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Development and validation of a maleimide-based enzyme-linked immunosorbent assay for the detection of tetrodotoxin in oysters and mussels

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Abbreviations

Acetic acid (AA), acetonitrile (ACN), anti-mouse IgG-horseradish peroxidase antibody (IgG-HRP), L-arginine (Arg), bovine serum albumin (BSA), correction factor (CF), decarbamoyl gonyautoxin 2&3 (dcGTX2&3), decarbamoyl neosaxitoxin (dcNEO), decarbamoyl saxitoxin (dcSTX), effective limit of detection (eLOD), electrospray ionization (ESI), equivalents (equiv.), ethylenediaminetetraacetic acid (EDTA), formic acid (FA), gonyautoxin 1&4 (GTX1&4), gonyautoxin 2&3 (GTX2&3), gonyautoxin 5 (GTX5), inhibitory concentration (IC), limit of detection (LOD), liquid chromatography-tandem mass spectrometry (LC-MS/MS), maleimide-based enzyme-linked immunosorbent assay (mELISA), monoclonal antibody (mAb), methanol (MeOH), 4-morpholineethanesulfonic acid (MES), multiple reaction monitoring (MRM), neosaxitoxin (NEO), N-sulfocarbamoyl gonyautoxin 2&3 (C1&2), paralytic shellfish poisoning (PSP), saxitoxin (STX), self-assembled monolayer (SAM), solid-phase extraction (SPE), 3,3',5,5'-tetramethylbenzidine (TMB), tetrodotoxin (TTX), voltage-gated sodium channel (VGSC).

Abstract

The recent detection of tetrodotoxins (TTXs) in puffer fish and shellfish in Europe highlights the necessity to monitor the levels of TTXs in seafood by rapid, specific, sensitive and reliable methods in order to protect human consumers. A previous immunoassay for TTX detection in puffer fish, based on the use of self-assembled monolayers (SAMs) for the immobilization of TTX on maleimide plates (mELISA), has been modified and adapted to the analysis of oyster and mussel samples. Changing dithiol for cysteamine-based SAMs enabled reductions in the assay time and cost, while maintaining the sensitivity of the assay. The mELISA showed high selectivity for TTX since the antibody did not cross-react with co-occurring paralytic shellfish poisoning (PSP) toxins and no interferences were observed from arginine (Arg). Moreover, TTX-coated maleimide plates stored for 3 months at -20 °C and 4 °C were stable, thus when pre-prepared, the time to perform the assay is reduced. When analyzing shellfish samples, matrix effects and toxin recovery values strongly depended on the shellfish type and the sample treatment. Blank oyster extracts could be directly analyzed without solid-phase extraction (SPE) clean-up, whereas blank mussel extracts showed strong matrix effects and SPE and subsequent solvent evaporation were required for removal. However, the SPE clean-up and evaporation resulted in toxin loss. Toxin recovery values were taken as correction factors (CFs) and were applied to the quantification of TTX contents in the analysis of naturally-contaminated shellfish samples by mELISA. The lowest effective limits of detection (eLODs) were about 20 and 50 µg/kg for oyster extracts without and with SPE clean-up, respectively, and about 30 µg/kg for mussel extracts with both protocols, all of them substantially below the eLOD attained in the previous mELISA for puffer fish (230 µg/kg). Analysis of naturally-contaminated samples by mELISA and comparison with LC-MS/MS quantifications demonstrated the viability of the approach. This mELISA is a selective and sensitive tool for the rapid detection of TTX in oyster and mussel samples showing promise to be implemented in routine monitoring programs to protect human health.

Keywords: Tetrodotoxin (TTX), solid-phase extraction (SPE) clean-up, oyster, mussel, maleimide-based enzyme-linked immunosorbent assay (mELISA), liquid chromatography-tandem mass spectrometry (LC-MS/MS).

1. Introduction

Tetrodotoxin (TTX) is a potent low-molecular-weight (319 Da) marine neurotoxin, named for the family of fish Tetraodontidae [1]. Tetrodotoxin possesses a unique structure, consisting of a positively charged guanidine group connected to a highly oxygenated carbon backbone [1-3]. Although TTX was originally found in the ovaries of puffer fish [4], several marine organisms have been shown to contain the toxin such as blue-ring octopus, ribbon worms, starfish and xanthid crabs [5], as well as terrestrial animals such as frogs and newts [6]. Unlike many other marine toxins, which are of microalgal origin, TTX production is thought to be produced by bacteria of the genera *Pseudomonas, Shewanella, Alteromonas* or *Vibrio* [7], in symbiosis with certain animals [8]. Recently, the marine dinoflagellate *Prorocentrum minimum* has been described to produce TTXs in cultures, with possible implication of endosymbiotic bacteria [9]. Tetrodotoxin has the ability to selectively bind to voltage-gated sodium channels (VGSCs), blocking the influx of sodium ions into the nerve cells, affecting neuromuscular transmission [10]. The consumption of puffer fish contaminated with TTX may result in mild gastrointestinal effects, numbness, respiratory failure, and even in death [11]. Human intoxication has been reported worldwide, mainly caused by the ingestion of contaminated puffer fish, served in Japan as a delicacy known as “fugu” [8, 11]. A toxic species of puffer fish, *Lagocephalus sceleratus*, recently reached the Mediterranean through the Suez channel [12], resulting in new reports of food poisoning in the Western Mediterranean and further migration towards eastern waters [13-15]. In Europe, the first toxicity report related with TTX-contaminated shellfish occurred in Spain in 2007 and it was caused by the ingestion of contaminated trumpet shells, although the shellfish was bought in Portugal [16]. Since then, TTXs have been detected in bivalve shellfish in different parts of Europe, including England [17], Greece [18] and the Netherlands [19]. In humans, according to case studies, between 0.18 and 0.2 mg of TTX have been reported to cause severe symptoms, and a fatality was reported after ingestion of around 2 mg of TTX [20]. Despite the fact that TTX is a toxin with a high fatality rate and worldwide distribution, neither a reference method nor regulatory limits have been specifically set for TTX. Nevertheless, in Japan a value of 2 mg TTX equiv./kg edible portion has been used as the acceptance criterion to consider puffer fish safe for consumption [21]. Moreover, in Europe, the Regulation (EC) no. 854/2004 stipulates that “fishery products derived from poisonous fish of the following families must not be placed on the market: Tetraodontidae, Molidae, Diodontidae and Canthigasteridae” [22]. Concern for TTX in Europe has been increasing, and just recently, the European Food Safety Authority has concluded that a concentration below 44

µg TTX equiv./kg shellfish meat, based on a large portion size of 400 g, is considered not to result in adverse effects in humans [19].

Given the occurrence of TTXs in European bivalve shellfish and the threat that this hazardous toxin poses to human health, the development of rapid, specific, sensitive, reliable and easy-to-use methods for their detection is a matter of utmost importance. Accordingly, several methods have been reported for the detection of TTXs, including immunoassays [23-33] and immunosensors [23, 34-41]. Although some of these immunochemical approaches have been applied to the analysis of puffer fish [32, 33, 36, 38, 40-41], newts [26], caddisflies [27], terrestrial flatworms [29], sea snails [38], urine [36] and milk/apple juice [37], none of them has been applied to the analysis of bivalve mollusks. Indeed, up to date only the LC-MS/MS method has been applied to the detection of TTXs in mussels and oysters [42, 43]. However, this technique requires trained personnel, sample pre-treatment and expensive equipment. Taking as a starting point an immunoassay previously developed for the determination of TTXs in puffer fish samples [33], the aim of this research was to illustrate the development of an improved bioanalytical tool for the analysis of oyster and mussel samples.

2. Materials and methods

Reagents and solutions

For mELISA, pure TTX standard was purchased from Tocris Bioscience (Bristol, UK) and standard solutions were prepared at 1 mg/mL in 10 mM acetic acid (AA). For LC-MS/MS analysis, pure TTX standard was purchased from Latoxan (Valence, France) and standard solutions were prepared at 1 mg/mL in 350 mM AA. Certified reference materials (CRMs), specifically gonyautoxin 1&4 (GTX1&4), gonyautoxin 2&3 (GTX2&3), decarbamoyl gonyautoxin 2&3 (dcGTX2&3), gonyautoxin 5 (GTX5), neosaxitoxin (NEO), decarbamoyl neosaxitoxin (dcNEO), saxitoxin (STX), decarbamoyl saxitoxin (dcSTX) and N-sulfocarbamoyl gonyautoxin 2&3 (C1&2), were obtained from the National Research Council of Canada (NRC, Halifax, NS, Canada). The anti-TTX monoclonal antibody (mAb) TX-7F was produced as described in Kawatsu et al. [25]. Pierce maleimide-activated plates were obtained from Thermo Fisher Scientific (Madrid, Spain). Ammonium hydroxide solution (NH_4OH, 25%), amorphous graphitized polymer carbon Supelco ENVI-Carb 250 mg/3 mL cartridges, anti-mouse IgG (whole molecule)-horseradish peroxidase antibody produced in rabbit (IgG-HRP), L-arginine (Arg), bovine serum albumin (BSA), cysteamine hydrochloride, ethylenediaminetetraacetic acid (EDTA), formaldehyde solution, 4-morpholineethanesulfonic acid (MES) hydrate, potassium phosphate dibasic, potassium phosphate monobasic and 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate were all supplied by Sigma-Aldrich (Tres Cantos, Spain). HPLC-grade acetonitrile (ACN), glacial AA and methanol (MeOH) were obtained from Chem-lab (Zedelgem, Belgium). Formic acid (FA) (98-100%) was purchased from Merck (Darmstadt, Germany). Ultra LC-MS ACN, Ultra LC-MS MeOH and Ultra LC-MS H_2O were purchased from Actu-All (Oss, The Netherlands). Ultrapure Milli-Q water (18.2 MΩ/cm^2) was used for the preparation of solutions (Millipore, Bedford, MA, USA).

Instrumentation

For toxin extraction, a water bath (model 6000138 600 W) purchased from J. P. Selecta S. A. (Barcelona, Spain), an Alegra X-15R centrifuge provided by Beckman Coulter (Barcelona, Spain) and a DVX-2500 multi-tube vortex mixer acquired at VWR International Eurolab S. L. (Barcelona, Spain) were used.

Extraction clean-up was performed with a Rapid Trace SPE workstation supplied by Caliper Life Sciences (Waltham, MA, USA).

Colorimetric measurements were performed with a Microplate Reader KC4 from BIO-TEK Instruments, Inc. with GEN 2.09 software (Winooski, VT, USA).

For LC-MS/MS analysis, the separation was performed on a Waters Acquity I-Class UPLC system (Waters, Milford, MA, USA) and mass spectrometric analysis on a Waters Xevo TQ-S (Waters, Milford, MA, USA).

Shellfish samples

For the evaluation of matrix effects and toxin recovery, Pacific oyster (*Crassostrea gigas*) and mussel (*Mytilus galloprovincialis)* samples from the Ebro Delta (Alfacs Bay, NW Mediterranean Sea) were used. These shellfish samples were determined as TTXs negative by LC-MS/MS analysis. For the analysis of naturally-contaminated shellfish, three oyster (*Crassostrea gigas*) and three mussel (*Mytilus edulis*) samples were obtained from production sites at the Oosterschelde in The Netherlands.

mELISA protocol

The protocol was similar to that previously described by our group [33], with some modifications regarding the TTX immobilization. The first step was the self-assembling of 1 mM cysteamine in phosphate buffer for 3h, followed by the direct immobilization of TTX (2 µg/mL) with formaldehyde (3.4 %) in the same buffer overnight. A competitive assay was then performed by incubating 50 µL of free TTX/shellfish extract and 50 µL of 1:3,200 anti-TTX mAb dilution in 1% BSA-phosphate buffer for 30 min. A blocking step was then performed with 200 µL of 1% BSA-phosphate buffer for 30 min and, finally, 100 µL of IgG-HRP at 1:1,000 dilution in 1% BSA-phosphate buffer was incubated for 30 min. Colorimetric response was measured at 620 nm after 10 min of TMB liquid substrate incubation.

Storage stability of TTX-coated maleimide plates

Tetrodotoxin was immobilized through cysteamine self-assembled on maleimide-activated plates as described in the section above, and TTX-coated maleimide plates were kept at 4 °C and -20 °C. Absorbance values of wells with mAb (maximum response) and without mAb (background) in the absence of free TTX were measured in triplicate at day 0 (reference value) and during several weeks up to 3 months.

Interference study

The selectivity of the mELISA was assessed by the analysis of the following Paralytic Shellfish Poisoning (PSP) toxins: GTX1&4, GTX2&3, dcGTX2&3, GTX5, NEO, dcNEO, STX, dcSTX and C1&2, as well as L-arginine (Arg). A concentration of 100 ng/mL was chosen taking into account that TTX almost completely inhibits the mAb binding. The protocol was the same as that explained in the mELISA protocol section, but replacing free TTX by PSP toxins or Arg prepared in 1% BSA-phosphate buffer in the competition step. Percentage of mAb binding was calculated with respect to the response obtained without toxin (maximum response).

Toxin extraction and SPE clean-up

For the analysis of toxins in shellfish by mELISA, extracts were obtained following the single dispersive procedure described in the literature [43] for other paralytic shellfish toxins, adapted to the amount of sample available. In brief: 1 g ± 0.1 g of shucked shellfish homogenate was weighed into a 15-mL tube and 1 mL of AA/H_2O (1:100, v:v) was added. After shaking the tube for 90 s on a multi-tube vortex mixer, samples were boiled in a water bath for 5 min at 100 °C. Tubes were then cooled and shaken again with the multi-vortex mixer for 90 s. Finally, samples were centrifuged at 4,500 rpm for 10 min, and the supernatants were filtered through 0.2-µm nylon filters and kept at -20 °C until analysis. The resulting extracts contained fresh shellfish matrix at a concentration of 1,000 mg equiv./mL.

For the clean-up of shellfish sample extracts, SPE was performed using graphitized polymer carbon ENVI-carb cartridges by adapting the automated protocol described by Boundy et al. [43]. Briefly, a 0.5-mL aliquot of the AA extract was transferred to a polypropylene tube and 1.25 µL of NH_4OH solution was added. The cartridges were conditioned with 3 mL of ACN/H_2O/AA (20:80:1, v:v:v), followed by 3 mL of H_2O/NH_4OH (1000:1, v:v). Then, 400 µL of sample extracts were loaded onto the conditioned cartridges and were washed with 700 µL of deionized H_2O. Finally, the retained TTX was eluted with 2 mL of ACN/H_2O/AA (20:80:1, v:v:v) and stored at -20 °C until analysis. Resulting extracts were at a shellfish matrix concentration of 200 mg equiv./mL. Further dilutions for mELISA experiments were performed in phosphate buffer. When required, shellfish sample extracts were evaporated for solvent exchange (from ACN/H_2O/AA to phosphate buffer).

Blank, TTX-spiked and naturally-contaminated shellfish sample extracts were analyzed by mELISA at 3 different stages: (1) after SPE clean-up, (2) after SPE clean-up, evaporation and

solvent exchange, and (3) directly after toxin extraction (without SPE clean-up nor evaporation and solvent exchange). The matrix effects of blank samples were evaluated and the TTX contents obtained in spiked and naturally-contaminated samples at each stage were determined.

LC-MS/MS analysis

For the analysis by LC-MS/MS, naturally-contaminated shellfish sample extracts were obtained as follows: 1 g of shellfish homogenate was accurately weighed, and 2 mL of H_2O/MeOH (50:50, v:v) containing 15 mM AA solution was added. First, TTX was extracted by a 15-min head-over-head extraction. The sample was then centrifuged for 10 min at 5,200 g and the supernatant was transferred to a volumetric tube. A second extraction was performed by adding 1.5 mL of extraction solvent and vortex mixing during 1 min. After centrifugation, the total volume was brought to 4 mL with the same extraction solvent. The extract was diluted 1:9 with ACN/H_2O (70:20, v:v) containing 6.7 mM AA. Subsequently, the diluted extract was centrifuged at 16,200 g during 5 min and the supernatant was transferred to a vial. For the construction of calibration curves, matrix-matched standards were prepared by spiking blank shellfish material with known concentrations of TTX, respectively 0, 20, 50, 75, 150 µg/kg.

Chromatographic separation was achieved using a UPLC system. The system consisted of a binary solvent manager, a sample manager and a column manager. The column temperature was at room temperature and the temperature of the sample manager was kept at 10 °C. For the analysis of TTX, a 10-µL injection volume was used. Mobile phase A was H_2O and B was ACN, both containing 50 mM FA. The analytical column used was a Tosoh Bioscience TSKgel Amide-80 column (250x2 mm, 5-µm particles). A flow rate of 0.2 mL/min was used. A gradient started at 30% A and after 1 min it was linearly increased to 95% A in 7.5 min. This composition was kept for 5 min and returned to 30% A in 0.5 min. An equilibration time of 6 min was allowed prior to the next injection. The effluent was directly interfaced in the electrospray ionization (ESI) source of the triple quadrupole mass spectrometer. The mass spectrometer operated in ESI positive ionization mode and two transitions for TTX were measured, *m/z* 320.1 > 162.1 and *m/z* 320.1 > 302.1.

Data analyses and statistics

Measurements were performed in triplicate for the mELISA experiments and singular in LC-MS/MS analysis. In the mELISA, calibration curves were background-corrected with respect to the controls with no mAb and adjusted to sigmoidal logistic four-parameter equations using SigmaPlot software 12.0 (Systat Software Inc., San José, CA, USA). From the equations, inhibitory concentrations (ICs) were calculated. Specifically, the midpoint (IC_{50}), the limit of detection (LOD) established as the IC_{20}, and the working range (IC_{20}-IC_{80}), were determined. In this work, the limit of quantification (LOQ) has been considered equal to the LOD.

To evaluate differences in the quantifications provided by the three approaches (mELISA with SPE clean-up/evaporated samples, mELISA with no SPE clean-up/not evaporated samples and LC-MS/MS analysis), a one-way analysis of variance was conducted using SigmaStat 3.1 software (Systat Software Inc., San José, CA, USA). Prior to the analysis, a normality and equal variance test was performed. Differences in the results were considered statistically significant at the 0.05 level.

3. Results and discussion

mELISA performance

Rapidity, low-cost and simplicity are key parameters for the success of immunoassays. To this purpose, the mELISA described previously elsewhere [33] was improved by using reagents readily available, reducing the protocol time and cost. The carboxylate-dithiol used for TTX-coating of the microtiter plate was replaced by cysteamine, which simplifies the protocol by eliminating 3 steps and shortens the analysis time by 90 min. Cysteamine can be readily purchased from different companies and it costs about 1,000-fold less than carboxylate-dithiol.

To enable the substitution of carboxylate-dithiol by cysteamine, the amount of cysteamine required for the SAM formation had to be determined, and according to the solubility of cysteamine, the solvent was changed from ethanol into an aqueous (phosphate) buffer. Thus, concentrations of 1, 10, 50 and 100 mM of cysteamine were tested in competition assays, using 2 µg/mL of TTX, 1:3,200 mAb dilution and 1:1,000 IgG-HRP dilution. All competitive assays showed appropriate trends according to the free TTX concentrations and provided similar IC_{50} values. Moreover, similar absorbance values were obtained for the positive (without free TTX) and negative (without mAb) controls, altogether indicating that 1 mM of cysteamine is a saturated concentration. Therefore, subsequent experiments were performed with 1mM cysteamine. Under the selected conditions, an IC_{50} of 8 ng/mL, an LOD, established as the IC_{20}, of 2 ng/mL and a working range (IC_{20}-IC_{80}) between 2 and 43 ng/mL were attained (**Fig. 1**), with an *R* from the sigmoidal adjustment of 0.996. The standard deviation (SD) values for the calibration points were lower than 8% of the mAb binding signal. The LOD attained with the mELISA described herein was similar to that obtained with the previous mELISA (using carboxylate-dithiol). In contrast, a narrower working range was obtained in the mELISA using cysteamine with respect to the previous mELISA using dithiol (2-43 *vs.* 2-95 ng/mL, respectively). This difference is attributed to the higher sensitivity of the cysteamine-based mELISA. The LOD provided by the mELISA described herein was in accordance with other immunoassays reported for TTX (~2 ng/mL [23, 25, 33]) and lower than others (5 ng/mL [32] and 10 ng/mL [28]).

Additionally, to investigate the possibility of further shortening the time of the mELISA protocol, the storage stability of TTX-coated maleimide plates at 4 and -20 °C was evaluated. The mAb binding signal was constant up to 3 months at both 4 °C and -20 °C, demonstrating the stability of the maleimide plates with immobilized TTX (**Fig. 2**). The great stability of the

TTX-coated plates significantly reduces the assay time, as multiple plates can be prepared on the same day and stored until use. Consequently, provided that TTX-coated plates are ready-to-use, the analysis of samples can be performed in less than 2 h on the same day, making a substantial improvement with respect to the previously reported mELISA [33]. Moreover, the preparation of multiple plates using the same solutions reduced the variability between assays, making the system more reproducible, particularly for a commercialization.

Interference studies

The protocol applied for TTX extraction from shellfish is also adequate to extract PSP toxins. Therefore, the TTX extraction process may extract PSP toxins, if present. As a consequence, prior to the analysis of naturally-contaminated samples, it is necessary to ensure that potential PSP toxins will not interfere in the TTX immunoassay performance. With this aim, the possible recognition of several PSP toxins (GTX1&4, GTX2&3, dcGTX2&3, GTX5, NEO, dcNEO, STX, dcSTX and C1&2) by the anti-TTX antibody was evaluated. As the mAb binding obtained for all toxins analyzed was close to 100% (**Fig. 3**), the cross-reactivity of the PSP toxins at a concentration of 100 ng/mL can be neglected, demonstrating the high specificity and selectivity of the mELISA. As a positive control, TTX tested at the same concentration resulted in a mAb binding decrease of more than 90% (**Fig. 1**). This study illustrates that those PSP toxins, for which standards are available, that may co-exist with TTX in shellfish extracts will not interfere with the immunorecognition of TTX in the assay.

Additionally, since multiple reaction monitoring (MRM) transitions for Arg have been found to suppress TTX response in the mass spectrometer source [42], the possible interference of this amino acid in the mELISA was also evaluated. As can be seen in **Fig. 3**, no significant effects of Arg on the mAb binding were observed.

Evaluation of matrix effects and establishment of matrix correction factors

Mussel and oyster matrix effects were studied by the analysis of blank shellfish extracts at different matrix concentrations (50, 75, 100 and/or 150 mg equiv./mL) at 3 different stages of the extraction protocol.

1) <u>After SPE clean-up without solvent evaporation</u>: Although SPE clean-up should reduce matrix effects, the highly acidic nature of the ACN/H_2O/AA solvent mixture used in the SPE protocol had a negative impact on the system, since mAb binding percentages obtained were

not consistent with the shellfish matrix concentration used. The detrimental effects observed in the mELISA were assumed to be due to the solvent composition rather than to the shellfish matrix. Even decreasing the solvent mixture percentage by 4 times respect to the extract arising from the SPE column (i.e. 12.5% of the total volume of the well), the solvent effect could not be avoided.

2) After SPE clean-up, evaporation and solvent exchange: SPE cleaned-up extracts were evaporated until dryness, dissolved in phosphate buffer and analyzed by mELISA at the same matrix concentrations. mAb binding percentages were between 92 and 108% in either oyster or mussel extracts, regardless of the matrix concentration used (**Table 1**). Taking into consideration that the highest RSD value obtained was of 10%, negligible matrix effects are assumed when mAb binding percentages are between 90 and 110%. Thus, results obtained with this protocol indicate that evaporation completely removes the previously observed undesirable effects. These results also reaffirm that when no evaporation was performed, the inhibition of the mAb binding was not due to the shellfish matrix, but to the solvent mixture presence, which could be harming the cysteamine and/or TTX immobilization. Therefore, mELISA enables loading up to 150 mg equiv./mL of shellfish matrix after SPE clean-up and evaporation.

3) Directly after toxin extraction (without SPE clean-up nor evaporation and solvent exchange): In order to evaluate if the SPE clean-up can be avoided to simplify the protocol or if, on the contrary, is a crucial step in the analysis of oyster and mussel extracts, extracts without SPE clean-up and not evaporated were tested. Under these conditions, differences between oyster and mussel extracts were observed (**Table 1**). In the case of oyster, no matrix effects were observed (i.e. mAb binding percentages were between 103 and108%), indicating that the mELISA tolerates up to 150 mg equiv./mL of oyster matrix without SPE clean-up and with no need of solvent exchange. These results indicate that whereas solvent evaporation is crucial for the analysis of SPE cleaned-up oyster extracts, oyster extracts without SPE clean-up containing up to 7.5 % of the extraction solvent do not to interfere with the assay performance and, therefore, solvent evaporation is not required for the analysis of these extracts. However, in the analysis of mussel, pronounced matrix effects were obtained for all matrix concentrations tested. Although loading a lower matrix concentration could in principle remove remaining matrix effects, this would compromise the effective LOD (eLOD) of the assay. While the reason for the different behavior between mussel and oyster extracts remains unclear, it is evident that in the analysis of mussel extracts, SPE clean-up and subsequent solvent exchange are recommended to avoid non-desired matrix interferences.

Toxin recovery in oysters and mussel samples

From the experiments performed with blank oyster extracts, it was concluded that up to 150 mg equiv./mL of oyster extract can be loaded on the immunoassay without requiring a clean-up step nor solvent exchange. Nonetheless, solvent evaporation is required if the oyster extract undergoes a SPE clean-up step. Mussel extract after SPE clean-up also requires solvent evaporation prior to analysis to avoid solvent interference in the immunoassay. However, mussel extracts without SPE clean-up and with no evaporation suffer from undesirable matrix effects even at 50 mg equiv./mL of mussel matrix. Consequently, oyster and mussel tissue homogenates were spiked and extracted, and toxin recovery was evaluated without SPE clean-up/not evaporated and after SPE clean-up/evaporated. Taking into account the matrix concentrations of the resulting extracts and to fit into the TTX calibration curves, extracts were spiked at 2 different levels of TTX in order to evaluate the toxin recovery.

1) After SPE clean-up, evaporation and solvent exchange: As SPE cleaned-up and evaporated extracts can be analyzed at 150 mg equiv./mL of shellfish matrix concentration, shellfish tissues were spiked at 75 µg TTX/kg (concentration that should provide about 50% of mAb binding inhibition when extract is analyzed at 150 mg equiv./mL of matrix concentration). Under these conditions, low toxin recovery values were obtained for both oyster and mussel tissue extracts (**Table 2**). These low recovery values can be attributed to toxin loss during the SPE clean-up and solvent exchange and/or interference of the shellfish matrix on the free toxin/antibody recognition. Nevertheless, these toxin recovery values can be taken as correction factors (CFs) and will be applied in the quantifications obtained in the subsequent analysis of naturally-contaminated samples extracted and treated under these conditions.

2) Directly after toxin extraction (without SPE clean-up nor evaporation and solvent exchange): Mussel extracts with no SPE clean-up showed higher matrix effects and, therefore, samples had to be analyzed at lower matrix concentrations. Consequently, to test these conditions, a higher TTX concentration (250 µg TTX/kg) was spiked in mussels (also for oysters, although it was not a requirement). Under these conditions, toxin recovery values were higher than those obtained for SPE cleaned-up extracts. Regarding oyster samples, the lower the matrix concentration, the higher the toxin recovery. The excellent toxin recovery at 75 mg equiv./mL (96%) indicates that these extracts do not suffer from toxin loss during SPE or solvent exchange. Consequently, the inhibition of mAb binding at higher matrix concentrations can only be due to the effect of the matrix on the free toxin/antibody recognition, which obviously decreases as the matrix concentration decreases. Again, these toxin recovery values can be taken as CFs and will be applied in the quantifications obtained in the subsequent analysis of

naturally-contaminated oyster samples extracted and treated under these conditions. Regarding mussel samples, only one matrix concentration (50 mg equiv./mL) was analyzed (matrix effects at higher matrix concentrations were considered too high to conduct spiking trials), and the toxin recovery obtained was very high (166%). In this case, it is evident that the mussel matrix inhibits the response (as observed in the previous experiment where no TTX was present), causing an overestimation of the TTX content. Taking into account the toxin recovery value (166%) and the percentage of mAb binding obtained in the analysis of blank mussel tissue at 50 mg equiv./mL (74%), a CF of 123% is obtained, which will be used in the quantifications obtained in the subsequent analysis of naturally-contaminated mussel samples extracted and treated under these conditions.

Once obtained all toxin recovery values, eLODs in µg TTX/kg shellfish were calculated for each shellfish matrix and for both protocols. These eLODS were calculated as the ratio of the LOD obtained in buffer (2 ng/mL ± SD) to the shellfish matrix concentration used and applying the corresponding CF (**Table 3**).

Regarding oysters, eLOD obtained for extracts without SPE clean-up and not evaporated were lower than for SPE cleaned-up and evaporated extracts. Consequently, SPE clean-up is certainly not worth conducting as this additional step increases the analysis time. Regarding mussels, similar eLODs were obtained after SPE clean-up at 150 mg equiv./mL of matrix and without SPE clean-up at 50 mg equiv./mL. These results reaffirm that SPE clean-up is not a requirement for the analysis of oyster and mussel extract samples.

The eLOD values obtained for both shellfish matrices are higher than those previously reported for TTX by LC-MS/MS (5 µg/kg in this work, 3 µg/kg [17], 7.2 µg/kg [18] and 15 µg/kg [43]), but still very acceptable. Notably, they are substantially below from the eLOD obtained for puffer fish with the previous mELISA (230 µg/kg) [33]. Moreover, the eLODS values obtained herein proved the capability of the mELISA of detecting TTX below or only slightly above the concentration of 44 µg TTX equiv./kg shellfish meat, level that is considered not to result in adverse effects in humans [19]. Therefore, the mELISA described herein is absolutely appropriate for the screening as well as for the quantification of TTX contents in natural shellfish samples.

Analysis of oyster and mussel samples and comparison with LC-MS/MS analysis

After evaluating the matrix effects and establishing the CFs according to the toxin recovery values obtained, the mELISA was applied to the analysis of 3 oyster and 3 mussel samples from

the Oosterschelde, The Netherlands. Oyster and mussel extracts were analyzed using the two different protocols (without SPE clean-up not evaporated and with SPE clean-up and evaporated). The corresponding CF values were applied to the TTX content determined in each sample, and resulting quantifications were compared to those obtained by LC-MS/MS analysis (**Table 4**).

Although the number of samples was too low for statistical treatment (due to the limited availability of natural samples of shellfish containing TTX), no significant differences (P=0.702) were observed in the quantifications provided by the three approaches (mELISA with SPE clean-up/evaporated samples, mELISA with no SPE clean-up/not evaporated samples and LC-MS/MS analysis). In the analysis of samples by LC-MS/MS, only TTX was detected (other TTX analogues were not found at detectable levels). This toxin profile, with only TTX, contributes to the similarity between techniques, even though they are based on different recognition principles.

The TTX content in the shellfish samples ranged from slightly below 44 µg/kg up to 4- or 5-fold higher. Thus, we provide the first immunoassay capable of screening and quantifying TTX in shellfish samples at levels that may be considered of concern for human health.

4. Conclusions

A modified SAM-based immunoassay has been applied to the determination of TTX in mussel and oyster samples. The replacement of dithiols by cysteamine for the SAM formation allowed decreasing the required time and cost, while maintaining the sensitivity of the previously reported mELISA (LOD of 2 ng/mL). Storage at -20 and 4 °C of the TTX immobilization up to at least 3 months, provided ready-to-use microtiter plates, enabling a user to perform the assay in less than 2 h. Additionally, as proven by the absence of interferences from PSP toxins and Arg, the mELISA is highly selective for TTX, and certainly TTX analogues, as demonstrated in our previous work [33, 40, 41, 44].

In the analysis of blank shellfish, oyster extracts did not show matrix effects even without the SPE clean-up step. The SPE clean-up of mussel extracts removed the strong matrix effects observed when no SPE was used. However, the SPE clean-up and the required solvent evaporation resulted in low toxin recovery percentages when analyzing TTX-spiked samples, probably because of toxin lost in the column and during the evaporation step. Toxin recovery values were obtained for all protocols and shellfish types, and can be used as CFs to be applied to the quantification of TTX contents in naturally-contaminated samples. Taking them into account, the lowest eLOD values obtained were about 20 and 50 µg TTX/kg for oyster extracts without and with SPE clean-up, respectively, and about 30 µg TTX/kg for mussel extracts with both protocols, substantially below the eLOD obtained by the previous mELISA for puffer fish (230 µg TTX/kg). This is in relatively good agreement with the level of 44 µg TTX equiv./kg shellfish meat, which is considered not to result in adverse effects in humans by the EFSA. Highly analogous results were determined on the comparison of the analysis of naturally-contaminated shellfish by mELISA with LC-MS/MS analysis.

Overall, the mELISA developed herein meets the requirements in terms of selectivity and sensitivity. Although toxin recovery values, and thus CFs, were obtained for all protocols, shellfish samples can be rapidly processed and analyzed by mELISA without SPE clean-up, which is a clear advantage over LC-MS/MS methodologies, where SPE is required. Therefore, the implementation of the mELISA for the screening of TTXs in bivalve shellfish samples in routine monitoring programs could be straightforward, providing a complementary analytical technique suitable for ensuring food safety and consumer protection.

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Figure legends

Figure 1. TTX calibration curve obtained by mELISA. Response is expressed as percentage of mAb binding, normalized to the signal when no TTX is present. Error bars represent the standard deviation values for 3 replicates.

Figure 2. Storage stability of TTX-coated maleimide plates. Bars represent the percentage of mAb binding obtained with plates stored at 4 °C (black bars) and at -20 °C (grey bars). Error bars represent the standard deviation values for 3 replicates.

Figure 3. Percentage of mAb binding obtained by mELISA with 100 ng/mL of PSP toxins and Arg. Error bars represent the standard deviation values for 3 replicates.

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Table 1. Percentages of mAb binding obtained in the analysis of blank oyster and mussel tissues extracted under different conditions (n=4 replicates).

Shellfish tissue	Protocol	[Matrix] (mg equiv./mL)			
		150	100	75	50
Oyster	SPE clean-up / not evaporated	X	X	X	X
	SPE clean-up / evaporated	106	92	97	-
	no SPE clean-up / not evaporated	103	110	108	108
Mussel	SPE clean-up / not evaporated	X	X	X	X
	SPE clean-up / evaporated	105	96	108	-
	no SPE clean-up / not evaporated	57	63	71	74

X: not consistent; -: not tested

Table 2. Percentages of toxin recovery obtained in the analysis of TTX-spiked oyster and mussel tissues extracted under different conditions (n=4 replicates).

Shellfish tissue	Protocol	TTX level (µg/kg)	[Matrix] (mg equiv./mL)			
			150	100	75	50
Oyster	SPE clean-up / evaporated	75	29	-	-	-
	no SPE clean-up / not evaporated	250	71	83	96	-
Mussel	SPE clean-up / evaporated	75	50	-	-	-
	no SPE clean-up / not evaporated	250	-	-	-	166

-: not tested

Table 3. eLODs (µg TTX/kg shellfish) determined for SPE cleaned-up evaporated and no SPE cleaned-up not evaporated oyster and mussel extracts at different matrix concentrations.

Shellfish tissue	Protocol	[Matrix] (mg equiv./mL)			
		150	100	75	50
Oyster	SPE clean-up / evaporated	47 ± 17	-	-	-
	no SPE clean-up / not evaporated	19 ± 7	25 ± 9	28 ± 10	-
Mussel	SPE clean-up / evaporated	27 ± 10	-	-	-
	no SPE clean-up / not evaporated	-	-	-	33 ± 12

-: not tested

Table 4. TTX content of 3 oyster and 3 mussel samples from the sanitary monitoring program of The Netherlands by the cysteamine-based mELISA under two different extraction protocols and comparison with LC-MS/MS analysis.

Method	Protocol	Oyster 1	Oyster 2	Oyster 3	Mussel 1	Mussel 2	Mussel 3
mELISA (µg TTX equiv./kg shellfish)	SPE clean-up / evaporated	82 ± 2	36 ± 6	67 ± 21	70 ± 11	146 ± 14	16 ± 4
	no SPE clean-up / not evaporated	61 ± 7	116 ± 16	86 ± 13	55 ± 1	227 ± 7	24 ± 5
LC-MS/MS* (µg TTX/kg shellfish)	-	113	51	79	93	172	41

*Samples were analyzed singular; during the intra laboratory validation of this method the repeatability error at 20 µg/kg was 15.7%.

Figure 1.

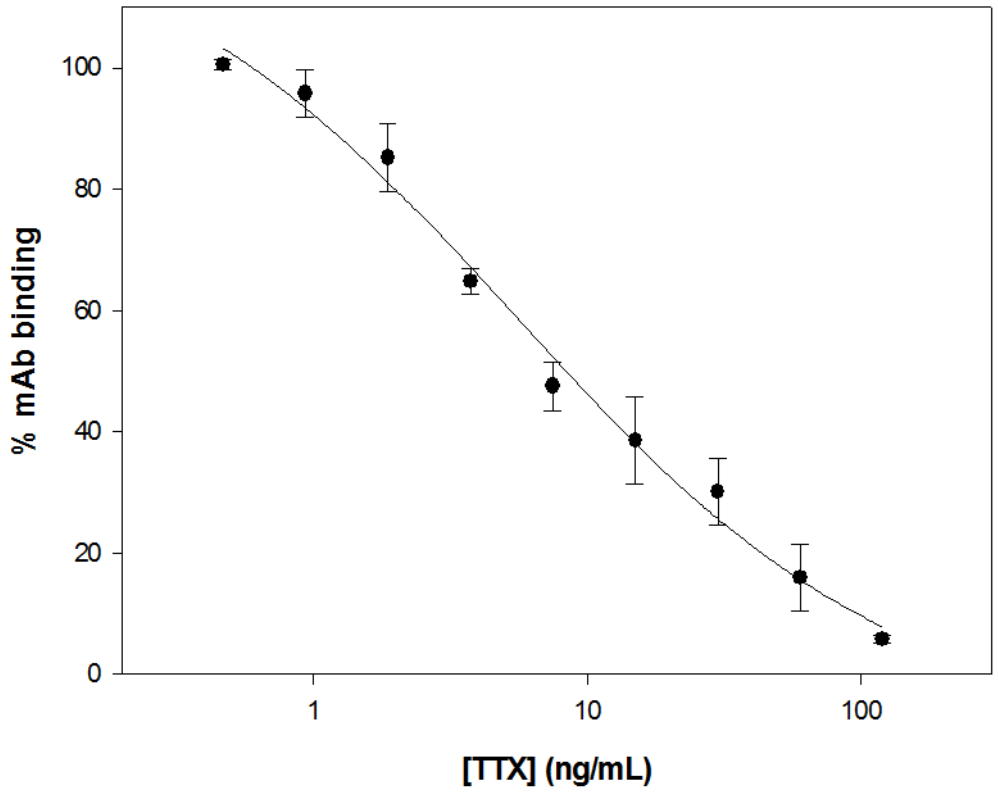


Figure 2.

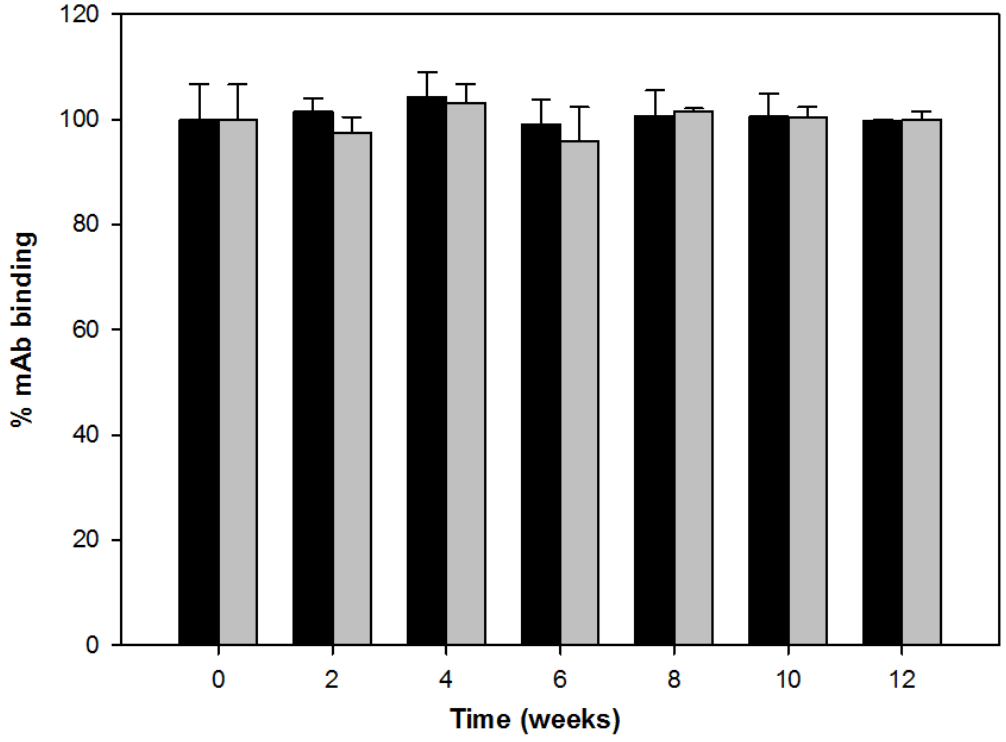
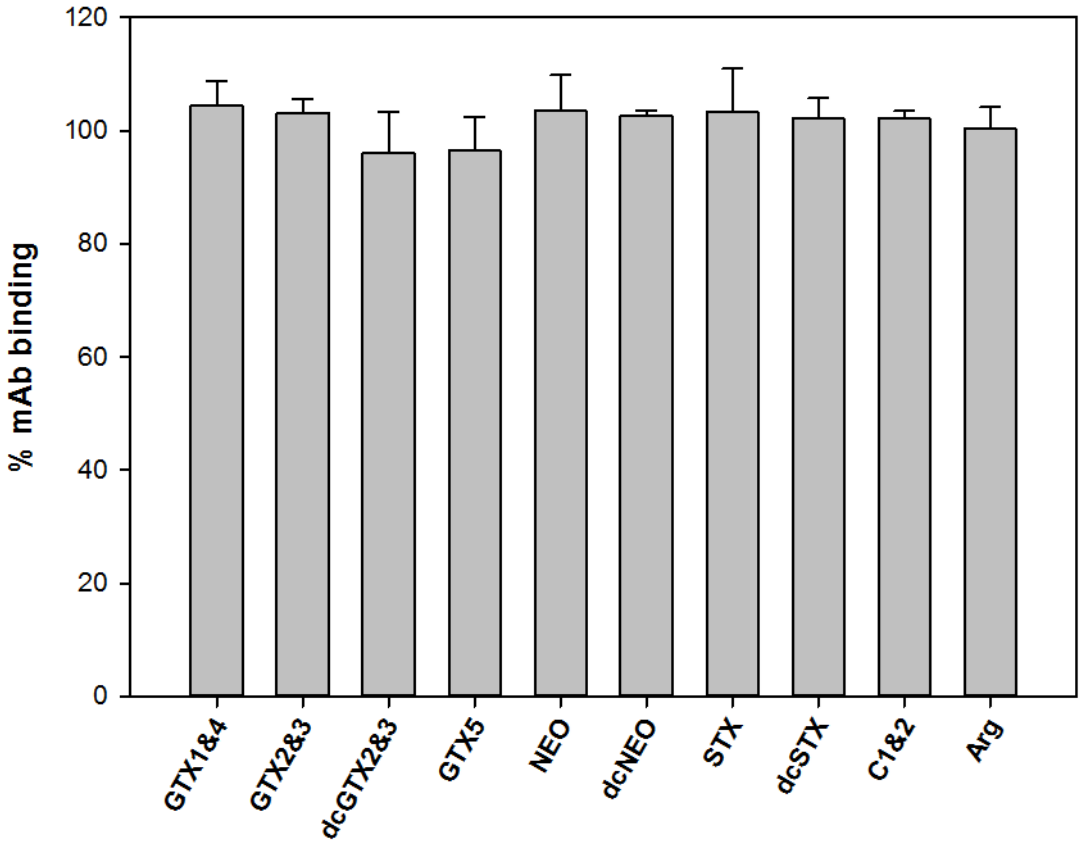
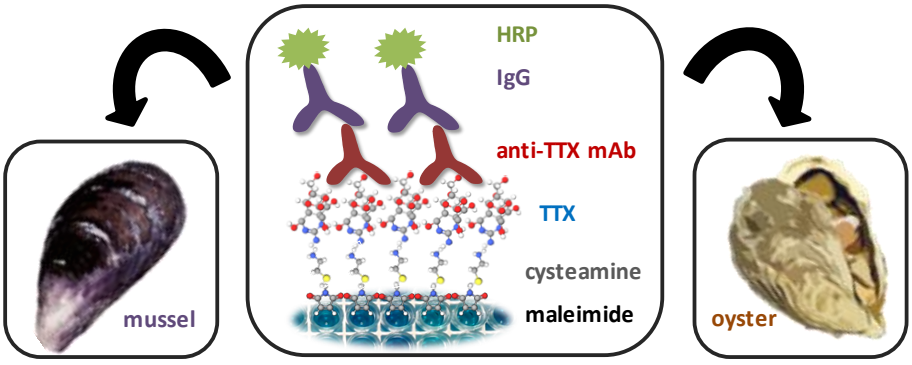


Figure 3.


% mAb binding
120
100
80
60
40
20
0
GTX1&4
GTX2&3
dcGTX2&3
GTX5
NEO
dcNEO
STX
dcSTX
C1&2
Arg

TOC


HRP
IgG
anti-TTX mAb
TTX
cysteamine
maleimide
mussel
oyster