection of DA in the media of algal cultures and in cell lysates prepared by sonicating either a portion of the diluted culture (cells plus media), or the cells resuspended in PBS after collection by centrifugation. Field samples (including seawater and phytoplankton collected by net tow or filtration (0.65 μm, Durapore), resuspended in PBS and sonicated) required at least a 6-fold dilution to avoid matrix effects on the ELISA. Maximum DA levels were found when a sample of the total culture was analyzed following dilution and ultrasonic disruption. The culture media often contained high levels of DA whereas the resuspended cells contained relatively little of this material, indicating significant secretion of the toxin from the cells. In keeping with this, DA was detected in cell-free seawater collected from an area with high *Pseudo-nitzschia* cell counts and toxic shellfish flesh. Media blanks gave a false positive signal when analyzed undiluted. Matrix effects were eliminated following dilution 1/4 in PBS.

Cultures of *Pseudo-nitzschia australis* isolated from samples collected at Marsden Point, Northland (CAWB02), which produced 2 pg DA per cell shortly after isolation (HPLC data, Rhodes *et al.*, 1998), now produce undetectable levels by HPLC after 1 year in culture. These were found to contain 45 fg DA per cell by ELISA. *P. fraudulenta* isolated from Matakana Bank, Bay of Plenty, produced 11–30 fg DA per cell following 14 days of growth in culture.

To assess the potential of the DA ELISA for the direct analysis of seawater and plankton samples (to determine whether toxic algae are present in sufficient number to be a risk to shellfish harvesting), a number of field samples, initially collected for cell counting, were analyzed. Samples (15 ml) were filtered and the collected algae re-suspended in PBS for analysis by ELISA. The original sample, which contained 6000 cells l^{-1} (approximately 1/10th the minimum cell count (50000–100000) above which shellfish harvesting is at risk), contained 3.2 ng DA. Shellfish flesh collected at this site contained DA at levels below the regulatory limit. Water and plankton samples from other sites at this time contained no, or only trace amounts of, DA (although non-toxic *Pseudo-nitzschia* were occasionally present). In accord with these findings, no DA could be detected in shellfish flesh collected at these sites. See Rhodes *et al.* (1997) for results of further studies on algal isolates and cultures.

CONCLUSIONS

The *N*-linked immunogen employed in this study generated antibodies providing a higher sensitivity ELISA than those generated using the carboxy-linked immunogen. Use of *N*-linked conjugates as the plate coater gave a wider working range than achieved with the carboxy-linked conjugate, but at the expense of assay sensitivity. Carboxy-linked DA conjugates were therefore used as plate coater in combination with antisera raised against *N*-linked DA conjugates.

The assay developed here showed similar or better sensitivities than those of Osada *et al.* (1995), who improved the assay of Newsome *et al.* (1991) to give a detection limit below 0.1 ng ml^{-1}. These authors reported the development of DA ELISAs, but did not apply the assay to field samples or shellfish flesh. Smith and Kitts (1995) demonstrated the utility of ELISA for analysis of shellfish extracts, but as their assay relied upon the limited resource of a serum from a single mouse it is unlikely that their assay would have widespread use in shellfish testing. Smith and Kitts did not report an LOQ, although they indicated that it would be below 0.25 μg ml^{-1} (0.5 μg g^{-1} shellfish).

In the present study, antibodies were raised against DA and ELISA methodology was developed. The ELISA had a conservatively-determined LOQ of 0.15 ng ml^{-1} and a working range of 0.15–15 ng ml^{-1} DA. The LOQ equates to 0.038 μg g^{-1} DA in shellfish flesh (assuming a 250-fold dilution during extraction), which is more than 500 times below the maximum permitted level of 20 μg g^{-1} flesh. Furthermore, the specificity of the DA ELISA is such that analogous compounds that are sometimes found in algae, such as KA (Sato *et al.*, 1996), do not interfere with the assay. The ELISA detection limit (<0.01 ng ml^{-1}) is considerably lower than the LOQ of 0.15 ng ml^{-1}, which was conservatively set at 80% maximum absorbance. Using an alternative approach, which mathematically applies a precision profile to the standard curve and determines the upper and lower limits of the standard curve, allowing estimation of concentrations with a less than 20% coefficient of variance (Cox, unpublished), the LOQ can be extended to approximately 0.02 ng ml^{-1}. The assay is robust, has been used successfully on a number of shellfish species, and is compatible with several different extraction methods

with little or no interference from matrix effects. It is therefore suitable for use in a screening program. With further validation, the DA ELISA should be suitable for routine use in the analysis of shellfish for regulatory purposes.

The successful development of the DA ELISA is a major step toward the provision of a shellfish screening system to ensure safe product for consumers. Use of the screening system in the shellfish certification procedure will have financial benefits to industry by reducing the costs of the current system.

An equally important, but longer term, aim is the provision of a test for detecting the onset of potentially toxic events. The very high sensitivity of the ELISA means that it could be a powerful aid in efforts to provide early warning of developing algal blooms, as it can be used to detect rising DA concentrations in seawater samples collected during the early stages of any bloom event without the need to identify and classify the *Pseudo-nitzschia* present as toxigenic or non-toxigenic.

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REFERENCES

AOAC (1997). AOAC Official Method 991.26. Domoic acid in mussels. Liquid chromatographic method. First action 1991. In: Cunniff P, ed.: *Official Methods of Analysis of AOAC International*, 16th edn, 3rd revn, vol 2. Maryland USA: AOAC International, chapter 35:9.

Bates SS, Bird CJ, de Freitas ASW, Foxall R, Gilgan M, Hanic LA, Johnson GR, McCulloch AW, Odense P, Pocklington R, Quilliam MA, Sim PG, Smith JC, Subba Rao DV, Todd ECD, Walter JA, Wright JLC (1989). Pennate diatom *Nitzschia pungens* as the primary source of domoic acid, a toxin in shellfish from eastern Prince Edward Island, Canada. *Can J Fish Aquatic Sci* **46**:1203–1215.

Garthwaite I, Ross KM, Miles CO, Hansen RP, Foster D, Towers NR (1997). An immunoassay for determination of domoic acid in shellfish and sea water. In: *VIII International Conference On Harmful Algae*. Vigo, Spain: Xunta de Galicia /IOC (of UNESCO) Paris, in press.

Giullard RRL (1975). Culture of phytoplankton for feeding marine invertebrates. In: Smith WH, Chanley MH, eds. *Culture of Marine Invertebrate Animals*. New York: Plenum Press, 29–60.

Iverson F, Truelove J (1994). Toxicology and seafood toxins: domoic acid. *Natural Toxins* **2**:334–339.

Lawrence JF, Charbonneau CF, Ménard C, Quilliam MA, Sim PG (1989). Liquid chromatographic determination of domoic acid in shellfish products using the paralytic shellfish poison extraction procedure of the Association of Official Analytical Chemists. *J Chromatogr* **462**:349–356.

Newsome H, Truelove J, Hierlihy L, Collins P (1991). Determination of domoic acid in serum and urine by immunochemical analysis. *Bull Environ Contam Toxicol* **47**:329–334.

Osada M, Marks LJ, Stewart JE (1995). Determination of domoic acid by two different versions of a competitive enzyme-linked immunosorbent assay (ELISA). *Bull Environ Contam Toxicol* **54**:797–804.

Ravn H (1995). Origin and occurrence. In: *Amnesic Shellfish Poisoning (ASP)*. IOC Manuals and Guides No.31. HAB Publication Series vol 1. Paris: UNESCO, Section 5, 1–15.

Rhodes L, White D, Syhre M, Atkinson M (1996). *Pseudonitzschia* species isolated from New Zealand coastal waters: domoic acid production *in vitro* and links with shellfish toxicity. In: Yasumoto T, Oshima Y, Fukuyo Y, eds. *Harmful and Toxic Algal Blooms*. IOC of UNESCO: pp 155–158.

Rhodes L, Scholin C, Garthwaite I, Haywood L, Thomas A (1997). Domoic acid producing *Pseudo–nitzschia* species deduced by whole cell DNA probe–based and immunochemical assays. In: *VIII International Conference On Harmful Algae*. Vigo, Spain: Xunta de Galicia /IOC (of UNESCO) Paris, in press.

Rhodes L, Scholin C, Garthwaite I (1998). *Pseudo–nitzschia* in New Zealand and the role of DNA probes and immunoassays in refining marine biotoxin monitoring programmes. *Natural Toxins* **6**:105–111.

Sato M, Nakano T, Takeuchi M, Kanno N, Nagahisa E, Sato Y (1996). Distribution of neuroexcitatory amino acids in marine algae. *Phytochemistry* **42**:1595–1597.

Smith DS, Kitts DD (1995). Enzyme immunoassay for the determination of domoic acid in mussel extracts. *J Agric Food Chem* **43**:367–371.

Still WC, Kahn M, Mitra A (1978). Rapid chromatographic technique for preparative separations with moderate resolution. *J Org Chem* **43**:2923–2925.

Towers NR, Garthwaite I, Miles CO, Borrell T, Busby P (1997). Towards an ELISA–based phycotoxin screening system. In: Abstracts and posters classification, *VIII International Conference On Harmful Algae*. Vigo, Spain: Xunta de Galicia, p202.

Van Dolah FM, Finley BL, Doucette GJ, Moeller PD, Ramsdell JS (1994). Development of rapid and sensitive high throughput pharmacologic assays for marine phycotoxins. *Natural Toxins* **2**:189–196.

Walter JA, Falk M, Wright JLC (1994). Chemistry of the shellfish toxin domoic acid: characterization of related compounds. *Can J Chem* **72**:430–436.

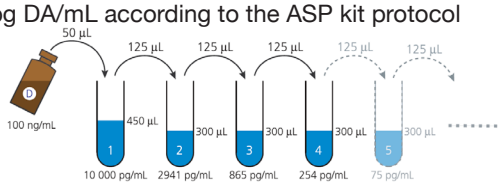
Wright JLC, Boyd RK, DeFreitas ASW, Falk M, Foxall RA, Jamieson WD, Laycock MV, McCulloch AW, McInnes AG, Odense P, Pathak VP, Quilliam MA, Ragan MA, Sim PG, Thibault P, Walter JA, Gilgan M, Richard DJA, Dwar D (1989). Identification of domoic acid, a neuroexcitatory amino acid, in toxic mussels from eastern Prince Edward Island. *Can J Chem* **67**: 481–490.

Biosense ASP ELISA, Quick guide

1. Preparation of standards and samples

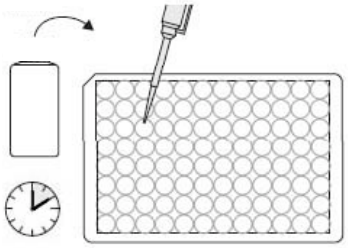
Prepare sample extracts and dilute the samples according to the ASP kit protocol (page 10).

Prepare the 10 Domoic acid calibration solutions in the range of 10 000-0.16 pg DA/mL according to the ASP kit protocol (page 9).



2. Pre-soak the wells

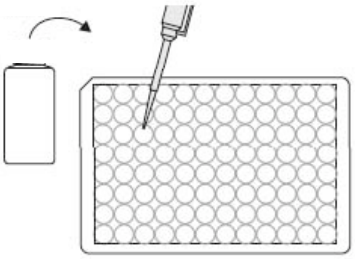
Pre-soak the wells for 5-10 minutes with 300 µl Washing buffer. Empty before use.



3. Addition of buffers to blank wells

Add 50 µl Standard/Sample buffer to the A_{max} and Blank wells.

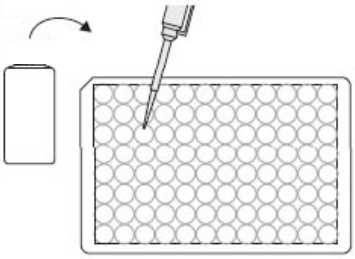
Add 50 µl of the Antibody-HRP buffer to Blank wells.



4. Addition of standards and samples

Transfer 50 µl of the 10 standard solutions (in duplicates) to the plate according to the plate layout (page13).

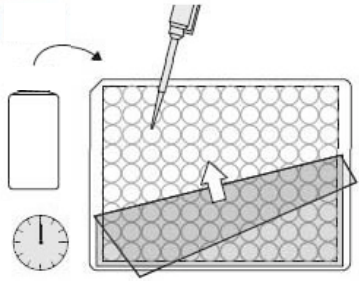
Transfer 50 µl of the diluted samples (in duplicates) to the plate according to the plate layout.



5. Addition of antibody-HRP conjugate

Dilute the concentrated antibody-HRP conjugate and add 50 µl to all wells except the Blank wells

Seal the plate and incubate for 60 minutes

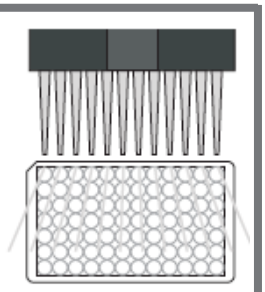


6. Wash the wells

After incubation, remove the covering and empty the contents of the wells.

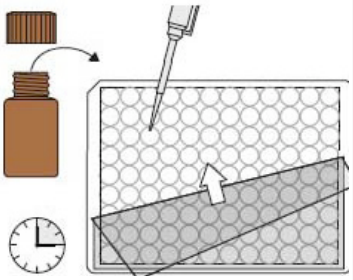
Wash all wells 4 times with 300 µl washing buffer.

Completely remove all liquids by patting the plate onto a stack of paper towels.



7. Addition of TMB solution

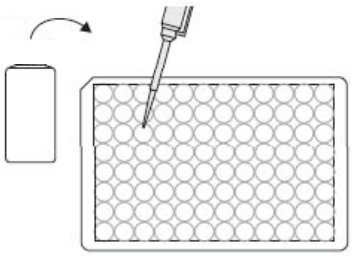
Add 100 µl of TMB solution to the individual wells succesively using a stepping pipette or a multi-channel pipette. Cover the wells with plate sealer and mix the contents by moving the strip holder in a gently circular motion on the benchtop. Be careful not to spill contents.



Incubate the strips for 15 minutes at room temperature (keep dark).

8. Addition of 0.3 M H_2SO_4 stop solution

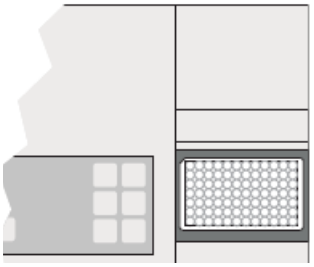
Add 100 µl of 0.3 M H_2SO_4 (stop solution) to the wells to stop the reaction using a stepping pipette or a multi-channel pipette.



9. Measurement of colour

Read the absorbance at 450 nm using a microplate ELISA reader or stripreader.

Calculate the concentrations using the Excel Macro EMA31 (4-p curve fit logistics).



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LABORATORIES

ASP ELISA KIT FOR QUANTITATIVE DETERMINATION OF DOMOIC ACID

PROD. NO.: A31300401

AOAC® *Official Method*℠ 2006.02

CONTENTS

A. INTRODUCTION

Domoic acid (DA) and DA derivatives are water-soluble neurotoxins produced by a number of marine algae, in particular by the microalgae of the genus *Pseudo-nitzschia* spp (Fig. 1). Blooms of *Pseudo-nitzschia* may lead to the accumulation of DA in shellfish filter feeders and other marine species [Scholin *et al*., 2000]. Ingestion of DA contaminated shellfish may lead to amnesic shellfish poisoning (ASP) by affecting the central nervous system, and has caused the death of both animal and human consumers in severe cases [Wright *et al*., 1989]. The European commision Directive 2002/226/EC implemented a maximum permitted level (MPL) of 20 mg DA equivalents/kg shellfish intended for human consumption. This MPL is adopted by most food safety authorities.

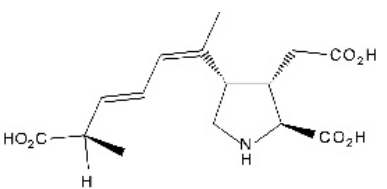

FIGURE 1:
DOMOIC ACID STRUCTURE

Enzyme Linked Immunosorbent Assay (ELISA) has proved to be a sensitive and rapid method for detection of DA in the marine environment [Garthwaite *et al*., 2001]. This quantitative DA ELISA was developed by AgResearch (Hamilton, New Zealand) for the detection of DA in water samples, shellfish and algal extracts, and is based on antibodies described by Garthwaite *et al*., 1998. The assay is specific for DA, with no cross-reactivity to non-toxic, structural analogues like kainic acid, L-glutamic acid, L-glutamine, formimino-L-glutamic acid, proline or g-aminobutyric acid (GABA). The assay is primarily intended for use in routine monitoring of DA levels in bivalve molluscs to comply with the regulatory MPL, but is also applicable for DA quantification in other marine matrixes like algal samples, seawater and body fluids of marine mammals. The assay has been subjected to comprehensive validation studies and is approved AOAC® *Official Method*℠ 2006.02 [*AOAC INTERNATIONAL*, 2006].

Assay principle

The ASP ELISA assay is in a direct competition format, where free DA in the sample competes with DA-conjugated protein coated on plastic wells for binding to anti-DA antibodies free in the solution (Fig. 2). The polyclonal ovine anti-DA antibodies are conjugated to horseradish peroxidase (HRP). Sample diluted in buffer is incubated in the wells with the anti-DA-antibody-HRP conjugate. After washing, the amount of conjugate remaining bound to the well is measured by incubation with a substrate that gives a blue product upon reaction with the HRP enzyme. Addition of acid stops the reaction and changes the product colour from blue to yellow. The colour intensity is measured spectrophotometrically on a plate-reader at 450 nm, and is inversely proportional to the concentration of DA in the sample solution. The assay is calibrated using dilutions of a DA calibration solution supplied with the kit. The calibrated range of the assay ($I_{20} - I_{80}$) is approximately 10 to 300 pg/mL of DA. The ASP ELISA is offered in a 8x12 strip well format. The ASP ELISA kit can be used in 2 separate rounds to analyze 12 samples each time, or the full plate can be used to analyze 36 samples in one round of analysis. The working range for ASP toxins in shellfish is 0.01mg/kg up to at least 250 mg/kg.

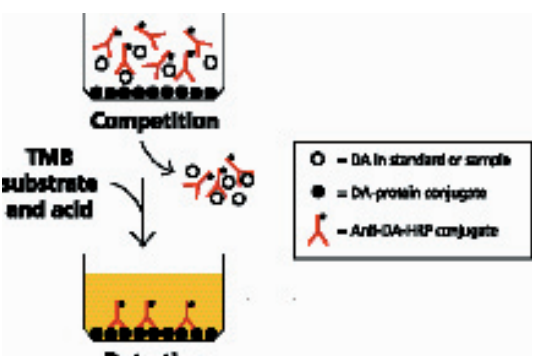

FIGURE 2: ASSAY FORMAT FOR THE COMPETITIVE ASP ELISA

METHOD OVERVIEW

Preparation of buffers

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Preparation of samples

Shellfish	*-page 10*
Algae	*-page 11*
Seawater	*-page 11*

Running the ASP ELISA

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Data calculation

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B. SAFETY INSTRUCTIONS

As all chemicals should be considered potentially hazardous, always wear suitable protective clothing during handling of the kit.

CAUTION: Domoic acid is a neurotoxin that is harmful by inhalation and ingestion. Avoid contact with skin, eyes and clothing. Wash hands thoroughly after handling.

Beware of the hazardous nature of methanol and sulfuric acid. Please refer to the manufacturers Material Safety Data Sheet for these reagents.

C. STORAGE AND STABILITY

Store the kit at 2-8°C upon arrival. Do not freeze. See expiry date on the kit box for stability of the kit.

D. WARRANTY AND LIMITATION OF REMEDY

Biosense Laboratories AS (hereafter: Biosense) makes no warranty of any kind, expressed or implied, including, but not limited to, the warranties of fitness for a particular purpose and merchantability, which extends beyond the description of the chemicals on the face hereof, except that the material will meet our specifications at the time of delivery.
Buyer's exclusive remedy and Biosense´s sole liability hereunder shall be limited to refund of the purchase price of, or at Biosense´s option, the replacement of, all material that does not meet our specifications. Biosense shall not be liable otherwise or for incidental or consequential damages, including, but not limited to, the costs of handling.
Said refund or replacement is conditioned on Buyer giving written notice to Biosense within thirty (30) days after arrival of the material at its destination, and Buyer treating the material as outlined in the product data sheet and/or kit insert after arrival. Failure of Buyer to give said notice within said thirty (30) days, or failure of Buyer in treating the material as outlined in the product data sheet and/or kit insert shall constitute a waiver by Buyer of all claims hereunder with respect to said material.
The responsibility of all patent considerations in the use of our products rests solely with the user.

E. KIT CONTENTS

		Number:
A)	12-well microplate strip modules (Precoated with DA-protein conjugate)	2 sealed pouches - 4 strips each
B)	Plate sealers	2
C)	PBS/Tween tablets	2
D)	Domoic Acid standard, 100 ng/mL (derived from NRC CRM-DA-e)	2 vials
E)	Anti-DA-HRP conjugate (6x concentrated)	2 vials
F)	Ovalbumin	2 vials á 60 mg
G)	TMB peroxidase substrate	2 vials

F. ADDITIONAL REAGENTS AND EQUIPMENT REQUIRED

In addition to the reagents supplied with the kit, the following reagents and equipment are required and/or recommended to perform the assay:

- Microplate spectrophotometer equipped with a 450 nm filter.
- Water; distilled and deionised (e.g. Milli-Q water, Millipore).
- Methanol (analytical grade).
- 0.3 M H_2SO_4.
- Vortex mixer.
- Micropipettes.
- Centrifuge.
- Kitchen blender.*

* For homogenizing shellfish samples.

G. IMPORTANT NOTES

1. Read the complete procedure before starting the assay.
2. Protect vials and microwell strips containing DA standard dilutions and samples from direct light during incubations.
3. The plate sealers are used to seal the strips during incubation and care must be taken when removing them from the strips.
4. Positive displacement pipettes (50 µL) are recommended for dispensing methanolic extracts.
5. As in every quantitative ELISA, consistent and precise pipetting at each step in the procedure is essential in order to obtain reliable results.
6. Reproducibility in any ELISA is also dependent upon consistent washing of the microwells.
7. After each wash, the wells are emptied by inverting the strips over a sink and then tap dry the wells against a pile of paper towels to remove all of the remaining liquid.
8. Avoid prolonged intervals between the working steps of the procedure, and do not allow the microwells to dry out totally during the assay procedure.

Definitions

Blank wells: Background absorbance of the TMB peroxidase substrate; approximately 0.05 A.U. (Absorbance Units).

A_{max} wells: Maximum absorbance; no standard or sample is added to these wells allowing maximum binding of the anti-DA-HRP conjugate to the plate-coated DA-conjugate; approximately 1.0 A.U. (Absorbance Units).

H. PREPARATIONS BEFORE THE ANALYSIS

a) Preparation of buffers and reagents

1. Washing buffer (PBS-T; 0.05% Tween 20 in PBS):
   Dissolve one tablet (C) in distilled water and dilute to 500 mL. May be stored at 4°C for one week.
2. Extraction solution (50% methanol in water):
   Prepare sufficient solution for the required number of samples by mixing equal volumes of methanol and distilled water. Prepare fresh each day.
3. Standard/Sample buffer (10% methanol in PBS-T):
   Mix 5 mL of methanol with 45 mL of Washing buffer. May be stored for 2-3 days at room temperature.
4. Antibody-HRP ovalbumin buffer (1% ovalbumin in PBS-T):
   Add 6 mL of Washing buffer to 60 mg of ovalbumin (vial F). Prepare fresh for each assay.

b) Preparation of Domoic acid calibration solutions

The 10-point calibration curve is ***freshly*** prepared using standard dilutions in the range of 10 000 – 0.16 pg DA/mL:

1. Prepare one Eppendorf tube containing 450 µL Standard/Sample buffer (10% methanol in PBS-T) - "tube 1", and 9 Eppendorf tubes containing 300 µL Standard/Sample buffer - "tubes 2-10".
2. Add 50 µL of the DA standard (100 ng/mL, vial D) to tube 1 and vortex, to obtain a 10 ng/mL DA solution.
3. Transfer 125 µL of the 10 ng/mL solution (tube 1) to tube 2 and vortex.
4. Complete the 3.4-fold dilution series by transferring 125 µL from tube 2 to tube 3 and vortex. Repeat this step for all tubes 3-10 (see Fig. 3).

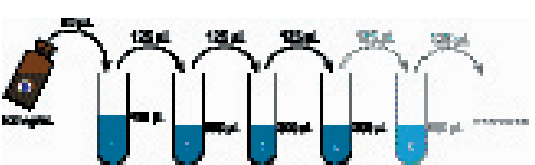

FIGURE 3. DOMOIC ACID STANDARD DILUTION SEQUENCE

I. PREPARATION OF SHELLFISH SAMPLES

a) Extraction of DA from shellfish samples

Shellfish flesh should be prepared as a finely blended homogenate. Preferably analyse fresh, but it may be stored frozen at -20°C for up to 14 days before use.

1. Prepare shellfish homogenate in a high speed blender (kitchen blender), from no less than 50 g shellfish flesh.
2. Accurately weigh 4 g into a 50 mL centrifuge tube.
3. Add 16 mL of Extraction solution (50% methanol).
4. Mix well by vigorous shaking on vortex for 1 min.
5. Centrifuge at 3000x*g* for 10 minutes at room temperature.
6. Retain the supernatant for further dilution prior to analysis. The extracts can be stored at -20°C for up to 14 days, although with a possible reduction in DA content.

b) Dilution of shellfish sample extracts

7. Prepare dilutions of the shellfish extract in Standard/Sample buffer (10% methanol in PBS-T) as follows:

1:20 dilution:	*50 µL shellfish extract*	*+ 950 µL buffer*
1:200 dilution:	*50 µL of the 1:20 dilution*	*+ 450 µL buffer*
1:2000 dilution:	*50 µL of the 1:200 dilution*	*+ 450 µL buffer*
1:20 000 dilution:	*50 µL of the 1:2000 dilution*	*+ 450 µL buffer*
1:200 000 dilution:	*50 µL of the 1:2000 dilution*	*+ 450 µL buffer*

Cap and *vortex* each dilution before proceeding to the next dilution step.

8. Analyze the sample dilutions according to the DA concentration range of interest (see Table 1), to give absorbance values within the calibration curve working range. It is recommended to analyze shellfish extracts diluted at 1:20 000 dilutions to comply with EC directive 2002/226/EC, for the quantification of DA

TABLE 1: SHELLFISH EXTRACT DILUTION FOR QUANTIFICATION OF DA

DA concentration range of interest [mg/kg]	Corresponding Sample Extract dilution to be analyzed
0.01 - 0.25	1:200 dilution (minimum dilution)
0.1 - 2.5	1:2000 dilution
1.0 - 25	1:20 000 dilution
10 - 250	1:200 000 dilution

up to the maximum permitted level at 20 mg/kg.

J. PREPARATION OF SAMPLES FROM ALGAL CULTURE AND SEAWATER

The analysis of samples from algal culture and seawater will depend on the amount of algae (cells/mL) and the amount of DA present in the algae and in the seawater or culture medium. The recommended procedure for preparation of samples is derived from Fehling *et al*., 2004.

1. Count the amount of algae (cells/mL) in your sample. If you want to analyze *total DA* and *extracellular DA* (DA released into the medium or seawater), divide each sample in duplicates with exact volumes.

2. *Total DA*: Sonicate the sample for 2 minutes (on ice) to disrupt the cells. Then filter the sample through a 0.2 µm disposable filter (surfactant free cellulose acetate membrane) to remove cell debris. Dilute the *total DA* filtrate in Standard/Sample buffer (see paragraph 3) before analysis . The sample result will be given as DA concentration in pg/mL, and DA per cell can be calculated by dividing the DA content with the cell numbers. The filtrate can be frozen at -20˚C for up to two weeks prior to analysis.

Extracellular DA: Gently filter the duplicate sample under low vacuum onto glass-fiber filters. Be carefull not to disrupt cells. Dilute the *extracellular DA* filtrate in Standard/Sample buffer (see paragraph 3) before analysis. The sample result will be given as DA concentration in pg/mL, and DA per cell can be calculated by dividing the DA content with the cell numbers. The filtrate can be frozen at -20˚C for up to two weeks prior to analysis.

Intracellular DA: Calculate the intracellular DA content by subtracting the *extracellular DA* content from the *total DA* content after analysis. The sample result will be given as DA concentration in pg/mL, and DA per cell can be calculated by dividing the DA content with the cell numbers.

3. Before analysis, dilute the *total DA* and *extracellular DA* filtrates in Standard/Sample buffer. For cell densities up to 100 000 cells/mL in culture medium or seawater, a minimum dilution of 1:25 in Standard/Sample buffer is required to avoid matrix effects.

K. ASSAY PROCEDURE

a) Incubation of standards and samples with antibody

Equilibrate pre-coated plate strips and all reagents to room temperature before use (1 hour max). See Figure 4 for a recommended plate layout for either using 4 strips in 2 rounds of analysis (4A), or all 8 strips at once (4B).

1. Open the packet(s) with pre-coated plate strips gently and place the strips in the strip frame. Label each strip *e.g.* A, B, C and D etc.
2. Add 300 µL Washing buffer to each well. Pre-soak the wells for 5-10 minutes.
3. Remove the Washing buffer by inverting the strips over a sink and tap against a pile of paper towels to remove all the remaining liquid.
4. Add 50 µL Standard/Sample buffer (10% methanol in PBS-T) to each of the duplicate Amax and Blank wells.
5. Add 50 µL Antibody-HRP ovalbumin buffer (1% ovalbumin) to the Blank wells.
6. Add 50 µL of each DA standard dilution to each of two wells.
7. Add 50 µL of each sample dilution to each of two wells.
8. Shake vial E briefly, and tap the vial gently on a hard surface to ensure that all the content is in the bottom of the vial. Transfer ***0.5 mL (for 4 strip assay)*** or ***1.0 mL (for 8 strip assay)*** from vial E (concentrated Anti-DA-HRP) to a Falcon type tube containing ***2.5 mL (for 4 strip assay)*** or ***5.0 mL (for 8 strip assay)*** Antibody-HRP ovalbumin buffer (1% ovalbumin). Vortex briefly.
9. Add 50 µL of the diluted Anti-DA-HRP conjugate to all wells ***except*** the Blank wells.
10. Seal the strips with the plate sealer (B) and incubate at room temperature (20-25°C) for 1 hour. Protect from light (*e.g.* cover with aluminium foil or place in a drawer).

	1	2	3	4	5	6	7	8	9	10	11	12
A	10 000 pg/ml	2941 pg/ml	865 pg/ml	254 pg/ml	75 pg/ml	22 pg/ml	6,5 pg/ml	1,9 pg/ml	0,56 pg/ml	0,16 pg/ml	Amax	Blank
B	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C	S1 1:20 000	S2 1:20 000	S3 1:20 000	S4 1:20 000	S5 1:20 000	S6 1:20 000	S7 1:20 000	S8 1:20 000	S9 1:20 000	S10 1:20 000	S11 1:20 000	S12 1:20 000
D	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

FIGURE 4A: SUGGESTED PLATE LAYOUT, USING 4 STRIPS, FOR THE QUANTIFICATION OF DA IN 12 SHELLFISH SAMPLES IN 2 SEPARATE ROUNDS. S1 = SAMPLE 1, S2 = SAMPLE 2, ETC.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10 000 pg/ml	2941 pg/ml	865 pg/ml	254 pg/ml	75 pg/ml	22 pg/ml	6,5 pg/ml	1,9 pg/ml	0,56 pg/ml	0,16 pg/ml	Amax	Blank
B	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C	S1 1:20 000	S2 1:20 000	S3 1:20 000	S4 1:20 000	S5 1:20 000	S6 1:20 000	S7 1:20 000	S8 1:20 000	S9 1:20 000	S10 1:20 000	S11 1:20 000	S12 1:20 000
D	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
E	S13 1:20 000	S14 1:20 000	S15 1:20 000	S16 1:20 000	S17 1:20 000	S18 1:20 000	S19 1:20 000	S20 1:20 000	S21 1:20 000	S22 1:20 000	S23 1:20 000	S24 1:20 000
F	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
G	S25 1:20 000	S26 1:20 000	S27 1:20 000	S28 1:20 000	S29 1:20 000	S30 1:20 000	S31 1:20 000	S32 1:20 000	S33 1:20 000	S34 1:20 000	S35 1:20 000	S36 1:20 000
H	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

FIGURE 4B: SUGGESTED PLATE LAYOUT, USING ALL 8 STRIPS, FOR THE QUANTIFICATION OF DA IN 36 SHELLFISH SAMPLES. S1 = SAMPLE 1, S2 = SAMPLE 2, ETC.

b) Developing and reading the microplate strips

11. Carefully remove the plate sealer. Wash the wells 4 times with 300 µL Washing buffer per well.

12. Add 100 µL of TMB peroxidase substrate (vial G) to all wells. Incubate at room temperature (20-25°C) for 15 minutes. Protect from light.

13. Stop the reaction by adding 100 µL 0.3 M H_2SO_4 to all wells.

14. After 2-5 minutes, read the absorbance in a microplate spectrophotometer using a 450 nm filter.

L. CALCULATION OF RESULTS

a) Calibration using the four-parameter logistic curve fit model

When the measured absorbance values of the standard dilutions are plotted on a linear scale (y axis) against the DA-concentrations of the standard dilutions on a logarithmic scale (x axis), a sigmoid (S-shaped) curve is obtained (see Fig. 5).
The non-linear 4-parameter logistic curve-fit model is extensively used for sigmoid curves, in order to get accurate quantification of samples and a good fit at the extremes of the curve. The following equation is given for a 4-parameter fitted curve:

$$y = (a-d)/[1+(x/c)^b]+d$$

where:

***x** is the concentration of DA in the standard/sample*

***y** is the absorbance of the standard/sample*

***a** is the y-value of the upper asymptote (A_{max})*

***b** is the relative slope of the curve at its center*

***c** is the x-value at the midpoint of the curve (I_{50})*

***d** is the y-value of the lower asymptote (Blank/A_{min})*

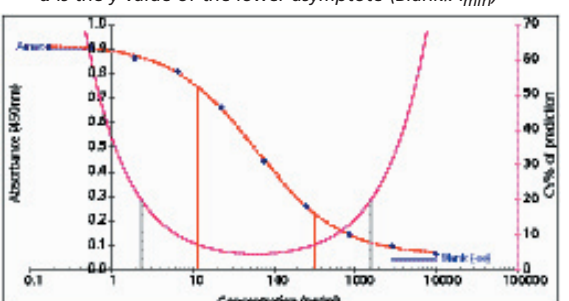

FIGURE 5. NON-LINEAR CALIBRATION CURVE PREPARED BY 4-PARAMETER LOGISTIC CURVE FIT.

b) Calculation formula

The following formula is used to convert ELISA results in pg/mL to shellfish concentrations in mg/kg:

$$\text{mg DA/kg} = \mu\text{g DA/g} = (\text{pgDA/mL}) \cdot D \cdot V \cdot \frac{1\ \mu g}{1\ 000\ 000\ pg} \Big/ M$$

where:

***pg DA/mL** is the concentration of DA in the diluted extract*

***D** is the dilution factor of the diluted extract*

***V** is the volume of the methanolic extract (16 mL plus 4 g of homogenate giving nominal 20 mL total volume).*

***M** is the mass of the shellfish homogenate (4 g).*

c) Excel macro EMA31 calculation of DA concentration in shellfish samples

For calculation of assay results, a spreadsheet has been developed implementing the calibration function and the conversion formula from pg/mL in the extract to mg DA/kg shellfish.

1. Open the provided Excel Macro EMA31, enable macros and install the Solver as described in the "Instructions" sheet of the Macro.
2. Copy the measured absorbance values (to at least 3 significant figures, *e.g.* 0.682) from the plate reading software result/report sheet and paste the values in the Excel Macro EMA31 "Data Entry" sheet.
3. Enter the correct dilution factor used for the samples, in the corresponding duplicate well windows.
4. Run the macro according to the instructions.
5. Go to the "Results" sheet. The results from the column "Shellfish sample DA eqv. (mg/kg)" give the concentration of DA in the shellfish samples.
6. Sample concentrations should only be calculated from datapoints that are within the valid working range of the standard curve as defined by the Excel macro. If more than one sample dilution hit the working range of the standard curve, we recommend that the dilution closest to the I_{50} value of the standard is used.

   Alternatively; another data analysis software (e.g. the software provided with the plate reader) may be used as long as it supports the 4-parameter logistic curve fit model.

d) Excel macro EMA31 calculation of DA concentration in Algal samples

1. Use the provided Excel macro EMA31 as described in the previous section.
2. Enter the correct dilution factor used for the algal samples, in the corresponding duplicate well windows.
3. The results from the column "Sample extract/solution (pg/mL)" will provide the DA concentration of the algae extracts as pg/mL.
4. If *Pseudo-nitzschia* cell counts are available for the filtered water sample, the results can be converted to pg DA/cell, taking into account the volume of water filtered and the extraction volume.

e) Excel macro calculation of DA concentration in seawater samples

1. Use the provided Excel macro EMA31 as described in the previous section.
2. Enter the correct dilution factor used for the seawater samples, in the corresponding duplicate well windows.
3. The results from the column "Sample extract/solution (pg/mL)" will provide the DA concentration of the seawater samples as pg/mL.

M. QUALITY ASSURANCE MEASURES FOR VALID ANALYSIS

- In order to qualify as a valid calibration curve suitable for accurate quantification of DA in samples, the requirements listed in Table 2 must be fulfilled.
- Sample concentrations should only be calculated from datapoints that are within the valid working range of the calibration curve as defined by the Excel macro.
- The estimated curve fit (%CV of prediction) for the calibration curve should be <20%, as indicated in the "Results" sheet of the Excel Macro EMA31.
- The concentration difference should not be more 15% between two duplicate wells for a given sample.

TABLE 2: QUALITY ASSURANCE REQUIREMENTS FOR VALID CALIBRATION CURVE

Calibration curve Parameter	Requirement
Maximum absorbance (A_{max})	> 0.8 A.U.
Blank/A_{min}	< 0.1 A.U.
Calibration curve I_{20} value	6-20 pg/mL
Calibration curve I_{50} value	35-80 pg/mL
Calibration curve I_{80} value	180-450 pg/mL

N. REFERENCES

- Garthwaite I, Ross KM, Miles CO, Briggs L, Towers N, Borell T, Busby P. (2001) *J. AOAC Int.* **84**, 1643-1648.
- Garthwaite I, Ross KM, Miles CO, Hansen RP, Foster D, Wilkins, AL, Towers N. (1998) *Wat. Toxins* **6**, 93-104.
- *Official Methods of Analysis of AOAC INTERNATIONAL* (2006) 19th Ed., AOAC INTERNATIONAL, Gaithersburg, MD USA, Official Method **2006.02**.
- Scholin CA, Gulland F, Douchette GJ *et al*. (2000) *Nature* **403**, 80-84.
- Wright JLC, Boyd RK, DeFreitas ASW, Falk M, Foxall RA, Jamieson WD, Laycock MV, McCulloch AW, McInnes AG, Odense P, Pathak VP, Quilliam MA, Ragan MA, Sim PG, Thibault P, Walter JA, Richard DJA, Dwar D. (1989) *Can. J. Chem.* **67**, 481-490.
- Fehling J, Davidson K, Bolch CJ, Bates SS. (2004) *J. Phycol.* **40**, 674-683.

O. QUICK GUIDE

1. Prepare dilutions of standard and samples.

2. Pre-soak the wells for 5-10 minutes with 300 µl Washing buffer. Empty before use.

3. Add 50 µL Standard/Sample buffer to the A_{max} and Blank wells.

4. Add 50 µl Antibody-HRP buffer to the Blank wells.

5. Transfer 50 µL of diluted standards and samples (in duplicate) to the plate.

6. Dilute the concentrated antibody-HRP conjugate and add 50 µL to all wells except the Blank wells.

7. Seal the plate and incubate at room temperature for 1 hour (keep dark).

8. Wash the wells.

9. Add 100 µL TMB peroxidase substrate to all wells.

10. Incubate at room temperature for 15 minutes (keep dark).

11. Add 100 µL of 0.3 M H_2SO_4 to all wells to stop the reaction.

12. Read the absorbance at 450 nm.

13. Calculate the concentrations using the Excel Macro EMA31.

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2005.2

FOOD CHEMICAL CONTAMINANTS

Single-Laboratory Validation of the Biosense Direct Competitive Enzyme-Linked Immunosorbent Assay (ELISA) for Determination of Domoic Acid Toxins in Shellfish

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Method validation was conducted for an enzyme-linked immunosorbent assay (ELISA) for the determination of domoic acid (DA) toxins, known to give amnesic shellfish poisoning (ASP) symptoms, in shellfish. The calibration curve range of the assay is approximately 10–260 pg/mL, with a dynamic working range for DA toxins in shellfish from 0.01 to at least 250 mg/kg. The ASP ELISA showed no significant cross-reactivity to structural analogs, and proved to be robust to deliberate alterations of the optimal running conditions. The shellfish matrix effects observed with mussels, oysters, and scallops were eliminated by diluting shellfish extracts 1:200 prior to analysis, leading to a limit of detection at 0.003 mg/kg. Thirteen blank shellfish homogenates were spiked with certified mussel material containing DA to levels in the range of 0.1–25 mg DA/kg, and analyzed in quadruplicate on 3 different days. The relative standard deviation (RSD) under intra-assay repeatability conditions ranged from 6.5 to 13.1%, and under interassay repeatability conditions the RSD ranged from 5.7 to 13.4%, with a mean value of 9.3%. The recoveries ranged from 85.5 to 106.6%, with a mean recovery of 102.2%. A method comparison was conducted with liquid chromatography with ultraviolet detection, using naturally contaminated scallop samples (n = 27) with DA levels at 0–244 mg/kg. The overall correlation coefficient was 0.960 and the slope of the regression was 1.218, indicating a good agreement between the methods.

Since the first outbreak of amnesic shellfish poisoning (ASP) on the Canadian coast in late 1987 following the ingestion of contaminated blue mussels, domoic acid (DA) has been identified as a potent neurotoxin, binding to the kainate type of synaptic glutamate receptors (1). The source of DA was shown to be the DA-producing diatom *Pseudo-nitzschia multiseries* (2), which accumulates in filter-feeding shellfish during *Pseudo-nitzschia* sp. blooms (3). The regulatory monitoring of shellfish for DA and its isomers to protect consumers from ASP is now well established in most countries. A general regulatory limit for human consumption has been set at 20 mg DA/kg edible parts of shellfish, as required by the European Directives 91/492/EEC and 2002/226/EC (4), and a liquid chromatography (LC) method with UV absorbance detection has been established as the reference method for this purpose (5, 6). As different species or strains of the genus *Pseudo-nitzschia* do not always produce significant concentrations of DA, blooms in coastal waters and laboratory cultures can range from highly toxic to essentially nontoxic (3, 7). Thus, direct analysis of phytoplankton or seawater samples will probably be the fastest and most reliable method to confirm the presence of DA in the marine environment. Although the implementation of routine marine toxin surveillance in matrixes other than shellfish is not a common practice, it may prove suitable as an early warning measure for DA entering the food web at levels sufficient to harm marine wildlife and perhaps humans. A fast and reliable analysis of DA levels in shellfish poses a significant preventive measure against poisoning incidents or product recalls and will provide a real-time control and ability to interpret subaction level trends taking place in the marine environment. Such early-warning and in-process control tools may add more predictability to the shellfish industry, possibly lowering both the economic risk involved and the risk to wildlife and public health. During the last decade, there has been an ongoing development of rapid assays, as an alternative to analytical instrumental methods, for the determination of marine biotoxins. Such assays offer advantages in sample turnover and accessibility over the instrumental methods. Assays described for DA determination include a receptor-binding assay (8) and immunological assays based on enzyme-linked immunosorbent assay (ELISA) technology (9–14). Preliminary investigations suggested that an indirect ELISA developed by the AgResearch Toxinology group in Hamilton,

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New Zealand (15) was a promising alternative to the current reference method using LC-UV for the determination of DA (16).

This study describes a reformatted version of this indirect ELISA to a rapid and more user-friendly, direct format using horseradish peroxidase-conjugated primary antibodies. We performed a single-laboratory validation (SLV) of the direct, competitive ASP ELISA for the determination of DA in different shellfish species, and compared the ELISA with LC-UV to assess if the ASP ELISA may be suitable for routine testing to comply with international regulatory limits.

Experimental

Apparatus and Reagents

(**a**) *Blender or ultrahomogenizer.*

(**b**) *Microtiter plate absorbance reader.*—Equipped with a 450 nm filter.

(**c**) *Solutions.*—All solvents were LC grade and were obtained from Rathburn (Walkerburn, Strathclyde, UK).

(**d**) *DA calibration solution.*—The working calibrant solution for the ASP ELISA is prepared by dilution of the NRC-CRM-DA-d solution (certified level of DA + epi-DA at 90.5 μg/mL) in acetonitrile–water (1 + 9, v/v) to a final concentration of 100 ng/mL (DA + epi-DA). The certified reference material (CRM) solution was obtained from the National Research Council of Canada's Certified Reference Materials Program (NRC CRMP; Halifax, Canada; http://imb-ibm.nrc-cnrc.gc.ca).

(**e**) *Certified mussel tissue reference material.*—The CRM-ASP-MUS-b mussel material used for spiking experiments was obtained from NRC CRMP.

Preparation of Samples

(**a**) *Extraction of shellfish samples.*—The shellfish samples were prepared according to the standard method of Quilliam et al. (6) with some modifications. The shellfish were thoroughly cleaned with fresh water before they were opened. No less than 50 g shellfish flesh was finely blended with an ultrahomogenizer, and 4.00 g (±0.10 g) was accurately transferred into a 50 mL polypropylene centrifuge tube. A 16 mL volume of 50% aqueous methanol was added, and the extract was mixed well on a Vortex mixer for 2 min. After subsequent centrifugation for 10 min at 3000 × *g* at room temperature, the supernatant was diluted stepwise 1:20, 1:200, 1:2000, and 1:20 000, with 10% methanol in phosphate-buffered saline (PBS)-Tween prior to analysis according to the ASP ELISA kit user's manual (17) in order to avoid unspecific matrix effects.

(**b**) *Algal samples.*—Cultured samples of nontoxic *Pseudo-nitzschia delicatissima* cells were prepared according to Fehling et al. (18) and the ASP ELISA kit user's manual (17). The cell count was determined before the cells were lysed by sonication and filtered through a 0.2 μm disposable filter to remove the cell debris. The filtrate, containing all the extracellular and intracellular DA content in addition to the disrupted cell components, was diluted with 10% methanol in PBS-Tween prior to analysis according to the ASP ELISA kit user's manual (17) in order to avoid unspecific matrix effects.

(**c**) *Seawater samples.*—Seawater samples were directly filtered through a 0.2 μm disposable filter to remove any debris. The filtered seawater was diluted with 10% methanol in PBS-Tween prior to analysis according to the ASP ELISA kit user's manual (17) in order to avoid unspecific matrix effects.

Assay Procedure

Following the sample preparation, 10 calibration standards were prepared freshly by serial dilution of the certified reference calibration solution NRC-CRM-DA-d in the range of 10 000–0.16 pg/mL. The assay was performed according to the ASP ELISA kit user's manual (17).

Calculation of Results

(**a**) *Calibration using the 4-parameter logistics curve fit model.*—The absorbance values (A_{450}) of the 10 standard dilutions were plotted on a linear scale (*y*-axis) against the DA concentrations of the standard dilutions on a logarithmic scale (*x*-axis) to obtain a sigmoid (S-shaped) curve. A 4-parameter logistic curve-fitting model (an Excel Macro provided with the ASP ELISA) was used to obtain the sigmoid calibration curve (Figure 1), with the following equation:

$$y\ (a - d)/[1 + (x/c)^b] + d$$

where *x* is the concentration of DA in the standard/sample; *y* is the absorbance of the standard/sample; *a* is the *y*-value of the upper asymptote (A_{max}); *b* is the relative slope of the curve at its center; *c* is the *x*-value at the midpoint of the curve (I_{50}); *d* is the *y*-value of the lower asymptote (A_{min}).

(**b**) *Calculation formula.*—The following formula was used to convert the ELISA results in pg/mL to DA concentrations in shellfish given in mg/kg:

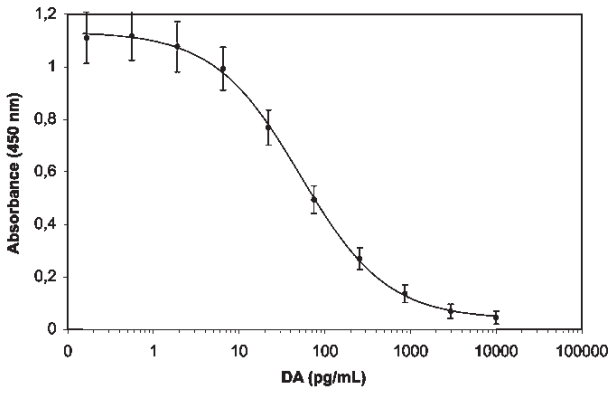

Figure 1. Representative calibration curve from the ASP ELISA, calculated by the mean of 17 independent calibration curves over a period of 9 weeks, as shown in Table 2.

$$mg\ DA/kg = \mu g\ DA/g = [1\ 000\ 000\ pg\ DA/mL \times D \times V]/M$$

where pg DA/mL is the calculated concentration of DA in the diluted extract; D is the dilution factor of the diluted extract; V (mL) is the volume of the methanol extract (16 mL + 4 g homogenate giving the nominal 20 mL total volume); M (g) is the mass of the shellfish homogenate (4 g).

Critical Control Points for System Suitability Check

(*1*) In order to qualify as a valid sigmoid calibration curve, defined parameter criteria for the anchoring points in the asymptotic curve (A_{max}, A_{min}, I_{20}, I_{50}, and I_{80}) must be met for the individual assay calibration curve (17).

(*2*) The valid working range of the calibration curve suitable for quantification of DA is defined by the most narrow concentration range between I_{20} and I_{80} values, and the part of the calibration curve where the % coefficient of variation (CV) estimate from the predicted calibration curve fit is <20%.

(*3*) The concentration variation between sample duplicate well values must be <15%.

Ruggedness

The ruggedness of the ASP ELISA was investigated by deliberately introducing modest variations into the procedure, as might arise in practice, and observing the effects. By following the "fractional factorial" design described by Wernimont (19), 7 different factors were subjected to suboptimal conditions in 8 run combinations. The effects of suboptimal conditions were estimated according to the AOAC method (19), based on changes in the calibration curve parameters and on the quantification of 2 mussel samples.

Validation

Blue mussels (*Mytilus edulis*), Pacific oysters (*Crassostrea gigas*), and king scallops (*Pecten maximus*) samples determined to be free of DA by LC-UV (6) were assayed to determine the respective matrix limit of detection (LOD) and limit of quantitation (LOQ). For precision and recovery experiments, shellfish samples free of DA (LOD at <0.003 mg/kg) were homogenized, and exact amounts of CRM-ASP-MUS-b were blended in to give spike levels in the range of 0.1–24 mg DA/kg shellfish. From the NRC certificate of analysis, the CRM-DA-d calibration standard and the CRM-ASP-MUS-b have different contents of epi-DA and iso-DA (Table 1). Because the ASP ELISA aimed to recognize total amount of DA toxins, the spike levels were based on an estimated amount of total DA displayed in Table 1. Subsamples for each spike level were prepared and kept frozen at –80°C until the day of analysis.

Intra- (n = 12) and interassay repeatability (n = 3) were assessed for all 3 shellfish species spiked with DA at levels from 0.123 to 24.55 mg/kg. Two analysts analyzed test portions of the 13 sample materials on 3 separate days. Because all the samples were spiked with CRM, the prepared sample material was also used to assess the recovery of the DA concentrations measured by the ASP ELISA.

Table 1. Domoic acid content in CRM-DA-d and CRM-ASP-MUS-b[a]

	CRM-DA-d, μg/mL	CRM-ASP-MUS-b, μg/g
DA	86.4	36.0
Epi-DA	1.3	3.0
Iso E[b]	0.24[b]	3.2[b]
Iso D[b]	0.80[b]	1.8[b]
Iso A[b]	1.8[b]	NM[c]
Iso F[b]		0.2[b]
Estimated total DA	90.54[b]	44.2[b]
Epimer and isomer fraction in "total DA"		
Epimer content, %	1.44	6.78
Isomer content[b], %	3.13	11.76
Epi-DA + iso-DA content, %	4.57	18.54

[a] NRC CRM program (1999).
[b] Isomer values are not certified.
[c] NM = Not measured.

For the purpose of method comparison, the ASP ELISA was compared to the current reference method using LC-UV (6), by analyzing naturally incurred scallop samples (n = 27) with DA contents in the range of 0.2–244 mg/kg. Shellfish samples were prepared as frozen subsamples, and the subsamples were homogenized and extracted at 2 different laboratories prior to analysis, using ASP ELISA at Biosense Laboratories and LC-UV at Integrin Advanced Biosystems (Oban, Scotland).

Results and Discussion

Calibration

The calibration of the assay was obtained using serial dilutions of the NRC CRM-DA-d calibration solution. Ten calibration points were processed using a 4-parameter logistic curve-fit, and the mean and between-day RSD were calculated from each of the 4 significant parameters: A_{max} (y-value of the upper asymptote), A_{min} (y-value of the lower asymptote), slope (the relative slope of the curve at its center), and I_{50} (the x-value at the midpoint of the curve). Processing the data points from representative calibration curves produced a typical calibration curve as shown in Figure 1. The valid calibration range for each assay is by convention defined as the concentration range between the parameters I_{20} (the x-value where the absorbance is 20% reduced relative to the A_{max} value) and I_{80} (the x-value where the absorbance is 80% reduced relative to the A_{max} value), usually ranging from 10 to 260 pg/mL. Every ASP ELISA calibration curve complied with the assay parameters quality assurance guidelines, according to the ASP ELISA kit manual (17). A representative data set from 13 calibration curves gathered from 3 different ASP ELISA production batches during a period of 8 weeks is

Table 2. Calibration curve variability within 3 ASP ELISA batches

		Significant 4 parameters				Working range	
Batch No.	Run date	A_{max}	A_{min}	Slope	I_{50}, pg/mL	I_{80}, pg/mL	I_{20}, pg/mL
B0202	Dec. 13, 2002	1.184	0.069	0.810	50.7	280.6	9.2
	Dec. 16, 2002	1.148	0.022	0.855	57.3	290.1	11.3
	Dec. 17, 2002	1.158	0.037	0.842	58.8	305.0	11.3
	Dec. 17, 2002	1.116	0.030	0.871	57.2	281.3	11.6
	Jan. 10, 2003	1.263	0.083	0.893	50.0	236.2	10.6
	Jan. 13, 2003	1.096	0.038	0.849	59.6	305.4	11.6
	Jan. 15, 2003	1.147	0.033	0.829	49.7	264.9	9.3
	Jan. 16, 2003	1.237	0.087	0.832	42.0	222.2	7.9
	Feb. 21, 2003	0.898	0.017	0.934	55.8	245.8	12.7
	Feb. 24, 2003	1.142	0.026	0.901	60.7	282.5	13.0
	Feb. 26, 2003	1.189	0.038	0.879	47.5	229.9	9.8
B0301	Feb. 21, 2003	0.924	0.011	0.898	46.0	215.4	9.8
	Feb. 24, 2003	1.139	0.017	0.864	53.3	265.3	10.7
	Feb. 26, 2003	1.183	0.027	0.850	49.1	251.1	9.6
B0302	Feb. 21, 2003	1.188	0.027	0.838	47.0	245.9	9.0
	Feb. 24, 2003	1.086	0.065	0.896	50.8	238.9	10.8
	Feb. 26, 2003	1.217	0.086	0.864	49.7	247.4	10.0
Mean		1.136	0.042	0.865	52.1	259.3	10.5
SD		0.097	0.026	0.032	5.3	27.8	1.3
%CV		8.52	61.36	3.70	10.28	10.74	12.87

shown in Table 2. The low variability between runs demonstrates that the ASP ELISA calibration is stable and well standardized.

Specificity

The antibody specificity observed with the ASP ELISA has been previously described for the indirect format (15). Furthermore, the ASP ELISA antibody has proved a low cross-reactivity (<1%) to the open-ring isomer iso-DA C (L. Briggs, unpublished results) reported to have a low neurotoxic potential (20), but there are no other cross-reactivity data on the epi-DA or the other DA isomers due to the poor availability of such compounds. However, based on the recovery of DA samples spiked with the CRM-ASP-MUS-b material, the results suggest that the antibody used in the ASP ELISA has a significant cross-reactivity with DA isomers. A slight overestimation of the total DA toxins is observed because the CRM-ASP-MUS-b spike material contains a higher portion of DA isomers than the CRM-DA-d solution used to define the calibration curve (Table 1).

Selectivity

Cross-reactivity to structural analogues.—The selectivity of the indirect version of the ASP ELISA has previously been described by Garthwaite et al. (15). Several structural analogs known to interfere with the receptor binding assay (4) were tested with no, or in some cases very weak, indications of cross-reactivity to the antibody used. In the current study, the cross-reactivity between DA and the most common structural analogs were determined, as well as tryptophan due to its interference with DA during LC/mass spectrometric (MS) analysis (Figure 2). Kainic acid appeared to cross-react weakly with the antibody, but only when the concentration was approximately 130 000 times higher than that of DA. However, this effect might also be caused by the low pH arising from both glutamic and kainic acid. The experiments also confirmed that the ASP ELISA cross-reacted minimally with glutamic acid. Overall, the ASP ELISA is very selective to DA and does not seem to be affected by structural analogs.

Matrix effects from shellfish.—Tissue extracts from blue mussels, Pacific oysters, and king scallops were investigated for adverse enhancement effects of the signal response (i.e., unspecific reduction of A_{450}). Blank shellfish samples, both raw and cooked, were processed according to method protocol, and the methanol extracts were serial diluted with sample buffer and loaded onto the plates. The unspecific reduction of the A_{max} parameter due to the shellfish matrix presence were gradually eliminated as the sample extracts were diluted, and a plateau was reached at about 1:100 dilution with blue mussels and Pacific oysters (Figure 3). Parallel shellfish extracts from raw and precooked

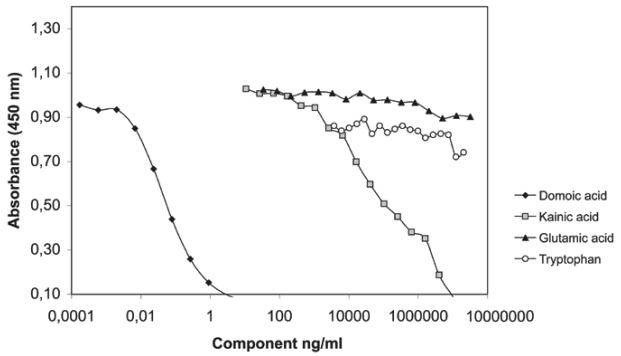

Figure 2. Competitive binding of potential interfering substances analyzed with the ASP ELISA. The high selectivity of the assay is demonstrated by extremely low cross-reactivity to such compounds, and their presence in high quantities will not interfere with the assay during normal use.

homogenates produced similar curves. The data suggest that a minimal dilution of 1:200 for the analysis of MeOH extracts from shellfish samples is required to eliminate the matrix effects, indicating an LOQ at 0.010 mg DA/kg shellfish, which is slightly lower than reported for the DA ELISA recently described by Yu et al. (13).

Matrix effects from algae and seawater.—To assess the suitability of the ASP ELISA as an early-warning tool for upcoming blooms of toxic *Pseudo-nitzschia* sp., the unspecific matrix effects from algal and seawater samples were investigated. Cultured samples of *P. delicatissima* were prepared according to Fehling et al. (18) and the ASP kit ELISA user's manual (17). Dilution series of filtrates from sonicated cells were prepared with 10% methanol in PBS-Tween, prior to ASP ELISA analysis (Figure 4). The culture cell density appeared to have insignificant effects on the matrix effects observed. Even for cell densities up to 98 000 cells/mL, the unspecific interference was similar to that observed with 5000 cells/mL and even the growth medium alone. The matrix effects observed were eliminated when samples were diluted 1:20, suggesting an LOQ at 200 pg/mL.

Direct analysis of mammal body fluids.—The increasing awareness of DA transfer in the marine food web has led to the monitoring of DA levels in marine mammals, seabirds, and other indicator species. Although ASP in humans is rare in areas with a well-managed routine monitoring program in place, a rapid and sensitive tool to screen body fluid samples from individuals who may have ingested DA-tainted shellfish or fish would aid in a rapid diagnosis and treatment. To demonstrate the broad sample applicability of the ASP ELISA, relevant human body fluids were investigated for unspecific matrix effects. Unprocessed blood, plasma, and urine from healthy humans were diluted directly with sample buffer and loaded onto the plates. The graph demonstrates that a plateau is reached at about 1:30 dilution for all samples, and that the matrix effects are negligible at 1:30 dilution (Figure 5). Although spike experiments were not conducted with these matrixes, the present data suggest that human body fluids can be analyzed for DA with an estimated detection limit at about 300 pg/mL, which is significantly lower than that reported for the DA ELISA previously described by Smith and Kitts (11).

LOD and LOQ for shellfish.—After investigations on how to eliminate unspecific matrix effect for shellfish samples, the LOD for 3 shellfish matrixes was calculated based on the "blank + 3 × SD" approach (21). Based on the standard deviation (SD) of 10 independent blank samples from each shellfish species, the LODs were calculated in the range of 1.1–5.3 μg DA/kg shellfish (Table 3). The results are in line with Figure 3, showing that scallops have a stronger matrix effect on the ASP ELISA as compared to mussels and oysters. The mean shellfish LOD was calculated to be 3.3 μg/kg, and the mean LOQ was 8.5 μg/kg based on the "blank + 10 × SD" approach (21). The LOD value is well below most of the current LC methods described (6, 22, 23).

Ruggedness

The ruggedness of the ASP ELISA was investigated by deliberately introducing modest variations into the procedure, as might arise in practice, and observing the effects. Based on the "fractional factorial" design described by Wernimont (19), Table 4 shows the effects of suboptimal conditions based on changes in the calibration curve parameters and on the quantification of 2 mussel samples. The factor combinations are being used to identify any variables causing large effects, and enable the control of conditions that may be critical to the assay results. The effect of each suboptimal factor variable is given as percent difference from the optimal condition (Table 4, footnote a). Despite occasional variations in the I_{20}

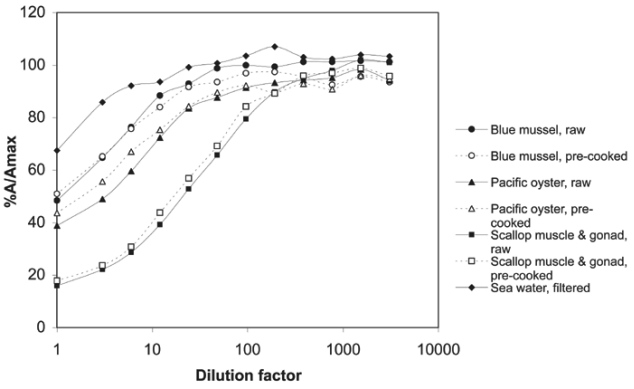

Figure 3. Dilution curves demonstrating the elimination of unspecific matrix effects from methanol extracts of raw and precooked shellfish free of DA. As the sample extracts are diluted, the unspecifc reduction of the absorbance is reduced until it is eliminated when samples are diluted at least 1:200.

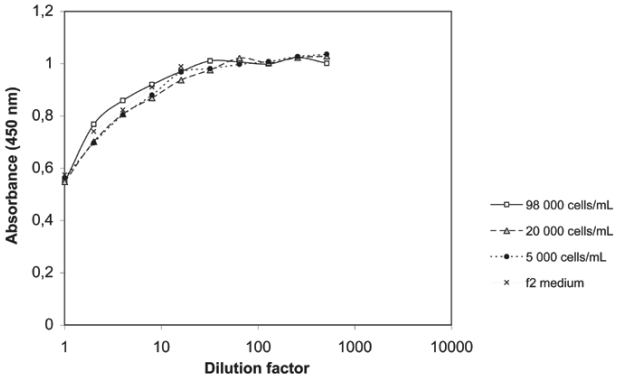

Figure 4. Dilution curves demonstrating the elimination of unspecific matrix effects from filtrates of sonicated cultures of nontoxic *P. delicatissima*. Regardless of culture cell densities up to 98 000 cells/mL, the matrix effects were eliminated when samples were diluted at least 1:30.

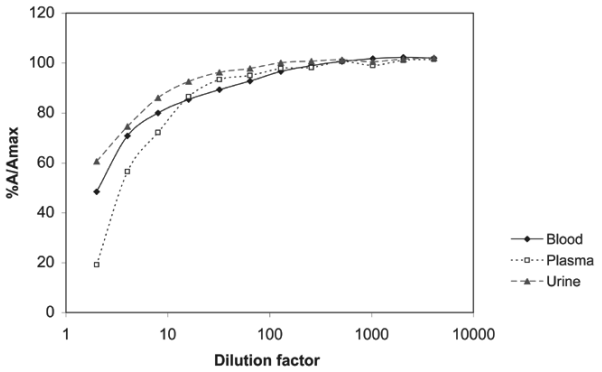

Figure 5. Dilution curves demonstrating matrix effects experienced with the ASP ELISA, upon the direct analysis of unprocessed blood, plasma, and urine from healthy humans. The graphs suggests that these matrixes can be analyzed directly for DA with a minimal dilution of 1:30 in sample buffer.

and I_{50} values for the calibration curves, the deviation from the quantification samples A and B was <15% except from one value (15.6%), but in total the ASP ELISA appears to be quite robust to deviations in the procedure with respect to incubation times and temperatures. The ruggedness analysis identified the incubation temperature during the competitive binding step as a critical parameter that should be kept under reasonably tight control. However, additional ruggedness experiments with incubation temperature have shown that the temperature may vary between 18 and 25°C with <15% deviation from assays conducted with the optimal incubation temperature at 22°C (results not shown).

Precision

To determine the within-day repeatability, blank blue mussel, Pacific oysters, and king scallop homogenates were spiked with CRM-ASP-MUS-b at 8 different concentrations covering the most relevant range for testing of shellfish for human consumption. CRM-ASP-MUS-b is estimated to contain a total of 44.2 ± 1.0 mg DA/kg, including epi-DA and the different iso-DAs (Table 1). The sample material at each spike level was further subsampled and stored at –80°C until analysis. Three series of runs were performed on different days, with 4 replicates analyzed in every run, and the RSD was calculated for each level on each day (Tables 5–7). The within-day repeatability RSD for the shellfish samples ranged from 6.5% for blue mussels to 13.7% for king scallops, and each shellfish species had a mean overall RSD_r <11%. The measurement precision did not appear to differ significantly between the 3 species, nor did the precision seem to be systematically affected by the concentration levels between 0.123 and 24.55 mg/kg.

The intra-assay data for each shellfish species from 3 different days were compiled to calculate the between-day repeatability (Table 8). The overall between-day repeatability RSD was 9.3%, and very similar to the intra-assay repeatability. These results are in line with the calibration data variability of the ASP ELISA observed over a period of 9 weeks (Table 2), where the RSD for the curve defining parameters I_{80}, I_{50}, and I_{20}, ranged from 10.28 to 12.87% within 3 different production batches. This suggests that the assay components are well defined and the method procedure is standardized in an easily

Table 3. Limits of detection and quantitation for shellfish

Matrix	Mean[a], pg/mL	SD	LOD[b], pg/mL	LOQ[c], pg/mL	Dilution factor[d]	Mean LOD, μg/kg	Mean LOQ, μg/kg
Blue mussels	1.149	±0.789	3.516	9.039	(5 × 200)		
Scallop, whole	1.473	±1.304	5.385	14.187	(5 × 200)		
Pacific oysters	0.549	±0.185	1.104	2.399	(5 × 200)		
						3.3	8.5

[a] Mean value of 10 independent blank shellfish samples.

[b] Mean blank value × 3 SD.

[c] Mean blank value × 10 SD.

[d] Extraction factor (1 + 5, v/v) × sample extract dilution factor (1:200).

Table 4. Ruggedness analysis of the ASP ELISA

Factor	Condition[a]	Difference resulting from suboptimal conditions, %				
		Sample A value	Sample B value	I_{80}	I_{50}	I_{20}
Prewash coated plate	Yes[a]					
	No[b]	15.6	13.9	15.3	13.5	11.9
Incubation temperature during Ab binding step	22°C[a]					
	30°C[b]	–4.8	0.6	–8.8	19.2	54.8
Incubation time during Ab binding step	1 h[a]					
	1 h 15 min[b]	14.3	13.0	–11.5	–6.9	–4.7
TMB temperature upon addition	22°C[a]					
	4°C[b]	–10.0	2.1	4.2	7.4	10.7
Incubation temperature during development	22°C[a]					
	30°C[b]	–4.2	1.1	–5.9	–11.7	–18.4
Incubation time during development	15 min[a]					
	20 min[b]	–6.7	–5.4	–2.0	3.6	10.5
Exposure to light during development	No[a]					
	Yes[b]	–4.6	0.1	–4.0	–1.8	7.9

[a] Optimal conditions.
[b] Altered condition.

Table 5. Intra-assay parameters calculated for spiked blue mussels samples

Day[a]	Spike value, mg/kg	Mean[a], mg/kg	SD	RSD, %	Mean within-day RSD, %
1	24.55	25.4	1.56	6.12	
	6.14	6.8	0.48	7.07	
	1.23	1.39	0.13	9.45	
	0.614	0.66	0.03	4.38	
	0.123	0.13	0.01	5.60	6.5
2	24.55	24.8	1.90	7.7	
	6.14	5.7	0.29	5.0	
	1.23	1.15	0.13	11.0	
	0.614	0.60	0.04	7.1	
	0.123	0.12	0.01	10.7	8.3
3	24.55	25.7	3.25	12.67	
	6.14	5.6	5.60	5.37	
	1.23	1.18	1.18	8.58	
	0.614	0.62	0.62	6.55	
	0.123	0.11	0.11	3.44	7.3

[a] Four replicates from each sample material were analyzed each day.

Table 6. Intra-assay parameters calculated for spiked Pacific oyster samples

Day[a]	Spike value, mg/kg	Mean[a], mg/kg	SD	RSD, %	Mean within-day RSD, %
1	24.55	26.5	1.29	4.85	
	6.14	6.0	0.85	14.13	
	1.23	1.34	0.14	10.59	
	0.614	0.66	0.03	5.33	
	0.123	0.14	0.02	14.73	12.9
2	24.55	28.5	1.91	6.7	
	6.14	6.8	0.72	10.6	
	1.23	1.37	0.11	7.8	
	0.614	0.61	0.06	9.6	
	0.123	0.13	0.01	8.5	8.6
3	24.55	27.2	2.84	10.45	
	6.14	7.5	0.35	4.7	
	1.23	1.34	0.03	2.57	
	0.614	0.61	0.02	4.05	
	0.123	0.12	0.03	12.97	10.5

[a] Four replicates from each sample material were analyzed each day.

reproducible manner. The overall precision level seems quite acceptable and is in the range of the target precision level described by AOAC for SLV studies (24).

Accuracy

To evaluate the method accuracy, the interassay data set was used to calculate the percent recovery of DA in the samples spiked with CRM-ASP-MUS-b. A total of 149 replicates analyzed with the ASP ELISA gave calculated recoveries from 85.8 to 106.6% (Table 8). Most of the determined values were slightly higher than the expected spike value with a mean recovery of 102.2% observed in the range of DA spike concentrations between 0.123–24.55 mg/kg, which is significantly higher than previously reported for other DA ELISAs (13). The sample extraction using the single-step dispersive extraction in 50% methanol has previously shown excellent recoveries (almost 98%) when analyzed by LC/MS (6). The slight overestimation of the DA sample values by the ASP ELISA indicates that the antibodies also recognize the epi-DA and/or iso-DAs in the spiked samples, because the CRM-ASP-MUS-b contains a higher portion of these components than the CRM-DA-d solution used to define the calibration curve (Table 1). This strongly suggests that the ASP ELISA determines the total DA toxin content in shellfish samples rather than merely the DA content alone. The recovery did not appear to be significantly influenced by the spike concentration levels, and the overall recovery at 102.2%

Table 7. Intra-assay parameters calculated for spiked king scallops samples

Day[a]	Spike value, mg/kg	Mean[a], mg/kg	SD	RSD, %	Mean within-day RSD, %
1	20.00	21.65	1.42	6.54	
	5.00	4.54	1.64	36.12	
	0.50	0.51	0.02	4.09	13.1
2	20.00	20.92	1.04	4.98	
	5.00	3.81	0.35	9.13	
	0.50	0.47	0.03	5.77	5.8
3	20.00	21.31	1.10	5.16	
	5.00	4.52	0.68	15.15	
	0.50	0.55	0.04	7.63	8.7

[a] Four replicates from each sample material were analyzed each day.

Table 8. Interassay ASP ELISA performance for the analysis of spiked mussels, Pacific oysters, and king scallops (*n* = 149)

Runs (*n*)	Replicates in total	Spike level, mg/kg	SD	Between-day RSD, %	Mean, mg/kg	Recovery, %
6	20	24.55	2.37	9.04	26.18	106.6
3	12	20.00	0.52	2.42	21.29	106.5
6	23	6.14	0.83	12.95	6.38	103.9
3	11	5.00	0.35	8.01	4.29	85.8
6	24	1.23	0.14	10.76	1.29	104.9
6	24	0.614	0.04	6.91	0.63	102.6
3	12	0.500	0.06	11.09	0.51	102.0
6	23	0.123	0.02	13.43	0.13	105.7
Overall mean RSD, %				9.3 ± 4		
Overall mean recovery, %						102.2 ± 7

is well in line with the requirements of the AOAC guidelines for this concentration level (24). Figure 6 shows the linear regression of the DA recovered from the spiked samples in Table 8 demonstrating a slope of 1.070 with excellent correlation to spike values ($R^2 = 0.998$).

Method comparison to LC-UV on natural samples.—Homogenates of naturally contaminated samples (n = 27) of king scallops were extracted and analyzed separately with the ASP ELISA and an accredited LC-UV method routinely used for analysis of shellfish for regulatory purposes (6). The 2 data sets from the ASP ELISA and the LC-UV compared well over a concentration range from 0.25 to 244 mg/kg (Figure 7). A good degree of correlation was demonstrated (R^2 = 0.960) between the 2 methods, considering the completely different detection technology and principles applied. The ASP ELISA gave sample values higher than the LC-UV method for 20 of the 27 samples analyzed, and the linear regression slope at 1.218 indicates a slight overestimation. This is in line with the slightly higher recovery levels observed (Table 3), again suggesting that the antibodies in the ELISA detect the DA isomers in addition to the DA and epi-DA. As the routine LC-UV method (6) only determined the levels of DA and epi-DA, the ASP ELISA appears to give slightly overestimated values because the remaining fraction of DA isomer toxins is taken into account.

We have validated the ASP ELISA rapid assay to assess the method as a suitable alternative to the current reference method LC-UV (6) for the determination of DA in shellfish. The method sample capacity is superior to the LC-UV methods in that the ELISA can analyze 36 samples during a 2 h run. The robustness of the method was demonstrated in that modest alterations of the optimal running conditions did not affect the assay significantly. By investigating the matrix effects from a wide variety of matrixes, we have shown that the ASP ELISA is a potential early-warning tool applicable for phytoplankton analysis, but may also prove to be a useful diagnostic tool. By diluting the shellfish extracts, the unspecific matrix effects were eliminated and did not pose a challenge to the performance of the assay. The LOD and LOQ prove to be the most sensitive compared to previous methods described (6, 9–14, 22, 23). The precision is a bit higher than that for the LC methods (6, 22, 23), but quite comparable to that recently described for an immunobiosensor using surface plasmon resonance technology (14). However, the between-day RSD is still at an acceptable level (9.3%), and the assay demonstrates good stability between production batches over a period of 9 weeks.

The method accuracy was very good with an overall recovery at 102.2% from the CRM spiked samples and was further supported by an excellent correlation slope of 1.070. The higher recovery estimate (>100%) is most likely due to the antibody recognition of DA isomers—a fraction not

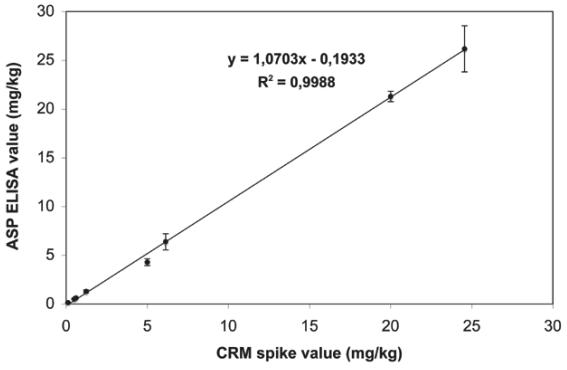

Figure 6. Correlation between shellfish spike values and the corresponding values from ASP ELISA determination. A total of 149 test portions, spiked with certified reference material CRM-ASP-MUS-b, were analyzed with the ASP ELISA in order to obtain an estimate of the method accuracy.

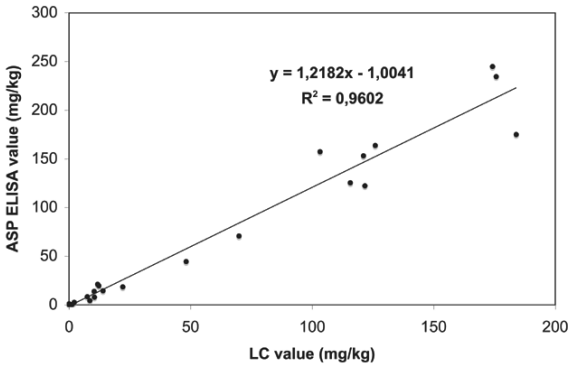

Figure 7. Correlation plot showing ASP ELISA values from analysis of naturally contaminated king scallop samples (*n* = 27), plotted against the corresponding values determined by LC-UV. The LC-UV analysis was performed at Integrin Advanced Biosystems (Oban, Scotland).

accounted for by most LC methods. This was also reported for the recently described immunobiosensor by Traynor et al. (14). This effect has probably also led to the positive bias of 21% observed when the ELISA was compared to LC-UV on the analysis of naturally contaminated scallops. Even though it is possible that the method comparison bias could have been reduced if the LC-UV method was also able to detect the DA isomers, the bias level still seems acceptable because the factors contributing to the variation as the analyses were made in different laboratories on separate sample extracts. The method comparison should ideally be carried out on the same sample extract at the same facility in order to compare the true analytical capabilities. However, the ASP ELISA demonstrated a good degree of correlation (R^2 = 0.960) with the reference LC-UV method, as compared to a recently developed DA immunobiosensor using a surface plasmon resonance system (14). The tendency for the ASP ELISA to slightly overestimate DA levels by providing an estimate of the total DA content will minimize the chance of generating false-negative results in shellfish testing and, thus, can reduce the risk of costly product recalls sometimes experienced by the shellfish industry today. The ASP ELISA holds several advantages over the current LC-UV method in terms of speed, simplicity, and daily sample capacity, and is therefore a promising alternative for on-site testing as part of industrial hazard analysis critical control point (HACCP) systems or local monitoring programs.

The validation study has proved the ASP ELISA to be an accurate and precise method suitable for the routine determination of DA in shellfish samples, and the method has therefore been subjected to a collaborative validation study (25).

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References

(1) Wright, J.L.C., Boyd, R.K., de Freitas, A.S.W., Falk, M., Foxall, R.A., Jamieson, W.D., Laycock, M.V., McCulloch, A.W., McInness, A.G., Odense, P., Pathak, V.P., Quilliam, M.A., Ragan, M.A., Sim, P.G., Thibault, P., Walter, J.A., Gilgan, M., Richard, D.J.A., & Dewar, D. (1989) *Can. J. Chem.* **67**, 481–490

(2) Bates, S.S., Bird, C.J., de Freitas, A.S.W., Foxall, R., Gilgan, M., & Hanic, L.A., Johnson, G.R., McCulloch, A.W., Odense, P., Pocklington, R., Quilliam, M.A., Sim, P.G., Smith, J.C., Subba Rao, D.V., Todd, E.C.D., Walter, J.A., & Wright, J.L.C. (1989) *Can. J. Fish. Aquat. Sci.* **46**, 1203–1215

(3) Bates, S.S., Garrison, D.L., & Horner, R.A. (1998) in *Physiological Ecology of Harmful Algal Blooms*, D.M. Anderson, A.D. Cembella, & G.M. Hallegraeff (Eds), Springer-Verlag, Heidelberg, Germany, pp 267–292

(4) EC Commisson Decision 2002/226/EC, Council Directive 91/492/EEC (2002) *Off. J. Eur. Commun.* **L75**, 62–63

(5) Lawrence, J.F., Charbonneau, C.F., Ménard, C., Quilliam, M.A., & Sim, P.G. (1989) *J. Chromatogr.* **462**, 349–356

(6) Quilliam, M.A., Xie, M., & Haardstaff, W.R. (1995) *J. AOAC Int.* **78**, 543–554

(7) Bates, S.S., de Freitas, A.S.W., Milley, J.E., Pocklington, R., Quilliam, M.A., Smith, J.C., & Worms, J. (1991) *Can. J. Fish. Aquat. Sci*. **48**, 1136–1144

(8) Van Dolah, F.M., Finley, B.L., Doucette, G.J., Moeller, P.D., & Ramsdell, J.S. (1994) *Nat. Toxins* **2**, 189–196

(9) Newsome, H., Truelove, J., Hierlihy, L., & Collins, P. (1994) *Bull. Environ. Contam. Toxicol*. **47**, 329–334

(10) Osada, M., Marks, L.J., & Stewart, J.E. (1995) *Bull. Environ. Contam. Toxicol.* **54**, 797–804

(11) Smith, D.S., & Kitts, D.D. (1995) *J. Agric. Food Chem.* **43**, 367–371

(12) Micheli, L., Radoi, A., Guarrina, R., Massaud, R., Bala, C., Moscone, D., & Palleschi, G. (2004) *Biosens. Bioelectron.* **20**, 190–196

(13) Yu, F.Y., Liu, B.H., Wu, T.S., Chi, F.T., & Su, M.C. (2004) *J. Agric. Food. Chem.* **52**, 5334–5339

(14) Traynor, I.M., Plumpton, L., Terence, L.F., Higgins, C., & Elliott, C.T. (2006) *J. AOAC Int.* **89**, 868–872

(15) Garthwaite, I., Ross, K.M., Miles, C.O., Hansen, R.P., Foster, D., Wilkins, A.L., & Towers, N. (1998) *Nat. Toxins* **6**, 93–104

(16) Young, M., Smith, E.A., Garthwaite, I., Gallacher, S., & Scott, A.C. (2002) in *Book of Abstracts,* 4th International Conference on Molluscan Shellfish Safety, Santiago de Compostela, Spain, p. 154

(17) Biosense Laboratories AS (2003) *A31300401-ASP ELISA User Manual,* Biosense Laboratories AS, Bergen, Norway, www.biosense.com

(18) Fehling, J., Davidson, K., Bolch, C.J., & Bates, S.S. (2004) *J. Phycol.* **40**, 674–683

(19) Wernimont, G.T. (1996) *Use of Statistics to Develop and Evaluate Analytical Methods*, 5th Ed., AOAC INTERNATIONAL, Gaithersburg, MD

(20) Holland, P.T., Selwood, A.I., Mountfort, D.O., Wilkins, A.L., McNabb, P., Rhodes, L.L., Doucette, G.J., Mikulski, C.M., & King, K.L. (2005) *Chem. Res. Toxicol.* **18**, 814–816

(21) Eurachem Guide (1998) *The Fitness for Purpose of Analytical Methods, A Laboratory Guide to Method Validation and Related Topics*, http://www.eurachem.ul.pt

(22) Hess, P., Gallacher, S., Bates, L.A., Brown, N., & Quilliam, M.A. (2001) *J. AOAC Int.* **84**, 1657–1668

(23) Holland, P. (2003*) Method Validation Report-ASP Toxins in Shellfish by LC-MS, Method 40.104*, Cawthron Institute, Nelson, New Zealand

(24) *Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements* (2003) AOAC INTERNATIONAL, Gaithersburg, MD

(25) Kleivdal, H., Kristiansen, S.I., Nilsen, M.V., Goksøyr, A., Briggs, L., Holland, P., & McNabb, P. (2007) *J. AOAC Int.* **90**, 1011–1027

FOOD CHEMICAL CONTAMINANTS

Determination of Domoic Acid Toxins in Shellfish by Biosense ASP ELISA—A Direct Competitive Enzyme-Linked Immunosorbent Assay: Collaborative Study

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A collaborative study was conducted on the Biosense amnesic shellfish poisoning (ASP) enzyme-linked immunosorbent assay (ELISA) for the determination of domoic acid (DA) toxins in shellfish in order to obtain interlaboratory validation data for the method. In addition, a method comparison study was performed to evaluate the ASP ELISA as an alternative to the current liquid chromatography (LC) reference method for DA determination. The study material comprised 16 shellfish samples, including blue mussels, Pacific oysters, and king scallops, spiked with contaminated mussel homogenates to contain 0.1–20 mg DA/kg shellfish flesh. The shellfish samples were extracted with 50% aqueous methanol, and the supernatants were directly analyzed. Sixteen participating laboratories in 10 countries reported data from the ASP ELISA, and 4 of these laboratories also reported data from instrumental LC analysis. The participating laboratories achieved interlaboratory precision estimates for the 8 Youden paired shellfish samples in the range of 10–20% for RSD_r (mean 14.8 ± 4%), and 13–29% for RSD_R (mean 22.7 ± 6%). The precision estimates for the ELISA data did not show a strong dependence on the DA concentration in the study samples, and the overall precision achieved was within the acceptable range of the Horwitz guideline with HorRat values ranging from 1.1 to 2.4 (mean HorRat 1.7 ± 0.5). The analysis of shellfish samples spiked with certified reference material (CRM)-ASP-MUS-b gave recoveries in the range of 88–122%, with an average recovery of 104 ± 10%. The estimate on method accuracy was supported by a correlation slope of 1.015 (R^2 = 0.992) for the determined versus the expected DA values. Furthermore, the correlation of the ASP ELISA results with those for the instrumental LC analyses of the same sample extracts gave a correlation slope of 1.29 (R^2 = 0.984). This indicates some overestimation of DA levels in shellfish by the ELISA, but it is also a result of apparent low recoveries for the LC methods. This interlaboratory study demonstrates that the ASP ELISA is suitable for the routine determination and monitoring of DA toxins in shellfish, and that it offers a rapid and cost-effective methodology with high sample throughput.

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Following the increasing awareness of the impact of harmful algal blooms on public health and the economy (1), an increasing number of marine samples will need to be analyzed on a routine basis. This is a challenge to the sample capacity of the test method regimens existing today and will be faced by the industry, regulatory authorities, and environmental surveillance agencies in the near future. The marine biotoxin content in shellfish intended for human consumption has traditionally been determined using mouse bioassays or time-consuming instrumental analysis. The analysis of shellfish for the domoic acid (DA) toxins causing amnesic shellfish poisoning (ASP) has been performed by several analytical methods based on liquid chromatography-ultraviolet detection (LC-UV), LC/mass

spectrometry (MS) and LC-fluorescence detection (2–8). The most frequently used method, described by Quilliam et al. (5), has been recommended as the international reference method by the Joint Food and Agriculture Organization of the United Nations/Intergovernmental Oceanographic Commission/World Health Organization (FAO/IOC/WHO) ad hoc Expert Consultation for Codex in Oslo, Norway (9). Although LC-UV analysis is accurate and provides high-resolution profiles, the instrumentation is expensive with high maintenance costs and requires highly skilled operators and a well-established laboratory infrastructure. Furthermore, the LC-UV method has a low daily sample throughput, due to the cleanup procedure and instrument run time. It is most often implemented in a centralized laboratory that requires the shipment of samples from the point-of-problem (the shellfish processing facility), adding significant delays due to logistics. As more legislations demanding in-process controls are being implemented by Food Safety Authorities, the number of marine biotoxin samples increases together with a growing need for more cost-effective and rapid alternative assays which allow real-time monitoring on-site, in accordance with the Hazard Analysis Critical Control Point (HACCP) concept. As a consequence of the immediate requirement for rapid assays, there has been an ongoing development of alternative methods to the traditional instrumental analysis during the last decade. Assays described for DA determination include a receptor-binding assay (10) and immunological assays based on enzyme-linked immunosorbent assay (ELISA) technology (11, 12). Some ELISA methods have been described for the analysis of shellfish, using monoclonal and polyclonal antibodies (13–15), but none of these studies reported collaborative validation data.

An indirect competitive ELISA for DA described by Garthwaite et al. (16) appeared to be appropriate for further refinement and laboratory validation. Recently, this ELISA was converted at AgResearch into a direct format using the same polyclonal ovine anti-DA antibodies, and was subsequently subjected to a single-laboratory validation (SLV) study at Biosense according to AOAC and Eurachem guidelines (17). The SLV results indicated that the ASP ELISA was accurate and reliable for DA toxin analysis in shellfish (18) and therefore suitable for a collaborative study. Prior to this study, the production of all assay components was carried out under strict quality control to ensure that the ASP ELISA kits produced were standardized under well-defined quality assurance criteria.

In the Biosense ASP ELISA kit, free DA in the sample competes with DA-conjugated bovine serum albumin (BSA) coated on plastic microtiter wells for binding to anti-DA antibodies free in the solution. The ovine antibodies are conjugated to horseradish peroxidase (HRP). Samples diluted in buffer are incubated in the wells with the anti-DA-antibody–HRP conjugate. After washing, the amount of conjugate remaining bound to the well is measured by incubation with a substrate that gives a blue product upon reaction with the HRP enzyme. Addition of acid stops the reaction and changes the product color from blue to yellow. The intensity is measured spectrophotometrically using a plate-reader at 450 nm, and is inversely proportional to the concentration of DA toxins in the sample solution. The assay is calibrated using dilutions of a standard solution of DA supplied with the assay kit. Each ASP ELISA kit can analyze up to 36 individual sample dilutions in duplicate, plus calibration solutions, controls, and blanks.

The present collaborative validation data strongly suggest that the described Biosense ASP ELISA is a suitable alternative to LC-UV as it offers advantages in sample turnaround and accessibility over the instrumental methods. Even though a high-quality analysis with an LC-UV system will provide accurate high-resolution profiles with better method precision, such methodology will require a costly facility with high running costs and training skills. The ASP ELISA can easily be operated in small, regional laboratories with little investment required, and will be a practical way of identifying negative samples as part of an effective HACCP system. The ASP ELISA uses a simple 50% aqueous methanol sample extraction protocol with no need to perform any cleanup or preconcentration steps as required for most LC protocols (5). Consequently, the ELISA has a significantly higher daily sample turnaround than most LC-UV systems, and can quantify 36 samples in about 2 h.

Collaborative Study

Certified Reference Material (CRM) and Calibration Solution

The National Research Council of Canada's Certified Reference Materials Program (NRC-CRMP) provided the CRM used in the study. The CRM-ASP-MUS-b is a naturally contaminated blue mussel homogenate certified to contain 39 ± 1 mg/kg DA + epi-DA, and the calibration solution CRM-DA-d (formerly DACS-1D) is certified to contain 87.7 ± 0.9 μg/mL DA + epi-DA. In addition to the DA and

Table 1. Calculation of DA content in CRM-DA-d and CRM-ASP-MUS-b (data compiled from NRC certificate of analysis)

	CRM-DA-d, μg/mL	CRM-ASP-MUS-b, μg/g
DA	86.4	36.0
Epi-DA	1.3	3.0
Epi-DA + DA certified content	87.7	39.0
Iso E[a]	0.24	3.2
Iso D[a]	0.80	1.8
Iso A[a]	1.8	NM[b]
Iso F[a]		0.2
Estimated total DA[a]	90.54	44.2

[a] Isomer values are not certified.

[b] NM = Not measured.

epi-DA, each of these CRMs contained an estimated amount of DA isomers, as shown in Table 1. Although these values are not certified, the sum of DA, epi-DA, and the listed DA isomers was used when calculating spike levels. The calibration standard solution (100 ng/mL DA + epi-DA) shipped with the kits as a sealed 1 mL ampule was prepared by dilution of CRM-DA-d (87.7 ± 0.9 μg/mL total DA toxins) into acetonitrile–water (1 + 9, v/v).

Study Design

The emphasis in the choice of the 16 spike recovery samples, which included flesh of blue mussels (*Mytilus edulis*), Pacific oysters (*Crassostrea gigas*), and king scallops (*Pecten maximus*), was to include the 3 major commercial shellfish species worldwide (*see* Table 2). The 16 individual samples were designed as 8 Youden pairs (split levels) of shellfish tissues (3 mussel, 3 scallop, and 2 oyster), fortified with mussel material containing DA toxins at levels from the regulatory limit at 20 mg/kg down to 0.1 mg/kg.

The spiked Youden pairs were prepared by a gravimetrically measured fraction of blank, precooked shellfish homogenate that was blended with a fraction of the NRC CRM-ASP-MUS-b, calculated to give 200 g homogenate containing the designated spike level. The blended homogenate was further homogenized using an ultrahomogenizer for 3 min, before aliquots of 4.00 g spiked homogenate were transferred to a 50 mL centrifuge tube (enough for a single sample extraction), sealed, marked with a random code number, and frozen at –80°C. The lower spiked shellfish homogenates of Youden split level pairs were prepared by adding an additional fraction of blank homogenate to the higher spike level. Following preparation of the shellfish study samples, a quality control was performed on at least 5% of the sample aliquots to ensure that the DA toxin concentration homogeneity was within the ASP ELISA single-laboratory repeatability precision (RSD_r) at 9%. The between-sample standard deviations were demonstrated to be negligible, as they did not affect the method's single-laboratory repeatability precision. A set of 5 familiarization samples, including blanks of each shellfish species, was also prepared for practice and to quality assure that the method was well established before the study samples were analyzed.

Shipment of the Study Material

All sample materials were shipped to the participants on dry ice, sufficient to keep the contents frozen for up to 5 days in room temperature conditions. The collaborators were to report the temperature and general conditions of the samples and kits once they arrived, before all sample materials were transferred to a conventional –20°C freezer until the day of analysis. Four of the 16 participating laboratories received the samples completely defrosted, and 3 full replacement sample shipments were processed successfully. The identity and content of the study samples were not released to the collaborators.

Organization of the Collaborative Study

The 16 participating laboratories from 10 different countries in Europe, North America, South America, and New

Table 2. Study sample spike levels (actual spike levels based on NRC CRMP certificate of analysis)

Shellfish	Sample	Estimated level [DA + epi-DA + iso-DA E, D, F, A], mg/kg	Certified level [DA + epi-DA], mg/kg
Blue mussels	S6	19.50	17.20 ± 0.44
	S14	20.20	17.80 ± 0.46
	S2	8.20	7.24 ± 0.19
	S7	8.60	7.59 ± 0.19
	S10	1.15	1.01 ± 0.026
	S16	1.10	0.97 ± 0.025
Scallops	S1	0.125	0.11 ± 0.003
	S4	0.12	0.106 ± 0.003
	S3	7.80	6.88 ± 0.18
	S8	7.50	6.62 ± 0.17
	S9	3.45	3.04 ± 0.078
	S11	3.30	2.91 ± 0.075
Oysters	S5	0.53	0.47 ± 0.012
	S12	0.51	0.45 ± 0.012
	S13	7.795	6.97 ± 0.18
	S15	8.091	7.24 ± 0.19

Zealand represented both regulatory authorities with monitoring responsibilities through government organizations or industry and academic institutions. The majority of the collaborators were familiar with DA analysis by LC-UV, but few were familiar with the ELISA technique.

For the collaborative trial, each participant received six coded samples of spiked blue mussels (3 Youden pairs spiked at 1.10, 1.15, 8.20, 8.60, 19.5, and 20.2 mg/kg); 6 coded samples of spiked king scallops (3 Youden pairs spiked at 0.120, 0.125, 3.30, 3.45, 7.50, and 7.80 mg/kg); 4 coded samples of spiked Pacific oysters (2 Youden pairs spiked at 0.510, 0.530, 7.90, and 8.20 mg/kg); 1 blank sample of each shellfish species; 2 practice samples of spiked blue mussels at 0.5 and 5.0 mg/kg; 10 ASP ELISA kits (5 spare kits); 1 amber vial of standard reference solution NRC-CRM-DA-d for the collaborators carrying out the parallel analyses by an LC method; ASP ELISA method protocol; a comprehensive study protocol with details on the following aspects: the practice samples to be carried out prior to the study, a recommended sequence for analysis of the main study samples, the reporting of results, the statistical analysis of the study data, and the reporting of data; an Excel macro for the analysis and reporting of data; and a data reporting form for the LC method analysis.

Analysis

Participants were instructed to analyze the Youden paired samples in numerical sequence on separate days. Each participant was required to prepare one extract (1 + 5, w/v, i.e., adding 16 mL to the preweighed 4 g sample) from each study material, and analyze the extract following the ASP ELISA method procedure. An ancillary method comparison study was conducted by 4 of the 16 participating laboratories. The 4 collaborators analyzed the same sample extracts in parallel with the ASP ELISA and their established LC methods. The spike levels for the study samples were based on the estimated level of total DA content in the CRM-ASP-MUS-b as described by the NRC CRMP. For the instrumental LC methods only, the certified level of DA + epi-DA was used to calculate the expected values.

Reporting of Data and Calculation

After completion of the main ELISA and ancillary LC studies, the collaborators forwarded their spreadsheets by e-mail to Biosense and Cawthron Institute. The results for each ELISA plate were contained in a spreadsheet which includes a macro and calculations to fit the calibration data and report concentrations of DA in the wells (ng/mL), extract (ng/mL), and shellfish tissue (mg/kg). The sheets were checked for obvious errors in sample naming, dilutions, and calculations. In some cases, the macro fitting the calibration curve had not worked correctly. The Study Director adjusted the macro to ensure the best fit of the calibration curve and forwarded the recalculated sheets to Cawthron. A number of laboratories had difficulty meeting all the quality control (QC) criteria for calibration of their plates or had samples where the extracts had not been sufficiently diluted to bring the results into the calibration range. In some cases, these laboratories provided additional spreadsheets containing repeat data for such samples or plates.

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Domoic Acid Toxins in Shellfish
Biosense ASP ELISA—A Competitive Direct Enzyme-Linked Immunosorbent Assay
First Action 2006

[Applicable to the determination of domoic acid (DA) toxins at levels >0.12 mg/kg in shellfish (mussels, scallops, oysters), with a method limit of detection (LOD) at 0.003 mg/kg and limit of quantitation (LOQ) at 0.009 mg/kg.]

Caution: DA is a neurotoxin that is harmful by inhalation and ingestion. Avoid contact with skin, eyes, and clothing. Wash hands thoroughly after handling. DA is subject to light degradation. Protect analytical work adequately from the daylight, and keep DA standard solutions protected from light by using amber glass vials or aluminium foil. Methanol and sulfuric acid are hazardous. Refer to the manufacturer's Material Safety Data Sheet for handling these reagents.

See Table **2006.02A** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

Shellfish flesh samples are homogenized, and 4 g homogenate is extracted with 16 mL of extraction solvent according to the method of Quilliam et al. (5). After centrifugation the sample extracts are diluted to a specified solvent concentration and added to the precoated Biosense amnesic shellfish poisoning (ASP) enzyme-linked immunosorbent assay (ELISA) microtiter wells. In the direct competitive ELISA, the free DA in the sample competes with DA-conjugated protein coated on the plastic wells for binding to anti-DA antibodies also added to the wells. The mixture is incubated for 1 h to allow the competitive binding step to reach an equilibrium. After all unbound components are washed away, a given portion of the antibodies conjugated to the reporter moiety horseradish peroxidase (HRP) is bound to the immobilized DA-conjugated protein. After washing, the amount of conjugate remaining bound to the well is measured by incubation with a substrate that gives a blue product upon reaction with the HRP enzyme. Addition of acid stops the reaction and changes the product color from blue to yellow. The intensity is measured spectrophotometrically on a plate-reader at 450 nm, and is inversely proportional to the concentration of DA in the sample solution. The assay is calibrated using dilutions of a standard solution of DA supplied with the assay kit. The absorbance values of the 10 calibration points are used to prepare a calibration curve by 4-parameter logistics analysis. The absorbance developed after addition of a given sample can be used to calculate the concentration of DA in the sample based on the defined working range of the calibration curve. Each 96 well plate can

Table 2006.02A. Results of interlaboratory study for determination of domoic acid toxins in shellfish by Biosense ASP ELISA

Youden pair	Matrix	Labs[a,b]	Mean, mg/kg	Recovery, %	S_r	RSD_r, %	S_R	RSD_R, %	HoRat[c]
S6–S14	Mussel	12 (0)	19.48	98.1	2.1	10.7	2.6	13.5	1.3
S2–S7	Mussel	12 (1)	9.43	112.4	1.4	15.3	2.0	21.5	1.9
S13–S15	Oyster	14 (0)	8.79	109.1	1.4	16.4	2.4	27.8	2.4
S3–S8	Scallop	12 (1)	7.63	99.8	0.84	11.0	1.5	20.0	1.7
S9–S11	Scallop	13 (0)	3.38	100.1	0.54	15.9	0.93	27.6	2.1
S10–S16	Mussel	13 (0)	1.14	101.0	0.17	14.9	0.20	17.3	1.1
S5–S12	Oyster	13 (0)	0.63	122.0	0.13	20.8	0.19	29.4	1.7
S1–S4	Scallop	13 (0)	0.11	88.0	0.015	13.5	0.027	24.8	1.1
Avg. recovery, %				103.9 ± 10					
Avg. RSD_r, %						14.8 ± 3			
Avg. RSD_R, %								22.7 ± 6	
Avg. HorRat									1.7 ± 0.5

[a,b] a = Number of laboratory data retained for analysis; b = value in parantheses is number of Cochran outlier laboratories.

[c] HorRat parameter may not be applicable for enzyme reactions (19), but is included by convention as an indication of method performance.

quantify 36 individual sample dilutions in duplicate, calibration standards, controls, and blanks.

B. Performance Standards

The ELISA uses antibodies raised against DA-conjugate immunogens, and DA-plate coater conjugates according to Garthwaite et al. (16). The defined calibration curve parameters (A_{max}, I_{20}, I_{50}, I_{80}, slope, A_{min}) should be within the required quality control guidelines as specified in the Biosense ASP ELISA kit protocol (Table **2006.02B**).

C. Apparatus

(**a**) *Blender or ultrahomogenizer.*

(**b**) *Microtiter plate absorbance reader.*—With 450 nm filter.

(**c**) *Benchtop centrifuge.*—50 mL tubes at 3000 × *g*.

(**d**) *Analytical balance.*—Sensitivity of 0.01 g.

(**e**) *Vortex minishaker.*—0–2500 rpm.

(**f**) *Volumetric glassware.*

(**g**) *Centrifuge tubes.*—50 mL; polypropylene.

(**h**) *Microcentrifuge polypropylene tubes.*—1.5 mL.

(**i**) *Variable volume pipettors and tips (as described or equivalent).*—Positive displacement pipet for dispensing methanolic extracts, single-channel pipets for standard and sample dilutions, and multichannel pipet or stepper for dispensing (antibody, substrate, etc.). All pipets should be routinely calibrated according to the manufacturer's recommendations.

D. Reagents

(**a**) *Methanol.*—LC grade.

(**b**) *Acetonitrile.*—HPLC grade.

(**c**) H_2SO_4.—0.3 M solution.

(**d**) *Water.*—Distilled or pretreated and passed through water purification system.

(**e**) *Extraction solvent.*—Methanol–water solution (1 + 1, v/v).

(**f**) *Phosphate-buffered saline with Tween 20 (PBST).*—pH 7.4.

(**g**) *Standard/sample buffer.*—10% methanol in PBST.

(**h**) *Antibody-HRP ovalbumin buffer.*—1% ovalbumin in PBST.

(**i**) *DA calibration solution (as described or equivalent).*—The working calibrant solution for the ASP ELISA is prepared by dilution of the NRC-CRM-DA-d solution (certified level of DA + epi-DA at 90.5 µg/mL) in acetonitrile–water (1 + 9, v/v) to a final concentration of 100 ng/mL [DA + epi-DA; National Research Council of Canada's Certified Reference Materials Program (NRC CRMP), Halifax, Canada, http://imb-ibm.nrc-cnrc.gc.ca].

Table 2006.02B. System suitability requirements for valid calibration curves

Parameter for sigmoid calibration curve	Requirement
Maximum absorbance (A_{max})	>0.8 AU
Blank and A_{min}	<0.1 AU
Calibration curve I_{20} value	6–20 pg/mL
I_{50} value	35–80 pg/mL
I_{80} value	180– 50 pg/mL

E. Extraction and Dilution of Shellfish Samples

(*1*) Thoroughly clean the outside of the shellfish with fresh water; then open the shellfish and homogenize no less than 50 g shellfish flesh until homogeneous. Accurately weigh 4.00 g (±0.10 g) of the homogenized shellfish into a 50 mL polypropylene centrifuge tube. Add 16 mL extraction solution (50% aqueous methanol), mix well on a Vortex mixer for 2 min, and centrifuge for 10 min at 3000 × *g* at room temperature. Retain the supernatant for further dilution prior to analysis.

(*2*) Prepare dilutions of extract supernatants in standard/sample buffer (10% methanol in PBST) using micropipets and microcentrifuge tubes as follows: 1:20 dilution: 50 µL shellfish extract + 950 µL buffer; 1:200 dilution: 50 µL of the 1:20 dilution + 450 µL buffer; 1:2000 dilution: 50 µL of the 1:200 dilution + 450 µL buffer; 1:20 000 dilution: 50 µL of the 1:2000 dilution + 450 µL buffer; 1:200 000 dilution: 50 µL of the 1:20 000 dilution + 450 µL buffer.

Cap and mix each dilution on a Vortex mixer before proceeding to the next dilution step.

(*3*) Analyze the sample dilutions according to the DA concentration range of interest (*see* Table **2006.02C**) to give absorbance values within the calibration curve working range. Shellfish extracts should be diluted at least 1:200 in standard/sample buffer before analysis in order to avoid matrix effects (nonspecific assay interferences due to other extract components). It is recommended to analyze shellfish extracts routinely at 1:20 000 dilutions for the quantification of DA from 1 to 25 mg/kg. This covers the maximum permitted limit of 20 mg/kg which is commonly set internationally. A further 1:200 000 dilution should also be included for the quantification of DA up to 250 mg/kg according to Table **2006.02C**.

F. Preparation of Domoic Acid Toxins Calibration Solution

The 10-point calibration curve solutions are prepared from a freshly opened DA standard glass ampule (vial D) containing 100 ng/mL DA + epi-DA (diluted CRM-DA-d calibration solution). The standard dilutions are prepared in the range of 10 000–0.16 pg/mL.

(*1*) Prepare one Eppendorf tube containing 450 µL standard/sample buffer (10% methanol in PBST; tube 1) and 9 Eppendorf tubes containing 300 µL standard/sample buffer (tubes 2–10).

(*2*) Add 50 µL of the calibration solution provided in vial D to tube 1 and mix on a Vortex mixer to obtain a 10 ng/mL DA toxin solution.

Table 2006.02C. Recommended shellfish extract sample dilutions for quantification

DA concentration range[a], mg/kg	Sample extract dilution
0.01–0.25	1:200
0.1–2.5	1:2000
1.0–25	1:20000
10–250	1:200000

[a] Based on a dynamic assay working range of 10–250 ng/mL.

(*3*) With a clean tip, transfer 125 µL of the 10 ng/mL solution (tube 1) to tube 2 and mix on a Vortex mixer.

(*4*) Complete the 3.4-fold dilution series by transferring 125 µL from tube 2 to tube 3 and mix on a Vortex mixer. Repeat this step for all tubes 3–10 to give the concentrations listed in Table **2006.02D**.

G. ELISA Procedure

(*1*) Equilibrate the precoated plate strips and all reagents to room temperature before use (1 h maximum), sufficient to run the number of samples required. *See* Figure **2006.02A** for the recommended plate layout for either using 2 strips for the calibration curve, and either 2 or 6 strips for the diluted samples.

(*2*) Open the sealed pouches containing the precoated 12 well strips gently, and place the strips in the plate frame. Label each strip, e.g., A, B, C, etc.

(*3*) Add 300 µL washing buffer (PBST) to each well. Presoak the wells for 5–10 min.

(*4*) Remove the washing buffer by inverting the plate frame over a sink and tap against a pile of paper towels to remove the remaining liquid completely before proceeding to the next step.

(*5*) Add 50 µL standard/sample buffer (10% methanol in PBST) to each of the duplicate A_{max} and blank wells in strips A and B, according to the suggested plate layout (Figure **2006.02A**).

(*6*) Add 50 µL blank antibody-HRP ovalbumin buffer (1% ovalbumin in PBST) to the blank wells.

(*7*) Add 50 µL of each DA calibration dilution to each of 2 wells in strips A and B.

(*8*) Add 50 µL of the 1:20 000 dilution from each sample to each of 2 wells in strips intended for the samples (C and D, E and F, etc.).

(*9*) Shake the vial containing concentrated anti-DA-HRP conjugate briefly, and tap the vial gently on a hard surface to

Table 2006.02D. Domoic acid toxin calibration solutions prepared

Tube	1	2	3	4	5	6	7	8	9	10
pg DA toxins/mL	10000	2941	865	254	75	22	6.5	1.9	0.56	0.16

	1	2	3	4	5	6	7	8	9	10	11	12
A	10000 pg/ml	2941 pg/ml	865 pg/ml	254 pg/ml	75 pg/ml	22 pg/ml	6,5 pg/ml	1,9 pg/ml	0,56 pg/ml	0,16 pg/ml	A_{max}	Blank
B	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C	Sample **1** 1:20 000	Sample **2** 1:20 000	Sample **3** 1:20 000	Sample **4** 1:20 000	Sample **5** 1:20 000	Sample **6** 1:20 000	Sample **7** 1:20 000	Sample **8** 1:20 000	Sample **9** 1:20 000	Sample **10** 1:20 000	Sample **11** 1:20 000	Sample **12** 1:20 000
D	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓

Figure 2006.02A. Layout recommended for using 4 strips to analyze 12 individual samples for the quantification of DA in shellfish samples in the range of 1–25 mg DA/kg (1:20 000 dilution).

ensure that all the content is in the bottom of the vial. Transfer 0.5 mL (for a 4 strip assay) or 1.0 mL (for an 8 strip assay) from vial E to a 15 mL polystyrene tube containing 2.5 mL (for a 4 strip assay) or 5.0 mL (for an 8 strip assay) antibody-HRP ovalbumin buffer (1% ovalbumin in PBST).

(*10*) Add 50 µL of the diluted anti-DA-HRP conjugate to all wells except the blank wells.

(*11*) Seal the strips with the self-adhesive plate-sealer and incubate at room temperature (20–25°C) for 1 h. Protect from light (e.g., cover with aluminium foil or place in a drawer).

H. Determination by Colorimetric Reaction

(*1*) Carefully remove the plate cover. Wash the wells 4 times with 300 µL washing buffer per well.

(*2*) Add 100 µL tetramethyl benzidine peroxidase substrate to all wells. Cover the plate with the plate sealer, and incubate at room temperature (20–25°C) for exactly 15 min. Protect from light.

(*3*) Carefully remove the plate sealer. Stop the reaction by adding 100 µL 0.3 M H_2SO_4 to all wells.

(*4*) After 2–5 min, read the absorbance in a microtiter plate reader using a 450 nm filter.

I. Calculations

(**a**) *Calibration using the 4-parameter logistics curve fit model*.—Plot the absorbance values (A_{450}) of the 10 standard dilutions on a linear scale (*y*-axis) against the DA concentrations of the standard dilutions on a logarithmic scale (*x*-axis) to obtain a sigmoid (S-shaped) curve. Use the 4-parameter logistic curve fitting model (e.g., the Excel macro provided with the ASP ELISA) to obtain a fitted sigmoid calibration curve (Figure **2006.02B**). The following equation is given for a 4-parameter fitted curve:

$$y = (a - d)/[1 + (x/c)^b] + d$$

where *x* is the concentration of DA in the standard/sample; *y* is the absorbance of the standard/sample; a is the *y*-value of the

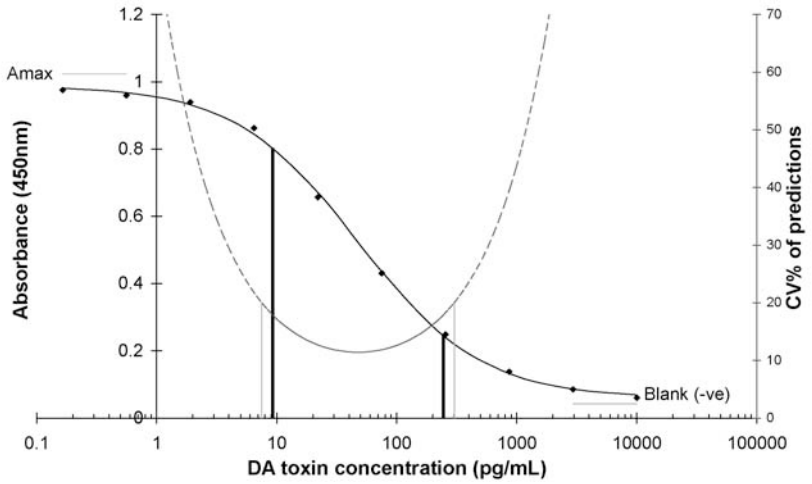

Figure 2006.02B. Representative example of an ASP ELISA calibration curve fitted with 4-parameter logistics. Bold vertical lines define the working range of this particular calibration curve.

Table 2006.02E. ASP ELISA limit of detection

Matrix	Mean, pg/mL (n = 10)	SD	Blank + 3 × SD	Matrix extraction and dilution factor	Matrix LOD, μg/kg	Mean matrix LOD, μg/kg
Blue mussels	1.149	0.789	3.516	(5 × 200)	3.51	
Scallops, whole	1.473	1.304	5.385	(5 × 200)	5.39	3.3
Pacific oysters	0.549	0.185	1.104	(5 × 200)	1.10	

upper asymptote (A_{max}); b is the relative slope of the curve at its center; c is the x-value at the midpoint of the curve (I_{50}); d is the y-value of the lower asymptote (blank/A_{min}).

(**b**) *Calculation formula.*—The following formula is used to convert the ELISA results in pg/mL to DA concentrations in shellfish given in mg/kg:

$$\text{mg DA/kg} = \mu\text{g DA/g} = [\text{pg DA/mL} \times \text{D} \times \text{V} \times (1\ \mu\text{g}/1\ 000\ 000\ \text{pg})]/\text{M}$$

where pg DA/mL is the calculated concentration of DA toxins in the diluted extract; D is the dilution factor of the diluted extract; V (mL) is the volume of the methanolic extract (16 mL + 4 g homogenate giving the nominal 20 mL total volume); M (g) is the mass of the shellfish homogenate (4 g).

(**c**) *Critical control points and system suitability requirements for acceptability of an assay.*—(*1*) In order to qualify as a valid sigmoid calibration curve, defined parameter criteria for the anchoring points in the asymptotic curve (A_{max}, A_{min}, I_{20}, I_{50}, and I_{80}) must be met for the individual assay calibration curve (Table **2006.02B**). (*2*) The valid working range of the calibration curve suitable for quantification of DA is defined by the most narrow concentration range between I_{20} and I_{80} values, and the part of the calibration curve where the %CV estimate from the predicted calibration curve fit is <20%. (*3*) The concentration variation between sample duplicate well values must be <15%.

J. ASP ELISA Limits of Detection and Quantitation

Limit of detection (LOD).—The LOD has been calculated based on the measurement of 3 blank shellfish matrixes, based on the "blank + 3 × SD" approach according to Eurachem guidelines (17). Based on 10 independent sample preparations of the sample blanks, the standard deviation (SD) of the blanks was calculated (Table **2006.02E**). From these numbers, the LOD was calculated for shellfish (18).

Mean shellfish LOD = blank + 3 × mean SD = 0.0033 mg/kg shellfish

Limit of quantitation (LOQ).—Based on the data set for the determination of LOD (Table **2006.02E**), the LOQ was calculated based on the "blank + 10 × SD" approach according to Eurachem guidelines (17).

Mean shellfish LOQ = blank + 10 × mean SD = 0.0085 mg/kg shellfish

Reference: *J. AOAC Int.* **90**, 1011(2007).

Results and Discussion

Collaborative Study Results

All 16 collaborators who received the study material completed the study and reported results. The laboratories first carried out their own analysis with the familiarization samples to ensure that the method was properly established. The Study Director assisted where there were problems meeting the quality assurance criteria for the calibration curves. After completion of the main ELISA and ancillary LC studies to their own satisfaction, participating laboratories forwarded their data reporting spreadsheets by e-mail to Biosense and Cawthron Institute. The reported results for each ELISA plate were contained in a spreadsheet which included a macro and calculations to fit the calibration data and report concentrations of DA toxins in the wells (pg/mL), extract (pg/mL), and shellfish tissue (mg/kg).

The reported spreadsheets were checked for obvious errors in sample naming, dilutions, and calculations. In some cases, the macro fitting the calibration curve had not worked correctly due to software incompatibility problems, and the Study Director then adjusted the macro and forwarded the recalculated report sheet to Cawthron Institute for further analysis. Of the 16 collaborators (coded as A–P), 4 laboratories had difficulty meeting the QC criteria (Table **2006.02B**) for calibration with either one or more of the 4 plates included in the study, or had samples where the extracts had not been sufficiently diluted to bring the results into the valid calibration working range. Three of these laboratories provided additional spreadsheets containing repeat data for such samples or plates. In 2 cases, the water from the participants' water purification system caused high background levels for the calibration curve (high A_{min}). There were no other indications of reduced water quality, but when the water source was changed, the high background levels were eliminated and the calibration curves were approved. Laboratory I had a systematic problem with their absorbance plate reader giving high background, and Laboratory P obtained consistently low I_{20}, I_{50}, and I_{80} values for the sigmoidal calibration curves. Table 3 shows the calibration curve data for the 3 plates used for the analysis of the reported sample values S1 to S16 for all the collaborators. Data that were >10% outside the QC criteria for acceptable calibration

Table 3. Summary of ASP ELISA calibration curve parameters

		Significant parameters (4-p logistics) working range						Calibration curve fit		
Lab	Standard curve	Slope	Maximum	Minimum	I_{50}, pg/mL	I_{20}, pg/mL	I_{80}, pg/mL	Mean %CV data points	Minimum %CV	ng/mL range were %CV <20%
A	1 (S1–S6)	0.796	0.952	0.000	61.5	10.8	351.0	29.8	34.34	None[b]
	2 (S7–S12)	1.019	0.883	0.075	52.5	13.5	204.6	63.0	22.42	None[b]
	3 (S13–S16)	0.870	0.807	0.000	46.3	9.4	227.6	22.3	23.78	None[b]
	Repeat	1.113	0.875	0.082	56.7	16.3	197.1	22.7	17.44	0.03–0.11
B	1 (S1–S6)	0.790	0.752	0.049	79.0	13.6	456.8	20.8	18.13	0.04–0.18
	2 (S7–S12)	0.921	0.792	0.067	74.8	16.6	336.7	22.1	7.96	0.01–0.71
	3 (S13–S16)	0.976	0.849	0.066	80.6	19.5	333.2	11.9	13.25	0.02–0.31
C	1 (S1–S6)	0.752	1.142	0.055	43.3	6.8	273.8	18.7	12.63	0.01–0.28
	2 (S7–S12)	0.756	0.976	0.051	72.6	11.6	454.5	16.7	7.37	0.00–1.28
	3 (S13–S16)	0.806	1.045	0.051	46.5	8.3	259.8	16.6	14.02	0.01–0.21
D	1 (S1–S6)	0.904	0.839	0.043	74.7	16.1	346.2	27.9	14.61	0.02–0.27
	2 (S7–S12)	0.904	0.839	0.043	74.7	16.1	346.2	27.9	14.61	0.02–0.27
	3 (S13–S16)	0.900	0.852	0.050	69.4	14.9	323.8	7.9	6.12	0.00–0.99
E	1 (S1–S6)	0.967	0.907	0.059	54.1	12.9	226.8	34.1	19.10	0.03–0.08
	2 (S7–S12)	0.812	0.900	0.033	44.0	8.0	242.3	13.2	6.06	0.00–0.85
	3 (S13–S16)	0.776	0.969	0.041	37.9	6.4	226.6	10.1	4.61	0.00–1.28
F	1 (S1–S6)	0.780	0.749	0.051	47.0	7.9	278.0	16.8	8.39	0.00–0.61
	2 (S7–S12)	0.849	0.843	0.052	59.8	11.7	305.6	9.9	7.25	0.00–0.79
	3 (S13–S16)	0.783	0.889	0.047	60.6	10.3	355.9	3.6	8.15	0.00–0.82
G	1 (S1–S6)	0.836	0.984	0.078	48.0	9.1	251.7	ND[a]	16.28	0.02–0.14
	2 (S7–S12)	0.687	0.993	0.043	45.0	6.0	338.3	15.2	23.22	None[b]
	3 (S13–S16)	0.838	1.064	0.076	41.5	7.9	217.2	27.1	10.61	0.01–0.31
H	1 (S1–S6)	0.856	0.958	0.078	56.8	11.2	287.2	13.1	6.41	0.00–0.88
	2 (S7–S12)	0.812	1.123	0.088	44.9	8.1	247.7	6.9	2.43	0.00–3.07
	3 (S13–S16)	0.835	1.125	0.091	53.5	10.2	281.5	19.0	2.00	0.00–4.18
I	1 (S1–S6)[b]	1.021[b]	0.963[b]	0.299[b,c]	69.9[b]	18.0[b]	271.7[b]	31.9[b]	27.14[b]	None[b]
	2 (S7–S12)[b]	0.869[b]	1.049[b]	0.279[b,c]	39.1[b]	7.9[b]	192.9[b]	38.9[b]	28.52[b]	None[b]
	3 (S13–S16)[b]	0.931[b]	0.993[b]	0.290[b,c]	49.1[b]	11.1[b]	217.5[b]	17.0[b]	31.21[b]	None[b]
J	1 (S1–S6)	0.884	0.922	0.070	82.0	17.1	393.7	12.9	14.60	0.02–0.33
	2 (S7–S12)	0.740	0.873	0.057	66.7	10.3	434.1	20.7	13.90	0.01–0.36
	3 (S13–S16)	0.966	0.906	0.077	77.9	18.6	327.1	10.9	4.66	0.00–1.29
	Repeat	0.800	0.786	0.066	65.5	11.6	370.7	24.1	9.84	0.01–0.61
K	1 (S1–S6)	0.877	0.962	0.047	46.8	9.6	227.5	40.7	18.18	0.02–0.10
	2 (S7–S12)[b]	0.797[b]	0.543[b,c]	0.043[b]	79.7[b]	14.0[b]	453.6[b]	19.1[b]	5.99[b]	0.00–1.66[b]
	3 (S13–S16)	0.756	1.019	0.042	45.3	7.2	283.2	16.1	7.06	0.00–0.85
L	1 (S1–S6)	0.800	1.108	0.041	66.2	11.7	374.7	7.1	12.10	0.01–0.42
	2 (S7–S12)	0.848	0.992	0.053	64.2	12.5	329.2	18.0	18.08	0.03–0.14
	3 (S13–S16)	0.843	1.017	0.059	80.1	15.5	415.0	11.4	13.91	0.02–0.35
M	1 (S1–S6)	0.797	0.936	0.038	38.1	6.7	216.9	10.6	10.10	0.00–0.34
	2 (S7–S12)	0.813	0.942	0.045	39.1	7.1	215.2	11.7	7.07	0.00–0.60
	3 (S13–S16)	0.899	0.899	0.054	45.9	9.8	214.5	9.2	8.33	0.00–0.43

Table 3. (*continued*)

Lab	Standard curve	Significant parameters (4-p logistics) working range						Calibration curve fit		
		Slope	Maximum	Minimum	I_{50}, pg/mL	I_{20}, pg/mL	I_{80}, pg/mL	Mean %CV data points	Minimum %CV	ng/mL range were %CV <20%
N	1 (S1–S6)	0.694	0.726	0.055	45.1	6.1	332.4	17.7	10.57	0.00–0.51
	2 (S7–S12)	0.707	0.815	0.050	44.4	6.2	315.4	12.8	12.67	0.01–0.32
	3 (S13–S16)	0.834	0.717	0.072	42.4	8.0	223.5	17.6	15.76	0.01–0.14
O	1 (S1–S6)	0.840	1.029	0.050	51.8	9.9	269.7	17.2	6.54	0.00–0.82
	2 (S7–S12)	0.890	0.962	0.054	41.8	8.8	198.3	18.2	10.61	0.01–0.28
	3 (S13–S16)	0.840	1.029	0.050	51.8	9.9	269.7	17.2	6.54	0.00–0.82
P	1 (S1–S6)[b]	0.823[b]	1.232[b]	0.068[b]	33.1[b,c]	6.1[b]	178.3[b,c]	15.6[b]	5.60[b]	0.00–0.69[b]
	2 (S7–S12)[b]	0.777[b]	1.161[b]	0.058[b]	25.2[b,c]	4.2[b,c]	150.2[b,c]	19.4[b]	20.00[b]	None[b]
	3 (S13–S16)[b]	0.558[b,c]	1.198[b]	0.000[b]	21.2[b,c]	1.8[b,c]	254.5[b]	11.8[b]	27.68[b]	None[b]
	Repeat[b]	0.740[b]	1.305[b]	0.035[b]	32.5[b,c]	5.0[b,c]	211.7[b,c]	17.6[b]	20.39[b]	None[b]
Avg.		0.839	0.938	0.068	54.8	10.7	290.0	18.9	13.4	
Standard deviation		0.094	0.135	0.060	15.3	4.0	77.3	10.3	7.7	
RSD_R (%CV)		11.19[c]	14.36[c]	88.16[c]	28.01[c]	37.37[c]	26.65[c]	54.44[c]	57.82[c]	

[a] ND = Not determined.

[b] Calibrations curves unsuitable for quantification of samples.

[c] Values >10% outside QC specifications.

and calibration curves that were considered unsuitable for quantification of the study samples are denoted in footnotes.

All the ELISA concentration data calculated on the basis of valid calibration curves, submitted by the 16 collaborators (coded as A–P) for the 3 different shellfish matrixes, are presented in Tables 4–6. These data sets were analyzed using the spreadsheet packages endorsed by AOAC for use with collaborative studies (Split Levels Version 1.6 and Blind Replicates Version 1.14; Joanna M. Lynch, Ithaca, NY, 2001). Following entry of the valid data for each pair of samples, the output of the spreadsheet was studied to identify outliers. The spreadsheet implements automatic detection of such outliers using progressive application of the Cochran and Grubbs criteria. Each flagged outlier data pair was removed following checking of the outlier statistic until no further outliers were identified in the remaining data. The statistical estimates for the method parameters obtained are summarized at the bottom of the data set for each sample pair in Tables 4–6. In 2 of the 8 data sets, one identified Cochran outlier pair was removed from 14 data pairs, which is in accordance with the AOAC guideline at 2 outliers from 9 data pairs. The data pairs formally identified as statistical outliers, and the data pairs that were based on invalid calibration, are marked in these tables and were not used for the statistical analysis.

Identification of outliers and the collaborative study protocol requires valid data from at least 8 laboratories for a given sample pair. This requirement was met for all sample pairs. Additionally, the requirement for valid analysis of split level data (Youden pairs) is that the mean levels differ by <5% and that the variances of the 2 levels are equivalent (95% confidence interval). These requirements were not met in some cases (*see* Pair delta in Tables 4–6) which requires some further discussion. Two sample pair data sets had mean levels differing by <5% and 6 by 5–10%. The sample pairs were all carefully prepared by weight to contain DA levels that differed by 3–4% (Table 2). The wider spread of differences between the Youden split level pairs found in the study, than as prepared (<5%), is attributed to the variability of the ELISA data (RSD_R 13–28%; RSD of mean 4–8%) rather than true systematic differences in the low levels of DA. In this case, the statistical analysis of the pairs will remain broadly valid, particularly because the differences did not exceed 10%. The variances of the 2 levels were equivalent (95% confidence interval) in all cases, and, overall, the parameters in Tables 4–6 derived from the statistical analysis are considered very adequate as a guide to the performance of the method, not withstanding these few concerns with the Youden pair statistics.

Performance Characteristics of the Method

A summary of the collaborative study results, showing the accuracy and precision estimates for the 8 pairs of shellfish samples, is given in Table **2006.02A**. The relative standard deviations (RSD) for within-laboratory repeatability (RSD_r) were in the range of 10–21% with a mean of 15%. Only the oyster sample pairs gave repeatability values >16%. The RSD for interlaboratory reproducibility (RSD_R) estimates were in the range of 13–29% with a mean of 23%. The precision

Table 4. Interlaboratory study results for determination of domoic acid toxins in mussel samples (Youden pair/split duplicates statistical treatment)

	Mussels total DA, mg/kg					
Sample	S6	S14	S2	S7	S10	S16
Spike value	19.5	20.2	8.20	8.60	1.15	1.10
Lab						
A	15.0	17.2	11.13	13.99	1.61	1.17
B	23.1	21.6	7.95	8.92	1.05	1.04
C	(20.6[a])	(19.4)[a,b]	7.40[a,c]	16.78[a,c]	1.51	1.18
D	22.1	15.6	10.51	7.99	1.06	1.00
E	16.9	16.2	9.49	9.31	1.41	0.81
F	19.5	19.4	7.77	6.45	0.97	1.16
G	18.6	19.4	7.65	7.90	1.08	1.01
H	20.9	22.3	13.62	9.16	1.22	1.28
I	(15.8)[a,d]	(20.6)[a,d]	(8.09)[a,d]	(7.86)[a,d]	(0.70)[a,d]	(0.95)[a,d]
J	23.1	23.1	7.84	8.40	1.46	1.28
K	15.6	21.4	(8.18)[a]	(8.01)[a,e]	(0.99)[a,e]	(1.00)[a]
L	21.6	20.0	7.98	8.99	1.14	0.89
M	19.3	20.2	10.96	10.62	1.16	1.17
N	(23.9)[a]	(24.9)[a,b,e]	12.98	10.26	1.07	0.99
O	16.4	19.1	9.40	7.03	0.79	1.02
P	(27.2)[a,f]	(29.9)[a,b,f]	(10.76)[a,f]	(11.11)[a,b,f]	(1.52)[a,f]	(1.09)[a,f]
	AOAC statistical analysis					
Mean level	19.34[c]	19.61[c]	9.77[c]	9.08[c]	1.20[c]	1.08[c]
n		12		12		13
Pair delta, %		1.4		7.0		9.9
Equivalent variance difference		OK		OK		OK
Recovery, %		98.1		112.4		100.1
s_r		2.1		1.4		0.17
s_R		2.6		2.0		0.20
RSD_r, %		10.7		15.3		14.9
RSD_R, %		13.5		21.5		17.3
r		5.8		4.1		0.47
R		7.4		5.7		0.55
HorRat[g]		1.3		1.9		1.1

[a] Values based on invalid calibration and removed from statistical analysis.
[b] Value outside defined I_{80}–I_{20} working range.
[c] Outlier pair removed from statistical analysis.
[d] $A_{min} > 0.1$.
[e] $A_{max} < 0.8$.
[f] Failing $I_{20}/I_{50}/I_{80}$ QC criteria.
[g] HorRat parameter may not be applicable for enzyme reactions (19), but is included by convention as an indication of method performance.

Table 5. Interlaboratory study results for determination of domoic acid toxins in scallop samples (Youden pair/split duplicates statistical treatment)

	Scallops total DA, mg/kg					
Sample	S3	S8	S9	S11	S1	S4
Spike value	7.80	7.50	3.45	3.30	0.125	0.120
Lab						
A	7.39	8.58	5.11	4.74	0.151	0.176
B	8.28	6.79	3.33	2.73	(0.125)[a,b]	(0.224)[a]
C	5.56[a,c]	11.58[a,c]	4.93	3.20	0.090	0.096
D	5.32	5.23	2.10	3.10	0.067	0.070
E	8.01	8.62	4.67	4.03	0.109	0.095
F	7.64	6.44	2.22	2.51	0.091	0.106
G	7.26	7.44	2.42	3.02	0.114	0.094
H	11.63	8.36	4.02	3.06	0.079	0.091
I	(5.69)[a,d]	(4.06)[a,d]	(2.89)[a,d]	(1.87)[a,d]	(0.090)[a,d]	(0.079)[a,d]
J	9.15	9.54	4.21	4.67	0.135	0.108
K	(6.84)[a]	(5.37)[a,e]	(1.69)[a,e]	(3.02)[a,e]	0.097	0.112
L	7.46	6.34	3.14	3.23	0.124	0.103
M	7.96	8.01	4.07	3.23	0.161	0.114
N	8.82	8.25	2.96	2.26	0.124	0.125
O	5.76	4.95	2.82	2.07	0.096	0.080
P	(8.63)[a,b,f]	(9.36)[a,b,f]	(4.49)[a,f]	(3.84)[a,f]	(0.111)[a,f]	(0.094)[a,f]
	AOAC statistical analysis					
Mean level	7.89	7.38	3.54	3.22	0.111	0.105
n		12		13		13
Pair delta, %		6.5		9.05		4.7
Equivalent variance difference		OK		OK		OK
Recovery, %		99.8		100.1		88.0
s_r		0.84		0.54		0.015
s_R		1.53		0.93		0.027
RSD_r, %		11.0		15.9		13.5
RSD_R, %		20.0		27.6		24.8
r		2.3		1.5		0.041
R		4.3		2.6		0.075
HorRat[g]		1.7		2.1		1.1

[a] Values based on invalid calibration and removed from statistical analysis.
[b] Value outside defined I_{80}–I_{20} working range.
[c] Outlier pair removed from statistical analysis.
[d] $A_{min} > 0.1$.
[e] $A_{max} < 0.8$.
[f] Failing $I_{20}/I_{50}/I_{80}$ QC criteria.
[g] HorRat parameter may not be applicable for enzyme reactions (19), but is included by convention as an indication of method performance.

Table 6. Interlaboratory study results for determination of domoic acid toxins in oyster samples (Youden pair/split duplicates statistical treatment)

	Oysters total DA, mg/kg			
Sample	S13	S15	S5	S12
Spike value	7.90	8.20	0.530	0.510
Lab				
A	6.27	10.02	1.188	0.788
B	10.24	9.32	0.589	0.719
C	15.88	11.88	0.594	0.589
D	5.37	6.26	0.583	0.781
E	6.16	6.45	0.640	0.734
F	6.19	7.93	0.501	0.415
G	8.11	7.96	0.527	0.397
H	9.43	11.39	0.904	0.586
I	(7.77)[a,b]	—[c]	(0.450)[a,b]	(0.399)[a,b]
J	11.40	10.83	0.940	0.694
K	10.08	10.27	(0.389)[a]	(0.438)[a,d]
L	6.04	9.44	0.617	0.709
M	9.28	10.78	0.573	0.599
N	8.95	6.85	0.452	0.573
O	6.33	6.94	0.380	0.422
P	(13.05)[a,e]	(12.80)[a,e]	(1.072)[a,e]	(0.571)[a,e]
	AOAC statistical analysis			
Mean level	8.55[f]	9.02[f]	0.653[f]	0.616[f]
n		14		13
Pair delta, %		5.2		5.7
Equivalent variance difference		OK		OK
Recovery, %		109.1		121.9
s_r		1.4		0.13
s_R		2.4		0.19
RSD_r, %		16.4		20.8
RSD_R, %		27.8		29.4
r		4.0		0.37
R		6.8		0.52
HorRat[g]		2.4		1.7

[a] Values based on invalid calibration and removed from statistical analysis.
[b] $A_{min} > 0.1$.
[c] — = Not determined.
[d] $A_{max} < 0.8$.
[e] Failing $I_{20}/I_{50}/I_{80}$ QC criteria.
[f] Outlier pair removed from statistical analysis.
[g] HorRat parameter may not be applicable for enzyme reactions (19), but is included by convention as an indication of method performance.

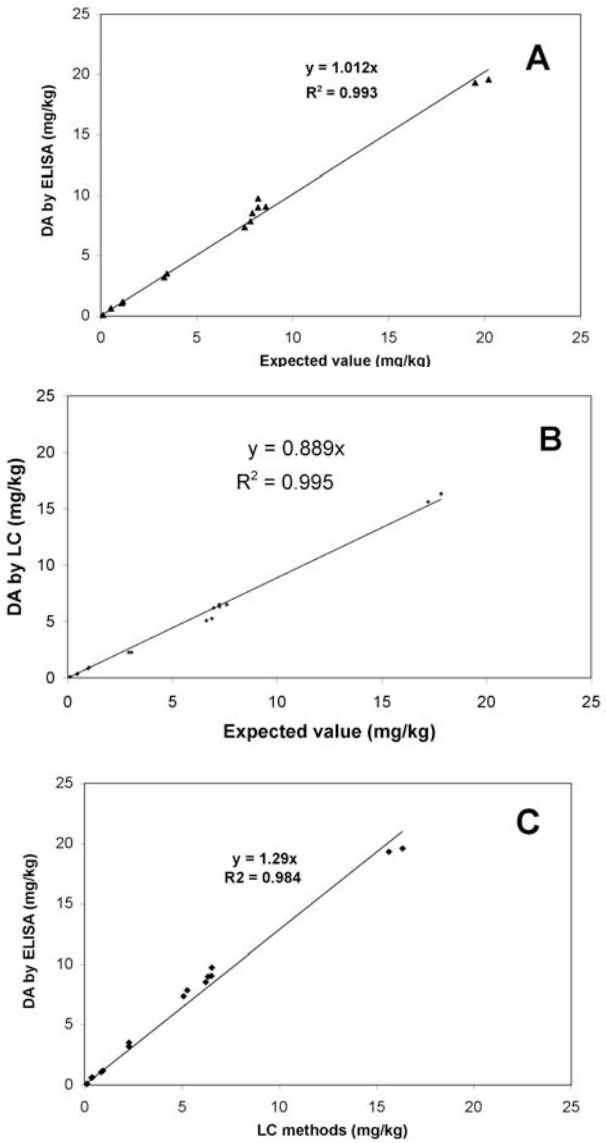

Figure 1. Correlation plots comparing the ASP ELISA collaborative study results with the reported results obtained with LC methods from the analysis of shellfish samples. (A) Correlation between total DA toxin levels measured by ELISA (*n* = 15) versus expected levels. Fit line is based on the mean data of sample values at each spike level. (B) Comparison of mean DA levels in shellfish samples by LC methods and expected levels (DA + epi-DA; Table 2006.02A). (C) Correlation between mean DA levels in shellfish samples by ELISA and instrumental LC methods (Table 7).

estimates for the oyster samples were somewhat lower than those for mussel or scallop, but these differences are probably not significant with only 2–3 sample pairs for each species. Overall, the RSD_R values do not show a strong dependence on the level of DA, i.e., they do not always follow the Horwitz bell curve which predicts less precision at lower concentrations. This may arise from the high levels of dilution used in this ELISA which tend to minimize matrix and concentration effects. The precision characteristics of the method, expressed as the HorRat values, range from 1.1 to 2.4, with an average mean of 1.7. Overall, the precision achieved is within the acceptable range for the guidance (HorRat <2) provided by AOAC for analytical methods. Even though Horwitz noted that the HorRat may not be applicable to enzyme methods (19), we have used this parameter by convention as an indication of method performance. For the ASP ELISA, the sources of variation are mainly operator dependent in achieving consistent and accurate dispensing of small volumes, particularly in the preparation of the dilution series, and the duplicate dispensing of the sample into the microwells.

The values for recovery of DA toxins derived from all the sample pairs ranged from 88 to 122%, with a mean value of 104% (Table **2006.02A**), based on the levels of fortification, assuming equal ELISA response to all the DA toxins present in the sample (Table 1). The lowest recoveries were obtained for the low spike scallop sample pair (S1/S4), and the highest recoveries were obtained for the low spike oyster sample pair (S5/S12). However, the recovery values do not show a strong dependence on the spike level or matrix. Figure 1A shows a correlation plot for each sample of the expected DA levels versus values obtained by ELISA analysis. The high correlation coefficient (r^2 = 0.993) and near unity slope (1.012) for the mean data demonstrate the method to be of excellent accuracy over the relevant concentration range of 0.1–20 mg/kg.

In order to compare the ASP ELISA method with the current reference method, all of the study samples were analyzed in parallel by 3 collaborators using LC-UV (5) and one collaborator using LC/MS (6). The 4 sets of data for LC analysis of the shellfish extracts are quite similar, as shown in Table 7. The average precision level is comparable to that of the ASP ELISA, but the precision of the LC methods appears to be slightly concentration dependent, giving relatively high RSD_R values (38–74%) for the reported low spike values (≤1.0 mg/kg). The mean recovery for DA from the shellfish samples is 87%, based on the spike levels calculated from DA and epi-DA, indicating that the LC instrumental methods slightly underestimate the DA levels in these study samples. The corresponding correlation (Figure 1B) between spike levels and reported LC data is quite good (r^2 = 0.995), and the slope (0.889) underscores the reduced recovery as revealed in Table 7. This indicates that the ASP ELISA has a higher level of accuracy than the LC methods in this study, when accounting for total DA toxins, rather than just the DA and epi-DA (Tables 1 and 2).

Despite the fact that the ASP ELISA and the LC methods rely on different principles of detection, the correlations between the mean DA levels measured for each sample by the 2 methods (Table 8) are in very good agreement over the full concentration range (Figure 1C). The ELISA test results closely track those from the expected values (Figure 1A) and those from the instrumental analysis (Figure 1C). It appears that the ELISA slightly overestimates the DA toxin levels compared to the levels of DA and epi-DA determined by the LC methods (slope at 1.29), suggesting that there could be some immuno-active compounds related to DA present in the contaminated shellfish CRM. However, the high dilution of

Table 7. Interlaboratory study results with LC-UV/LC/MS for determination of domoic acid toxins in shellfish

	Reported values (DA + epi-DA), mg/kg				Method performance			
Sample	Lab G LC/MS (Ref. 6)	Lab H LC-UV (Ref. 5)	Lab N LC-UV (Ref. 5)	Lab D LC-UV (Ref. 2)	Expected[a], mg/kg	Mean, mg/kg	Recovery, %	RSD_R, %
Blue mussels								
S6	17.13	13.89	17.19	14.30	17.2	15.6	90.7	11.4
S14	18.12	15.40	16.62	15.20	17.8	16.3	91.6	8.2
S2	6.49	5.92	7.64	6.10	7.24	6.5	89.8	11.8
S7	7.53	5.58	6.59	6.34	7.59	6.5	85.6	12.4
S10	1.08	0.805	0.778	1.11	1.01	0.94	93.1	18.7
S16	1.015	0.803	0.711	ND[b]	0.97	0.84	86.6	18.5
Scallops								
S3	5.18	5.10	6.53	4.27	6.88	5.3	77.0	17.8
S8	5.77	4.90	5.62	4.06	6.62	5.1	77.0	15.4
S9	2.58	2.02	2.63	1.87	3.04	2.3	75.6	16.9
S11	2.76	2.00	2.47	1.85	2.91	2.3	79.0	18.5
S1	0.08	0.195	0.07	ND	0.11	0.113	102.4	64.0
S4	0.08	0.219	0.06	ND	0.106	0.118	111.6	74.0
Oysters								
S13	6.57	5.81	6.52	6.01	6.97	6.23	89.4	6.1
S15	7.07	6.07	6.07	6.12	7.24	6.33	87.4	7.7
S5	0.38	0.41	0.386	ND	0.47	0.39	82.9	4.1
S12	0.425	0.415	0.195	ND	0.45	0.35	77.8	38.0
Avg. rec., %							87.3	
Avg. RSD_R, %								22.1

[a] Based on certified level of DA + epi-DA (Table 2).
[b] ND = Not determined.

sample extract prior to analysis and low response for blank shellfish rules out an effect from shellfish tissues. The ELISA overestimation suggests that the antibodies recognize some other DA derivatives present in the certified mussel material used for spiking. An unspecific signal caused by antibody binding to structural analogs of DA is not likely, as Garthwaite et al. (16) reported negligible cross-reactivity to kainic acid and glutamate. The ASP ELISA has a low cross-reactivity (<1%) to the open-ring isomer iso-DA C (L. Briggs, unpublished results) reported to have a low neurotoxic potential (20), but there are no cross-reactivity data on the epi-DA or the other DA isomers reported as present in the certified mussel material (Table 1). The extraction procedure used in the study using 50% aqueous methanol with a single-step dispersive extraction with an ultrahomogenizer was reported to give a recovery of 93% (5). The reported mean recovery of 87% from the LC methods in this study is, therefore, lower than expected and the ELISA overestimation can be partly attributed to an underestimation of DA by the LC methods (Table 8, Figure 1B). The low recovery values reported for the LC methods account for approximately half of the discrepancy between the 2 methods. This may be attributed to partial sample degradation or transformation from DA or epi-DA to DA toxins that the ASP ELISA may still respond to. In particular, the low spike samples gave significantly high variability with the LC methods, indicating that this might have been the case. Previously, there have been some reports on inefficient extraction of DA from the reference material binding of the toxin to the pasteurized tissue (7). However, as the same sample extract after the methanol extraction step was used for the analysis by both methods, the difference in mean sample recovery values can only arise from the cleanup (LC method) and determination steps.

In general, the ASP ELISA compares well with analytical instrumental LC techniques, both the LC-UV as the preferred analytical technique during the last decade (2–5, 8), and the more recently described use of LC/MS for determination of DA in shellfish (6, 7). Previously reported mean bias values from LC method validations have ranged from 3 to 5% (5, 6, 8), as compared to the reported mean bias at 4% in the present study (Table **2006.02A**). Previously, a correlation between LC-UV and LC/MS at 0.995 (slope 0.93) was reported (8), and a recent limited interlaboratory study of

Table 8. Comparison of ASP ELISA interlaboratory study results with LC-UV/LC/MS for determination of domoic acid toxins in shellfish

	LC-UV and LC/MS method performance (n = 4)				ASP ELISA method performance (n = 15)			
Sample	Expected[a], mg/kg	Mean, mg/kg	Recovery, %	RSD_R, %	Expected[b], mg/kg	Mean, mg/kg	Recovery, %	RSD_R, %
				Blue mussels				
S6	17.2	15.6	90.7	11.4	19.5	19.3	98.1	13.5
S14	17.8	16.3	91.6	8.2	20.2	19.6		
S2	7.24	6.5	89.8	11.8	8.2	9.77	112.4	21.5
S7	7.59	6.5	85.6	12.4	8.6	9.08		
S10	1.01	0.94	93.1	18.7	1.15	1.20	100.1	17.3
S16	0.97	0.84	86.6	18.5	1.1	1.08		
				Scallops				
S3	6.88	5.3	77.0	17.8	7.8	7.89	99.8	20.0
S8	6.62	5.1	77.0	15.4	7.5	7.38		
S9	3.04	2.3	75.6	16.9	3.45	3.54	100.1	27.6
S11	2.91	2.3	79.0	18.5	3.3	3.22		
S1	0.11	0.113	102.4	64.0	0.125	0.11	88	24.8
S4	0.106	0.118	111.6	74.0	0.12	0.11		
				Oysters				
S13	6.97	6.23	89.4	6.1	7.9	8.55	109.1	27.8
S15	7.24	6.33	87.4	7.7	8.2	9.02		
S5	0.47	0.39	82.9	4.1	0.53	0.65	121.9	29.4
S12	0.45	0.35	77.8	38.0	0.51	0.62		
Avg. recovery, %			87.3				103.9	
Avg. RSD_R, %				22.1				22.7

[a] Based on certified level of DA + epi-DA (Table 2).
[b] Based on level of total DA toxins (Table 2).

LC/MS for ASP toxins in shellfish extracts achieved a repeatability at 9% and reproducibility at 23% (21). Again, this compares well with the present collaborative data and demonstrates that the reported correlation between the ASP ELISA and LC methods is quite acceptable, considering that it compares 2 fundamentally different methods—an immunoassay and an instrumental technique. Even though ELISA methods are generally regarded as less reproducible than instrumental methods, the between-laboratory precision estimates achieved for the ASP ELISA are generally very acceptable in the ng/g range. The slight reduction in precision compared to instrumental methodologies will be compensated by a more rapid methodology with higher sample throughput capacity. There are no requirements for highly specialized and expensive instrumentation, nor elaborate precleanup or concentration steps. Following the simple extraction procedure, the direct analysis enables the determination of total DA in shellfish with high recovery and acceptable precision, due to the high sensitivity and selectivity of the method. The low assay LOD (0.01 mg/kg) indicates that the ASP ELISA may also be a valuable early-warning tool for the monitoring of shellfish growing and harvesting areas, as well as an effective tool for the biomonitoring of DA in marine mammals and human populations (22–24).

Collaborators' Comments

Many collaborators had little or no previous experience with the ELISA technique and very few were trained ELISA analysts. However, most collaborators found the method easy to establish in their laboratory. Initially, there were some occasional difficulties obtaining acceptable calibration curve parameters, but almost all laboratories achieved very good method performance after running a few assays. Some collaborators also experienced problems installing the Excel macro spreadsheet for data treatment and sample calculation, but this was resolved by consultation with the Study Director. One collaborator reported A_{max} values below the system suitability requirement (0.8 AU) for calibration curve, but the problem was overcome when the water sources were changed.

Recommendations

This interlaboratory study was successfully completed by 15 of the 16 participating laboratories representing 10 different countries from around the world. Collaborators were able to accurately quantify DA at concentrations from 0.12 mg/kg up to the regulatory level at 20 mg/kg with high recovery and acceptable precision, demonstrating that the Biosense ASP ELISA is suitable for the routine determination of DA in mussels, oysters, and scallops. It is recommended that this method be accepted by AOAC INTERNATIONAL as Official First Action for the quantitative determination of DA toxins in mussels, oysters, and scallops at levels >0.12 mg/kg, with a method LOD at 0.003 mg/kg and LOQ at 0.009 mg/kg.

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References

(1) Hallegraeff, G.M. (1995) in *Manual on Harmful Marine Microalgae*, IOC Manuals and Guides No. 33, G.M. Hallegraeff, D.M. Anderson, A.D. Cembella, & H.O. Enevoldsen (Eds), UNESCO, Paris, France, pp 1–22

(2) *Official Methods of Analysis* (2000) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **991.26**

(3) Lawrence, J.F., Charbonneau, C.F., & Ménard, C. (1991) *J. AOAC Int.* **74**, 68–72

(4) Quilliam, M.A., Sim, P.G., McCulloch, A.W., & McInnes, A.G. (1989) *Int. J. Environ. Anal. Chem.* **36**, 139–154

(5) Quilliam, M.A., Xie, M., & Hardstaff, W.R. (1995) *J. AOAC Int.* **78**, 543–554

(6) Holland, P.T., McNabb, P., Selwood, A.I., & Neil, T. (2003) *J. AOAC Int.* **86**, 1095–1100

(7) Hess, P., Gallacher, S., Bates, L.A., Brown, N., & Quilliam, M.A. (2001) *J. AOAC Int.* **84**, 1657–1668

(8) Pocklington, R., Milley, J.E., Bates, S.S., Bird, C.J., de Freitas, A.S.W., & Quilliam, M.A. (1990) *Int. J. Environ. Anal. Chem.* **38**, 351–368

(9) Report on the Joint FAO/IOC/WHO ad hoc Expert Consultation on Biotoxins in Bivalve Molluscs (September 26–30, 2004) Oslo, Norway, http://unesdoc.unesco.org/images/0013/001394/139421e.pdf

(10) Van Dolah, F.M., Finley, B.L., Doucette, G.J., Moeller, P.D., & Ramsdell, J.S. (1994) *Nat. Toxins* **2**, 189–196

(11) Newsome, H., Truelove, J., Hierlihy, L., & Collins, P. (1994) *Bull. Environ. Contam. Toxicol.* **47**, 329–334

(12) Osada, M., Marks, L.J., & Stewart, J.E. (1995) *Bull. Environ. Contam. Toxicol.* **54**, 797–804

(13) Smith, D.S., & Kitts, D.D. (1995) *J. Agric. Food Chem.* **43**, 367–371

(14) Kawatsu, K., Hamano, Y., & Noguchi, T. (2000) *J. AOAC Int.* **83**, 1384–1386

(15) Yu, F.Y., Liu, B.H., Wu, T.S., Chi, T.F., & Su, M.C. (2004) *J. Agric. Food Chem.* **52**, 5334–5339

(16) Garthwaite, I., Ross, K.M., Miles, C.O., Hansen, R.P., Foster, D., Wilkins, A.L., & Towers, N. (1998) *Nat. Toxins* **6**, 93–104

(17) Eurachem Guide (1998) *The Fitness for Purpose of Analytical Methods, A Laboratory Guide to Method Validation and Related Topics*, http://www.eurachem.ul.pt

(18) Kleivdal, H., Kristiansen, S.I., Nilsen, M.V., & Briggs, L. (2007) *J. AOAC Int.* **90**, 1000–1010

(19) *AOAC Definitions and Calculations of HorRat Values from Interlaboratory Data* (2005) http://www.aoac.org/dietsupp6/Dietary-Supplement-web-site/HORRAT_SLV.pdf

(20) Holland, P.T., Selwood, A.I., Mountfort, D.O., Wilkins, A.L., McNabb, P., Rhodes, L.L., Doucette, G.J., Mikulski, C.M., & King, K.L. (2005) *Chem. Res. Toxicol.* **18**, 814–816

(21) McNabb, P., Selwood, A.L., & Holland, P.T. (2005) *J. AOAC Int*. **88**, 761–772

(22) Maucher, J.M., & Ramsdell, J.S. (2005) *Toxicon* **45**, 607–613

(23) Maucher, J.M., & Ramsdell, J.S. (2005) *Environ. Health Perspect.* **113**, 461–464

(24) Hesp, B.R., Harrison, J.C., Selwood, A.I., Holland, P.T., & Kerr, D.S. (2005) *Anal. Bioanal. Chem.* **383**, 783–786

AOAC INTERNATIONAL

The Scientific Association Dedicated to Analytical Science ®

August 15, 2006

Hans Kleivdal, Ph.D
Biosense Laboratories AS
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Dear Dr. Hans Kleivdal:

We are pleased to inform you that the AOAC Methods Committee on Natural Toxins and Allergens adopted your method, **Determination of Domoic acid Toxins in Shellfish by Biosense ASP ELISA - a Direct Competitive Enzyme-linked Immunosorbent Assay** as a First Action *Official Method*SM in June 2006. The method was assigned an *Official Method*SM number **2006.02**. A notice of the adopted method was published in the July/August 2006 issue of the AOAC magazine, *Inside Laboratory Management*, and will be published in "For Your Information" in the AOAC *Journal*. AOAC staff editors will prepare the collaborative study manuscript for publication in the *Journal*. The method will be published as part of the collaborative study and will be included in the next online revision of the *Official Methods of Analysis*.

AOAC would like to take this opportunity to thank you for all your contributions in the development of this method.

Sincerely,

Robert Rathbone
Director of Publications, *Official Method*SM Programs
AOAC INTERNATIONAL

COMMISSION REGULATION (EC) No 1244/2007

of 24 October 2007

amending Regulation (EC) No 2074/2005 as regards implementing measures for certain products of animal origin intended for human consumption and laying down specific rules on official controls for the inspection of meat

(Text with EEA relevance)

THE COMMISSION OF THE EUROPEAN COMMUNITIES,

Having regard to the Treaty establishing the European Community,

Having regard to Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption [1], and in particular Article 16 and Article 18(3), (7) and (12) thereof,

Whereas:

(1) Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin [2], Regulation (EC) No 854/2004, and Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules [3] lay down the health rules and requirements regarding food of animal origin and the official controls required.

(2) Implementing rules for those Regulations are laid down in Commission Regulation (EC) No 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004 [4].

(3) In accordance with Regulation (EC) No 854/2004, the competent authority may decide that the official veterinarian need not be present at all times during post-mortem inspections in certain slaughterhouses or game handling establishments identified on the basis of a risk analysis. In such cases, an official auxiliary is to perform the post-mortem inspection, which might contribute to reducing the financial burden for establishments with a low throughput.

(4) The criteria for such derogations should be determined on the basis of a risk analysis. In particular, establishments carrying out discontinuous slaughter or game handling activities fulfil a social and economic function in rural communities. It should therefore be possible for those establishments to benefit from such derogations provided that they comply with the legal and hygiene requirements.

(5) In accordance with Regulation (EC) No 854/2004 the competent authority may decide that fattening pigs housed under controlled housing conditions in integrated production systems since weaning need only undergo visual inspection. More specific requirements should be laid down for the conditions under which such reduced, but risk-based meat inspection procedures should be allowed.

(6) On 24 February 2000, the Scientific Committee on Veterinary Measures relating to Public Health adopted an opinion on 'Revision of meat inspection procedures', which deals with the general principles relating to meat inspections. It concludes that current meat inspection systems can be improved when supplemented with information from the complete production chain, use of the Hazard Analysis, Critical Control Point (HACCP) principles in the slaughter plant and microbiological monitoring of faecal indicator organisms.

[1] OJ L 139, 30.4.2004, p. 206, as corrected by OJ L 226, 25.6.2004, p. 83. Regulation as last amended by Council Regulation (EC) No 1791/2006 (OJ L 363, 20.12.2006, p. 1).

[2] OJ L 139, 30.4.2004, p. 55, as corrected by OJ L 226, 25.6.2004, p. 22. Regulation as last amended by Regulation (EC) No 1791/2006.

[3] OJ L 165, 30.4.2004, p. 1, as corrected by OJ L 191, 28.5.2004, p. 1. Regulation as last amended by Regulation (EC) No 1791/2006.

[4] OJ L 338, 22.12.2005, p. 27. Regulation as amended by Regulation (EC) No 1664/2006 (OJ L 320, 18.11.2006, p. 13).

(7) On 20 and 21 June 2001, the Scientific Committee on Veterinary Measures relating to Public Health adopted an opinion on 'Identification of species/categories of meat-producing animals in integrated production systems where meat inspection may be revised'. It concludes that there are already a number of production systems in Member States where the criteria for application of a simplified meat inspection system are fulfilled.

(8) On 14 and 15 April 2003, the Scientific Committee on Veterinary Measures relating to Public Health adopted an opinion on 'Revision of meat inspection in veal calves', which states that visual inspection of veal calves reared in integrated systems is sufficient for routine inspection, but that as long as bovine tuberculosis has not been eradicated, surveillance for bovine tuberculosis should be maintained in bovine animals at both holding and abattoir levels.

(9) On 26 November 2003, the European Food Safety Authority (EFSA) adopted an opinion on 'Tuberculosis in bovine animals: risks for human health and control strategies', which concludes that efficient post-mortem examination of specified lymph nodes and of the lungs represents an important element of national bovine tuberculosis eradication programmes, as well as being an integral part of veterinary meat inspection programmes aimed at the protection of human health.

(10) On 1 December 2004, the EFSA adopted an opinion on 'Revision of meat inspection for beef raised in integrated production systems', which states that the incision of lymph nodes should continue as part of a revised post-mortem meat inspection system in order to be able to detect tuberculous lesions.

(11) On 18 May 2006, the EFSA adopted an opinion on 'An assessment of the public and animal health risks associated with the adoption of a visual inspection system in veal calves raised in a Member State (or part of a Member State) considered free of bovine tuberculosis'. It states that in case of veal calves reared in integrated production units and in officially bovine tuberculosis-free herds, post-mortem inspection can be restricted to observation and palpation of lymph nodes.

(12) On 22 April 2004, the EFSA adopted an opinion on 'Meat inspection procedures for lambs and goats'. It states that the important pathological conditions seen at meat inspection of lambs and goat kids can be diagnosed by visual inspection, thus preventing cross-contamination by less manipulation.

(13) On 27 and 28 September 2000, the Scientific Committee on Veterinary Measures relating to Public Health adopted an opinion on 'The control of taeniosis/cysticercosis in man and animals'. It specifies the prerequisites necessary to ensure cysticercosis-free conditions.

(14) On 26 and 27 January 2005, the EFSA adopted an opinion on 'The risk assessment of a revised inspection of slaughter animals in areas with low prevalence of *Cysticercus*'. It emphasises the need for risk profiling of the different calf production systems. Simplified post-mortem inspection can be applied for calves coming from integrated production systems previously assessed as of low-risk profile.

(15) Based on those scientific opinions the conditions for a reduced, but risk-based meat inspection procedure of ruminants of a young age should be laid down.

(16) The availability of food chain information 24 hours in advance of slaughter should be a prerequisite for a risk-based meat inspection without incision procedures. Consequently, whenever such a simplified meat inspection procedure is applied, the food business operator should not be able to benefit from the transitional arrangements laid down in Commission Regulation (EC) No 2076/2005 of 5 December 2005 laying down transitional arrangements for the implementation of Regulations (EC) No 853/2004, (EC) No 854/2004 and (EC) No 882/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004 [1].

(17) Regulation (EC) No 2074/2005 establishes the analytical methods for the detection of the amnesic shellfish poison (ASP) content of edible parts of molluscs. The 2006.02 ASP ELISA Method, as published in the AOAC Journal of June 2006, should be considered as an alternative screening method to the high-performance liquid chromatography (HPLC) method for the detection of ASP in bivalve molluscs. The ELISA method has the advantage of being able to screen a large number of samples in a relatively cheap way.

(18) Part D of Chapter IX of Section IV of Annex I to Regulation (EC) No 854/2004 provides for that, where appropriate, solipeds are to be examined for glanders. A detailed post-mortem examination for glanders should be mandatory for those solipeds or meat thereof that originates from countries that are not free of the disease.

[1] OJ L 338, 22.12.2005, p. 83. Regulation as last amended by Regulation (EC) No 479/2007 (OJ L 111, 28.4.2007, p. 46).

(19) Regulation (EC) No 2074/2005 should therefore be amended accordingly.

(20) The measures provided in this Regulation are in accordance with the opinion of the Standing Committee on the Food Chain and Animal Health,

HAS ADOPTED THIS REGULATION:

Article 1

Regulation (EC) No 2074/2005 is amended as follows:

1. the following Article is inserted:

'*Article 6b*

Requirements concerning official controls for the inspection of meat for the purpose of Regulation (EC) No 854/2004

Requirements concerning official controls for the inspection of meat are laid down in Annex VIb.';

2. Chapter II of Annex III is amended in accordance with Annex I to this Regulation;

3. the text in Annex II to this Regulation is inserted as Annex VIb.

Article 2

This Regulation shall enter into force on the 20th day following its publication in the *Official Journal of the European Union*.

This Regulation shall be binding in its entirety and directly applicable in all Member States.

Done at Brussels, 24 October 2007.

For the Commission
Markos KYPRIANOU
Member of the Commission

ANNEX I

In Annex III to Regulation (EC) No 2074/2005, Chapter II is replaced by the following:

'CHAPTER II

AMNESIC SHELLFISH POISON (ASP) DETECTION METHOD

The total content of amnesic shellfish poison (ASP) of edible parts of molluscs (the entire body or any part edible separately) must be detected using the high-performance liquid chromatography (HPLC) method or any other internationally recognised method.

However, for screening purposes, the 2006.02 ASP ELISA method as published in the AOAC Journal of June 2006 may also be used to detect the total content of ASP of edible parts of molluscs.

If the results are challenged, the reference method shall be the HPLC method.'

ANNEX II

'ANNEX VIb

REQUIREMENTS APPLICABLE TO THE OFFICIAL CONTROLS FOR THE INSPECTION OF MEAT

1. For the purpose of this Annex, the following definitions shall apply:

   (a) "controlled housing conditions and integrated production systems" means a type of animal husbandry where animals are kept under conditions in compliance with criteria set out in the Appendix;

   (b) "young bovine animal" means a bovine animal of either gender, which is not older than eight months;

   (c) "young ovine animal" means an ovine animal of either gender, not having any permanent incisor erupted and not older than 12 months;

   (d) "young caprine animal" means a caprine animal of either gender, not older than six months of age;

   (e) "herd" means an animal or group of animals kept on a holding as an epidemiological unit; if more than one herd is kept on a holding, each of these herds shall form a distinct epidemiological unit;

   (f) "holding" means any establishment, construction or, in the case of an openair farm, any place situated within the territory of the same Member State, in which animals are held, kept or handled;

   (g) "establishment carrying out discontinuous slaughter or game handling activities" means a slaughterhouse or game handling establishment designated by the competent authority on the basis of a risk analysis, in which, in particular, the slaughter or game handling activities do not take place either during the entire working day or during subsequent working days of the week.

2. Post-mortem inspections in establishments carrying out discontinuous slaughter or game handling activities.

   (a) In accordance with point 2(b) of Chapter II of Section III of Annex I to Regulation (EC) No 854/2004, the competent authority may decide that the official veterinarian need not be present at all times during post-mortem inspection, provided that the following conditions are complied with:

      (i) the establishment concerned is an establishment carrying out discontinuous slaughter or game handling activities and has sufficient facilities to store meat with abnormalities until a final post-mortem inspection by the official veterinarian can take place;

      (ii) an official auxiliary carries out the post-mortem inspection;

      (iii) the official veterinarian is present in the establishment at least once a day when slaughter activities take place or have taken place;

      (iv) the competent authority has put in place a procedure to assess on a regular basis the performance of official auxiliaries in these establishments, including:

         — monitoring individual performance,

         — verification of documentation with regard to inspection findings and comparison with the corresponding carcasses,

         — checks of carcasses in the storage room.

   (b) The risk analysis carried out by the competent authority as referred to in point 1(g) to identify the establishments that may benefit from the derogation as laid down in point 2(a) shall at least take account of the following elements:

      (i) the number of animals slaughtered or handled per hour or per day;

      (ii) the species and class of animals slaughtered or handled;

      (iii) the throughput of the establishment;

      (iv) the historical performance of slaughter or handling activities;

(v) the effectiveness of any additional measures in the food chain for procurement of animals for slaughter taken to guarantee food safety;

(vi) the effectiveness of the HACCP-based system in place;

(vii) audit records;

(viii) the competent authority's historical records of ante-mortem and post-mortem inspections.

3. Requirements for a risk-based meat inspection without incisions.

(a) In accordance with point 2 of Part B of Chapter IV of Section IV of Annex I to Regulation (EC) No 854/2004, the competent authority may limit the post-mortem inspection procedures of fattening pigs to a visual inspection, provided that the following conditions are complied with:

(i) the food business operator ensures that the animals are kept under controlled housing conditions and integrated production systems as laid down in the Appendix to this Annex;

(ii) the food business operator does not benefit from the transitional arrangements with regard to food chain information as laid down in Article 8 of Commission Regulation (EC) No 2076/2005;

(iii) the competent authority implements or orders the implementation of regular serological and/or microbiological monitoring of a selected number of animals based on a risk analysis of food safety hazards which are present in live animals and relevant at the holding level.

(b) By way of derogation from the specific requirements of Chapters I and II of Section IV of Annex I to Regulation (EC) No 854/2004, the post-mortem inspection procedures of young bovine, ovine and caprine animals may be reduced to a visual inspection with limited palpation, provided that the following conditions are complied with:

(i) the food business operator ensures that young bovine animals are kept under controlled housing conditions and in an integrated production system as laid down in the Appendix to this Annex;

(ii) the food business operator ensures that young bovine animals are reared in an officially bovine tuberculosis-free herd;

(iii) the food business operator does not benefit from the transitional arrangements with regard to food chain information as laid down in Article 8 of Regulation (EC) No 2076/2005;

(iv) the competent authority implements or orders the implementation of regular serological and/or microbiological monitoring of a selected number of animals based on a risk analysis of food safety hazards which are present in live animals and relevant at the holding level;

(v) post-mortem inspection of young bovine animals includes at all times palpation of the retropharyngeal, bronchial and mediastinal lymph nodes.

(c) In the case of any abnormality detected, the carcass and offal shall be subjected to a full post-mortem inspection as provided for in Chapters I and II of Section IV of Annex I to Regulation (EC) No 854/2004. However, the competent authority may decide on the basis of a risk analysis that meat with certain minor abnormalities as defined by the competent authorities, which pose no risk to animal or human health, does not need to be subjected to a full post-mortem inspection.

(d) Young bovine, ovine and caprine animals and weaned pigs that do not go directly from the holding of birth to a slaughterhouse may be moved on one occasion to another holding (for rearing or fattening) prior to dispatch to a slaughterhouse. In such cases:

(i) regulated assembly centres may be used for young bovine, ovine or caprine animals between the holding of origin and the rearing or fattening holding, as well as between these holdings and the slaughterhouse;

(ii) traceability shall be ensured at the level of the individual animal or batch of animals.

4. Additional requirement for the post-mortem examination of solipeds.

(a) Fresh meat from solipeds reared in countries not free of glanders in accordance with Article 2.5.8.2 of the *Terrestrial Animal Health Code* of the World Organisation for Animal Health shall not be placed on the market, unless such meat is derived from solipeds examined for glanders in accordance with point D of Chapter IX of Section IV of Annex I to Regulation (EC) No 854/2004.

(b) Fresh meat from solipeds in which glanders has been diagnosed shall be declared unfit for human consumption as provided for in point D of Chapter IX of Section IV of Annex I to Regulation (EC) No 854/2004.

Appendix to Annex VIb

For the purposes of this Annex, "controlled housing conditions and integrated production systems" means that the food business operator needs to comply with the criteria set out below:

(a) all feed has been obtained from a facility which produces feed in accordance with the requirements provided for in Articles 4 and 5 of Regulation (EC) No 183/2005 of the European Parliament and of the Council [1]; when roughage or crops are provided to the animals as feed, it shall be treated appropriately, and where possible, dried and/or pelleted;

(b) an all-in/all-out system is applied as far as possible. Where animals are introduced into the herd, they shall be kept in isolation as long as required by the veterinary services to prevent introduction of diseases;

(c) none of the animals has access to outdoor facilities unless the food business operator can show by a risk analysis to the satisfaction of the competent authority that the time period, facilities and circumstances of outdoor access do not pose a danger for introduction of disease in the herd;

(d) detailed information is available concerning the animals from birth to slaughter and their management conditions as laid down in Section III of Annex II to Regulation (EC) No 853/2004;

(e) if bedding is provided for the animals, the presence or introduction of disease is avoided by appropriate treatment of the bedding material;

(f) holding staff comply with the general hygiene provisions as laid down in Annex I to Regulation (EC) No 852/2004;

(g) procedures are in place that control access to the premises where animals are kept;

(h) the holding does not provide facilities for tourists or for camping unless the food business operator can show by a risk analysis to the satisfaction of the competent authority that the facilities are sufficiently separated from the animal rearing units that direct and indirect contact between humans and animals is not possible;

(i) animals do not have access to garbage dumps or household garbage;

(j) a pest management and control plan is in place;

(k) silage feeding is not used unless the food business operator can show by a risk analysis to the satisfaction of the competent authority that the feed can not transmit any hazards to the animals;

(l) effluent and sediment from sewage treatment plants are not released in areas accessible to the animals or be used for fertilising pastures used to grow crops, which are used to feed animals, unless treated appropriately and to the satisfaction of the competent authority.

[1] OJ L 35, 8.2.2005, p. 1.'