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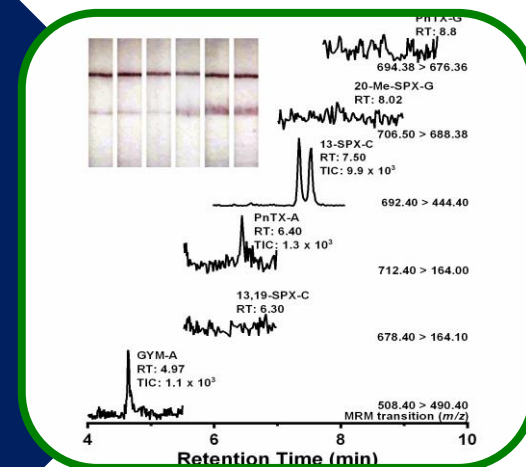
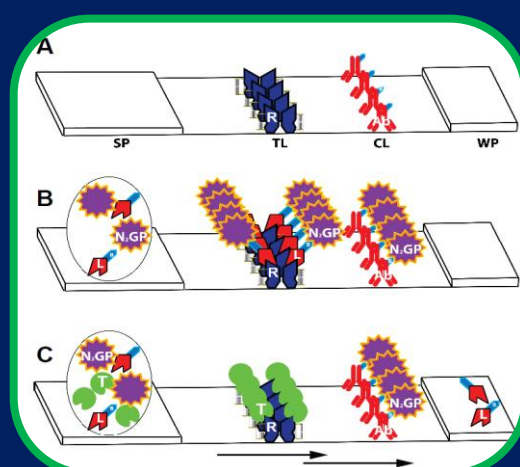
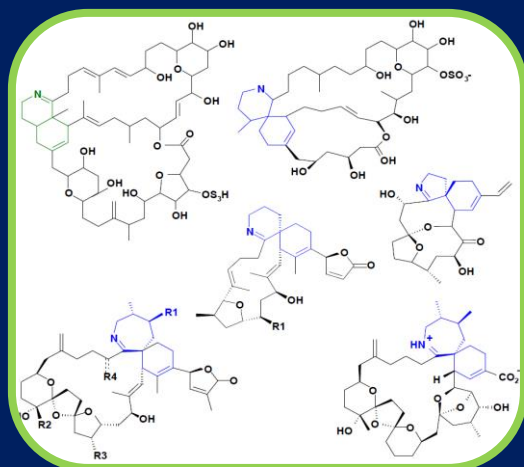
# Analytica Chimica Acta

## NeuroTorp, a Lateral Flow Test Based on Toxin-Receptor affinity for in-situ early detection of cyclic imine toxins --Manuscript Draft--

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<b>Manuscript Region of Origin:</b>	FRANCE
<b>Abstract:</b>	<p>The emergent cyclic imine toxins produced by marine dinoflagellates are potent antagonists of nicotinic acetylcholine receptors. Harmful algal blooms dominated by toxic dinoflagellates producing cyclic imine toxins have prompted in the past the closure of shellfish farms worldwide. Shellfish accumulate cyclic imine toxins following filter-feeding on toxic dinoflagellates vectoring them to humans. Herein is presented a lateral flow test for the detection of cyclic imine toxins based on three new concepts for test strips: i) the immobilization of lipoprotein vesicles in the test line, ii) the high affinity of neurotoxins for their receptor targets and iii) the use of high porosity glass fiber filter membranes as support for the fabrication of the lateral flow test NeuroTorp (WO2017108115)1. Since nicotinic acetylcholine receptors represent ~30% of the total protein of the electric organ from <i>Torpedo marmorata</i>, the purified electrocyte membrane vesicles were used as a source of receptor and immobilized on the test line. Biotin-<math>\alpha</math>-bungarotoxin was used as toxin tracer for the LFT binding assay given its high affinity for nicotinic acetylcholine receptors. Finally, it is the first time that GF/C glass fiber membranes are used as support for the LFT NeuroTorp that ensures both, the immobilization of a complex lipoprotein sample and the capillary migration of the mobile phase. In absence of a nicotinic toxin, Biotin-<math>\alpha</math>-bungarotoxin binds the nicotinic acetylcholine receptor and the use of neutravidin nanogold particle conjugate enables its visual detection. When nicotinic toxins are in the sample, they will enter in competition with the toxin tracer for binding the receptor. The inhibition degree is proportional to the concentration of the toxins in the sample. NeuroTorp is a ready-to-use low-cost point-of-care warning device for rapid and early detection of nicotinic neurotoxins in freshwater and marine environments by end-users.</p>
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He is a leading expert in natural products chemistry focusing in marine toxins. He is interested in detecting marine biotoxins by analytical and immunological methods. His research involves toxin discovery and the characterization of their structure and their mode of action.



## **NeuroTorp, a Lateral Flow Test Based on Toxin-Receptor affinity for *in-situ* early detection of cyclic imine toxins**

Fanny Noirmain<sup>1</sup>, Julie Dano<sup>2</sup>, Nathalie Hue<sup>3</sup>, Jesús M. Gonzalez-Jartin<sup>4</sup>, Luis M. Botana<sup>4</sup>,  
Denis Servent<sup>1,5</sup> Stéphanie Simon<sup>2</sup> and Rómulo Aráoz\*<sup>1,5</sup>

### **Highlights:**

- The lateral flow test NeuroTorp is based on the high affinity of cyclic imine toxins for nicotinic acetylcholine receptors.
- NeuroTorp introduces for the first time the use of GF/C glass microfiber membranes as the stationary phase for Lateral Flow Tests.
- NeuroTorp introduces the immobilization of a complex lipoprotein mixture, namely, the *Torpedo marmorata*-erythrocyte membrane vesicles that contains ~400 different proteins instead of a single protein target immobilized in the test-line opening the way to immobilizing multiprotein complexes and even cellular membranes on the test-line.
- NeuroTorp can detect several members of the cyclic imine toxin family directly in shellfish extracts.
- The method was validated by UPLC-MS/MS putting in evidence the presence of 13-desmethyl spirolide-C in canned cockles. The latter indicates that 13-desmethyl spirolide-c is able to resist the sterilization process to which canned cockles are submitted prior to commercialization processes.
- NeuroTorp is a fast and cost-effective early warning device for sensitive detection of cyclic imine toxins by end-users.

**NeuroTorp, a Lateral Flow Test Based on Toxin-Receptor affinity for *in-situ* early  
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## ABSTRACT

1  
2  
3 The emergent cyclic imine toxins produced by marine dinoflagellates are potent antagonists of  
4  
5 nicotinic acetylcholine receptors. Harmful algal blooms dominated by toxic dinoflagellates  
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11 dinoflagellates vectoring them to humans. Herein is presented a lateral flow test for the  
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13 detection of cyclic imine toxins based on three new concepts for test strips: *i*) the  
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57 <sup>1</sup>WO2017108115. Device for detecting neurotoxins and process for manufacture thereof. Aráoz  
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59 R. et al. (2017).  
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Keywords: Lateral flow test, nicotinic acetylcholine receptors, receptor ligand binding assay,  
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## 1. Introduction

Marine phytoplankton plays a major role on biomass and O<sub>2</sub> production and CO<sub>2</sub> reduction at a global scale. Phytoplankton growth is characterized by episodic blooming covering large ocean surfaces at very high densities affecting the food-web dynamics and the eco-climatic parameters within aquatic environments [1]. When these harmful algal blooms (HABs) are dominated by toxin-producer species, they may be detrimental for marine wildlife, economical activities and Public Health. Shellfish that filter feed on toxic phytoplankton are primary vectors for the transfer of toxins to humans, provoking a series of health syndromes, some of them with fatal outcomes [2]. As a consequence, some marine toxins like saxitoxin, domoic acid, okadaic acid, pectenotoxin, yessotoxin and azaspiracid are regulated. Toxin monitoring applies to phytoplankton producers and shellfish, fish and seafood that accumulate and vectorize harmful toxins to humans [3, 4]. Hence, the frequency of HABs events is increasing worldwide as a consequence of global warming [5]. Cyclic imine toxins, ciguatoxins, tetrodotoxins and palytoxins are considered emergent toxins in Europe [6].

Cyclic imine toxins (CiTXs) are fast acting neurotoxic compounds that kill mice by respiratory arrest within minutes after intraperitoneal injection or by gavage at lethal toxin doses [7, 8]. They are potent competitive antagonists of muscle and neuronal nicotinic acetylcholine receptors (nAChR) with affinities in the nanomolar range [9, 10]. Muscle nAChRs mediate fast neurotransmission at the neuromuscular junction enabling muscle contraction and escape from predation, and play a crucial role at the autonomous nervous system for respiration control. Therefore, they constitute a target of choice for a wide array of toxins from diverse origin including dinoflagellates (*i.e.*: CiTXs [11]) and cyanobacteria (*i.e.*: anatoxin-a [12]). Even if cyclic imine toxins are worldwide distributed and are often reported in shellfish for human consumption, these are not regulated since no human fatalities are associated with CiTXs [3]. However, it is of concern that certain CiTXs congeners like 13-

1 desmethyl spirolide-C (13-SPX-C), gymnodimine-A (GYM-A) and pinnatoxin-G (PnTX-G)  
2 are known to cross the brain-blood barrier [13, 14] and the placental barrier [15]. Further,  
3  
4 human subpopulations suffering from neuromuscular disorders like myasthenia gravis or  
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6 Lambert-Eaton myasthenic syndrome can be vulnerable to CiTXs.  
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10 Several liquid chromatography mass spectrometry methods and bioassays were  
11 developed for the detection of this class of emergent neurotoxins in Europe [16-19], however  
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13 their use is restricted to reference laboratories for marine biotoxins. Lateral flow tests (LFT) are  
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15 ready-to-use, low-cost, point-of-care diagnostic tools that are based on the use of antibodies,  
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17 enzymes, nucleic acid probes or aptamers for auto-diagnose of pregnancy, blood glucose,  
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19 infectious diseases, and for the detection of food contaminants, drugs, heavy metals and toxins  
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21 [20-22]. In the present work, we report the development of a LFT to detect fast-acting  
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23 neurotoxins based on three new approaches for this technology, that is, the high affinity of  
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25 neurotoxins for their receptor targets, the use of glass microfiber GF/C filter as support for LFT  
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27 and the immobilization of complex lipoprotein membranes in the test line.  
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## 35 2. MATERIAL AND METHODS

### 36 2.1 Reagents

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39 Cellulose fiber sample pads, laminated cards 60 mm × 301 mm and Estapor®  
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41 microspheres were from MerckMillipore (Darmstadt, Germany). Whatman® GF/C glass  
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43 microfiber filters, BSA, sodium, phosphate buffer, NHS-LC-Biotin, HAuCl, trisodium citrate,  
44  
45 were bought from Sigma-Aldrich (St Louis, MO, USA). Monoclonal antibody RAB37,  
46  
47 neutravidin,  $\alpha$ -bungarotoxin ( $\alpha$ -BgTX), Biotin- $\alpha$ -BgTX conjugate were purchased from  
48  
49 Thermo Fisher Scientific (Waltham, MA, USA). GYM-A and 13-SPX-C were purchased from  
50  
51 NRC (Institute for Marine Biosciences, Halifax, NS, Canada). 13,19-didesmethyl spirolide-C  
52  
53 (13,19-SPX-C) and 20-methyl spirolide-G (20-met-SPX-G) were bought from CIFGA (Lugo,  
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Spain). Pinnatoxin-A (PnTX-A) and PnTX-G were synthesized in the Laboratory of Prof. Dr. A. Zakarian (Dep. of Chemistry and Biochemistry, University of California, Santa Barbara, CA, USA).

## 2.2 Preparation of *Torpedo*-electrocyte membranes vesicles for the test-line

*Torpedo marmorata* electrocyte membrane vesicles were prepared as previously described [8]. The membranes were conserved at -80°C in 5 mM glycine until use.

## 2.3 Preparation of biotinylated RAB37 monoclonal antibody-latex bead microspheres conjugate for control-line

100 µL of monoclonal antibody RAB37 (1 mg mL<sup>-1</sup>) prepared in 10 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS) were mixed with 400 µL of 100 mM borate buffer, pH 9.5. After mixing, 6 µL of NHS-LC-Biotin (1 mg mL<sup>-1</sup> in *N,N*-dimethyl formamide) was added to the antibody solution. The whole was incubated for 30 min at room temperature in the dark. Thereafter, 100 µL of 1M Tris buffer, pH 8 was added to the reaction mix and it was further incubated for 15 min as previously described. The protein concentration was calculated from the OD at 280 nm.

A volume of, 200 µL of biotinylated RAB37 antibodies was mixed with 200 µL of 1% polystyrene Estapor® microspheres prepared in PBS. The whole was incubated overnight in a rotatory shaker at 4°C. Free biotinylated RAB 37 antibodies were discarded by centrifugation (20 000g for 30 min at 4°C). The pelleted microbead-immobilized biotinylated RAB 37 antibodies were resuspended in 400 µL PBS buffer. The protein concentration was calculated from the difference between the OD at 280 nm of the biotinylated RAB37 solution and the OD at 280 nm of the supernatant.

## 2.4 Preparation of the colloidal neutravidin nanogold particle (NGP) conjugate

1 The nanogold particles were synthesized by trisodium citrate reduction of HAuCl<sub>4</sub>. The  
2 size of the gold nanoparticles was controlled by dynamic light scattering. For the labelling of  
3  
4 neutravidin with nanogold particles, 100 μL of neutravidin (1 mg mL<sup>-1</sup>) in 50 mM PBS, pH 7.5  
5  
6 was added to 1 ml colloidal gold. The whole was incubated at room temperature in a rotating  
7  
8 shaker for 1 h in the dark. Then, 100 μL of 50 mM PBS, 0.1% BSA, pH 7.5 was added and the  
9  
10 mixture was centrifuged at 14 000 rpm for 40 min. The supernatant was discarded and the pellet  
11  
12 was suspended in 1 mL of 50 mM PBS, 0.1% BSA, pH 7.5. The mix was sonicated, vortexed  
13  
14 and centrifuged as described. The neutravidin-NGP conjugate was resuspended in 250 μL of  
15  
16 50 mM PBS, 0.1% BSA, pH 7.5, aliquoted and stored at 4°C until use.  
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## 22 2.5 Construction of the LFT NeuroTorp

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25 A GF/C microfiber membrane strip of 30 cm long × 2.5 cm height was soaked in TBS  
26  
27 (50 mM Tris, 150 mM NaCl, pH 7.5) for 10 min. The GF/C filter was dried for an hour at 37°C  
28  
29 in an air oven and was placed over a polystyrene sticking backing card. To dispense the reagents  
30  
31 onto the GF/C microfiber membrane, 500 μL of *Torpedo*-electrocyte membrane (500 μg mL<sup>-1</sup>  
32  
33 total protein) in TBS was filled in the test-line container of the Benchtop Dispenser Workstation  
34  
35 XYX3050 (BioDot, UK). Similarly, 100 μg mL<sup>-1</sup> of RAB37-Biotin conjugated with latex bead  
36  
37 microspheres was filled in the control-line container. *Torpedo*-nAChR and RAB37-Biotin  
38  
39 conjugated antibody were dispensed onto the GF/C membrane at a flowrate of 1 μL cm<sup>-1</sup>. After  
40  
41 the membrane was dried as described, it was soaked in TBS, 0.1% BSA, pH 7.5 for 30 min at  
42  
43 room temperature. Afterwards, the membrane was washed twice with water and then soaked in  
44  
45 the preserving buffer TBS, 0.1% Tween, 7.5% glucose, pH 7.4. Thereafter, the membrane was  
46  
47 dried for 1 h at 37°C in an air oven. Once the sample and absorbent pads were placed onto the  
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49 card (Scheme 1) 5 mm-wide strips were cut with a BioDot CM4000 Batch Cutting System. The  
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51 membranes were stored in sealed bags at 4°C until use.  
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## 2.6 Toxin extraction from shellfish

Two shellfish homogenates and a canned cockle were provided by CIFGA (Spain). Four canned cockles were opened and 10 g of cockles were homogenized using a waring blender and proceeded as described in [8, 18] according to Table 1. In parallel, 10 ml of canned cockle supernatant was mixed with an equal volume of acetone and toxin extraction was performed as described [18]. CiTX extraction from shellfish homogenates and from the canned cockle supernatant was performed as described [18]. Briefly, the shellfish homogenates and the supernatant were mixed with acetone (see Table 1), vortexed three-times for one min and Roller vortexed for 15 min at room temperature in the dark. The suspensions were centrifuged for 15 min at 4 000 rpm at 20°C. The pellets were re-extracted with acetone as described, the supernatants were pooled and evaporated under a stream of azote at 40°C. The dried material was resuspended in a volume of methanol and conserved ay -20°C until.

## 2.7 NeuroTorp protocol and data analysis

Fifty  $\mu\text{L}$  of TBS-BSA, or TBS-BSA containing toxin sample were applied to the sample pad. Once the whole was entirely migrated, 50  $\mu\text{L}$  of the toxin tracer Biotin- $\alpha$ -BgTX (80 nM) was dispensed onto the sample pad. Following complete migration of the toxin tracer, 100  $\mu\text{L}$  of neutravidin-NGP, extemporarily diluted 10-times with EIA-buffer (100 mM potassium phosphate, 150 mM NaCl, 0.1% BSA, 0.01%  $\text{NaN}_3$ , 0.5% Tween 20, pH7.4), was added to the sample pad. To improve the contrast 100  $\mu\text{L}$  EIA-buffer was added to the sample pad. The results were evaluated by naked-eye after 15 min. Since NeuroTorp is a competitive inhibition test, the presence of a nicotinic toxin will displace the *toxin-tracer* in a concentration dependent manner: At 100% inhibition, the test control-line is colorless. In absence of inhibition, the test control line is red with a similar intensity as the control-line. For quantitation purposes, the NeuroTorp LFT strips were scanned with an LFT-reader (NgBiotech, Brest, France). The inhibition percent was calculated according to:

$$\text{Inhibition \%} = 100 \times [(\text{Control LFT signal} - \text{Sample LFT signal}) / (\text{Control LFT signal})]$$

## 2.9 UPLC-MS/MS analysis of the sample extracts

UPLC-MS/MS analysis of shellfish samples in the multi reaction mode was performed as described [18]. For quantitation purposes, calibration curves for GYM-A, 13,19-SPX-C, PnTX-A, 13-SPX-C, 20-met-SPX-G and PnTX-G in the range of 100 pM to 1  $\mu$ M were built. The UPLC-chromatographic protocol was designed for the simultaneous detection and quantification of six cyclic imine toxin standards in a 10-min run.

## 3. RESULTS AND DISCUSSION

### 3.1 Choice of an LFT membrane for immobilizing *Torpedo*-electrocyte lipoprotein vesicles

NeuroTorp introduces for the first time the use of GF/C glass microfiber membranes as the stationary phase for LFT. Most of the polymer membranes used for LFT, namely, nitrocellulose (NC), polyvinylidene fluoride (PVDF) or nylon membranes, are oriented towards the immobilization of purified proteins (polyclonal or monoclonal antibodies, enzymes), nucleic acids or aptamers in the test-line. However, these membrane supports are not suitable to immobilize *Torpedo*-electrocyte membranes which are lipoprotein vesicles rich in nAChR of muscle type among other membrane proteins [23]. When applied onto a NC, PVDF or nylon membrane, the *Torpedo*-electrocyte membranes forms tiny droplets that are not absorbed by these membranes.

Since GF/C glass microfiber membranes are widely used for radioactive and non-radioactive filter binding assays, in which, the complex receptor-ligand is captured by the GF/C membrane following vacuum filtration for scintillation counting or color development [10, 24], the capacity of GF/C membranes to immobilize *Torpedo*-nAChRs was tested. Thus, when *Torpedo*-electrocyte membrane vesicles are applied by vacuum filtration or with the automatic dispenser BioDot, these highly hydrophobic membrane vesicles readily impregnate the GF/C

1 glass fiber filter forming a continuous straight line of ~1 mm large [25] (Fig. 1). Pioneering  
2 works proposed, albeit without success, the use of GF/C glass microfiber membranes previously  
3 treated with polybrene as transfer membranes for Western-Blot given that peptides and proteins  
4 bound preferentially glass hydrophilic surfaces rather than polypropylene hydrophobic surfaces  
5 depending on the ionic strength and the presence of surfactants [26-28]. Actually, the glass  
6 microfiber GF/C filter is an inert porous membrane (1.2  $\mu\text{m}$  pore size) with low unspecific  
7 binding and a capillary flow rate of  $\sim 1 \text{ cm min}^{-1}$ . Therefore, GF/C membranes are traditionally  
8 used for preparing the conjugate pad that uniformly release the detector reagent into the mobile  
9 phase in current LFT technology [29]. The primary binding mechanisms for NC and nylon  
10 membranes is electrostatic, while for PDFV membranes the immobilization of the target  
11 substrate is through hydrophobic interactions [29]. The mechanism by which *Torpedo*-  
12 electrocyte membranes are immobilized by GF/C glass microfiber membranes is not known.  
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### 3.2. Optimization of NeuroTorp LFT

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33 The following factors influencing the interaction *toxin-receptor* were considered: *i*) the  
34 receptor concentration, *ii*) the *toxin-tracer* concentration, *iii*) the conjugate to visualize the  
35 ligand binding, and *iv*) the compatibility of buffers and solvents with LFT conditions.  
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41 Nicotinic acetylcholine receptor of muscle-type is naturally overexpressed (~300-times  
42 its level in skeletal muscle) in the *Torpedo*-electrocyte organ which allowed crystallographic  
43 approaches for characterizing nAChRs [23, 30]. The nicotinic receptors represent ~40% of the  
44 total protein content of the *Torpedo*-electrocyte membrane vesicles and are surrounded by a  
45 native lipidic and lipoproteic environment that ensures the stability and the functionality of the  
46 receptors [18, 23, 31]. To determine the optimal concentration of the receptor, we performed a  
47 series of saturation binding experiments using different concentrations of *Torpedo*-electrocyte  
48 membranes vesicles immobilized in the test line of the LFT (100, 300, 500, 700 and 1400 ng  
49 total protein). The receptors were exposed to different concentrations of Biotin- $\alpha$ -BgTX  
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1 ranging from  $1 \times 10^{-9}$  to  $1 \times 10^{-6}$  M. The half maximal effective concentration ( $EC_{50}$ ) values of  
2 Biotin- $\alpha$ -BgTX were of the same order of magnitude ranging from 20 – 93 nM (Table 1).  
3  
4 Considering an appropriate signal-to-noise ratio, the optimal protein concentration of *Torpedo*-  
5 electrocyte membranes immobilized on the test-line of NeuroTorp strips was 500 ng which  
6 represents ~200 ng of *Torpedo*-nAChR (Table 1). By using 200 ng of *Torpedo*-nAChR  
7 immobilized on the test-line, the  $EC_{50}$  of Biotin- $\alpha$ -BgTX binding to *Torpedo*-nAChR was 39.5  
8 nM (95% confidence interval = 18 - 85 nM; Fig. 2A). The  $EC_{50}$  obtained for the LFT NeuroTorp  
9 compares well the binding affinities of Biotin- $\alpha$ -BgTX for *Torpedo*-nAChR reported before [8,  
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22 For radioligand binding assays under equilibrium, it is recommended that the receptor  
23 concentration should be less than 10% of the ligand's  $K_D$ , implying that 10% of the ligand will  
24 be bound to the receptor to avoid ligand depletion [33]. However, ligand-binding under  
25 equilibrium is not compatible with high throughput miniaturized binding assays in which higher  
26 receptor concentrations are used while radioligand concentrations are kept to a minimum which  
27 may induce ligand depletion [34]. Receptor binding assay under LFT conditions does not reach  
28 the equilibrium. The competition between the CiTXs and Biotin- $\alpha$ -BgTX for binding *Torpedo*-  
29 nAChR takes place under fluidic conditions while the *toxin-tracer* migrates over the test-line  
30 for a short period of time. The capillarity flow rate of GF/C filter membranes is slow for a LFT  
31 strip (~1 min  $cm^{-1}$ ) which is good because it increases the reaction time between the toxin  
32 complexes and the receptor [29]. A *toxin-tracer* concentration of 80 nM Biotin- $\alpha$ -BgTX (~70  
33 ng) was experimentally determined from the saturation binding curve (Fig. 2A). This  
34 concentration is twice the  $EC_{50}$  of the toxin tracer for *Torpedo*-nAChR and it is ~3-times lower  
35 than the *Torpedo*-nAChR concentration (~200 ng).  
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57 Neutravidin-NGP conjugate was used to visualize Biotin- $\alpha$ -BgTX-binding to *Torpedo*-  
58 nAChRs. Neutravidin is a deglycosylated form of avidin (pI of 6.3) that conserve the high  
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1 affinity and specificity for biotin in solution ( $K_a \approx 10^{15} \text{ M}^{-1}$ ) with lower non-specific binding  
2 for lipids and carbohydrates than avidin. Thus, the use of Neutravidin-NGP is not a limiting  
3 step for CiTXs detection [35].  
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7 The buffer composition, the pH and the ionic strength were similar to the microplate  
8 receptor-binding assay [8, 18, 32]. A rigorous study has showed that methanol up to 10% did  
9 not interfere with *Torpedo*-nAChRs binding assay in the microplate format [8, 32]. Therefore,  
10 methanol concentration in the loading sample for LFT was kept  $\leq 10\%$ .  
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### 18 3.2.2 Inhibition of *toxin-tracer* binding to *Torpedo*-nAChRs by $\alpha$ -bungarotoxin 19 20

21 Once the proper conditions for the binding assay under LFT conditions were  
22 determined, a homologous competition binding experiment was performed using non-labeled  
23  $\alpha$ -BgTX (Fig. 2B). Given that the monoclonal Ab RAB37-Biotin was immobilized on the  
24 control line of NeuroTorp LFT to serve as internal control to monitor the binding of neutravidin-  
25 NGP to the biotin moiety of Ab RAB37, two series of LFT strips were run per triplicate. Figs.  
26 2C-H show a set of three sample strips for each  $\alpha$ -BgTX dose in the left side, and a set of 3  
27 control strips in the right side, which were loaded with the same sample solution exempt of  
28 toxin. Inhibition binding was calculated from the data obtained from the test-line of the  $\alpha$ -BgTX  
29 treated strips against the test-line of the control strips according to Eq. 1. The competitive  
30 inhibition binding of Biotin- $\alpha$ -BgTX by  $\alpha$ -BgTX was dose dependent with an  $IC_{50}$  of 38.2 nM  
31 (95% confidence interval = 20.1 – 70.2 nM). The obtained  $IC_{50}$  compares well the inhibition  
32 values obtained by radioactive and non-radioactive ligand binding [24, 36] indicating that the  
33 *Torpedo*-nAChRs were stable and functional under LFT conditions.  
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### 53 3.2.3 Inhibition binding by CiTX under LFT conditions. 54 55

56 The inhibition of Biotin- $\alpha$ -BgTX binding to *Torpedo*-nAChRs under LFT conditions by  
57 20-methyl SPX-G was dose dependent with an  $IC_{50}$  of 1,03  $\mu\text{M}$  (95% confidence interval =  
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0,64 – 1,8  $\mu\text{M}$ ) (Fig. 3A, inset). This value was lower than the  $IC_{50}$  (20-me SPX-G) obtained by microplate-RBA (25 nM; [18]). The latter may be explained because in the microplate-RBA the incubation of the *Torpedo*-nAChR with the toxin is performed at equilibrium (overnight). In contrast, the LFT NeuroTorp is never performed under equilibrium conditions. The interaction between the nicotinic ligands and *Torpedo*-nAChR takes place for a brief period of time while the mobile phase is drifting by capillarity over the test-line. Yet, the affinity of CiTXs for *Torpedo*-nAChR enables the detection of CiTXs by LFT (Figs. 2, 3).

In naturally contaminated shellfish it is usual to find several CiTXs analogues in the same sample [16, 17, 37, 38]. Therefore, a non-contaminated mussel extract was spiked with GYM-A, 13,19 SPX-C, PnTX-A, 13-SPX-C, 20-me-SPX-G and PnTX-G to a final concentration of 1  $\mu\text{M}$ , each ([CiTX-mix] = 6  $\mu\text{M}$ ). A series of dilutions were prepared using non-contaminated mussel extract that contained 600, 300, 150 and 75 nM CiTX-mix, each. Their methanol concentration was 10, 5, 2.5 and 1.5%, respectively. The inhibition binding by the CiTXs-spiked mussel samples was dose-dependent with an  $IC_{50}$  of 108,6 nM (95% confidence interval = 94,1 – 125,2 nM) (Fig 3B) that suggests toxin synergy. The inhibition binding of the spiked mussel sample as well as their respective controls are depicted in Figs. 3C-F. These experiments showed a reduced effect of the shellfish matrix on the performance of the LFT NeuroTorp. Further, 10% methanol did not interfere with toxin detection (see the control strips of Fig. 3F). The reduced shellfish matrix effect and the tolerance to methanol of NeuroTorp strips correlates with CiTXs detection by microplate-RBA [18, 32].

NeuroTorp could be applied to the detection of competitive antagonists of nAChRs like the peptide neurotoxin  $\alpha$ -BgTX (Fig. 2B) or the CiTX alkaloids (Fig. 3A and 3B), and freshwater cyanobacterial agonist anatoxin-a (data not shown). The degree of the inhibition is dependent on the potency of the neurotoxin tested.

3.2.4. CiTXs detection in shellfish extracts by NeuroTorp and by Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS).

Two shellfish homogenates and a canned cockle provided by CIFGA (Spain) were used to test NeuroTorp strips on real samples (Table 2). They were tested at 4 different dilutions containing 10, 5, 2.5 and 1.25% methanol, respectively. All the samples including the canned cockle (meat and supernatant) were positive for CiTXs, but, to a different extent (Fig. 4A). The test and control NeuroTorp strips illustrate that the inhibition binding was dose dependent. Figs. 4B-E illustrate that methanol neither interferes with CiTXs detection nor with neutravidin-NGP binding to the biotinylated RAB37 antibody at the tested concentrations (1.25, 2.5, 5 and 10%).

All the shellfish samples were analyzed by UPLC-MS/MS for the simultaneous detection and quantification of GYM-A, 13,19-SPX-C, PnTX-A, 13-SPX-C, 20-me-SPX-G and PnTX-G. MS/MS fragmentation was optimized to determine the MRM transitions for each of the CiTX standards (Table 3). Linear calibration curves in the working range with an  $r^2 \geq 0.999$  were obtained for each CiTX standard. The limits of detection (LOD) and limits of quantification (LOQ) were comparable with the reported literature [8, 16-18] (Table 3). ALTX-01 was contaminated with  $0.3 \mu\text{g kg}^{-1}$  GYM and  $0.72 \mu\text{g kg}^{-1}$  13-SPX-C and with traces of PnTX-A. ALTX-02 contained  $3.2 \mu\text{g kg}^{-1}$  GYM-A and  $72 \mu\text{g kg}^{-1}$  13-SPX-C. In the canned cockles for human consumption, both, the supernatant and cockles' flesh were contaminated with  $1.2$  and  $4.7 \mu\text{g kg}^{-1}$  13-SPX-C, respectively. Traces of PnTX-A were detected in both samples, while traces of GYM-A could also be detected in the cockles flesh samples (Table 3, Fig. 5A-D). Since CiTX are not regulated, it is usual to detect several members of this family of neurotoxin in shellfish for human consumption [8, 16, 18]. The thermal stability of PnTX-A was studied [39]. It was seen that after heating at  $100^\circ\text{C}$  for 24 h only 20% of the PnTX-A underwent hydrolysis. The present results indicate that 13-SPX-C can resist the sterilization process to which canned cockles are submitted prior to commercialization. As the initial amount

1 of 13-SPX-C are not known, the stability of this toxin cannot be calculated. This is the first  
2 report in which the presence of CiTXs is reported for canned shellfish.  
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## 5 **Conclusions**

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8 Global Climate Change, water eutrophication and maritime activities have increased the  
9 frequency of HABs occurrence and the spreading of toxic species worldwide. The emergent  
10 CiTXs are often found in shellfish intended for human consumption since they are not regulated  
11 [8, 16, 18]. Of concern is the high affinity of CiTXs for muscle and neuronal nAChRs subtypes  
12 given that they can cross the intestinal, brain blood and placental barriers [11, 14, 15]. The  
13 French agency for food safety (ANSES) has recently recommended the inclusion of pinnatoxins  
14 in the list of marine regulated biotoxins establishing a guidance level for PnTX-G content in  
15 shellfish of 23  $\mu\text{g kg}^{-1}$  [40]. The current methods for detecting CiTXs for the shellfish industry  
16 are LC-MS and receptor-binding assay. These methods are performed in laboratories implying  
17 a time-gap between shellfish/ phytoplankton sampling and results delivery. There is therefore  
18 a real need to dispose of a simple and inexpensive method for detecting CiTXs. NeuroTorp  
19 provides the means for early, rapid and quantitative detection of nicotinic toxins in the field by  
20 end-users.  
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41 NeuroTorp is based on the affinity of CiTXs for the nAChRs that introduces two novel  
42 aspects for LFT technology: *i*) the use of a complex lipoprotein mixture, namely, the *Torpedo*  
43 *marmorata*-electrocyte membrane vesicles that contains ~400 different proteins [23] instead of  
44 a single protein target immobilized in the test-line, and *ii*) the use of glass microfiber  
45 membranes to trap larger lipoprotein vesicles as a support for LFT. *Torpedo*-electrocyte  
46 membranes is a validated source of nAChRs of muscle-type. The use of a solely *Torpedo*  
47 *marmorata* electric fish as donor of electrocyte membranes provides enough material to  
48 manufacture hundred thousand of LFT NeuroTorp units. Thus, the number of animals sacrificed  
49 for toxicity screening in shellfish samples is highly reduced. Herein is demonstrated that it is  
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1 not necessary to functionalize the GF/C microfiber membrane to immobilize *Torpedo*-nAChR  
2 on the test-line. Once applied the electrocyte membrane vesicles are trapped in the porous TBS-  
3 treated membrane. The choice of GF/C glass microfiber as the membrane support for  
4 NeuroTorp opens the way for immobilizing multiprotein complexes and even cellular  
5 membranes on the test-line of LFT.  
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12 We have developed a fast and cost-effective early warning LFT device for field  
13 detection of cyclic imine toxins by end-users that could also be applied for  $\alpha$ -conopeptide  
14 discovery and for monitoring freshwater cyanobacterial anatoxins.  
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**Table 1**

*EC*<sub>50</sub> of Biotin- $\alpha$ -BgTX to Torpedo-nAChR under different receptor concentrations.

<i>Torpedo</i> -electrocyte membranes* (ng)	<i>Torpedo</i> -nAChR** (ng)	<i>EC</i> <sub>50</sub> (nM)
1400	560	71.9
700	280	20.1
500	200	18.2
300	120	93.4
100	40	21.7

\* Total protein concentration; \*\* Calculated concentration of nAChR considering that the nicotinic receptors represent ~40% of the total protein concentration of *Torpedo*-electrocyte membranes.

**Table 2**

Shellfish samples: origin, processing and analyzed volumes.

Sample	Origin	Sample processing				
		Masse (g)	ACN (mL)*	MeOH (mL)	to LFT (µL)	to UPLC (µL)
<b>Mussels ALTX 01</b>	CIFGA, Spain	5	20	2	1.25 – 10	5
<b>Mussels ALTX 02</b>	CIFGA, Spain	2.5	10	1	1.25 – 10	5
<b>Cockles (can) SS</b>	CIFGA, Spain	10	40	4	1.25 – 10	5
<b>Cockles (can) Flesh</b>	CIFGA, Spain	10 mL	40	4	1.25 – 10	5

\* Shellfish samples were extracted twice with acetonitrile. Abbreviations: ACN: acetonitrile,

MeOH: methanol, LFT: lateral flow test, UPLC: ultra-performance liquid chromatography.

**Table 3**

UPLC-MS/MS conditions for CiTXs quantification.

	<b>GYM-A</b>	<b>13,19-SPX-C</b>	<b>PnTX-A</b>	<b>13-SPX-C</b>	<b>20-me-SPX-G</b>	<b>PnTX-G</b>
<b>MRM trans. (m/z)</b>	508.4 > 490.4	678.4 > 164.1	712.4 > 164.0	692.4 > 444.4	706.5 > 688.4	694.4 > 676.4
<b>Cone voltage (V)</b>	50	50	65	60	65	75
<b>Coll. energy (eV)</b>	32	40	48	35	30	30
<b>Ret. time (min)</b>	4.83	6.19	6.23	7.31	8.54	8.99
<b>LOD</b>	10 pM or 0.010 pg/col.	100 pM or 0.14 pg/ col.	1 pM or 0.0014 pg/col.	100 pM or 0.14 pg/ col.	100 pM or 0.14 pg/col.	1 pM or 0.0014 pg/col.
<b>LOQ</b>	1 nM or 1.0 pg/col.	1 nM or 1.35 pg/col.	10 pM or 0.014 pg/col.	1 nM or 1.38 pg/col.	1 nM or 1.41 pg/col.	10 pM or 0.014 pg/col.
<b>R<sup>2</sup></b>	0.9999	0.9999	1.0000	0.9999	1.0000	0.9998

Abbreviations: UPLC-MS/MS: ultra-performance liquid chromatography-tandem mass spectrometry, GYM-A: gymnodimine-A, 13,19-SPX-C: 13,19-didesmethyl spiroside-C, PnTX-A: pinnatoxin-A, 13-SPX-C: 13-desmethyl spiroside-C, 20-me-SPX-G: 20-methyl spiroside-G, PnTX-G: pinnatoxin-G. MRM trans.: multiple reaction-monitoring mode transitions, Coll. Energy: collision energy, Ret. Time: retention time, LOD: limit of detection, LOQ: limit of quantification.



**Table 4**CiTX content in  $\mu\text{g kg}^{-1}$  shellfish determined by UPC-MS/MS

Sample	GYM-A ( $\mu\text{g kg}^{-1}$ )	13,19-SPX-C ( $\mu\text{g kg}^{-1}$ )	PnTX-A ( $\mu\text{g kg}^{-1}$ )	13-SPX-C ( $\mu\text{g kg}^{-1}$ )	20-me-SPX-G ( $\mu\text{g kg}^{-1}$ )	PnTX-G ( $\mu\text{g kg}^{-1}$ )	Inhib. % (5% MeOH)
<b>Mussels ALTX 01</b>	0.32	n.d.	Traces	0.72	n.d.	n.d.	41.9 $\pm$ 11
<b>Mussels ALTX 02</b>	3.2	n.d.	n.d.	72	n.d.	n.d.	76.4 $\pm$ 3.9
<b>Cockles (can) SS</b>	n.d.	n.d.	Traces	1.2	n.d.	n.d.	7.8 $\pm$ 5.7
<b>Cockles (can) Flesh</b>	Traces	n.d.	Traces	4.7	n.d.	n.d.	41.6 $\pm$ 4.8

Abbreviations: GYM-A: gymnodimine-A, 13,19-SPX-C: 13,19-didesmethyl spirolide-C,

PnTX-A: pinnatoxin-A, 13-SPX-C: 13-desmethyl spirolide-C, 20-me-SPX-G: 20-methyl spirolide-G, PnTX-G: pinnatoxin-G.

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## Figure Legends.

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3 **Figure 1.** NeuroTorp LFT schema. (A) NeuroTorp strip at time zero. (B) Control NeuroTorp  
4 strip. The LFT strip is exposed to Biotin- $\alpha$ -BgTX and to neutravidin-NGP in sample buffer.  
5 Both, the test-line and the control-line will be colored. (C) Sample problem NeuroTorp strip.  
6 The LFT strip is exposed to a toxic sample (CiTX standard or shellfish extract), to Biotin- $\alpha$ -  
7 BgTX and to neutravidin-NGP in sample buffer. The CiTXs in the sample, if any, will displace  
8 the *toxin-tracer*. The intensity of the color of test-line will depend on the concentration of  
9 CiTXs, while the control-line will be colored. Abbreviations: SP: sample pad, TL: test-line, C-  
10 L control-line, WP: wicking pad, R: *Torpedo*-nAChR, Ab: monoclonal antibody RAB37, B:  
11 biotin moiety, L: Biotin-  $\alpha$ -BgTX, N<sub>2</sub>GP: neutravidin-NGP, T: CiTX. The arrows indicate the  
12 path of capillary migration.  
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**Figure 2.** Saturation binding and homologous competitive inhibition binding of Biotin- $\alpha$ -BgTX. (A) Saturation curve of Biotin- $\alpha$ -BgTX binding to 500 ng *Torpedo*-electrocyte membranes immobilized in the test-line of NeuroTorp LFT strips. (B) Competitive inhibition binding of Biotin- $\alpha$ -BgTX to *Torpedo*-nAChRs by non-labelled  $\alpha$ -BgTX. (C-H) LFT NeuroTorp strips showing the dose-dependent inhibition of Biotin- $\alpha$ -BgTx binding to *Torpedo*-nAChRs by  $\alpha$ -bungarotoxin. For each dose, two series of LFT strips were run simultaneously per triplicate. The test-strips were exposed to a given dose of  $\alpha$ -bungarotoxin (left side of each panel), while the control-strips were exposed to TBS-BSA (right side of each panel). (C) 2.5 nM  $\alpha$ -BgTX. (D) 120 nM  $\alpha$ -BgTX. (E) 370 nM  $\alpha$ -BgTX. (F) 1  $\mu$ M  $\alpha$ -BgTX. (G) 3  $\mu$ M  $\alpha$ -BgTX. (H) 10  $\mu$ M  $\alpha$ -BgTX. Abbreviations:  $\alpha$ -BgTX:  $\alpha$ -bungarotoxin, C: Control-line, in which the monoclonal antibody Biotin-RAB37 was immobilized, T: Test-line, in which 500 ng of *Torpedo*-electrocyte membrane vesicles were immobilized. Three independent experiments performed by triplicate for each dose were repeated at different dates.

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**Figure 3.** 20-methyl SPX-G and shellfish extracts inhibition binding under LFT conditions. (A) Dose-response inhibition of Biotin- $\alpha$ -BgTX binding to *Torpedo*-nAChR by 20-me-SPX-G. Inset: NeuroTorp test-lines showing the dose-dependent inhibition binding of 20-me-SPX-G: (a) 39 nM 20-me-SPX-G. (b) 78 nM 20-me-SPX-G. (c) 156  $\mu$ M 20-me-SPX-G. (d) 312 nM 20-me-SPX-G. (e) 625 nM 20-me-SPX-G. (f) 2.5  $\mu$ M 20-me-SPX-G. (g) 5  $\mu$ M 20-me-SPX-G. (h) 10  $\mu$ M 20-me-SPX-G. (B) Dose-response inhibition of Biotin- $\alpha$ -BgTX binding to *Torpedo*-nAChR by a mussel extract spiked with GYM-A, 13,19-SPX-C, PnTX-A, 13-SPX-C, 20-me-SPX-G and PnTX-G at a final concentration of 1  $\mu$ M each. (C-F) LFT NeuroTorp strips showing the dose-dependent inhibition of Biotin- $\alpha$ -BgTx binding to *Torpedo*-nAChRs by CiTXs-spiked mussels. For each dose, two series of LFT strips were run simultaneously per triplicate. The test-strips were exposed to a given dose of CiTXs-spiked mussel extracts (left side of each panel). Control strips are depicted at the right side of each panel. (C) 75 nM CiTXs-containing spiked mussels (D) 150 nM CiTXs-containing spiked mussels (E) 300 nM CiTXs-containing spiked mussels (F) 600 nM CiTXs-containing spiked mussels. Abbreviations: Spk Mus: Spiked Mussels, CiTX: cyclic imine toxins, CL: Control-line, in which the monoclonal antibody Biotin-RAB37 was immobilized, T: Test-line, in which 500 ng of *Torpedo*-electrocyte membrane vesicles were immobilized. Three independent experiments performed by triplicate for each dose were repeated at different dates.

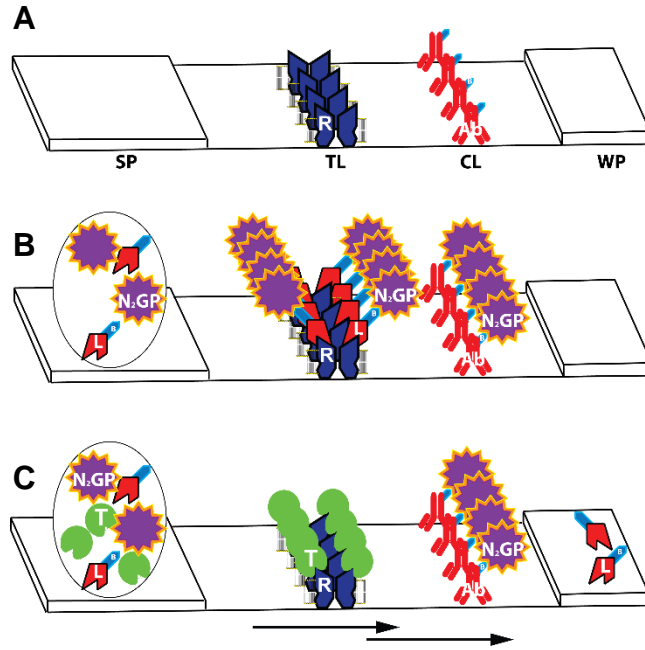
**Figure 4.** Detection of CiTX in methanolic shellfish extracts by the LFT NeuroTorp.

(A) Competitive inhibition binding of Biotin- $\alpha$ -BgTX to *Torpedo*-nAChRs by shellfish extracts at 10, 5, 2.5 and 1.25% methanol. Inhibition binding was dependent on the concentration of CiTX in each of the samples. (B-E) LFT NeuroTorp strips showing the inhibition of Biotin- $\alpha$ -BgTx binding to *Torpedo*-nAChRs by shellfish extracts. For each dose, two series of LFT strips were run simultaneously per triplicate. The test-strips were exposed to a given shellfish extract (left side of each panel). Control strips are depicted at the right side of each panel. (B) Inhibition binding of Biotin- $\alpha$ -BgTX to *Torpedo*-nAChRs by ALTX mussel 01 sample (5% methanol). (C) Inhibition binding of Biotin- $\alpha$ -BgTX to *Torpedo*-nAChRs by ALTX mussel 02 extract (5% methanol). (D) Inhibition binding of Biotin- $\alpha$ -BgTX to *Torpedo*-nAChRs by the supernatant of canned cockles extract (10% methanol) (E) Inhibition binding of Biotin- $\alpha$ -BgTX to *Torpedo*-nAChRs by canned cockles extract (10% methanol). Abbreviations: Mussels 01: methanolic extract of ALTX mussels 01 provided by CIFGA, Mussels 02: methanolic extract of ALTX mussels 02 provided by CIFGA, Cockles SS: extract of the supernatant of canned cockles for human consumption provided by CIFGA, Cockles PP: extract of canned cockles flesh for human consumption provided by CIFGA, CL: Control-line, in which the monoclonal antibody Biotin-RAB37 was immobilized, T: Test-line, in which 500 ng of *Torpedo*-electrocyte membrane vesicles were immobilized. Three independent experiments performed by triplicate for each dose were repeated at different dates.

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**Figure 5.** UPLC-MS/MS simultaneous detection of gymnodimine-A (GYM-A), 13,19-didesmethyl spirolide-C (13,19-SPX-C), pinnatoxin-A, (PnTX-A), 13-desmethyl spirolide-C (13-SPX-C), 20-methyl spirolide-G (20-met-SPX-G) and pinnatoxin-G (PnTX-G) in the shellfish extracts studied. (A) ALTX 01 mussels extract. (B) ALTX 02 mussels extract. (C) Canned cockles flesh extract. (D) Canned cockles supernatant extract. Calibration curves were obtained using toxin standard mixtures in the range from 1 pM to 1  $\mu$ M freshly prepared for the quantitation. All samples were run three times, and two blank runs (5  $\mu$ L of methanol) were intercalated between different samples to prevent carry-over.

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Figure 2

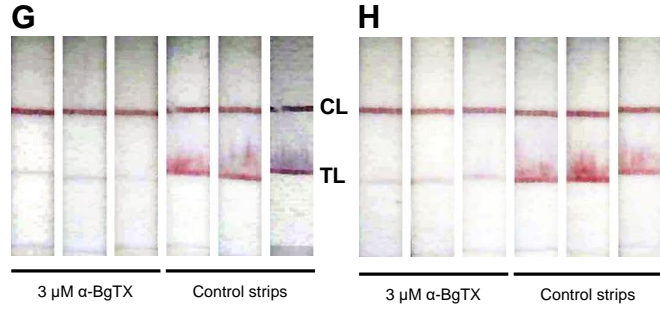
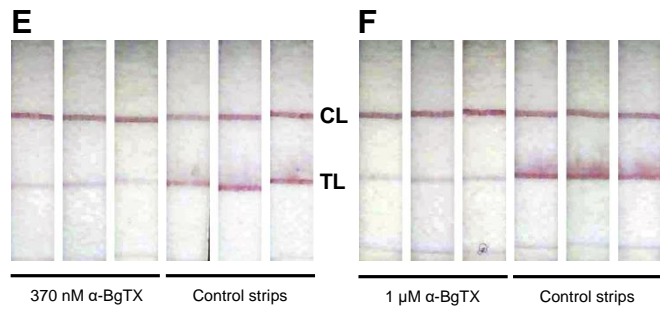
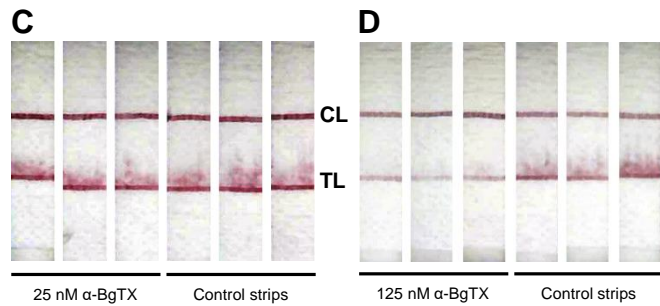
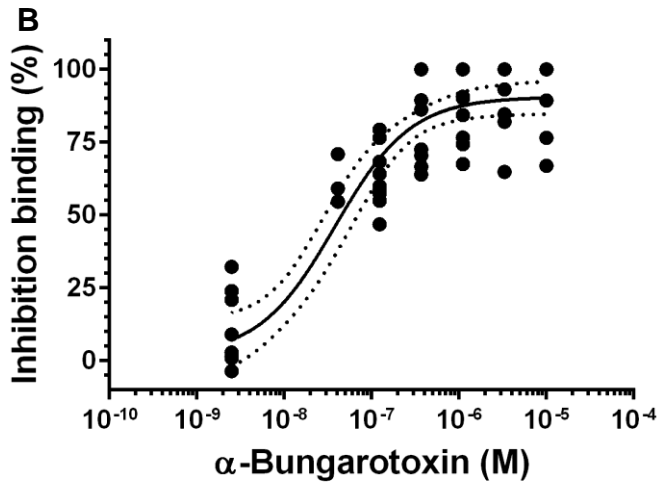
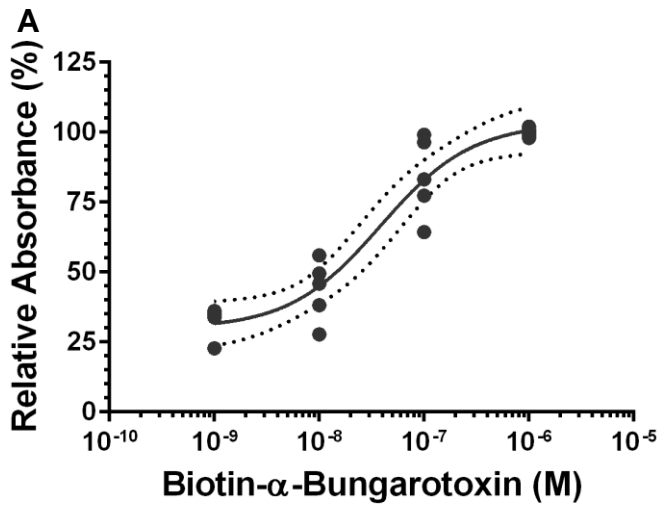
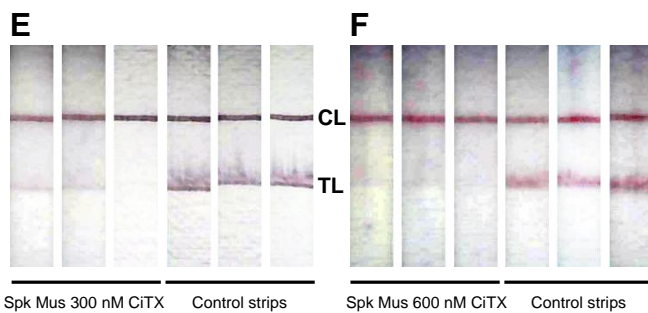
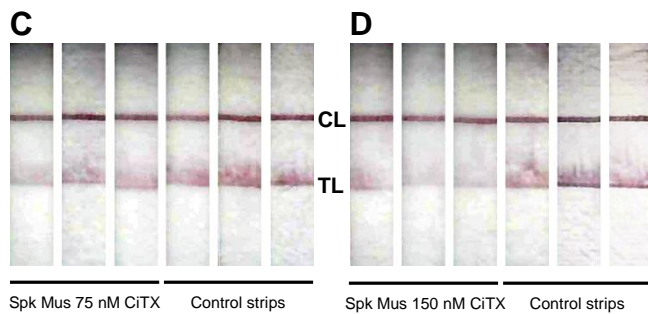
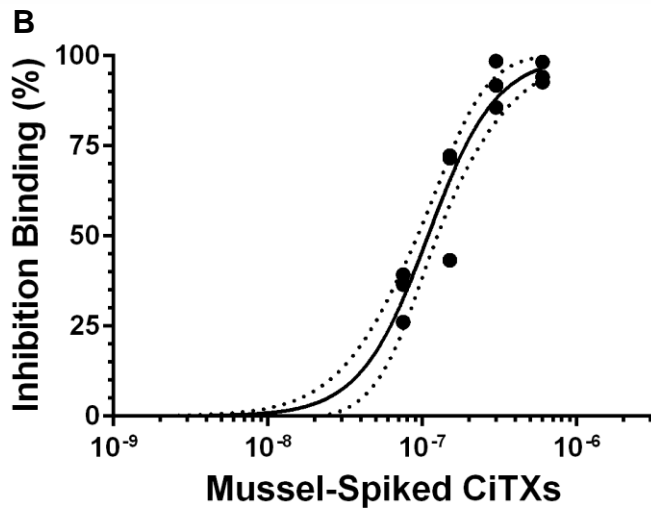
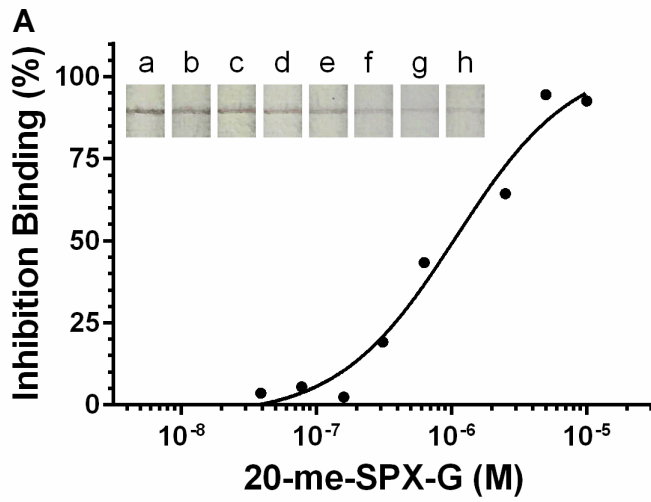
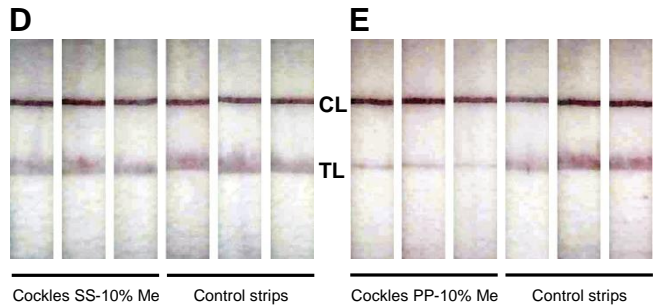
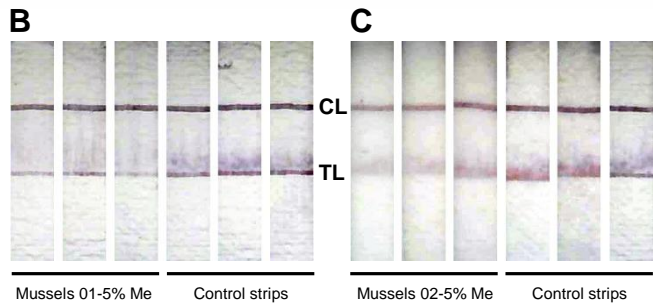
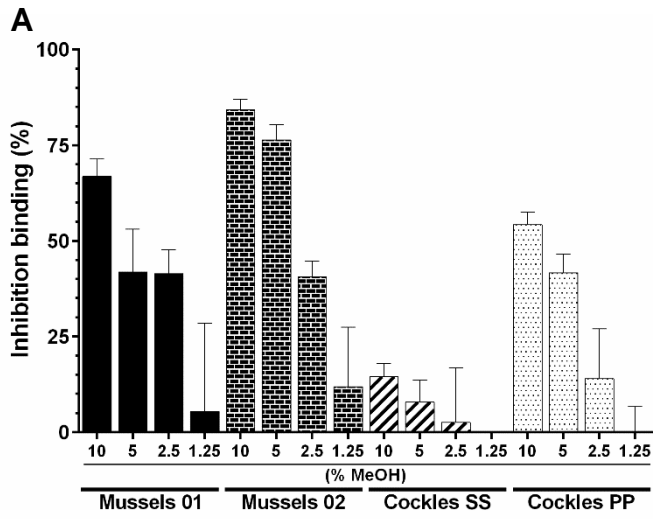


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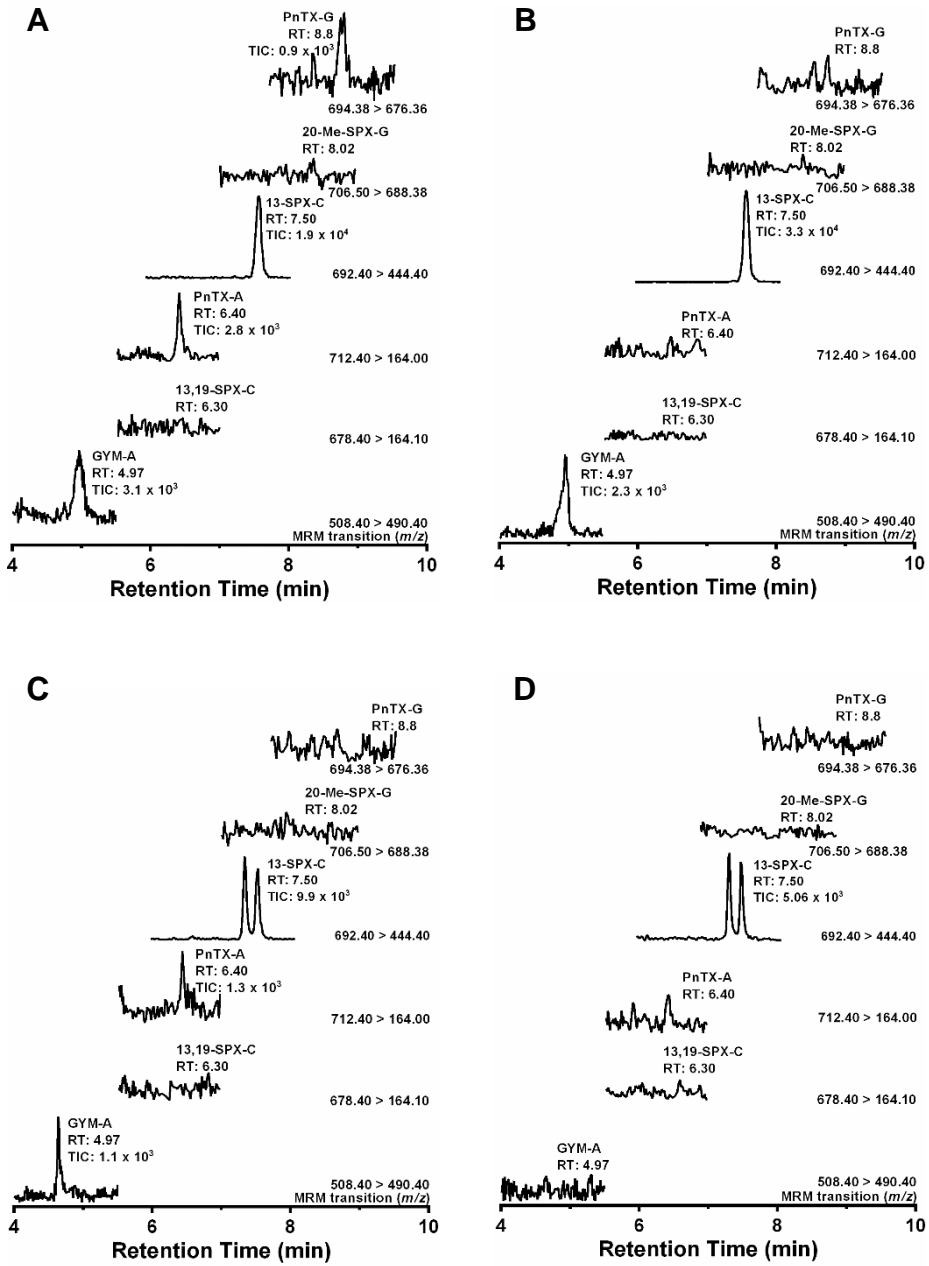
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