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RESEARCH PAPER

Development and single laboratory validation of an optical biosensor assay for tetrodotoxin detection as a tool to combat emerging risks in European seafood

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Abstract Tetrodotoxin (TTX) is a potent neurotoxin emerging in European waters due to increasing ocean temperatures. Its detection in seafood is currently performed as a consequence of using the Association of Analytical Communities (AOAC) mouse bioassay (MBA) for paralytic shellfish poisoning (PSP) toxins, but TTX is not monitored routinely in Europe. Due to ethical and performance-related issues associated with this bioassay, the European Commission has recently published directives extending procedures that may be used for official PSP control. An AOAC-accredited high-performance liquid chromatography (HPLC) method has now been accepted by the European Union as a first action screening method for PSP toxins to replace the MBA. However, this AOAC HPLC method is not capable of detecting TTX, so this potent toxin would be undetected; thereby, a separate method of analysis is required. Surface plasmon resonance (SPR) optical biosensor technology has been proven as a potential alternative screening method to detect PSP toxins in seafood. The addition of a similar SPR inhibition assay for TTX would complement the PSP assay in removing the MBA. The present report describes the development and single laboratory validation in accordance with AOAC and IUPAC guidelines of an SPR method to be used as a rapid screening tool to detect TTX in the sea snail *Charonia lampas lampas*, a species which has been implicated in 2008 in the first case of human TTX poisoning in Europe. As no current regulatory limits are set for TTX in Europe, single laboratory validation was undertaken using those for PSP toxins at 800 μg/kg. The decision limit (CCα) was 100 μg/kg, with the detection capability (CCβ) found to be ≤200 μg/kg. Repeatability and reproducibility were assessed at 200, 400, and 800 μg/kg and showed relative standard deviations of 8.3, 3.8, and 5.4 % and 7.8, 8.3, and 3.7 % for both parameters at each level, respectively. At these three respective levels, the recovery of the assay was 112, 98, and 99 %.

Keywords Tetrodotoxin · Optical biosensor · Gastropods · Validation · Puffer fish

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Introduction

Tetrodotoxin (TTX), described recently as having molecular mystique, is a low-molecular-weight neurotoxin (319 Da) [1], with numerous known analogues of varying toxicities (Fig. 1), found in various marine organisms [2]. For the last century, scientists have investigated the producer, chemical properties, and molecular interactions of this naturally occurring toxic compound [3–8]. It is not commonly produced

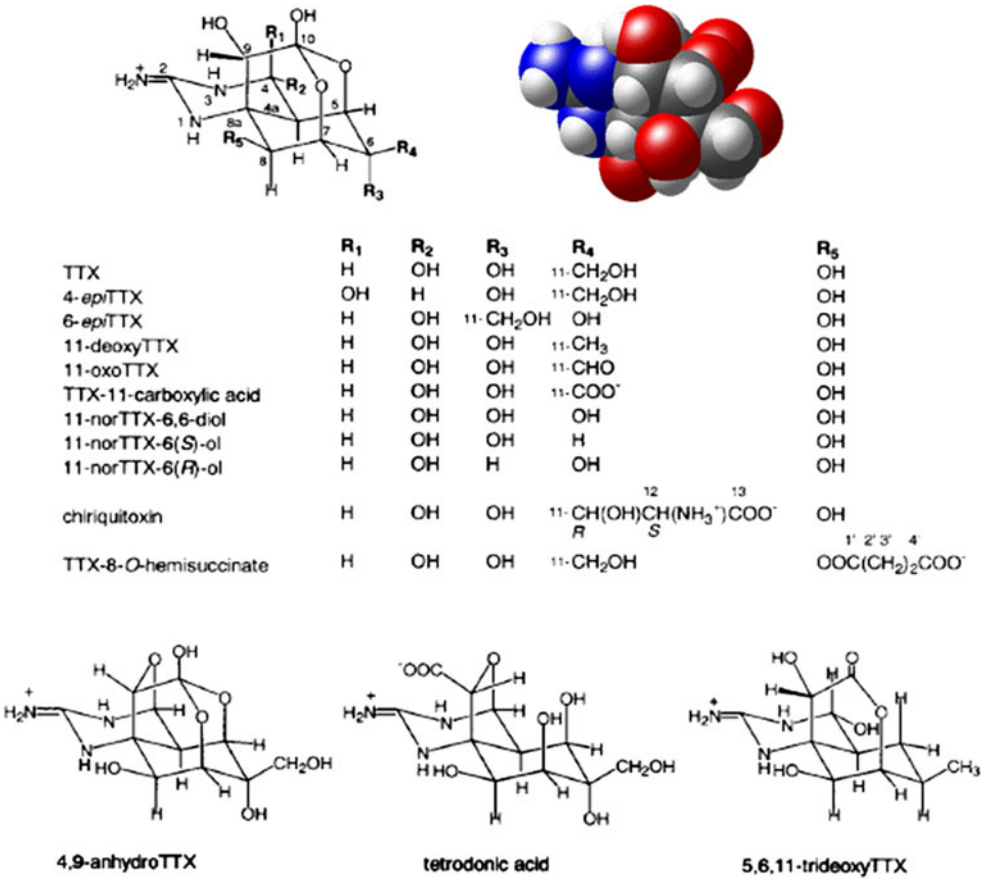


Fig. 1 Structure of TTX and analogues

by algae but believed to be mainly produced by bacteria from species in the following genera: *Actinomyces*, *Aeromonas*, *Alteromonas*, *Bacillus*, *Pseudomonas*, *Vibrio*, and *Shewanella algae* and *Shewanella putrefaciens* [1, 9, 10]. The proposed mechanisms of TTX accumulation state that either TTX produced by certain marine algae and bacteria is excreted into the sediment whereby detritivores and scavengers such as zooplankton, worms, gastropods, shrimp, or starfish feed and accumulate the toxin or through parasitism or symbiosis these bacteria enter species such as puffer fish or gastropods whereby they continue to produce the toxin [9].

TTX, like saxitoxin, a potent paralytic shellfish poison, has the ability to bind to site 1 of voltage-gated sodium channels [3, 5]. These toxins interfere with the passive influx of sodium ions and can result in numbness, tingling, respiratory paralysis, and even death of the affected individual. TTX has an LD_{50} in mammals of 2–10 μg/kg intravenously, 10–14 μg/kg subcutaneously, and an oral median lethal dose for mice of 334 μg/kg [11]. Seafood such as "fugu" or gastropods then entering the food chain with toxic levels can pose a severe threat to human health. The potential occurrence of TTX in seafood has important consequences relative to the food security, hygiene, and safety aspects for the economic protection of fisheries.

TTX poisoning episodes are more prevalent from seafood captured from the warmer waters of the Pacific and Indian oceans, the consumption of "fugu" in Japan as a delicacy and in the USA derived from misidentified or illegal importation of puffer fish as monkfish [12]. This is currently difficult to detect other than through the use of speciation tests. The Japanese government has set regulatory limits for TTX in food of 2 mg/kg TTX equivalents [13], while the USA has zero tolerance, with the exception of one legally imported species [14]. However, the distribution of TTX has, in recent years, been reported in European waters with the first confirmed toxic episode in the Mediterranean in 2008 following the ingestion of gastropods [15, 16]. Lessepsian migrant puffer fish were found travelling from the Suez Canal to warm Greek waters with high levels of TTX in the skin, muscle, and liver [17–19], and other consumable seafood species were determined to contain TTX off the coast of Portugal [20]. This may suggest that, as this toxin is normally found in warmer waters, European seafood is at risk of contamination of this toxin due to increasing water temperatures. Currently, there are no European Union (EU) regulatory limits specifically set for TTX, but only for muscle-paralyzing toxins such as the paralytic shellfish poisoning (PSP) toxins. The legal limit is stated as 800 μg saxitoxin equivalents/kg

shellfish meat. To date, as no regulatory limits are set specifically for TTX and thereby monitoring is not obligatory, this legislation does not provide sufficient protection to human health. Regulation (EC) no. 854/2004 [21] stipulates that: "Fishery products derived from poisonous fish of the following families must not be placed on the market: Tetraodontidae, Molidae, Diodontidae and Canthigasteridae. Fishery products containing biotoxins such as ciguatoxin or muscle paralysing toxins must not be placed on the market. However fishery products such as gastropods complying with Regulation 853/2004 may be placed on the market." This regulation refers that gastropods should, therefore, be analyzed as for PSP toxins with the reference method being the Association of Analytical Communities (AOAC) mouse bioassay (MBA) [22]. This assay is notorious as it determines sample toxicity based on the correlation between the time of death of the mouse following administration of the sample. It is used worldwide as the standard detection technique for paralytic poisons [23]. The inhumane characteristics and poor performance of the MBA has promoted the development of analytical techniques such as AOAC 2005.06 high-performance liquid chromatography with fluorescence detection (HPLC-FLD) as a first action screening test in Europe for the detection of PSP toxins [24]. This, however, leaves a gap in the detection of TTX as this method does not detect TTX or its analogues. Therefore, to cover the EU legislation 853/2004 for the provision of gastropods fit for human consumption, this single screening method is not adequate

Alternative methods to the MBA for testing TTX in seafood have been extensively reported, such as the receptor binding assay [25], various HPLC-FLD [26], and mass spectrometric detection (LC/MS) [14, 19, 20, 27], though drawbacks are identified in the need for titration reagents, instrumentation, trained personnel, and organic solvents for what are described as laborious methods. Immunological methods for TTX detection in puffer fish such as enzyme-linked immunosorbent assay (ELISA) have been offered for the rapid sensitive analysis of this toxin [28–31]. However, automated surface plasmon resonance (SPR) biosensors offering less manual labor-intensive methods for the detection of this compound have been utilized for TTX detection in milk, apple juice, and puffer fish [14, 32, 33]. To date, the ELISA and SPR immunological methods that have been applied are only to puffer fish as seafood and require lengthy repetitive sample preparation steps. SPR sensors allow for real-time and label-free analysis without the use of deleterious solvents, dangerous radiolabels, or animal bioassays and in addition using minimal toxin standards. In addition to these advantages, SPR is also versatile and has been used for many analytes including bacteria, toxins, vitamins, drug residues, and allergens. The recently developed and validated optical SPR methods for domoic acid [34] and okadaic acid [35] and additionally single laboratory [36] and interlaboratory piloted PSP analyses [37] demonstrated the versatility and robustness of this technology for marine biotoxin analysis, but only in shellfish.

Current opinion thus appears to indicate that the development of a convenient, quick, and cost-effective method for detecting all paralytic toxins of regulatory relevance remains a critical need for the monitoring of these compounds. The aim of this research was the development and single laboratory validation of a rapid, simple, and one-step extraction SPR assay for the detection of TTX in gastropods that could be employed as a validated method in the routine monitoring program for marine biotoxin screening of seafood.

Materials and methods

Instrumentation

An optical biosensor (Biacore™ Q) with Control Software (Version 3.0.1) and BIAevaluation software version 4.1 and CM5 sensor chips were obtained from GE Healthcare Bio-Sciences (Uppsala, Sweden).

Materials

TTX standard material was obtained from Biomol/Affiniti Research Products, Exeter, UK and Latoxan, Valence, France. HBS-EP buffer (pH 7.4; 0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.005 % Surfactant P20) and an amine coupling kit (containing 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 0.1 M *N*-hydroxysuccinimide (NHS), and 1 M ethanolamine; pH 8.5) were provided by GE Healthcare, Little Chalfont, UK. Boric acid, ethylenediamine, formaldehyde, and jeffamine were obtained from Sigma-Aldrich (Dorset, UK). Sodium acetate anhydrous, glacial acetic acid, 1 M hydrochloric acid (HCl), and sodium dodecyl sulfate (SDS) were all supplied by Fisher Scientific UK Ltd. (Leicestershire, UK).

Sample collection

Charonia lampas lampas (gastropod) samples (n=27) were provided by Centro Interdisciplinar de Investigacao Marinha e Ambiental (CIIMAR), Porto, Portugal. The samples were collected by fishermen near Angeiras, in the Northern Portuguese coast. The whole animal was removed from the shell and the muscle and viscera homogenized using a Waring® benchtop laboratory blender until the sample was homogeneous. The homogenate was stored at −20 °C in a temperature-monitored freezer until required. Puffer fish samples (Lessepsian migrant puffer fish *Lagocephalus sceleratus*) were obtained from the National Reference Laboratory for Marine Biotoxins in Greece.

Assay development

Immobilization of TTX on sensor chip surfaces

The biosensor chip surface was prepared in a similar manner to the saxitoxin chip for the SPR PSP assay, with some modifications [38]. In brief, the carboxymethylated surface of a CM5 certified grade biosensor chip (GE Healthcare) was equilibrated to room temperature and activated using an amine coupling kit. Briefly, EDC (0.4 M) and NHS (0.1 M) from the kit were mixed (1:1, *v*/*v*) and applied to the chip surface for 30 min to activate the surface. Excess solution was removed and ethylenediamine (0.1 M) in borate buffer pH 8.5 was added to the chip surface and allowed to react for 1 h. Excess activated groups on the chip surface were deactivated by exposure to ethanolamine (1 M) for 30 min, and the chip washed with water. TTX in 1 M sodium acetate buffer was then immobilized onto the amine surface via amino–amino coupling. The sensor chip surface was then washed with deionized water, dried using a stream of nitrogen gas, and stored desiccated at 4 °C when not in use.

Antibody production

A monoclonal antibody (MAb) designated TX-7F raised to TTX–bovine serum albumin protein conjugate was used with the production and characterization of this MAb by ELISA described in detail elsewhere [28]. The antibody was produced by a cell line and concentrated in cell culture media using Viva spin centrifuge tubes (30,000 MWCO) followed by purification via affinity chromatography using a protein G-sepharose gel column (MAbTrap Kit) followed by dialysis, over 24 h in saline solution with three solution changes, performed to remove any chemicals which may interfere with antibody coupling. The concentration of MAb was monitored by absorbance at 280 nm using a spectrophotometer.

Preparation of the assay calibration curve

The sensitivity of the MAb to TTX was assessed using the Biacore Q biosensor. A stock solution of TTX (1 mg/ml) in 10 mM acetic acid was prepared and used to prepare TTX calibrants ranging in concentration from 0, 1, 2.5, 5.0, 10, and 20 ng/ml in pH 7.4 HBS-EP buffer. These working standards were equivalent to 0, 120, 300, 600, 1,200, and 2,400 μg TTX/kg seafood. These working standards were used to produce calibration curves based on dose–response on the biosensor. A calibration curve is determined for each set of analysis, and the concentration of TTX in the samples is interpolated from this curve.

Extraction method

An extraction procedure proposed by Bates and coworkers [39] and employed for the SPR analysis of PSP toxins in shellfish [36] was employed. Briefly, 1 g of gastropod or puffer fish homogenate was weighed into 20 ml disposable Sterilin centrifuge tubes and 5 ml of sodium acetate buffer pH 5.0 added. Each tube was vigorously vortex mixed for 10 s followed by roller mixing for 30 min. After mixing, samples were centrifuged at 3,600×*g* for 10 min at room temperature. The supernatant was decanted, collected, and diluted 1:20 in HBS-EP buffer pH 7.4 (50 μl extract to 950 μl buffer).

SPR analysis: instrumental parameters

The assay protocol was based on that developed previously for the SPR analysis of PSP toxins in shellfish [36]. Briefly, the TTX antibody was diluted 1:300 with HBS-EP buffer. The working antibody solution was mixed with equal amounts of either the calibrant solution or sample and was injected over the chip surface at a flow rate of 12 μl/min for 2 min. A reading of instrument response units (RU) was taken 10 s before and 30 s after each injection to determine the difference in relative RU for each calibrant or sample. Calibrants and samples were analyzed in duplicate. Report points were recorded before (5 s) and after (30 s) each injection and the relative RU determined. The chip surface was regenerated with a 1:1 volume mixture of hydrochloric acid (10 mM) and 1 % SDS solution of pH 1.75. A typical analytical cycle was completed in approximately 6 min and each standard was analyzed in duplicate.

Validation

The method was validated considering the guidelines required by IUPAC [40], AOAC [41], and the Commission Decision 2002/657/EC [42] concerning the performance of analytical methods.

Sensitivity and selectivity

The sensitivity (IC_{50}) and dynamic range (IC_{20}–IC_{80}) of the assay and calibration curves in buffer only, gastropod, and puffer fish using known negative samples spiked and then extracted were analyzed as described to compare matrix effects in the procedure to establish the applicability of the method.

Selectivity is the ability of the method to distinguish between the target analyte (TTX) and the presence of other potentially interfering analytes [14]. This was assessed by analyzing working standards of a range of shellfish toxins. The toxins assessed included those currently covered by EU legislation and which could co-extract with TTX. Solutions

of yessotoxin, pectenotoxin-2, okadaic acid, azaspiracid-1, azaspiracid-2, and azaspiracid-3, dinophysistoxin-1 and dinophysistoxin-2, domoic acid, neosaxitoxin-c, decarbamoyl neosaxitoxin-b, gonyautoxin 1,4-c, gonyautoxin 5-b, gonyautoxin 2,3-c, decarbamoyl gonyautoxin 2,3-b, saxitoxin diacetate, decarbamoyl saxitoxin, and C1,2 were prepared in HBS-EP buffer at 200 ng/ml (10 times the highest concentration of the calibration curve). The responses obtained from the toxins were compared with that of the TTX calibration curve. The percent cross-reactivity of the TTX antibody with each potential cross-reactant was calculated using the IC_{50} values.

Recovery

To measure the recovery of the TTX under assay conditions from known negative homogenized gastropod samples using this extraction procedure, three aliquots of 1 g for seven samples were fortified with TTX at 200, 400, and 800 μg/kg and analyzed on three different days.

Analysis of known uncontaminated gastropods for interference effects (decision limit (CCα))

This is the limit at and above which it can be concluded with an error probability of α that a sample contains TTX [42]. The determination of CCα, while not compulsory as part of the 2002/657/EC guidelines, is essential in order to calculate the β error for the detection capability. The CCα was obtained by the duplicate analysis of 21 individual blank gastropod samples over 3 days. The decision limit was calculated as the mean of the calculated concentration+(2.33×the standard deviation [SD]). This provides a 99 % confidence level that a sample producing a result above this decision limit does contain TTX.

Detection capability (CCβ)

The detection capability (CCβ) is the smallest content of the substance that may be detected, identified, and/or quantified in a sample with an error probability of β [42]. As no regulatory limit is currently set within EC legislation, a permitted level of 800 μg/kg was assumed for the purposes of assessing the performance of the method. This value corresponds to the current level in PSP toxins. Noncontaminated gastropod samples (n=21) were analyzed over 3 days, seven samples per day. Each sample was fortified with TTX at 200, 400, and 800 μg/kg and analyzed. This level was chosen for fortification as it is above the calculated decision limit and one quarter of the permitted level for PSPs which have a similar mechanism of action of selective blocking of sodium channels. The same 21 gastropod samples were analyzed without fortification, in parallel.

Precision—repeatability and within-laboratory reproducibility

Repeatability of the method was assessed by the analysis of 10 replicates (n=10) of a single gastropod sample. Each replicate was extracted and analyzed in duplicate during a single biosensor run. The repeatability study was carried out at three levels of toxin fortification, 200, 400, and 800 μg/kg.

Reproducibility of the method was assessed by the analysis of a gastropod sample on three separate days (n=3 days). On each of the 3 days, an aliquot of the same gastropod homogenate was extracted and analyzed in duplicate at three levels of toxin fortification, 200, 400, and 800 μg/kg. For both repeatability and reproducibility, the mean concentration, SD, and the relative standard deviation (RSD) were calculated using the response value of each analysis.

Stability

Stability of TTX in HBS-EP solution, antibody in HBS-EP solution (working assay dilution), and gastropod extract were assessed.

TTX in HBS-EP solution

Working calibrant solutions of TTX in HBS-EP solution were prepared and analyzed using a freshly prepared working antibody solution. Aliquots of each calibrant solution were then stored at 4–8 and −20 °C and analyzed on day 3 and weeks 1, 2, 4, and 9 using freshly prepared antibody solution each day. The stored calibrants were compared against calibrants which had been freshly prepared on each day of analysis.

Antibody in HBS-EP solution

Working antibody solution in HBS-EP was prepared and analyzed using freshly prepared TTX calibration solutions. Aliquots of the antibody solution were then stored at 4–8 and −20 °C and analyzed on day 3 and weeks 1, 4, and 9 using freshly prepared TTX calibrant solutions each day. The stored working antibody solutions were compared against a working antibody solution which had been freshly prepared on each day of analysis.

Gastropod extract

Gastropod samples were fortified at 0, 200, 400, and 800 μg/kg with TTX and extracted. Aliquots of the gastropod extract were stored at 4–8 and −20 °C and analyzed on days 0 and 3 and weeks 1, 2, 4, and 9 using freshly prepared TTX calibrants and working antibody solution on each day. The stored gastropod extracts were compared against the same gastropod sample freshly extracted on each day of analysis.

Ruggedness trial

The ruggedness of the method was tested by applying the Youden ruggedness trial [41]. Briefly, the use of the Youden method allows seven factors to be investigated, which could influence the outcome of a result, in a single run with eight separate analyses. For this study, it was decided to investigate the possible factors which could influence the extraction procedure. Table 1 shows each of the seven factors investigated. A high value and a low value for each factor were assigned, with the high value given a capital letter and the low value given a small letter.

The assigned high and low values had been determined as values with a reasonable possibility of occurring because of human error. The high and low values for each factor were combined in specific combinations, as stipulated in the Youden approach during eight experiments. The value of each experiment relates to each factor investigated and was used to calculate the parameter difference of each factor [41]. If the SD of the parameter differences of the ruggedness trial is significantly larger than the SD of the within-laboratory reproducibility, then all factors together have an effect on the result even if every single factor does not show a significant effect and that the method is not sufficiently robust against the chosen modified factors [14]. The ruggedness investigation was carried out using a single gastropod sample which had been fortified at 200 μg/kg.

Analysis of samples

Gastropod samples (n=27) were analyzed in duplicate by the SPR method and confirmed using a previously developed mass spectrometry method (27). As no true naturally contaminated gastropod samples were available, the method was further evaluated using naturally contaminated puffer fish samples from Greece. Different tissues were assessed. The puffer fish samples were analyzed in duplicate by SPR and compared with MBA data previously determined using the AOAC method (18).

Results and discussion

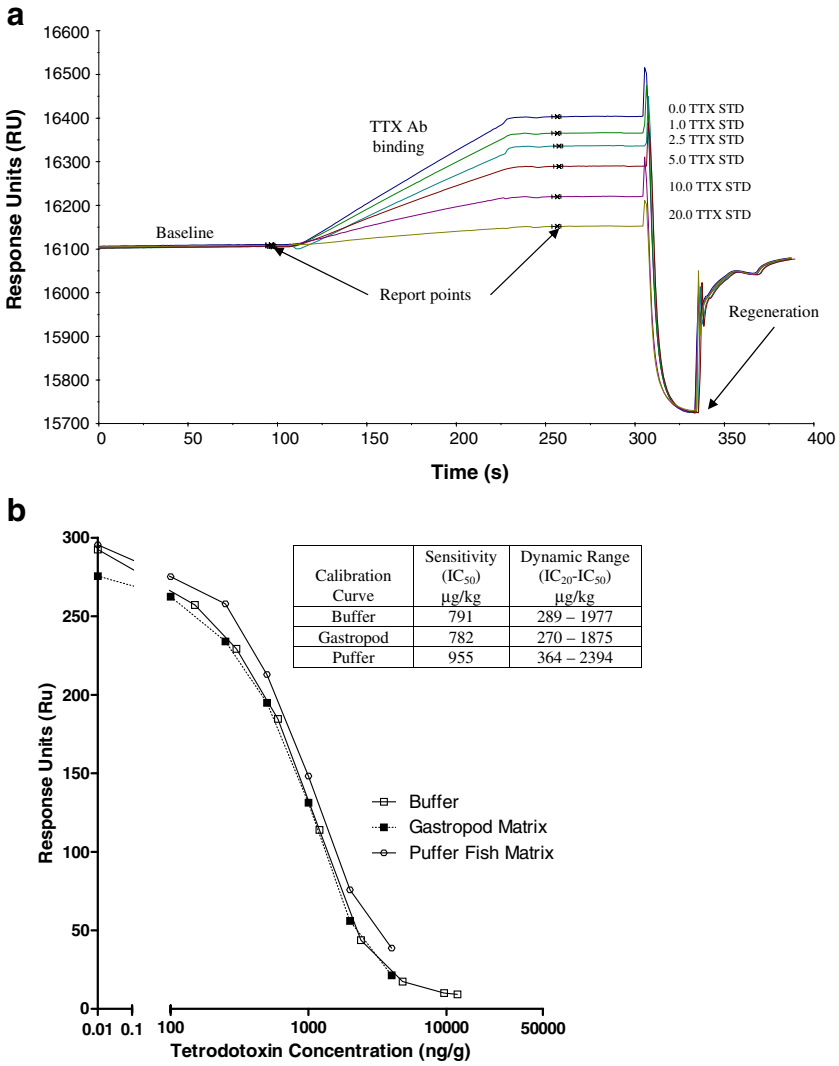
The antibody was purified, and the concentration was determined to be 1.1 mg/ml in 0.15 M saline from the absorbance measured at 280 nm. It should be noted that varying the biosensor parameters, such as flow rate, antibody to sample ratio, and regeneration solution, used to construct the assays can enhance the sensitivity. In deciding which parameters would be the best fit for the assay setup, some general "rules of thumb" are taken into consideration, e.g., dynamic range of curve ~300 RU, sensitivity (based on midpoint) must be as low as possible, and the amount of critical reagents (TTX Ab and calibrants) used in the assay must be kept to a minimum. It was the basis of these factors that the assay conditions were selected considering the assay for PSP toxins [36]. To further save on the volumes of precious TTX used in the assay, the final calibrant range was selected as 0, 1.0, 2.5, 5, 10, and 20 ng/ml. For reusable biosensing methods, an essential requirement is regeneration of the sensor chip surface for removal of all noncovalently bound material while maintaining the structural integrity of the attached ligand. For this MAb, following a process of regeneration scouting, a 1:1 volume mixture of 100 mM HCl and 1 % SDS at pH 1.75 achieved total removal of the antibody without causing deterioration of the immobilized TTX. The chip can be regenerated 200 times per flow cell.

Sensitivity and selectivity

Figure 2a illustrates an overlay of the typical sensorgrams obtained for a TTX calibration curve. The IC_{50} of the buffer curve was 6.6 ng/ml with a dynamic range of 2.4–16.5 ng/ml which, although displaying marginally better sensitivity, correlates with the ELISA data previously obtained [28]. Figure 2b illustrates a response versus TTX concentration calibration curve obtained in HBS-EP buffer and different seafood matrices. Gastropod matrix had no effect on the assay, whereas the puffer fish calibration curve was shifted slightly to the right whereby these samples analyzed to a

Table 1 Effects of various factors on ruggedness of the assay

Factor (assay parameter)	Height value	Low value	Parameter difference (μg/kg)
pH (5.0)	A=5.2	a=4.8	−38.36
Roller mix time (30 min)	B=40	b=20	−24.24
Centrifuge time (10 min)	C=12	c=8	−8.8
Weight of test portion (1 g)	D=1.1	d=0.9	−17.49
Centrifuge speed (3,600×g)	E=4,000	e=3,200	6.82
Volume of extraction buffer (5 ml)	F=5.2	f=4.8	−13.74
Vortex time (10 s)	G=12	g=8	−2.19
	Ruggedness SD		14.8
	Within-laboratory reproducibility SD		15.3



Calibration Curve	Sensitivity (IC_{50}) µg/kg	Dynamic Range (IC_{20}-IC_{50}) µg/kg
Buffer	791	289 – 1977
Gastropod	782	270 – 1875
Puffer	955	364 – 2394

Fig. 2 **a** Real-time response curves for TTX calibration standards. **b** Typical TTX toxin concentration versus response calibration curves illustrating the comparison of the matrix effects for different seafood species

buffer curve would show a slight decrease in toxicity. Nevertheless, as minimal matrix effects were observed between buffer, gastropods, and puffer fish standards, a buffer calibration curve was established for this screening assay which avoids the need to find confirmed negative matrix matched materials for assay maintenance. The respective IC_{50} values determined were 791, 782, and 955 µg/kg. The matrix effects between species were most likely due to the dispersive nature of the gastropod extract in the extraction solvent compared to the nondispersive nature of the meatier textured fish.

For specificity, no other TTX analogues could be evaluated due to their unavailability. Data obtained from biosensor analysis with standards prepared at 200 ng/ml showed that, at this concentration, an IC_{50} could not be calculated from any of the NRC reference marine toxin standards as listed in the methods, so calculating a value for cross-reactivity could not be performed. Taking into account the dilution factor associated with the extraction procedure, it can be reported that the percentage cross-reactivity of the other toxins with the TTX antibody is ≤3.3 % at levels of 24 mg/kg. This demonstrates that the assay is specific for TTX and not for other marine biotoxins that could occur in certain seafoods. Even though there is similarity in structure to PSP toxins, the antibody did not display any cross-reactivity to these toxins. The most likely site of conjugation through the guanidine group of TTX, which is the structural component of the molecule similar to STX, eliminates this site as the antigen-presenting site; thereby, the antigen raised will be specific for other structural components on TTX and not STX.

Recovery

The seven samples fortified at the three levels of toxin fortification, 200, 400, and 800 µg/kg, and analyzed over 3 days measured on average at 224±45, 393±47, and 793±90 µg/kg, displaying recoveries of 112±22, 98±12, and 99±11 %, respectively.

Decision limit (CCα) results

The effect on the assay of possible interference from noncontaminated gastropod samples was determined by the analysis of 21 TTX-free samples analyzed over 3 days. Using the calculation stated previously, the decision limit was calculated to be 100 μg/kg. This is the noise level of the assay and it can be concluded with a 99 % confidence level that any sample which gives a response above 100 μg/kg contains TTX. The CCα value represents possible effects of

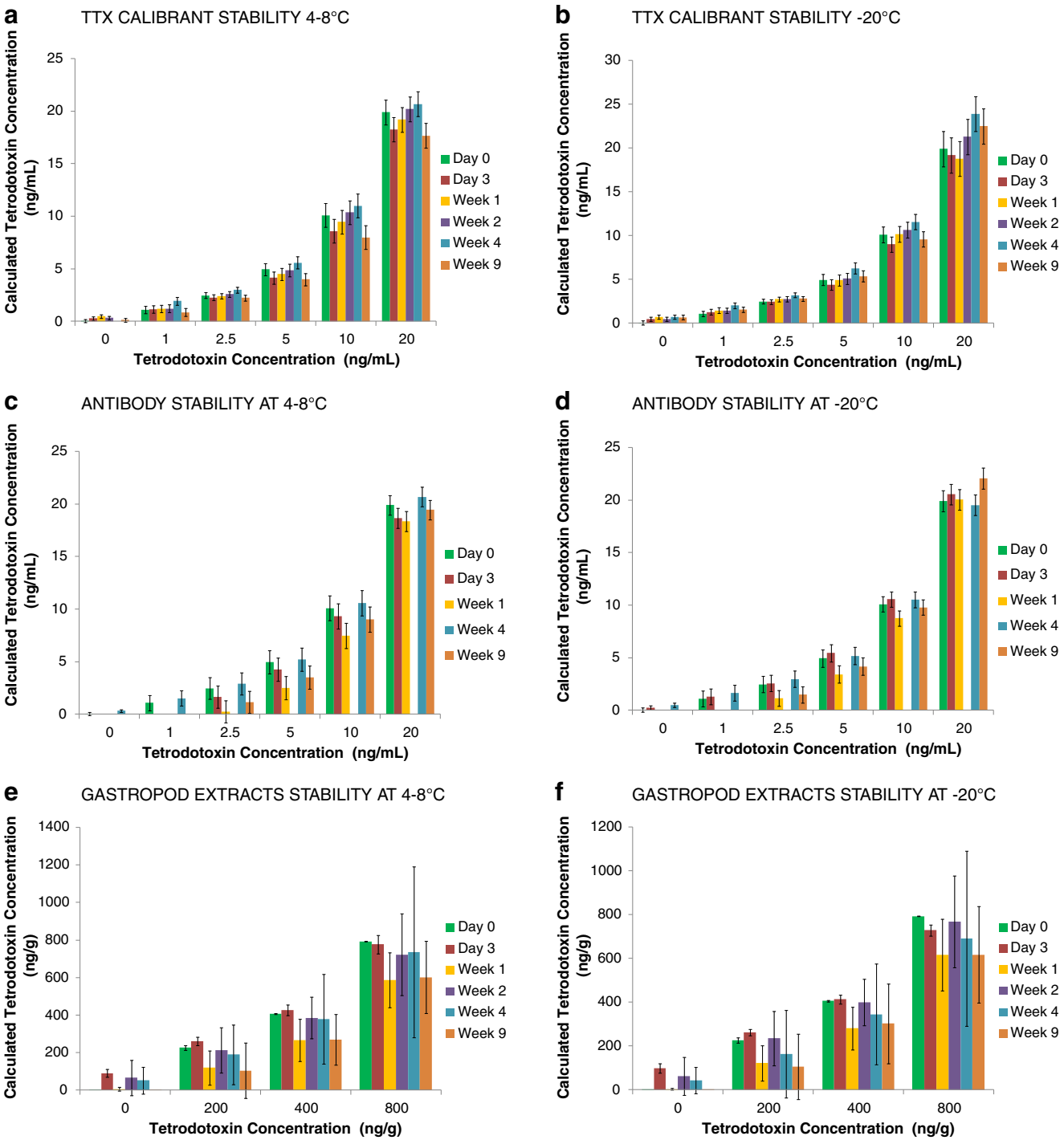


Fig. 3 Bar charts illustrating antibody stability, TTX extract stability, and calibrant stability at 4–8 and −20 °C over the TTX concentration range. **a** TTX calibrant stability at 4–8 °C, **b** TTX calibrant stability at −20 °C, **c** antibody stability at 4–8 °C, **d** antibody stability at −20 °C, **e** gastropod extracts stability at 4–8 °C, **f** gastropod extracts stability at −20 °C

interfering compounds found in the noncontaminated gastropod samples. The method demonstrates that a sample will contain TTX if the result is above 100 μg/kg at a confidence level of 99 %.

Detection capability (CCβ) data

The detection capability (CCβ) was determined by the analysis of 21 gastropod samples fortified with TTX at 200 μg/kg over 3 days, 7 samples per day. The same 21 gastropod samples were analyzed without fortification, in parallel. The 21 unfortified samples produced responses ranging from 0.01 to 100 μg/kg. The 21 gastropod samples which had been fortified at a level of 200 μg/kg produced responses ranging from 131 to 290 μg/kg. It can be seen from these results that none of the responses from the unfortified samples overlap with any of the responses from the fortified samples. Therefore, it can be concluded that the detection capability (CCβ) of this TTX screening method is ≤200 μg/kg. The lowest response from the fortified samples is 131 μg/kg and can be set as the cutoff level for the method. Therefore, any sample giving a response greater than 131 μg/kg is deemed to be a positive screen and exceeds the CCβ of the screening method. As the only country to have a regulatory limit for TTX, Japan employs regulatory limits of 2 μg/g (2,000 μg/kg) [13]. The single laboratory validation of this method shows that it can perform to a level 10 times more sensitive than the regulatory limit set in Japan.

Precision—repeatability and within-laboratory reproducibility

The validation study showed acceptable results for repeatability and within-laboratory reproducibility of the assay. The repeatability variations (%RSD) of a gastropod sample fortified at levels of 200, 400, and 800 μg/kg TTX were found to be 8.3, 3.8, and 5.4 %, respectively. The within-laboratory reproducibility variations (%RSD) of a gastropod sample fortified at levels of 200, 400, and 800 μg/kg TTX were found to be 7.8, 8.3, and 3.7 %, respectively. The %RSD values for both repeatability and reproducibility fall within the acceptable limits laid out in the AOAC single laboratory validation document [41] at each concentration of fortification.

Stability results

For the duration of the 9-week trial, the TTX calibrants in HBS-EP buffer showed no major deterioration when compared against freshly prepared calibrants at both storage temperatures (Fig. 3a, b). This is an important feature of the assay because of the high cost of TTX for the preparation of standards. The antibody in HBS-EP solution showed no significant deterioration between day 0 and week 4 at both storage temperatures (Fig. 3c, d). In general, it could be concluded that the antibody is stable over the 4-week period. The stability of the gastropod extracts at all levels of fortification is questionable after 3 days at both storage temperatures (Fig. 3e, f). After 3 days, there is a significant fluctuation in the average amount of TTX detected in the samples at both storage temperatures, indicating the instability of the sample. The fluctuation can be caused by matrix components decaying in the sample that bind nonspecifically to the antibody or to the chip surface, causing increases and decreases in toxin value. As, however, the general trend is that the average TTX levels drop from day 3 to week 4 both at 4 and −20 °C, it would appear that the TTX is decomposing in the gastropod extracts. The results have demonstrated that extraction of the sample and sensor analysis is best performed on the same day.

Ruggedness trial results

Table 1 shows the effects that the high and low values have on a gastropod sample fortified with TTX at 200 μg/kg. The factors with the larger parameter differences are the factors which are most influenced by deviating from the normal factor value. The SD of the parameter differences for the ruggedness trial is lower than that of the within-laboratory reproducibility which indicates that all factors together do not have an effect and that the method is sufficiently robust.

Analysis of samples

For the gastropod samples (n=27), all were deemed by SPR to be lower than the decision limit (CCα) of 100 μg/kg and confirmed by LC-MS to be negative for TTX [27]. By LC-MS the multiple reaction monitoring transitions for TTX were monitored as 319.92 to 302.00 and 319.92 to 161.80 [27]. Although, these samples were less than 100 μg/kg by SPR some exhibited levels greater than zero and by LC-MS exhibited the latter transition 319.92 to 161.8 only. These results may be indicative of matrix effects on the antibody or the presence of low levels of TTX analogues that the antibody-based method could detect. However, no standards were available for TTX analogues to investigate this

Table 2 Comparison of MBA and biosensor assay for TTX detection in puffer fish tissues as available naturally occurring samples

Sample	Biosensor (μg/g)	MBA [18] (μg/g)
Muscle	<0.01	<1.1
Skin	0.20±0.07	<1.1
Liver	8.36±0.04	10.84
GI tract	6.09±0.06	6.31
Gonads	1.34±0.02	1.49

conclusion in detail. Due to the lack of certified reference material and gastropod material naturally contaminated with TTX, puffer fish samples received from Greece were tested and compared with MBA data previously published (Table 2). The correlation between these limited samples was extremely good, demonstrating 100 % reliability, with higher levels detected in the liver and gastrointestinal tract.

Conclusions

The present study has demonstrated that a robust stable optical biosensor chip can be produced on immobilization of TTX, whereby an assay was developed and extensively validated for the screening of TTX in gastropods and puffer fish. As the chip is capable of running >200 analyses per flow cell amounting to 800 per chip, this ensures that the cost of analysis is competitive. The detection capability achieved was 10 times lower than the regulatory limit required for Japan, but thereby can be offered as an early warning monitoring tool of low-level TTX outbreaks. A one-step extraction procedure was applied to promote the rapidity of the test. This simple sample preparation allows 40 samples per hour to be extracted with a rapid detection of 6 min per analysis, providing the capability of a high-throughput rapid format. This SPR technology could be a first action screen in monitoring laboratories for this toxin. Advances in SPR technology in multichannel instruments would allow the amalgamation of both the PSP toxin assay [36] and TTX assay to be performed simultaneously on different samples using the same extraction procedure, thus reducing the MBA as a screen but not missing these potent paralytic toxins.

Safety

TTX is responsible for incidents of paralytic poisoning (PSP). Therefore, when using TTX standard solutions, special care should be taken. Gloves and eye protection should be worn at all times. Appropriate disposal methods should also be utilized.

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