Talanta 116 (2013) 663–669

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Development and validation of the first high performance-lateral flow immunoassay (HP-LFIA) for the rapid screening of domoic acid from shellfish extracts

Waqass Jawaid [a,b,*], Julie Meneely [b], Katrina Campbell [b], Mark Hooper [a], Karrie Melville [a], Stephen Holmes [a], Jennifer Rice [c], Christopher Elliott [b]

[a] Neogen Europe Limited, The Dairy School, Auchincruive, Ayr, KA6 5HW, Scotland, UK
[b] Institute for Global Food Security, School of Biological Sciences, Queen's University, David Keir Building, Stranmillis Road, Belfast BT9 5AG, UK
[c] Neogen Corporation, 620 Lesher Place, Lansing, MI 48912, USA

ARTICLE INFO

Article history:
Received 10 March 2013
Received in revised form
6 July 2013
Accepted 13 July 2013
Available online 24 July 2013

Keywords:
Amnesic shellfish poisoning (ASP)
Domoic acid (DA)
High performance
Lateral flow immunoassay (LFIA)
Screening
Rapid tests

ABSTRACT

A lateral flow immunoassay (LFIA) has been developed and fully validated to detect the primary amnesic shellfish poisoning (ASP) toxin, domoic acid (DA). The performance characteristics of two versions of the test were investigated using spiked and naturally contaminated shellfish (mussels, scallops, oysters, clams, and cockles). The tests provide a qualitative result, to indicate the absence or presence of DA in extracts of shellfish tissues, at concentrations that are relevant to regulatory limits. The new rapid assay (LFIA version 2) was designed to overcome the performance limitations identified in the first version of the assay. The improved test uses an electronic reader to remove the subjective nature of the generated results, and the positive cut-off for screening of DA in shellfish was increased from 10 ppm (version 1) to 17.5 ppm (version 2). A simple extraction and test procedure was employed, which required minimal equipment and materials; results were available 15 min after sample preparation. Stability of the aqueous extracts at room temperature (22 °C) at four time points (up to 245 min after extraction) and across a range of DA concentrations was 100.3 ± 1.3% and 98.8 ± 2.4% for pre- and post-buffered extracts, respectively. The assay can be used both within laboratory settings and in remote locations. The accuracy of the new assay, to indicate negative results at or below 10 ppm DA, and positive results at or above 17.5 ppm, was 99.5% (n=216 tests). Validation data were obtained from a 2-day, randomised, blind study consisting of multiple LFIA lots (n=3), readers (n=3) and operators (n=3), carrying out multiple extractions of mussel tissue (n=3) at each concentration (0, 10, 17.5, and 20 ppm). No matrix effects were observed on the performance of the assay with different species (mussels, scallops, oysters, clams, and cockles). There was no impact on accuracy or interference from other phycotoxins, glutamic acid or glutamine with various strip incubations (8, 10, and 12 min). The accuracy of the assay, using naturally contaminated samples to indicate negative results at or below 12.5 ppm and positive results at or above 17.5 ppm, was 100%. Variability between three LFIA lots across a range of DA concentrations, expressed as coefficient of variation (% CV), was 1.1 ± 0.4% (n=2 days) based on quantitative readings from the electronic reader. During an 8 week stability study, accuracy of the method with test strips stored at various temperatures (6, 22, 37 and 50 °C) was 100%. Validation for both versions included comparisons with results obtained using reference LC–UV methods.

1. Introduction

Phycotoxins can have serious implications for both the economy and public health [1]. As a result, routine monitoring for a number of recognised marine biotoxins in edible shellfish is a regulatory requirement in many areas worldwide, including the European Union, USA, Canada, New Zealand and Australia [2,3].

One of the prevalent toxins that can be present in filter-feeding marine organisms, such as shellfish, is domoic acid (DA); this was first isolated from the red algae *Chondria armata* [4]. Clinical symptoms that may occur due to consumption of DA-contaminated shellfish include permanent short term memory loss, disorientation, confusion, in addition to more typical food poisoning symptoms associated with the gastrointestinal tract (vomiting and diarrhoea). In severe cases, death may result [5,6]. DA has been widely referred to as the primary amnesic shellfish poisoning (ASP) toxin, based on a

* Corresponding author at: Neogen Europe Limited, The Dairy School, Auchincruive, Ayr, KA6 5HW, Scotland, UK. Tel.: +44 1292 525553; fax: +44 1292 525601.
E-mail addresses: wjawaid@gmail.com, w.jawaid@neogeneurope.com (W. Jawaid).

0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.07.027

characteristic neurological symptom observed as a result of the 1987 crisis in Canada [7]; maximum permitted levels of, typically, 20 mg DA/kg shellfish tissue (20 ppm) are imposed for most species.

Ensuring consumer safety via monitoring of such toxins has, historically, relied upon animal-based assays to provide an indication of sample toxicity [8]. However, in recent years there has been increasing focus to develop and adopt non animal-based methods. This is due not only to moral and ethical concerns but performance issues, such as reproducibility, sensitivity and specificity [9,10]. Analytical chemistry methods involving the use of liquid chromatography (LC) [11] have been widely accepted and adopted for the accurate detection of DA from shellfish extracts [12]; however, the requirement of skilled personnel and expensive equipment, which require time and maintenance, is a limitation of such methods. Another accepted methodology, which offers the potential benefits of providing accurate and specific assays, and which can be used by official monitoring laboratories in areas such as the EU, are immunoassays [13]. This includes immunobiosensors, enzyme-linked immunosorbent assays (ELISAs) and lateral flow immunoassays (LFIAs). Biosensors adopted for this purpose require high initial costs for equipment and are currently limited to laboratory-based settings [14,15]; whereas, ELISAs are not truly rapid, simple and practical screening tools [16–18]. LFIAs are widely acknowledged as providing a low cost and simple means to rapidly screen samples. Due to these attributes, in addition to minimal requirements for equipment, LFIAs can be tested off-site or in remote locations. This would provide rapid results, and could potentially complement official, established marine biotoxin monitoring methods. However, performance issues associated with many LFIAs have been widely reported within the literature and include accuracy, sensitivity, interference, matrix effects, stability, ruggedness, robustness, reproducibility and lot variability [19–21]. In addition to the innate strengths of the LFIA method (portable, simple, rapid, and low cost), the ideal LFIA should also address the performance issues commonly encountered with the method.

In this publication, the development and validation of the first high performance-LFIA (HP-LFIA) for DA is reported, which fully meets the demands of both industrial and regulatory end users.

2. Materials and methods

2.1. Toxins and other spiking materials

DA certified standards (CRM-DA-f, CRM-Mus-C, and CRM-Mus-D) and saxitoxin dihydrochloride (CRM-STX-e and CRM-STX-f) were obtained from the National Research Council of Canada (NRCC, Halifax NS, Canada). Larger quantities of DA were also obtained from BioVectra Inc. (PE, Charlottetown, Canada), to produce spiked shellfish samples. DA concentrations were verified using LC–UV prior to use in spiked studies. Okadaic acid was obtained from LC laboratories (Woburn, MA, US). Glutamic acid and L-glutamine were obtained from Sigma (US).

2.2. Liquid chromatography–ultraviolet (LC–UV) analysis

Accepted reference methods were employed for quantitative sample analysis and data generation during the validations. The protocols were based on a previously published method, in which DA was extracted from the shellfish using 50% methanol and the extracts were analysed using reverse-phase LC–UV with diode array detection and monitoring at a wavelength of 242 nm [11].

2.3. Sources and treatment of samples

Certified negative mussel samples (CRM-Zero-Mus) were obtained from the NRCC. LC–UV verified negative and DA contaminated samples (mussels, oysters, scallops, clams and cockles) were obtained from the Agri-Food and Biosciences Institute (Northern Ireland, UK), the California Department of Public Health (CDPH, CA, US), or were locally sourced. Locally sourced fresh samples were cleaned and opened by cutting the adductor muscles. Tissue was removed from the shell, transferred to strainers and drained prior to homogenisation using a blender or Ultra Turrax®. Homogenates were distributed into aliquots and all homogenates that were not used immediately were frozen at −20 °C until analysis.

2.4. Principle of LFIA test strips

The assay developed was a single-step LFIA based on a competitive immunoassay format, as shown in Fig. 1. The test strips consist of several key components, which are adhered onto a backing strip and over-laminated using a cover strip. The sample is wicked from the sample pad through to a reagent pad containing antibodies specific for DA that have been conjugated to coloured particles, which produce the visible signals. If DA is present in the sample, the toxin will be captured by the particle–antibody complex. The complex is then wicked onto a membrane, which contains a stationary test zone containing a DA-protein conjugate (target material zone). This zone captures free conjugated antibody (antibody that has not bound with DA within the sample). Therefore, as the concentration of DA in the sample increases, the test zone intensity decreases. The membrane also contains a stationary control zone, which will always form regardless of the concentration of DA. Excess sample is absorbed onto a wicking pad, which acts as a waste reservoir to prevent backflow.

2.5. LFIA readers

Portable electronic LFIA readers (Reveal® AccuScan III and AccuScan® PRO) were supplied by Neogen Corporation (MI, US). Reveal® AccuScan III was employed for LFIA version 1 validations.

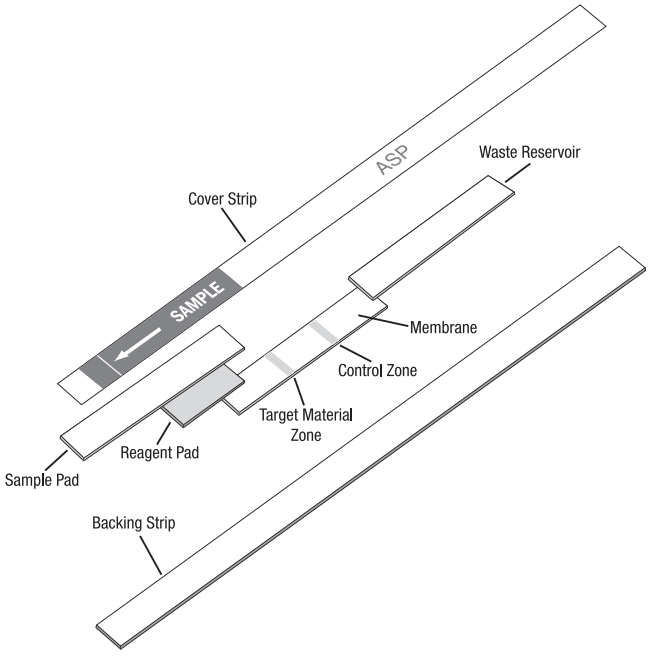

Fig. 1. Components of LFIA test strip.

AccuScan® PRO generated lower mean coefficient of variations (% CVs) across a range of DA concentrations and was employed for key LFIA version 2 validations.

2.6. LFIA assay design goals

2.6.1. LFIA version 1

The goals were to produce a qualitative assay to allow for the screening of shellfish samples (mussels, oysters, scallops, clams, and cockles) containing DA at suspect levels. Results were interpreted visually and the target cut-off for positive results was 10 ppm, whilst ensuring a minimal number of false positives below this concentration. A polyclonal antibody was employed for the assay [14].

2.6.2. LFIA version 2

The goals of the improved assay were to: (1) produce a reader-based qualitative assay to eliminate any subjective element to the generation of results and (2) increase the positive screening cut-off to 17.5 ppm, whilst ensuring no false negatives at 20 ppm and a minimal number of false positives below 17.5 ppm. A monoclonal antibody was employed [15].

2.7. Sample preparation

1 g of homogenised shellfish tissue was weighed from larger pools of homogenates (> 100 g) into each screw-cap plastic container. These samples were spiked with fixed volumes of the relevant standard concentration of DA toxin for the preparation of spiked matrix samples.

2.8. Procedure for extracting toxins

2.8.1. LFIA version 1 procedure

Distilled water (20 mL) was added to 1 g of weighed homogenised shellfish tissue, which was then mixed by hand for 30 s. The extract was poured into a 280 μM micro-perforated filter bag (BagPage®+100, Interscience, France) and a metal-seam roller applied for 30 s to ensure maximal toxin recoveries and a homogenate mixture. Subsequently, 100 μL of the filtrate was added to distilled water (20 mL) in a plastic screw-cap vial.

2.8.2. LFIA version 2 procedure

Distilled water (30 mL) was added to 1 g of shellfish tissue, which was then mixed by hand for 30 s. The extract was poured into a filter bag and a metal-seam roller applied for 30 s. Subsequently, 100 μL of filtrate was added to a pH 7 buffer solution containing various salts and surfactants optimised for the assay (1.3 mL).

2.9. Analysis of DA by the LFIA

2.9.1. LFIA version 1 analysis

100 μL of the sample extract was added to a sample cup or microwell. A test strip was placed within the sample cup or microwell for 10 min. A qualitative result (negative/positive) was visually interpreted by use of a reference card, as shown in Fig. 2.

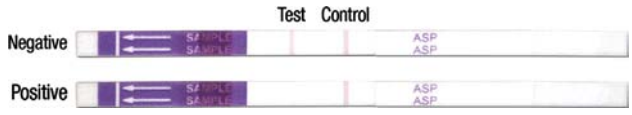

Fig. 2. LFIA version 1 reference card. The test line intensity determines the result. The test line intensity on the 'Positive' test strip marks the cut off: any test strip with a darker test line is negative, whereas any test strip with a line equal to or lighter than the 'Positive' deemed positive.

To represent the results graphically, test and control line readings via an electronic reader were also taken during key studies.

2.9.2. LFIA version 2 analysis

100 μL of the sample extract was added to a microwell. A test strip was placed within the microwell for 10 min, prior to placement into an LFIA reader. The qualitative result generated was derived from quantitative test and control line readings obtained from the test strip.

2.10. LFIA method validation

Serial dilutions using CRM-DA-f were prepared to obtain the spiked buffer dose-response graphs and hence determine the optimal matrix dilution factor, which was required to effectively screen samples containing suspect levels of DA. The dilution factors were selected based on three criteria: the region on the curve which would (1) provide sufficient resolution at the desired cut-off, (2) allow for a simple and practical dilution scheme and (3) be large enough to reduce or eliminate non-specific interference on assay performance from various shellfish matrices. DA standard concentrations ranged from 0 to 800 ng/mL. DA spiking concentrations in shellfish typically ranged from 0 to 40 μg/g (ppm), with LC–UV verification of samples carried out during key stages to determine the accuracy of data. LC–UV verified naturally contaminated samples (mussels, oysters and scallops) were used as part of the validation to determine the accuracy and toxin recovery with true samples. Pools of mussel tissue containing DA at concentrations ranging from 0 to 20 ppm were prepared (CRM-Mus-Zero and CRM-Mus-D) and aliquoted for use in multiple LFIA version 2 studies (sample extract stability, test strip stability, LFIA lot comparisons and robustness validations). Concentrations and homogeneity were verified using LC–UV using randomly selected aliquots ($n=3$) from each sample pool (0, 10, 17.5, and 20 ppm). Robustness and ruggedness studies were carried out to determine the extent and key sources of uncertainty, associated with the method, to various intrinsic and extrinsic factors (operators, days, LFIA lots, extractions, readers, species, assay run time, ambient temperature and humidity).

3. Results and discussion

3.1. LFIA version 1

3.1.1. Spiked buffer study

The linear portion of the dynamic range of the assay was approximately 0.25–50 ng/mL DA, as shown in Fig. 3. To ensure samples were effectively screened at the desired 10 ppm cut-off, a considerable matrix dilution factor was selected (more than 4000 fold). In shellfish, 10 ppm equated to approximately 2.5 ng/mL based on the dilution factor; where 95% inhibition of response was observed. At the equivalent of 20 ppm, there was 99% inhibition of response. The theoretical visual limit of detection (LOD) in shellfish, estimated to occur at 50% response, was 2 ppm DA.

3.1.2. Accuracy study

A randomised, blind study was conducted by comparison of the colour intensity on the test line against a reference card, as shown in Fig. 2. Five different shellfish species (mussels, scallops, oysters, clams and cockles) were extracted and analysed by two operators at various DA concentrations (0, 2, 10, 20, and 40 ppm) using two LFIA lots ($n=5$ tests per condition). The overall accuracy, to indicate negative results at or below 2 ppm and positive results at or above 10 ppm, was 100% ($n=500$ tests). Although maximal accuracy was obtained in this study, the 2 and 10 ppm DA sample

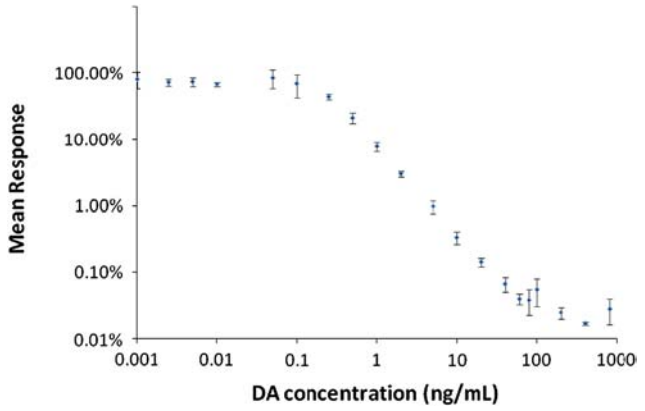

Fig. 3. Spiked buffer dose–response graph (LFIA version 1). Plots of mean response ± SD ($n=3$) for various concentrations of DA. Linear dynamic range between approximately 0.25 and 50 ng/mL DA.

tests were visually close to the threshold of detection (the positive test strip on the reference card) in a number of cases.

3.1.3. Naturally contaminated samples and comparisons with LC–UV

Studies evaluating naturally contaminated samples, which were verified using LC–UV, were conducted using the LFIA method, to assess whether the simple aqueous extraction procedure was effective at recovering toxins from real samples in addition to spiked materials. Samples included mussels and scallops (whole, muscle only, gonads and muscle) with DA concentrations ranging from 1.1 to 106 ppm ($n=29$ samples). Results are shown in Table 1. The accuracy, to indicate negative results at or below 4 ppm and positive results at or above 9.3 ppm, was 100% ($n=87$).

3.1.4. Specificity (interference)

A study to assess the specificity of the assay was conducted using three matrices (mussels, oysters, and clams) spiked at 0, 10 and 40 ppm DA. Each sample was also spiked with one of four potentially interfering compounds: okadaic acid (10 ppm), glutamic acid (100 ppm), glutamine (100 ppm) or saxitoxin (5 ppm). Each condition was tested using five test strips. Results indicated no evidence of interference by any of the compounds. The overall accuracy of the study, to indicate negative results at 0 ppm and positive results at 10 or 40 ppm, was 100% ($n=180$).

3.1.5. Ruggedness – temperature/humidity

Assay ruggedness trials with respect to five different indoor and outdoor ambient conditions (temperature and humidity ranging from 4 to 50 °C and 15% to 83%, respectively) were conducted using mussel samples spiked at various DA concentrations (0, 2 and 20 ppm). Each condition was tested in triplicate. The test strips were found to function in all evaluated conditions, although it was observed that the tests took longer to clear background antibody-particle conjugates at colder temperatures; taking up to 9 min at 4 °C and under 5 min at 50 °C. The overall accuracy of the study with all conditions, to indicate negative results at 0 and 2 ppm and positive results at 20 ppm, was 100% ($n=45$ tests).

3.1.6. Ruggedness – test strip run time

Assay ruggedness trials with respect to various incubation times (8, 10 and 12 min) were conducted using three matrices (mussels, oysters, and clams) spiked at 0, 10 and 40 ppm DA ($n=40$ tests per sample, multiple operators). No impacts on performance were observed with the various conditions; the overall accuracy, to indicate negative results at 0 ppm and positive results at 10 or 40 ppm, was 94.7% ($n=1080$ tests). The accuracy for the 8, 10 and 12 min conditions were 94.4%, 94.7% and 95%, respectively. All 0 and 40 ppm results were correctly identified. 10 ppm tests were at the threshold of detection, as intended, but some tests deemed positive by operators (laboratory 2, US) and accuracy to indicate positive results at 10 ppm was 83.3%, 84.2% and 85% ($n=120$) at 8, 10 and 12 min, respectively.

Table 1
LFIA results from naturally contaminated sample evaluations and comparative data obtained with LC–UV reference methods (LFIA version 1).

Sample source	LC–UV laboratory	Shellfish	Sample	LC–UV result (ppm DA)	LFIA results	
					# Tests	# Positives
US	Canada	Mussels	1	16.4 ± 0.3[a]	3	3
			2	15.4 ± 0.1[a]		3
			3	14.5 ± 0.1[a]		3
			4	15.7 ± 0.2[a]		3
			5	15.1 ± 0.04[a]		3
			6	14.6 ± 0.03[a]		3
			7	13.7 ± 0.1[a]		3
			8	15.2 ± 0.1[a]		3
			9	14.0 ± 0.1[a]		3
			10	11.9 ± 0.1[a]		3
EU	EU	Scallops	11	53	3	3
			12	30		3
			13	57		3
			14	4		0
			15	106		3
			16	3		0
			17	3		0
			18	26		3
NI	NI	Scallops	19	50	3	3
			20	9.26		3
			21	12.4		3
			22	88 ± 8[b]		3
			23	1.6 ± 0.4[b]		0
		Mussels	24	1.1		0
Scotland	Scotland	Scallops	25	26.7	3	3
			26	39.8		3
			27	19.8		3
			28	34.7		3
			29	18.1		3

[a] Mean ± SD based on 3 determinations from a single extraction.
[b] Mean ± SD based on 2 extractions.

3.1.7. Stability

A 12 month stability trial was conducted with test strips stored refrigerated (2–8 °C) and at room temperature (20–25 °C). Test strips were evaluated at various time points (months 1, 2, 3, 4, 6, 8, 10 and 12) using spiked buffers containing the equivalent of 0, 2 and 20 ppm DA in shellfish; with *ad hoc* checks using 2 and 15 ppm DA spiked mussel extracts ($n=5$ tests per condition). The accuracy of the study, to indicate negative results at 0 or 2 ppm and positive results at 15 or 20 ppm, was 100% ($n=325$) based on the reference card.

3.1.8. Inter-laboratory evaluations

A randomised, blind study involving four mussel samples (<0.5, 10.3, 20.4 and 41 ppm DA) and two scallop samples (1.2 and 94 ppm DA) was conducted by five external participants, within the EU and US, who had no prior experience with the test. Each sample was to be run using five test strips each and a result reported for each test strip based on the reference card ($n=30$ tests per site). The accuracy for the study, to indicate negative results at or below 1.2 ppm and positive results at or above 10.3 ppm, was 100% ($n=150$ tests).

3.1.9. Quality control (QC) and lot reproducibility

Twelve lots of LFIAs were produced between 2010 and 2012. QC testing of the manufactured LFIA lots was performed at both in-process and finished product stages. In-process testing consisted of balancing the antibody-particle conjugate for optimal test and control line intensity, and testing the device membrane for proper test and control line placement by running negative samples. Manufacturing proved to be a laborious and technical challenge to maintain performance characteristics, whereby: (1) 2 ppm samples always produced a negative result and 10 ppm samples produced a positive result at the threshold of detection; whilst (2) ensuring line intensities matched the reference card. Both of the aforementioned criteria had to be met in order to ensure maximum performance of the assay.

3.1.10. LFIA version 1 summary

The assay proved to be an effective tool for the rapid and simple screening of DA from shellfish. Validations from six different LFIA lots demonstrated 100% accuracy in obtaining positive results at or above 20 ppm samples and negative results at or below 2 ppm. However, there were several limitations identified with LFIA version 1: (1) the subjective nature of the test; (2) unwanted positives from some industrial end-user applications, which occurred with samples containing around 10 ppm DA; (3) tedious manufacturing processes and (4) limited supply of polyclonal antibody. To completely eliminate the subjective nature of the test and to also further improve the assays resolution around the regulatory limit, a new version of the assay was developed employing a more robust manufacturing process and the embodiment of a monoclonal antibody (LFIA version 2).

3.2. LFIA version 2

3.2.1. Spiked buffer study

The linear portion of the dynamic range of the assay was approximately 5–200 ng/mL DA, as shown in Fig. 4. To ensure samples were effectively screened at the desired 17.5 ppm cut-off, a matrix dilution factor of more than 400 fold was selected. In shellfish, 17.5 ppm equated to approximately 42 ng/mL based on the dilution factor; where 30% response was observed. The theoretical LOD for DA in shellfish was approximately 2 ppm, where 80% response was observed.

3.2.2. Sample extract stability and toxin recovery

A study to determine the stability of the aqueous sample extracts at room temperature (22–25 °C) was conducted using mussels containing DA at 0, 10, 17.5 or 20 ppm. The pre-buffered and buffered extracts were both tested at various time points (within 15 min of extraction; 45, 145, and 245 min after extraction) and were left at room temperature in plastic screw-cap vials. Pre-buffered extracts were diluted in the running buffer at the specified time points. The overall accuracy for both the pre-buffered and buffered sample extracts, to indicate negative results at 0 or 10 ppm and positive results at 17.5 or 20 ppm, was 100% ($n=160$). Test and control line readings demonstrated the stability of extracts, relative to data from fresh extractions across the range of concentrations. The stability was determined as $100.3 \pm 1.3\%$ and $98.8 \pm 2.4\%$ ($n=4$) for pre-buffered and buffered extracts, respectively.

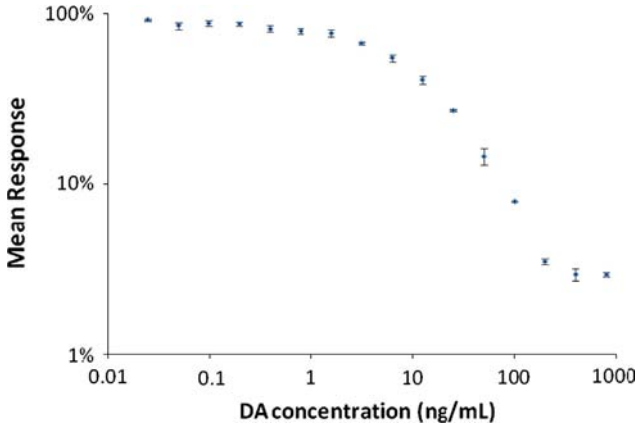

Fig. 4. Spiked buffer dose–response graph (LFIA version 2). Plots of mean response ± SD ($n=3$) for various concentrations of DA. Linear dynamic range between approximately 5 and 200 ng/mL DA.

3.2.3. Accuracy study A (laboratory 1)

A study to determine the accuracy of the method was conducted using five matrices (mussels, oysters, scallops, clams, and cockles) spiked at 0, 10, 17.5 and 20 ppm DA ($n=5$ tests per condition). No matrix effects were observed. A two operator follow-up study using pools of homogenate mussel tissues (CRM-Zero-Mus and CRM-Mus-D) using the same DA concentrations was also conducted ($n=5$ tests per condition). The accuracy, to indicate negative results at 0 or 10 ppm and positive results at 17.5 or 20 ppm, was 100% ($n=140$).

3.2.4. Accuracy study B (laboratory 2)

A second accuracy study was conducted and involved preparation of spiked matrices (mussels, oysters, and clams) at 0, 10, 17.5 and 20 ppm DA. The overall accuracy of the method was 98.2% ($n=225$ tests). For mussels and oysters, accuracy of the assay was 100% ($n=150$). For clams, accuracy of the assay was 94.7% ($n=75$), as only 80% of samples containing 17.5 ppm DA were classed as positive ($n=20$). All other samples produced accurate results.

3.2.5. Naturally contaminated samples and comparisons with LC–UV reference methods

Studies involving naturally contaminated samples, which were verified using LC–UV, were conducted using the LFIA method. Samples included mussels and oysters with toxin concentrations ranging from 2.3 to 30.6 ppm DA ($n=21$ samples), plus a control mussel sample (CRM-Mus-D). LC–UV data for the control sample indicated a recovery of 97.6%, based on the certified value by NRCC (Canada). Results are shown in Table 2. All samples containing DA at or below 12.6 ppm DA produced negative results ($n=70$) and all samples containing at or above 17.6 ppm DA produced positive results ($n=25$). Mussel samples quantified using LC–UV to contain 16.0 and 16.8 ppm DA (samples 17 and 19, respectively) were correctly classified as negative in seven tests ($n=10$), suggesting the assay's cut off to reside at or around these concentrations.

3.2.6. Ruggedness – LFIA lot

Assay ruggedness trials with respect to LFIA lot were conducted using mussels prepared at 0, 10, 17.5 and 20 ppm DA. Three lots of test strips were evaluated on two separate days ($n=10$ tests per condition, selected from a random selection of different cards from each LFIA lot). The overall accuracy of the method was 100% with all lots ($n=120$ tests). Variability between the three LFIA lots across the range of DA concentrations, expressed as % CV, was $1.1 \pm 0.4\%$ ($n=2$ days) based on the test and control line readings.

3.2.7. Ruggedness – test strip run time

Assay ruggedness trials with respect to incubation times were conducted using three matrices (mussels, oysters, and clams) spiked at 0, 10 and 20 ppm DA. Various incubations of the test strip (8, 10 and 12 min) were evaluated. The overall accuracy, to

indicate negative results at 0 and 10 ppm and positive results at 20 ppm, with all conditions was 100% ($n=384$ tests).

3.2.8. Robustness study

A 2-day randomised, blind study consisting of multiple LFIA lots ($n=3$), readers ($n=3$) and operators ($n=3$) carrying out multiple extractions of mussel tissue ($n=3$) at each concentration (0, 10, 17.5, and 20 ppm) was conducted. Mussel samples were prepared at each concentration and the blended pools aliquoted into 21 portions for the study, three of which were randomly selected for LC–UV analysis to ensure homogeneity and accuracy. Results are shown in Table 3. LC–UV results demonstrated the four samples to contain $100.4 \pm 2.5\%$ of intended concentrations. The accuracy of the LFIA, to indicate negative results at or below 10 ppm DA and positive results at or above 17.5 ppm, was 99.5% ($n=216$ tests); with one inaccurate result occurring at 17.5 ppm (operator 1, day 1, extraction 1, reader 1, and LFIA Lot 3). The overall mean CV of the method across the four DA concentrations, based on quantitative test and control line readings, was $10.4 \pm 1.8\%$, indicating the cumulative sum of inter- and intra-variability (operators, readers, extractions, LFIA lots and days).

Table 2
LFIA results from contaminated sample evaluations and comparative data obtained with the LC–UV reference method (LFIA version 2).

Source	Shellfish	Sample	LC–UV result (ppm DA)	LFIA results	
				# Tests	# Positives
US/Canada	Oysters	1	3.0	5	0
		2	28.2		5
		3	11.4		0
		4	1.9		0
		5	3.3		0
		6	5.1		0
		7	7.0		0
		8	17.6		5
		9	8.9		0
	Mussels	10	12.6	5	0
		11	30.6		5
		12	10.1		0
		13	4.7		0
		14	6.1		0
		15	17.6		5
		16	8.1		0
		17	16.0		2
		18	5.4		0
		19	16.8		1
		20	2.3		0
		21	29.6		5
NRCC	Mussels[a]	22	47.8	5	5

[a] CRM-Mus-D certified concentration: 49 ppm DA (NRCC).

3.2.9. Specificity (interference)

A study to assess the specificity of the assay was conducted using three matrices (mussels, oysters, and clams) spiked at 0, 10 and 20 ppm DA. Each sample was also spiked with one of four potentially interfering compounds: okadaic acid (10 ppm), glutamic acid (100 ppm), glutamine (100 ppm) or saxitoxin (5 ppm). Results indicated no evidence of interference by any of the compounds. The overall accuracy of the study, to indicate negative results at 0 or 10 ppm and positive results at 20 ppm, was 100% ($n=180$).

3.2.10. Stability

A 2 month accelerated stability study involving LFIA storage at four different temperature conditions (4–8 °C, 20–25 °C, 37 °C and 50 °C) was conducted at various time points (weeks 0, 1, 2, 3, 4, 6 and 8). Extracts were prepared from mussel samples containing various concentrations of DA (0, 10 and 20 ppm). Each extract was analysed using five test strips for each condition. The overall accuracy of the study, to indicate negative results at 0 or 10 ppm and positive results at 20 ppm, was 100% ($n=375$).

3.2.11. Inter-laboratory evaluations

A randomised blind study involving six mussel samples, two samples each at concentrations of <0.5, 10 and 20 ppm DA, was conducted by four external participants. Each sample was to be

Table 3
LFIA robustness study results and comparative data using the LC–UV reference method (LFIA version 2).

Concentration (ppm DA)	LC–UV result[a] (ppm DA)	Operator	Day	# Extractions	# Tests	# Positive results		
						LFIA lot 1	LFIA lot 2	LFIA lot 3
0	< LOD	1	1	3	9	0	0	0
			2			0	0	0
		2	1			0	0	0
			2			0	0	0
		3	1			0	0	0
			2			0	0	0
10	10.3 ± 0.7	1	1	3	9	0	0	0
			2			0	0	0
		2	1			0	0	0
			2			0	0	0
		3	1			0	0	0
			2			0	0	0
17.5	17.8 ± 0.5	1	1	3	9	3	3	2
			2			3	3	3
		2	1			3	3	3
			2			3	3	3
		3	1			3	3	3
			2			3	3	3
20	19.4 ± 0.2	1	1	3	9	3	3	3
			2			3	3	3
		2	1			3	3	3
			2			3	3	3
		3	1			3	3	3
			2			3	3	3

[a] Mean $\pm$ SD based on three extractions carried out on sub-aliquots from each sample homogenate pool.

run using two test strips each and a result reported for each test strip ($n=12$ tests per site). The accuracy for the study, to indicate negative results at or below 10 ppm and positive results at 20 ppm, was 100% ($n=48$ tests).

3.2.12. Quality control (QC) and lot reproducibility

QC testing of manufactured LFIA lots was performed at both in-process and finished product stages. Devices passed set criteria, using mussel samples containing 0, 10, 17.5 and 20 ppm DA, whereby 100% accuracy was obtained using a representative number of test strips selected at random from multiple cards. Defined CV limits, based on quantitative test and control line readings, were to be met to ensure maximal performance from the test strips.

3.2.13. LFIA version 2 summary

The use of LFIAs has been previously exploited for detection of marine biotoxins. However, two major weaknesses for some end-users have been the occurrence of false positive results at concentrations considerably lower than the regulated limits for DA [22] and the subjective nature of interpretation. LFIA version 2 was designed as a reader-based assay to eliminate any subjective element, with a positive cut off for DA in shellfish markedly closer to the regulatory limits at 17.5 ppm DA. Substantial validation data demonstrated the occurrence of no false negative results with samples containing 20 ppm DA or greater and no false positives with samples containing up to 12.5 ppm DA. Furthermore, the new assay has simpler and more robust manufacturing procedures.

4. Conclusions

A robust and rapid high performing (HP) LFIA has been reported which demonstrates practical, simple and accurate screening for DA contamination in shellfish. The assay can be utilised as a potential early warning detection system for use within the shellfish industry including shipboard or remote locations, providing added protection of shellfish consumers. Results demonstrate a rapid single-step assay, which requires minimal materials, that provides the simplest DA extraction and detection system reported to date. More importantly, the assay has demonstrated high performance characteristics with respect to accuracy, recovery, specificity, matrix effects, robustness, ruggedness, reproducibility and stability. There is also a significant potential for this method to be used in regulatory laboratories to replace the costly LC–UV based tests that are routinely employed as a part of the national monitoring programmes. Such simple to use and low cost methods are ideal for laboratories in developing countries where the ability to purchase, run and maintain expensive LC–UV equipment is very difficult.

Acknowledgements

The authors express their thanks to the UK national reference laboratory for marine biotoxins, AFBI for provision of samples, assisting with method validations and for performing LC–UV analyses; CDPH (Canada) for provision of samples; and NRCC for performing LC–UV analyses. The authors would also like to thank (in alphabetical order): BioPol (Iceland), The Center for Food Safety and Applied Nutrition (CFSAN), FDA (MD, US), CVUA Sigmaringen (Germany), Loch Fyne Seafarms (UK), MacDuff Shellfish (UK), The National Oceanic and Atmospheric Administration (US) and Shetland Seafood Quality Control (UK) for participation in the inter-laboratory trials; James Clarke, Tim Goldy, Frank Klein and Steven Schadler (Neogen Corporation) for key technical input; Oscar Caballero, Jill Feldpausch, R. Lucas Gray, Jake Knickerbocker and Mark Mozola (Neogen Corporation) for the generation of additional validation data; Nate Banner and Sharon Graham (Neogen Corporation) for their valuable efforts and input; and Paul Muirhead (Neogen Corporation) for performing LC–UV analyses. The authors would like to acknowledge that the work was part-funded by a Knowledge Transfer Partnership (KTP) programme.

Note: organisations listed above do not endorse or promote the LFIA.

References

[1] J. Sobel, J. Painter, Clin. Infect. Dis. 41 (2005) 1290.
[2] European Commission Regulation (EC) No. 2074/2005 under regulations (EC) nos. 853/2004 and 854/2004, Off. J. Eur. Comm., vol. 358, 2005, pp. 27.
[3] Codex Alimentarius, Codex standard 292, Standard for live and raw bivalve molluscs, Codex Alimentarius Commission, Rome, Italy 2008.
[4] T. Takemoto, K. Daigo, Jpn. Chem. Pharm. Bull. 6 (1958) 578.
[5] L. Mos, Environ. Toxicol. Pharmacol. 9 (2001) 79.
[6] A.L. Lefebvre, A. Robertson, Toxicon 56 (2010) 218.
[7] J.L.C. Wright, R.K. Boyd, A.S.W. De Freitas, M. Falk, R.A. Foxall, W.D. Jamieson, M.V. Laycock, A.W. McCulloch, A.G. McInnes, P. Odense, V.P. Pathak, M. A. Quilliam, M.A. Ragan, P.G. Sim, P. Thibault, J.A. Walter, M. Gilgan, D.J. A. Richard, D. Dewar, Can. J. Chem. 67 (1989) 481.
[8] I. Garthwaite, K.M. Ross, C.O. Miles, R.P. Hansen, D. Foster, A.L. Wilkins, N. R. Towers, Nat. Toxins 6 (1999) 93.
[9] P. McNabb, A.I. Selwood, P.T. Holland, J. AOAC Int. 88 (2005) 761.
[10] B. Christian, B. Luckas, Anal. Bioanal. Chem. 391 (2008) 117.
[11] M.A. Quilliam, M. Xie, W.R. Hardstaff, J. AOAC Int. 78 (1995) 543.
[12] Anonymous, Approaches to validation and regulatory acceptance of alternative methods to the mouse bioassay as method of reference, Bunderinstitut fur risikobewertung, Berlin, Germany, 2005.
[13] European Commission (EC), Decision 2002/226/EC, Council Directive 91/492/EEC, Off. J. Eur. Comm., vol. 75, 2002, pp. 62.
[14] I.M. Traynor, L. Plumpton, T.L. Fodey, C. Higgins, C.T. Elliott, J. AOAC Int. 89 (2006) 868.
[15] T.F. McGrath, K. Andersson, K. Campbell, T.L. Fodey, C.T. Elliott, Biosens. Bioelectron. (2013) 96.
[16] M. Osada, L.J. Marks, J.E. Stewart, Contam. Toxicol. 54 (1995) 797.
[17] R.W. Litaker, T.N. Stewart, B.-T.L. Eberhart, J.C. Wekell, V.L. Trainer, R.M. Kudela, P.E. Miller, A. Roberts, C. Hertz, T.A. Johnson, G. Frankfurter, G.J. Smith, A. Schnetzer, J. Schumacker, J.L. Bastian, A. Odell, P. Gentien, D. Le Gal, D. R. Ardison, P.A. Tester, J. Shellfish Res. 27 (2008) 1301.
[18] I. Garthwaite, Trends Food Sci. Technol. 11 (2000) 235.
[19] R.C. Wong, H.Y. Tse (Eds.), Humana Press, New York, USA, 2008.
[20] G.A. Posthuma-Trumpie, J. Korf, J.A.V. Amerongen, Anal. Bioanal. Chem. 393 (2009) 569.
[21] L. Anfossi, C. Baggiani, C. Giovannoli, G. D'Arco, G. Giraudi, Anal. Bioanal. Chem. 28 (2012) 1.
[22] C. Rafuse, A.D. Cembella, M. Laycock, J. Jellett., Harmful Algae (2002).