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Development and Validation of a Novel Lateral Flow Immunoassay (LFIA) for the Rapid Screening of Paralytic Shellfish Toxins (PSTs) from Shellfish Extracts

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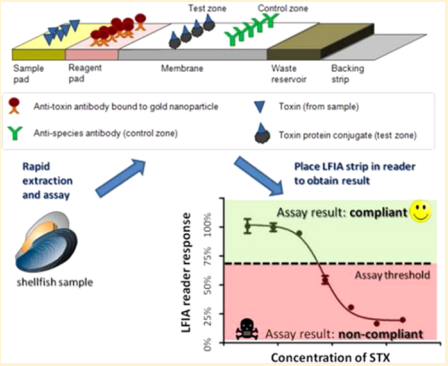
Supporting Information

ABSTRACT: A single-step lateral flow immunoassay (LFIA) was developed and validated for the rapid screening of paralytic shellfish toxins (PSTs) from a variety of shellfish species, at concentrations relevant to regulatory limits of 800 μg STX-diHCl equivalents/kg shellfish meat. A simple aqueous extraction protocol was performed within several minutes from sample homogenate. The qualitative result was generated after a 5 min run time using a portable reader which removed subjectivity from data interpretation. The test was designed to generate noncompliant results with samples containing approximately 800 μg of STX-diHCl/kg. The cross-reactivities in relation to STX, expressed as mean ± SD, were as follows: NEO: 128.9% ± 29%; GTX1&4: 5.7% ± 1.5%; GTX2&3: 23.4% ± 10.4%; dcSTX: 55.6% ± 10.9%; dcNEO: 28.0% ± 8.9%; dcGTX2&3: 8.3% ± 2.7%; C1&C2: 3.1% ± 1.2%; GTX5: 23.3% ± 14.4% (n = 5 LFIA lots). There were no indications of matrix effects from the different samples evaluated (mussels, scallops, oysters, clams, cockles) nor interference from other shellfish toxins (domoic acid, okadaic acid group). Naturally contaminated sample evaluations showed no false negative results were generated from a variety of different samples and profiles (n = 23), in comparison to reference methods (MBA method 959.08, LC-FD method 2005.06). External laboratory evaluations of naturally contaminated samples (n = 39) indicated good correlation with reference methods (MBA, LC-FD). This is the first LFIA which has been shown, through rigorous validation, to have the ability to detect most major PSTs in a reliable manner and will be a huge benefit to both industry and regulators, who need to perform rapid and reliable testing to ensure shellfish are safe to eat.



1. INTRODUCTION

The presence of harmful algal blooms (HABs) in seawater can result in toxins entering the food chain and give rise to a range of different toxin poisoning symptoms to humans.[1,2] One of the key vectors of clinical relevance which ingests and concentrates algal toxins is bivalve molluscs (shellfish). The toxins are sequestered mainly in the digestive glands of these filter feeders. The global economic impact due to marine biotoxins has been estimated at four billion USD per year.[3] However, under suitable conditions and with adequate time, shellfish can in some cases be detoxified and made safe to eat. Due to the severity of illness that may result in humans due to consumption of contaminated shellfish, and to help save loss of product, the presence of marine biotoxins in shellfish is monitored and regulated in many countries.[4,5]

One of the most dangerous and widespread shellfish poisoning symptoms in humans is paralytic shellfish poisoning (PSP). Clinical symptoms can appear several minutes to several hours after ingestion and can include nausea, vomiting, diarrhea, abdominal pain, burning in the mouth, respiratory distress, and fatal muscular paralysis.[6,7] Paralytic shellfish toxins (PSTs) can be produced by dinoflagellates of various genera including *Alexandrium* and *Gymnodinium*.[8] PSTs include saxitoxin (STX) and its structural analogues, which are all hydrophilic alkaloids of low molecular weight.[9] The chemical structures of key groups of PSTs are shown in Figure S-1, where substitutions at four specific positions on the generic molecule determine the different toxin analogues and toxic potency. The carbamate group is generally the most toxic on a molar basis; the decarbamoyl group consists of toxins of mostly intermediate toxicity; and the *N*-sulfocarbamoyl (or sulfamate) group is considered the least toxic. Many regions worldwide, such as the European Union (EU) and the USA, have therefore

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established action limits (AL), which are typically 800 μg STX-diHCl equivalents/kg shellfish meat (800 μg STX eq/kg).

The internationally recognized method for determination of PSTs is the mouse bioassay (MBA, AOAC OMA 959.08), which possesses a key advantage over alternative methods in that results will directly reflect sample toxicity.[10−13] The method has a reported detection limit of around 400 μg STX eq/kg and can be performed in a relatively short period of time. However, this bioassay is acknowledged as having several important limitations including poor sensitivity, variability, possibility of under- or over-estimating sample toxicity, and poor correlation with human oral toxicity. Furthermore, there has been increasing pressure to reduce use of the method due to the moral and ethical concerns involved with such *in vivo* toxicity models.[14−18]

Several analytical methods have been developed and adopted for the monitoring of PSTs, which help overcome some of the issues associated with the MBA. Although they require expensive instrumentation, skilled personnel, and use of certified reference standards, the methods are highly sensitive and specific laboratory based tools which allow for a medium throughput of samples to be analyzed. The use of liquid chromatography with fluorescent detection (LC-FD) is the most widely employed analytical methodology. The first internationally validated LC-FD method involved precolumn oxidation and is referred to as the "Lawrence" method, AOAC OMA 2005.06.[19] The method was subsequently accepted as an alternative to the MBA in the EU and has been since used as the primary screening method for the monitoring of PSTs by some EU laboratories.[6,12] However, the method is cumbersome, is resource intensive, and requires a high level of data processing. An LC-FD method, involving postcolumn oxidation, has also been internationally validated as AOAC OMA 2011.02 and subsequently approved in the United States by the ISSC (NSSP).[20] OMA 2011.02 provides a simpler overall method, including easier data processing, in comparison to OMA 2005.06. However, the method involves a more complex chromatographic setup, and the correlation with the MBA is not as good.[21]

Other analytical methodologies for the determination of PSTs include the use of liquid chromatography tandem-mass spectrometry (LC-MS/MS). Although LC-MS/MS based methods have not been internationally validated, they require only a single analysis to obtain a full toxin profile without derivatization of any toxins, in addition to providing high sensitivity and specificity.[22] When using chemical methods, toxicity equivalency factors (TEFs) are applied to each toxin congener to allow the total toxicity value to be calculated (Table S-1). TEFs applied by laboratories are usually based on values derived from Oshima's toxicity studies or those stipulated by the EFSA panel.[6,23−25]

Other methods which try to overcome some of the drawbacks associated with biological or analytical methods include molecular/functional assays. One such example, which has recently gained some interest, is the receptor binding assay (RBA), AOAC OMA 2011.27.[26] The method involves a competitive assay based on interactions of radiolabeled toxins with voltage-gated sodium channels (the natural binding sites of PSTs in mammalian nerve cells). Unlike the MBA, such methods do not require use of live animals. The method provides a result as STX eq and has greater sensitivity than the MBA. The assay is generally faster than analytical methods, taking less than 4 h to perform. However, there is still a requirement of animals (albeit a lower quantity than the MBA) for the preparation of the receptors, in addition to specialist equipment and materials.[27]

Though there are several different methods which have been validated for the screening of PSTs, there is an acknowledged requirement for more portable and cost-effective methods that are faster and simpler. The use of PST specific antibodies has provided a possible option to achieve this using a suitable immunoassay format, such as an enzyme-linked immunosorbent assay (ELISA) or lateral flow immunoassay (LFIA).[28] There have been several ELISAs which have been developed and validated for PSTs, many of which focus mainly on STX. Results can be produced usually within 2−3 h, with relatively simple data processing required. Recently, a method has been reported in the literature that can analyze a sample from homogenate in approximately 40 min.[29]

However, the simplest immunoassay format in terms of time, equipment, and operator expertise is the LFIA, which uses immunochromatographic test strips. Unlike ELISA, this format does not usually require the use of any standards, requires no data processing, and usually requires the least number of steps (often a single-step assay). The fastest reported PSP LFIA allowed a test result to be obtained in approximately 35 min from sample extraction.[30] However, one of the main challenges with immunoassays is that antibodies employed detect toxins based on structure and not function (i.e., toxicity). For many years the focus of immunoassays has mainly concentrated on STX, but this has proven to be a poor marker of PST presence due to the many different toxin profiles observed in naturally contaminated shellfish. As a result of this, developed immunoassays have had the potential to grossly overestimate toxicity with samples rich in C1&C2 and/or GTX5 (by up 10-fold or more); and more worryingly from a food safety perspective to underestimating toxicity with samples rich in toxins such as NEO, dcNEO, and/or GTX1&4 (by up to tens or hundreds of folds). The results in practice, though, will seldom show inaccuracies at such extremes due to the profiles of toxic samples often consisting of various mixes of different PSTs.[31,32]

The performance of all methodologies for PSTs can be influenced by a variety of parameters. Tissue specific interference, or matrix effects, is another issue which has been observed with all methods, including immunoassays, though to varying extents.[33−36] Another important consideration for immunoassays is the stability of the assay, which has raised concerns with some PST immunoassays.[30] Although there are rapid immunoassays which are accepted as regulatory screening tools for toxins such as domoic acid (Reveal 2.0 ASP); no method to date has proven to be a similarly rapid, simple, low cost, and effective means for the screening of PSTs.[38,39] This is due to aforementioned reasons including poor correlation with reference methods due to, e.g., cross-reactivity or matrix effects in addition to lack of speed, portability, or ease of use.[27,37]

The present study was designed to develop and fully validate a simple and rapid assay which required minimal materials and equipment and, even more importantly, correlated well to accepted reference methods (MBA and LC-FD) with a range of samples consisting of different species, profiles, and concentrations.

2. MATERIALS AND METHODS

2.1. Toxins. Certified toxin standards (CRM-STX-f, CRM-NEO-c, CRM-GTX1&4-c, CRM-GTX2&3-c, CRM-dcSTX-b,

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Table 1. Ruggedness Parameters

Parameter	Method Value	Low Value(s)	High Value(s)
Roller extraction	30 s	0, 15 s	60 s
Mixing time (buffered sample)	30 s	0, 5, 15 s	60 s
Mixing speed (buffered sample)	Fast*	Slow (0.5 rotation/s)	NA
Sample extract volume to buffer	100 μL	80, 90 μL	110, 120 μL
Buffered sample volume to well	100 μL	80, 90 μL	110, 120 μL
Strip run time	5 min	2, 3, 4 min	6, 7, 8 min

*Shaken vigorously by hand (no objective unit of measure to quantify speed).

CRM-dcNEO-c, CRM-dcGTX2&3-b, CRM-C1&C2-b, CRM-GTX5-b, CRM-GTX5-c) were obtained from the National Research Council of Canada (NRCC, Halifax, Canada).

2.2. Reference Methods. LC-FD (AOAC OMA 2005.06) and/or MBA (AOAC OMA 959.08) were the main methods employed for quantitative sample analyses during the validations. Except where stated, LC-FD data was not recovery corrected.

2.3. Sources and Treatments of Samples. Certified negative mussel tissues (CRM-Zero-Mus), in addition to certified positive mussel tissues for amnesic shellfish poisoning (ASP) and diarrheic shellfish poisoning (DSP) toxins (CRM-ASP-Mus-D, CRM-DSP-Mus-B, RM-ASP-Mus), were obtained from the NRCC. LC-FD certified (AOAC OMA 2011.02) PST contaminated oyster tissues (PO PST CRM 1101) were obtained from CEFAS (England, UK). LC-FD (AOAC OMA 2005.06) and/or MBA verified negative and PST contaminated samples (mussels, oysters, scallops, clams and cockles) were obtained from the Agri-Food and Biosciences Institute (Northern Ireland, UK), the Cawthron Institute (Nelson, NZ), 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 homogenization 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. Sample Preparation. An aliquot (1 g) of homogenized shellfish tissue was weighed from larger pools of homogenates into each screw-cap plastic container. These samples were spiked with fixed volumes of the relevant standard concentration of PSTs for the preparation of spiked matrix samples. Three concentrations of samples were used for robustness, ruggedness, interlaboratory and extract stability evaluations: (1) Negative samples, assigned a nominal 0% AL concentration, were prepared using LC-FD and MBA verified oysters which contained no detectable levels of PSTs; (2) samples at 20% AL were prepared by mixing the 0% AL sample with PST contaminated material (PO PST CRM) in ratios calculated to result in a sample containing 160 μg STX eq/kg; and (3) samples at 100% AL were prepared using 1.2 g PO PST CRM (equivalent to 802 ± 76 μg STX eq/kg, based on the certification).

2.5. Principle of the Method. The LFIA was a single-step test based on a competitive immunoassay format (Figure S-2). The sample was wicked through a reagent zone, containing antibodies specific for PSTs that were conjugated to colloidal gold particles. If PSTs were present, they would be captured by the particle-antibody complexes. The complexes wicked onto a nitrocellulose membrane, which contained a stationary capture zone of a toxin-protein conjugate (test zone). The test zone would capture any particle-antibody complexes which had not bound to toxin. Therefore, as the concentration of toxins in the sample increased, the test zone intensity decreased. The membrane also contained a stationary control zone, consisting of antispecies antibodies, which would always form regardless of the level of toxins.

2.6. Reader. The portable electronic readers (Reveal AccuScan PRO) used for the validations were obtained from Neogen Corporation (MI, US). The qualitative result (negative/positive) generated was derived from test and control line intensity readings obtained from the test strip.

2.7. Procedure for Extraction and Analysis of Toxins.

2.7.1. Standard Extraction Protocol. 280 μM microperforated filter bags (BagPage +100, Interscience, France) and metal-seam rollers (Trade1st, UK) were employed to perform the manual extraction. 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 one side of a filter bag and a roller applied by hand for 30 s.

2.7.2. Analysis of Samples. An aliquot (100 μL) of filtrate was removed, from the opposite side of the filter bag to which the extract was poured, added to approximately 15 mL buffered saline solution and mixed. From this buffered extract, 100 μL was subsequently added to a microwell and a test strip added to the microwell for 5 min, prior to placement into the LFIA reader to obtain the result.

2.8. Method Validation. The certified PST standards were diluted over a range of concentrations, typically from 0.01 to 10 ng/mL, to obtain dose−response plots. The optimal matrix dilution factor to effectively screen samples containing suspect levels of PSTs (approximately 85% AL STX-diHCl) was determined based on the dose−response data at a designated region of the curve (IC_{65}). The region on the curve was selected on the basis that it provided (1) an optimal cross-reactivity profile, (2) sufficient resolution at the target qualitative cutoff for positive results, (3) a simple and practical dilution scheme and (4) a large enough matrix dilution factor to minimize nonspecific interference on assay performance from various shellfish matrices.

Single-batch evaluations, using negative and PST spiked shellfish, were conducted using all certified PSTs to determine the extent of matrix effects; in addition to possible loss of toxins during the extraction procedure. Twelve negative samples consisting of different species (mussels, scallops, oysters, clams, cockles) and one chemical blank (buffer only) were initially evaluated (n = 20 tests per sample). The toxin spiking concentrations in shellfish typically ranged from 0 to approximately 2000 μg STX eq/kg.

A two-day robustness study consisting of multiple LFIA lots (n = 3), readers (n = 3) and operators (n = 3) was conducted. Each operator carried out multiple extractions (n = 4) of three different shellfish tissues (0%, 20%, 100% AL) each day (n = 3 tests per condition).

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Table 2. Toxin Profiles of PSP Contaminated Shellfish Tissues as Determined by LC-FD (AOAC OMA 2005.06)[a]

Sample ID	STX-diHCl	NEO	GTX1&4	GTX2&3	dcSTX	dcNEO	dcGTX2&3	C1&C2	GTX5
1	4%	<LOD	6%	9%	<LOD	<LOD	<LOD	28%	54%
2	2%	<LOD	14%	8%	<LOD	<LOD	<LOD	47%	29%
3	1%	<LOD	<LOD	19%	0.1%	<LOD	0.5%	53%	27%
4	95%	2%	<LOD	2%	0.5%	<LOD	<LOD	<LOD	1%
5	<LOD	<LOD	<LOD	<LOD	16%	<LOD	71%	11%	3%
6	1.2%	0.7%	9%	9%	0.2%	<LOD	0.3%	37%	42%
7	<LOQ	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOD	62%	38%
8	<LOQ	<LOD	<LOD	9%	<LOD	<LOD	24%	25%	42%
9	<LOQ	<LOD	<LOD	15%	<LOD	<LOD	<LOD	51%	34%
10	10%	<LOD	<LOD	81%	<LOD	<LOD	<LOD	<LOQ	9%
11	<LOQ	<LOD	15%	7%	<LOD	<LOD	<LOD	66%	13%
12	<LOQ	<LOD	3%	<LOQ	<LOD	<LOD	<LOD	90%	7%
13	<LOD	<LOD	<LOD	<LOD	38%	<LOD	62%	<LOD	<LOQ
14	<LOD	<LOD	<LOD	<LOD	43%	<LOD	57%	<LOD	<LOQ
15	<LOD	<LOD	<LOD	<LOD	57%	<LOD	43%	<LOD	<LOQ
16	<LOD	<LOD	<LOD	18%	<LOD	<LOD	<LOD	54%	28%
17	<LOQ	<LOD	12%	16%	<LOD	<LOD	<LOD	48%	25%
18	1%	<LOD	11%	16%	<LOD	<LOD	<LOD	46%	25%
19	46%	<LOD	<LOD	54%	<LOD	<LOD	<LOD	<LOQ	<LOD
20	8%	20%	36%	<LOQ	<LOD	<LOD	<LOD	36%	<LOQ

[a]LOD = limit of detection. LOQ = limit of quantification. Samples 21−23 were free of PSP toxins.

Table 3. LFIA Results from Naturally Contaminated Shellfish Samples in Comparison to Reference Methods

Sample ID	Species	DSP LC-MS/MS (μg OA eq/kg)	ASP LC-UV (mg DA/kg)	PSP MBA, μg STX eq/kg	PSP LC-FD, μg STX eq/kg (EFSA TEFs)	LFIA lot 1 result	LFIA lot 2 result	LFIA lot 3 result	LFIA lot 4 result
1	Mussels	ND	ND	ND	1,282	Positive	Positive	Positive	Positive
2	Mussels	ND	ND	ND	3,744	Positive	Positive	Positive	Positive
3	Mussels (hepatopancreas)	ND	ND	ND	14,396	Positive	Positive	Positive	Positive
4	Clams	ND	ND	ND	13,457	Positive	Positive	Positive	Positive
5	Clams	ND	ND	ND	2,258	Positive	Positive	Positive	Positive
6	Mussels (hepatopancreas)	ND	ND	ND	17,211	Positive	Positive	Positive	Positive
7	Pacific Oyster	ND	ND	540	114	Negative	Negative	Negative	Negative
8	Mussels	ND	ND	540	340	Positive	Positive	Negative	Positive
9	Pacific Oyster	ND	ND	700	389	Positive	Positive	Positive	Positive
10	Scallop (muscle and roe)	ND	ND	100−400	518	Negative	Negative	Negative	Negative
11	Scallop (muscle and roe)	ND	ND	2300	346	Positive	Positive	Positive	Positive
12	Scallop (muscle and roe)	ND	ND	1020	265	Positive	Positive	Positive	Positive
13	Clams	ND	ND	430	202	Negative	Negative	Negative	Negative
14	Clams	ND	ND	520	247	Negative	Negative	Negative	Negative
15	Clams	ND	ND	790	378	Negative	Negative	Negative	Negative
16	Mussels	ND	ND	440	104	Negative	Negative	Negative	Negative
17	Mussels	ND	ND	1120	408	Positive	Positive	Positive	Positive
18	Mussels	ND	ND	6580	2,516	Positive	Positive	Positive	Positive
19	Mussels	ND	ND	ND	230	Negative	Negative	Negative	Negative
20	Pacific Oyster[a]	ND	ND	ND	690	Positive	Positive	Negative	Negative
21	Mussels	<LOD	49	ND	<LOD	Negative	Negative	Negative	Negative
22	Mussels	<LOD	98	ND	<LOD	Negative	Negative	Negative	Negative
23	Mussels	11400	<LOD	ND	<LOD	Negative	Negative	Negative	Negative

[a]Certified as 668 ± 63 μg STX eq/kg via LC-FD, AOAC OMA 2011.02 (CEFAS); ND = no data; LOD = limit of detection.

Ruggedness of the method was determined by the introduction of parameter changes into the method and subsequent assessment of the effects these changes had on assay performance. Three different sample types (0%, 20%, 100% AL) were used for the study (n = 5 tests per condition). Six experimental parameters were chosen for the study, as shown in Table 1, which were those thought to most likely affect the performance of the method.

A single-batch study to determine the stability of both the unbuffered aqueous sample extracts and the buffer-diluted

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Table 4. External Laboratory-Sample Toxin Profiles as Determined by LC-FD (AOAC OMA 2005.06)[a]

Sample ID	STX-diHCl	NEO	GTX1&4	GTX2&3	dcSTX	dcNEO	dcGTX2&3	C1&C2	GTX5
24	3.7%	ND	ND	0.8%	7.7%	4.4%	2.1%	26.4%	54.8%
25	4.7%	ND	3.8%	0.8%	6.7%	5.0%	1.9%	26.3%	50.8%
26	6.3%	ND	3.0%	1.0%	7.2%	4.3%	1.6%	17.8%	58.8%
27	4.7%	ND	2.7%	1.1%	9.3%	3.6%	1.5%	7.7%	69.4%
28	9.3%	ND	1.5%	1.0%	8.0%	2.1%	1.3%	9.0%	67.8%
29	6.2%	ND	2.5%	1.0%	10.7%	5.8%	1.5%	4.1%	68.2%
30	12.6%	ND	ND	0.9%	8.9%	ND	1.0%	4.1%	72.5%
33	1.2%	ND	ND	14.6%	0.8%	ND	1.2%	36.2%	46.0%
45	5.3%	4.1%	47.7%	15.3%	ND	ND	ND	27.6%	ND
46	10.1%	ND	53.2%	36.7%	ND	ND	ND	ND	ND
47	3.7%	2.6%	51.6%	31.2%	ND	ND	ND	10.9%	ND
48	18.2%	11.6%	42.7%	27.5%	ND	ND	ND	ND	ND
49	100.0%	ND	ND	ND	ND	ND	ND	ND	ND
50	74.5%	ND	ND	ND	ND	ND	ND	ND	25.5%

[a]ND = not detected. Samples 39−44 and 51−56 were free of PSP toxins. Sample 34 contained PSP <25% AL. No LC-FD data was available for samples 31−32 and 35−38 from the external laboratory.

extracts (0%, 20%, 100% AL) at room temperature (RT, 20−25 °C) was conducted. The unbuffered and buffered extracts were both tested (n = 5 test per condition) at various time points (0, 1, 4, 24, 72 h after extraction) and were left at RT in plastic screw-cap vials. Unbuffered extracts were diluted in the running buffer at the specified time points.

An interlaboratory study was conducted by five external sites involving six oyster samples (two samples each at concentrations of 0%, 20% and 100% AL). Samples were extracted and assayed (n = 3 tests per condition) by participants with no prior experience of the assay.

A nine-week, single-batch stability study involving LFIA storage at three different temperature conditions (6 °C, 22 °C, 37 °C) was conducted at various time points (weeks 0, 1, 2, 4, 6 and 9). Spiked buffer samples were employed to determine stability of the assay using dose-response plots for each of the evaluated PSTs.

All validation studies were conducted randomized blind and, except where stated, all samples were evaluated in duplicate per each condition. All LFIA batches were produced following the same manufacturing procedures, whereby key reagents were titered for optimal performance. Accuracy of the LFIA, where stated, was defined as the percentage of tests which correctly identified samples containing 0% or 20% AL as negative (compliant) and samples containing 100% AL as positive (noncompliant).

3. RESULTS

3.1. Assay Sensitivity and Cross-reactivity in Buffer. The mean concentration of STX required to generate a result deemed to be a noncompliant response was 0.1 ± 0.01 ng/mL; which equated to approximately 80% AL in shellfish, based on the dilution factors employed. The cross-reactivity in relation to STX, expressed as means ± SD, were: NEO: 128.9% ± 29%; GTX1&4: 5.7% ± 1.5%; GTX2&3: 23.4% ± 10.4%; dcSTX: 55.6% ± 10.9%; dcNEO: 28.0% ± 8.9%; dcGTX2&3: 8.3% ± 2.7%; C1 and C2:3.1% ± 1.2%; GTX5:23.3% ± 14.4% (n = 5 LFIA lots).

3.2. Negative and Spiked Matrix (Matrix Effects and Toxin Recovery). All tests from the 13 PST samples compliant by reference methods (LC-FD and/or MBA) produced compliant results (n = 260). Results showed negligible matrix effects with mean intrasample CVs, based on test and control line readings, calculated as 8.6% ± 1.5%.

Results from PST spiked mussel evaluations indicated neither any matrix effects nor loss of toxins during extraction, with results closely correlating to buffer data. The mean concentration of toxins, in μg/kg, required to generate a noncompliant response in isolation were: STX-diHCl: 680; NEO: 580; GTX1&4: 8055; GTX2&3: 2830; dcSTX: 1375; dcNEO: 2925; dcGTX2&3: 7450; C1&C2:10855; GTX5:2400 (n = 3).

3.3. Naturally Contaminated Samples. Naturally contaminated samples (n = 23) consisting of mussels, scallops, oysters and clams were assayed using the test. The toxin profiles for each PST contaminated sample as identified by LC-FD is shown in Table 2, and the comparative analysis by LC-FD, LC-MS/MS, MBA and LFIA is shown in Table 3. Results indicated no false negative results in comparison to reference method data.

Samples 11, 12, and 17 were found to be compliant (based on EC regulations) via LC-FD (346, 264, 408 μg STX eq/kg, respectively) but noncompliant by both MBA and LFIA. Sample 9 was deemed to be compliant by both MBA (700 μg STX eq/kg) and LC-FD (389 μg STX eq/kg) but produced a non compliant result by LFIA. Sample 8 was recorded as being compliant by both MBA (540 μg STX eq/kg) and LC-FD (340 μg STX eq/kg) but produced a noncompliant response by three LFIA lots. Sample 20 (CRM PO PST 1101, certified as containing 84% ± 8% AL) as expected, generated non-compliant responses with two LFIA lots.

Results between the four LFIA lots evaluated indicated identical qualitative results with all samples evaluated, except for samples 8 (68% AL) and 20 (84% AL). Based on test and control line readings, it was noted that these samples produced data close to the assay's qualitative cut off. There were no indications of matrix effects from the different species evaluated (mussels, scallops, oysters or clams) nor interference from samples containing DA or OA-group toxins.

Dilutions of PST contaminated sample extracts, using sample extracts containing no detectable PSTs, were also undertaken. Based on LC-FD data, samples containing greater than 20% AL were prepared to the theoretical equivalents of 100% AL and/or 20% AL, before being assayed using a single LFIA batch. The overall accuracy, to generate compliant results at 20% AL and noncompliant results at 100% AL, was 100%.

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Table 5. LFIA Results from Naturally Contaminated Toxic Samples in Comparison to Reference Methods (External Laboratory)

Sample ID	Species	DSP LC-MS/MS (μg OA eq/kg)	ASP LC-UV (mg DA/kg)	PSP MBA, μg STX eq/kg	PSP LC-FD, μg STX eq/kg (EFSA TEFs)	LFIA Result
24	Cockles	ND	ND	2230	3200	Positive
25	Cockles	ND	ND	2710	4318	Positive
26	Cockles	ND	ND	1150	2428	Positive
27	Cockles	ND	ND	2150	2789	Positive
28	Cockles	ND	ND	990	2003	Positive
29	Cockles	ND	ND	1250	2238	Positive
30	Cockles	ND	ND	960	1096	Positive
31	Mussels	ND	ND	800	ND	Positive
32	Mussels	ND	ND	2228	ND	Positive
33	Mussels	ND	ND	392	575	Positive
34	Mussels	ND	ND	ND	<200	Negative
35	Mussels	ND	ND	1448	ND	Positive
36	Cockles	ND	ND	325	ND	Negative
37	Mussels	ND	ND	500	ND	Negative
38	Mussels	ND	ND	696	ND	Negative
39	Scallops	ND	106	ND	Negative	Negative
40	Scallops	ND	3	ND	Negative	Negative
41	Scallops	ND	3	ND	Negative	Negative
42	Scallops	ND	57	ND	Negative	Negative
43	Scallops	ND	3	ND	Negative	Negative
44	Scallops	ND	53	ND	Negative	Negative
45	Mussels	ND	ND	858	1993	Positive
46	Mussels	ND	ND	440	658	Negative
47	Mussels	ND	ND	1154	2630	Positive
48	Mussels	ND	ND	810	838	Positive
49	Cockles	ND	ND	ND	51	Negative
50	Scallops	ND	ND	ND	201	Negative
51	Mussels	189	ND	ND	Negative	Negative
52	Mussels	61	ND	ND	Negative	Negative
53	Mussels	176	ND	ND	Negative	Negative
54	Mussels	130	ND	ND	Negative	Negative
55	Mussels	176	ND	ND	Negative	Negative
56	Mussels	181	ND	ND	Negative	Negative

[a]Recovery corrected; ND = no data.

3.4. Naturally Contaminated Samples (External Laboratory and Samples). Negative (n = 6) and naturally contaminated (n = 33) samples were assayed using the test by an external laboratory using their library of samples. The toxin profiles for each PSP contaminated sample as identified by LC-FD is shown in Table 4, and the comparative analysis by LC-FD, LC-MS/MS, MBA and LFIA is shown in Table 5. Samples deemed compliant for regulated toxins (ASP, DSP, PSP) consisted of different species (mussels, oysters and clams) and in all cases generated compliant results using the LFIA. Results from naturally contaminated samples indicated no false negative results in comparison to PST reference method data. Sample 33 was recorded as being compliant with the regulated limits by both MBA (392 μg STX eq/kg) and LC-FD (575 μg STX eq/kg) but produced a noncompliant result using the LFIA. There were no indications of matrix effects from the different species evaluated (mussels, scallops, oysters, clams, cockles) nor interference from samples containing DA or OA-group toxins.

3.5. Robustness (Method Uncertainty). The robustness evaluations indicated that the overall accuracy of the method was 99.7% (n = 648 tests). All compliant and 20% AL (156 ± 14 μg STX eq/kg) samples generated compliant results. Samples containing 100% AL (802 ± 76 μg STX eq/kg) generated 99.1% noncompliant results (n = 216). The overall mean CV of the method across the three samples, based on test and control line readings, was 8.2 ± 2.6%. This indicated the sum of inter- and intra- variability (operators, readers, extractions, LFIA lots, days) associated with the method.

3.6. Ruggedness (Parameter Variability). The evaluations undertaken indicated that three parameter changes had impacts on the accuracy of the assay; while the overall accuracy of the method with all other evaluated conditions (n = 25) was 100%. (1) When the 30 s mixing time (buffered sample) parameter was omitted completely, the assay generated compliant results for all samples (0%, 20%, 100% AL). This was likely due to lack of sample homogeneity, since 100 μL extract was slowly added to a much larger volume (~15 mL buffer) without any subsequent mixing. (2) When the sample extract volume added to buffer parameter was reduced by 10%, from 100 to 90 μL, the accuracy was 93.3% since one 100% AL sample generated a compliant result. (3) When the buffered sample volume added to well parameter was increased by 20%, from 100 to 120 μL, the accuracy was 93.3% since one 100% AL sample generated a compliant result.

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3.7. Extract Stability. The study undertaken indicated no impacts on assay performance with any of the samples (0%, 20%, 100% AL) during any of the evaluated time points (0, 1, 4, 24, 72 h after extraction). The overall accuracy of the method, for both the unbuffered and buffered sample extracts, was 100% (n = 150). Therefore, results indicated that sample extracts, whether in aqueous solution or diluted in buffer, were stable for at least 72 h at RT.

3.8. Interlaboratory Evaluations. All participants conducted the evaluations in three rounds, with each round consisting of two unknown samples extracted and assayed in triplicate. All participants were able to complete a round of testing (generating six results, including sample extraction) within 20 min. The overall accuracy of the interlaboratory study was 98.6% (n = 72 tests); one test with a 100% AL sample generated a compliant result. All 0% and 20% AL samples correctly generated compliant results.

3.9. Assay Stability. The overall mean stability of the negative sample across the different conditions (6 °C, 22 °C, 37 °C), throughout the duration of the 9 week study was calculated. The stability of the negative sample, relative to week 0, was 97.4% ± 3.0% based on test and control line readings. The mean concentration of STX, required to generate a positive response, was 0.11 ± 0.02 ng/mL across the different conditions. The overall mean % cross-reactivity of the other PSTs were calculated as 103.8% ± 23% (NEO), 3.8% ± 0.5% (GTX1&4), 21.4% ± 4.4% (GTX2&3), 44.4% ± 4.6% (dcSTX), 24.4% ± 5.3% (dcNEO), 6.4% ± 1.1% (dcGTX2&3), 4.3% ± 0.8% (C1&C2) and 10.4% ± 2.1% (GTX5) across the different conditions.

4. DISCUSSION

The cross-reactivity profile of the reported LFIA in buffer and spiked matrices indicated that the nonsulfated carbamate toxins (STX, NEO) possessed the highest % cross-reactivity, followed by nonsulfated toxins of the decarbamoyl group (dcSTX, dcNEO) and the *N*-sulfocarbamoyl toxin GTX5. The sulfated toxins generally displayed the lowest % cross-reactivity (GTX1&4, GTX2&3, dcGTX2&3, C1&C2). Results implied that the assay may under- and overestimate toxicity by greater than 2-fold with samples rich in sulfated toxins (GTX1&4 or dcGTX2&3) and GTX5, respectively. However, all naturally contaminated samples, including those rich in GTX5 (e.g., sample 1 which had a toxin profile that included 54% GTX5), correctly generated compliant results when samples were diluted to concentrations of 20% AL. In addition, samples rich in sulfated PSTs such as dcGTX2&3 (e.g., sample 5, which had a toxin profile that included 71% dcGTX2&3, 16% dcSTX, and 11% C1&C2) correctly generated noncompliant results, when samples were diluted to 100% AL. External laboratory evaluations demonstrated that the method produced accurate results, in comparison to the reference methods, with all four samples rich in GTX1&4 (samples 48−51, consisting of 43−53% GTX1&4). Although the impacts of mixes of PSTs may help partially explain the observed accuracy of the method with such samples, another plausible factor involved may be differences in recovery rates of, at least some, PSTs between the LFIA method in comparison to LC-FD.[32,40]

The LFIA developed by Jellett et al. (2002) has been widely evaluated as a screening tool, offering simple and rapid analysis of samples in the field within only 35 min.[31] However, the assay was shown to possess a high cross-reactivity of some PSTs in relation to the TEFs (e.g., C1&C2 and GTX5 at 33% and 40%, respectively) which, coupled with a low qualitative cut off for STX-diHCl (around 120 μg/kg), was likely to cause a high rate of false positive results.[28,37,41] In addition, as with many LFIAs, the results were interpreted by the visual determination of line intensities, which introduced a level of subjectivity. In the present study, a reader was used to removing subjectivity in results interpretation; cross-reactivity to C1&C2 and GTX5 was closer to the TEFs (approximately 3% and 23%, respectively), and the cut off for STX-diHCl was at 680 μg/kg. The biosensor immunoassay method of Campbell et al. (2010) performed well in the analysis of naturally contaminated samples in comparison to reference methods (MBA, LC-FD), but the biosensor equipment employed was very expensive and not suited for field use.[42] The reported assay, due to an assay speed of less than 10 min (from sample homogenate to result) along with the simplicity of the procedure, is suitable for on-site screening. There have been a number of ELISA methods published which offer low cost testing, but limitations include analysis time of several hours, and the cross-reactivity profiles of the antibodies employed have been problematic in terms of generating inaccurate results, especially with samples containing complex profiles.[43−45]

5. CONCLUSIONS

A LFIA has been reported which demonstrates a practical and simple means for the screening of PSTs in shellfish. The assay can be utilized as a rapid screening tool (less than 10 min including sample extraction) for use within the shellfish industry including shipboard or remote locations, providing added protection of shellfish consumers. There is also a significant potential for this method to be used in regulatory laboratories to complement the costly analytical or biological based tests that are routinely employed as part of national monitoring programs. Such simple to use and low cost methods are ideal for laboratories in developing countries where the ability to purchase, run, and maintain expensive analytical equipment is very difficult or the MBA method not feasible or desirable to conduct routinely.

5.1. Safety. PSP group toxins are responsible for incidents of PSP. Therefore, when using PSP toxin standard solutions or contaminated shellfish, special care should be taken. Gloves and eye protection should be worn at all times. Appropriate disposal methods should also be utilized.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b00608.

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Notes

The authors declare no competing financial interest.

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