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Research Article

Rapid quantitative detection of saxitoxin using peroxide oxidation derivatization coupled with fluorescence spectrophotometry

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Abstract
Often found in North America, Europe, and Asia, saxitoxin (STX) is a powerful natural marine neurotoxic that is categorized as a biological weapon. By preventing regular cellular activity, STX has been shown to cause paralytic shellfish poisoning in both humans and animals.. In the realm of STX analysis, the time required to detect and quantify STX in water is a crucial concern with implications for public health and regulatory compliance. Therefore, the peroxide oxidation derivatization coupled with fluorescence spectrophotometry method for the detection and analysis of STX was developed in this study. This study was divided into two parts, namely method optimization and method validation. For optimization part, the optimum instrumental parameters, excitation and emission spectral bandwidths were fixed at 5 nm and 20 nm, respectively. While the optimum excitation wavelengths of oxidized STX was determined as 334 nm. Moreover, the optimum parameters of peroxide oxidation process, concentration of NaOH and time of reaction were determined as 0.4 M and 180 s, respectively. For the validation part, the linear equation for the five-point STX calibration curve (40 – 200 µg/L) was y = 0.3321x + 5.0021 with a correlation coefficient, R^2 of 0.9979. Based on the linear regression, limit of detection (LOD=3.3 σ/S, with σ as the standard deviation of calibration curve and S as the slope of calibration curve) and limit of quantification (LOQ=10 σ/S) were found to be 11.92 and 36.11 µg/L, respectively, were at par with the guideline values of recreation water (30 µg/L). The precision and accuracy results were studied at three concentrations of STX, 50, 100, and 150 µg/L. For precision study, relative standard deviations were determined in the range of 0.35-1.10% for intra-day precision and 0.51-2.81% for inter-day precision. For accuracy study, relative recoveries were determined, ranging from 101-103%. This developed analytical methods for the STX detection offered these advantages of rapidity (approximately 4 min), sustainable, high precision, and high accuracy alternative to conventional methods.

Keywords: Saxitoxin, peroxide oxidation, fluorescence spectrophotometry, paralytic shellfish poisoning, analytical eco-scale

Introduction

Harmful algal blooms (HABs) are naturally occurring events that occur when algae or dinoflagellates multiply rapidly, usually as a result of favourable environmental conditions such as nutrient enrichment and warm temperatures. These blooms can cause certain species such as *Alexandrium minutum* and *Pyrodinium bahamense var. compressum* to produce saxitoxin (STX), the most dangerous toxin among the 57 recognized paralytic shellfish toxins (PSTs) [1, 2]. When marine creatures, specifically filter-feeding shellfish, consume these STX-producing species, they accumulate STX in their tissues. This bioaccumulation mechanism can lead to significant levels of STX in shellfish, presenting serious health concerns to those who consume contaminated seafood. For instance, within 30 min after the consumption of contaminated water supply or shellfish, symptoms of paralytic shellfish poisoning (PSP) in human like fainted, breathing difficulty, nausea, numbness, dizziness and headache might appear. For severe PSP cases, respiratory paralysis typically ensues within a timeframe of 2-12 h after ingestion, and even fatal [3]. In order to conduct effective risk assessments on STX, Food and Agricultural Organization of the United Nations [4] had documented oral consumption for both minimal nonlethal doses and lethal doses of STX in human, as

they were 120 to 4128 µg/person and 456-12400 µg/person, respectively. The guideline values (GVs) for seafood industry, drinking water, and recreational water also were established based on large database of human PSP cases. In the seafood industry, European Union Regulation No 853/2004 had implemented a PSP health alert level at 800 µg/kg to regulate and track seafood quality to limit human exposure to PSTs [5]. As for the GVs for drinking water (3 µg/L) and recreational water (30 µg/L), they are set by the World Health Organization [6]. In short, effective and rapid detection of STX in water and the seafood industry is essential to reducing its adverse impact on the environment and human health. This includes making sure that GVs are followed and evaluating in any associated health and environmental hazards.

PSP is a common marine biotoxin intoxication which had been reported in many countries [1]. Annually, there were approximately 2000 PSP outbreaks, associating with 15% of fatality rate were reported [7]. It mainly restricted to temperate climates, with most reported outbreaks in North America, Europe, and Asia countries. In the United States, 20 PSP cases were reported by the Oregon Heath Authority's Public Health Division in the year of 2024, in which 20 patients with PSP intoxication symptoms were hospitalized after consuming contaminated mussels from the Oregon's coast [8]. In Portugal, two victims with severe PSP symptoms were hospitalized after consuming mussels from Portuguese southwest coast during the fall of 2018 [9]. At the same year, a PSP case was also reported in Japan and one patient was hospitalized due to the consumption of contaminated geoduck [10]. In the Philippines, a total of 31 PSP outbreaks were reported in the year of 2013, including 23 people who suffered from mild PSP symptoms where 8 people were hospitalized, and two deaths were reported due to the congestion of contaminated green mussel broth in Western Samar [11]. The first PSP incident in Malaysia, with 202 poisonings and seven deaths, was reported along the Sabah's western coast in 1976 [12]. Since then, PSP cases have been regularly observed and reported in Sabah and Peninsular Malaysia, including Kuantan Port, Melaka, and Kelantan [1]. The most recent incident occurred in 2024, as reported by the Negeri Sembilan State Health Department where nine food poisoning cases encompassing PSP symptoms, were hospitalized after consuming contaminated green mussel [13].

Initially, mouse bioassay (MBA) standard method (AOAC 959.08) was internationally recognized for quantifying STX. However, this method was unfavourable and slowly replaced by other validated method due to the ethical issues [14]. Currently, more analysis methods for STX are being developed and they are receptor binding assay (RBA), high-performance liquid chromatography-fluorescence detection (HPLC/FLD), liquid chromatography-tandem mass spectrometry (LC-MS/MS), cell viability assay (CVA) and competitive enzyme-linked immunosorbent assays (ELISAs). RBA is remarkable for its simplicity and cost effectiveness; however, it requires radioactive labelling, depending on the cell of living organism, and longer analysis duration [15]. For instance, Van Dolah et al. [15] reported the validation work showed that, RBA is reasonably sensitive with an acceptable limit of detection (LOD) (8.75 µg/L) and relative recovery (RR) (121%). However, the incubation process before quantifying the STX was time consuming which was approximately took 1 h. In addition, the usage of radioactive tritiated STX also raised health issues. While LC-MS/MS possess high sensitivity (0.02 µg/L) and precision, it requires more time for flushing column, injecting sample and separating studied components based on their interacting ability with stationary phase. Large usage of organic solvents as mobile phase also appeared as a major problem as organic solvents are harmful to the environment [16]. CVA shows high sensitivity but it shares similar problems as RBA as it depends on cell of living organism and requires long analysis duration [17]. For example, Cañete & Diogène [17] reported a high sensitivity neuroblastoma-2a cell viability assay with low LOD (0.34 µg/L). Nonetheless, the cell incubation and toxin exposure of cells were time-consuming which was approximately 24 h. ELISAs methods are also endowed with high sensitivity but it depends on the availability of complex antibodies and require long analysis duration [18]. Cho et al. [18] developed a highly sensitive molecular imprinted polymer (MIP)-based indirect competitive ELISAs with low LOD (3.17 µg/L) and high RRs (98.6-116.9%). However, the preparation of MIP is complicated and time-consuming which was more than a day. Besides, the MIP immobilization and toxin exposure process were also laborious. Hence, there is a critical need for the development of rapid, simple and sensitive method for the STX detection.

HPLC/FLD is also known as the gold standard for STX detection due to its high robustness and fluorescence sensitivity [19, 20]. The high fluorescence sensitivity arises from its capability to detect analyte structured with fluorophore which can exhibit fluorescence when exposed to the light source. However, it is non-user friendly to non-fluorophore analyte for STX. Researchers had developed innovative derivatization and fluorescence labelling to modify the structure and tune it to be fluoresced. Fluorescence labelling method is deemed as accurate and sensitive, but it requires complex and longer time of sample preparation [21]. Zhu et al. [21] implemented the concept of fluorescence labelling and proposed a sensitive magnetic fluorescence

aptasensor for the detection of STX with low LOD (0.6 nM) and high RRs (82.0-102.6%). However, the construction of the probe is complicated, requiring exhaustive preparation steps, and time consuming which required approximately 41 h. For derivatization, which was implemented in HPLC/FLD, there are two methods that can be used, including peroxide and periodate oxidations. Peroxide oxidation is more favoured for its higher sensitivity, simplicity, and rapidness [19]. For instance, Lawrence et al. [22] compared the performance between two different oxidation methods of STX by using precolumn oxidation chromatographic method. As a result, peroxide oxidation of STX was more favourable by yielding stronger chromatographic peaks, meanwhile, STX is poorly detected when periodate oxidation is used [23]. Besides that, the AOAC 2006.05 standard method also showed that peroxide oxidation was relatively faster, which was 3 min, as compared to periodate oxidation, which was 11 mins, for completing STX derivatization [19].

The concept of green analytical chemistry (GAC) was introduced in 1999. Recently, GAC was widely implemented by analytical chemists in green analytical chemistry in order to alleviate and eliminate the hazardous substances, the electricity consumption, analysis duration, and the waste production through each analysis [24, 25]. However, some important analytical aspects, including complexity of sample matrices, diversity of solvent, analytical methods and other crucial analytical aspects complexified the evaluation of analytical approaches in terms of sustainability [26]. Hence, analytical chemists actively developed comprehensive ways to evaluate the greenness of analytical methods. Galuszka et al. [24] developed a novel comprehensive approach, Analytical Eco-Scale, to evaluate the greenness of the analytical methodology. This green chemistry assessment is widely used due to its simplicity and applicable for different analytical methods [26-28].

Since monitoring marine and freshwater ecosystems for these toxins is vital for assessing environmental and health risks, development of a rapid and straightforward analytical technique for STX would be highly beneficial. To address this, a simple, rapid, and sustainable screening method using fluorometric detection was introduced by implementing the concept of HPLC/FLD. The best to knowledge, this is the first report that developed peroxide oxidation derivatization coupled with fluorescence spectrophotometry for STX detection. The objectives of this research were to optimize and validate peroxide oxidation derivatization coupled with fluorescence spectrophotometry method, specifically for STX detection. Besides, comparative studies with available analytical methods were conducted by emphasizing the potential advantages and limitations of our analytical approaches. Additionally, green analytical chemistry assessment was evaluated for all the available methods via analytical Eco-scale. In short, this approach promises rapid results, high sustainability, high consistency, exceptional accuracy, and reasonable sensitivity as compared to current methods. Successful development of this method could significantly impact public health, food safety, and environmental monitoring in a positive way.

Materials and Methods

Chemicals

Certified reference material of STX dihydrochloride (61.4 µmol/L) was obtained from National Research Council of Canada (Canada) (CAS number: 3554-08-6) and stored at 4 °C before used. The peroxide oxidation derivatization reagents, acetic acid reagent plus >99% and sodium hydroxide (NaOH) pellets were obtained from Sigma-Aldrich (Malaysia), while 30% hydrogen peroxide (H_2O_2) solution was obtained from Merck (Germany). Stock solution (1000 µg/L) of STX was prepared using deionized water (DI, Barnstead, USA) and a series of standard solution (40, 50, 100, 150, and 200 µg/L) were prepared by diluting the stock solution with DI water.

Derivatization of STX: Peroxide oxidation

The STX derivatization method was adapted from AOAC 2005.06 standard method [19]. H_2O_2 solution (10%, 25 µL) and NaOH solution (1 M, 250 µL) were measured and added to an Eppendorf tube (1.5 mL). Then, the mixture was mixed well via vortexing (5 s) and spiked with the STX standard solution (100 µL). The mixture was vortexed again (5 s) and left for reacting at room temperature (25 °C) for 120 s. After that, glacial acetic acid (20 µL) was added into the mixture and vortexed (5 s) for terminating the oxidation reaction [19]. The process of STX peroxide oxidation was depicted in **Figure 1**. Since the oxidized STX was stable at maximum of 12 h, thus, the derivatized standard solutions were prepared freshly and right before the fluorescence spectrophotometry detection [19].

Setting up of fluorescence spectrophotometer

A Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, USA) equipped with an 80 Hz xenon flash lamp was used for the detection of STX. After derivatization, the oxidized STX (300 µL) was transferred into a quartz micro-cuvette (50-2000 µL) (CotsLab, UK). The instrumental parameters such as excitation wavelength, range of emission wavelength, excitation spectral bandwidths, emission spectral bandwidths, and scan rate were set up as 334 nm, 300-500 nm, 5 nm, 10 nm, 600 nm/min, respectively [29]. The software used was Cary WinFLR. The voltage detector sensitivity was set as medium (600 V).

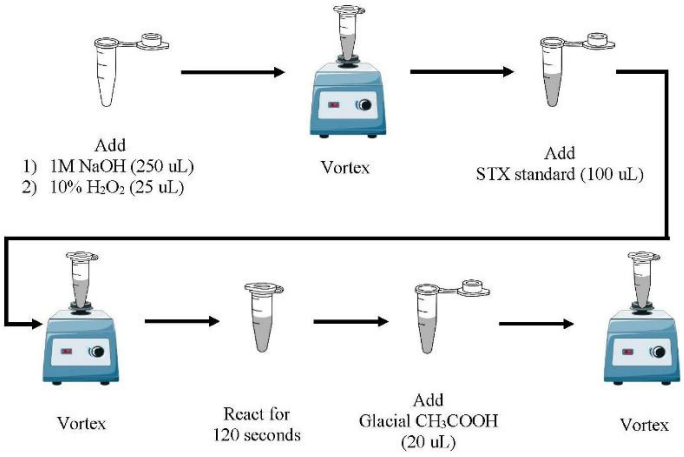

Figure 1. Procedure of STX peroxide oxidation

Method optimization

The method optimization stage was divided into two parts: the first part was performed to optimize the instrumental parameters while the second part was to optimize the parameters of peroxide oxidation process. For instrumental parameters, three parameters were optimized, they were emission spectral bandwidths (5, 10 and 20 nm), excitation spectral bandwidths (5, 10 and 20 nm) and excitation wavelength of oxidized STX (330, 332, 334, 336 and 338 nm). Besides, two parameters were optimized in the prosses of peroxide oxidation, namely time of reaction (60, 120, 180, 240 and 300 s) and the concentration of NaOH (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 M). The optimization part was performed with sample prepared from distilled water spiked with 200 µg/L of STX standard in duplicate. Means, standard deviations, and relative standard deviations of the signals were calculated and recorded.

Method validation

The peroxide oxidation derivatization coupled with fluorescence spectrophotometry analysis were validated with external calibration at STX concentrations of 40, 50, 100, 150 and 200 µg/L whereby the validated analytical parameters included linearity of the calibration graph, LOD, LOQ, precision (repeatability and intermediate precision studies) and accuracy of the method. Both precision and accuracy studies were performed using water samples spiked with 50, 100 and 150 µg/L STX. LOD (1) and LOQ (2) were calculated using the following formula:

$$LOD = 3.3\ (\frac{\sigma}{S}) \quad \text{(Eq. 1)}$$

$$LOQ = 10\ (\frac{\sigma}{S}) \quad \text{(Eq. 2)}$$

where σ as the standard deviation of calibration curve and S as the slope of calibration curve. Relative standard deviation (RSD) for precision study (3) and relative recovery (RR) for accuracy study (4) were calculated using the following formula:

$$RSD\ (\%) = \frac{\sigma}{Mean} \times 100\% \quad \text{(Eq. 3)}$$

$$RR\ (\%) = (\frac{Calculated\ concentration\ of\ analyte}{Initial\ concentration\ of\ analyte}) \times 100\% \quad \text{(Eq. 4)}$$

Green analytical chemistry metrics

In this study, a simple chemistry metric system, analytical Eco-scale, proposed by Galuszka et al. [24] was utilized to quantitatively evaluate the greenness of analytical methods. In this assessment, the greenness evaluation of the analytical method was based on the analytical Eco-Scale total score, which was calculated by subtracting the total penalty points (PPs) from 100. Three criteria were used to allocate PPs: the energy consumption of equipment, the danger categories offered by reagents (including the volume used in the analytical procedure), and additional factors including waste generation and occupational hazards [24]. The PPs of reagent was assigned based on the most up-to-date classification, Globally Harmonize System of Classification and Labelling of Chemicals [24]. A perfect score of 100 represents an ideal green analytical method; scored more than 75 was ranked as excellent green analysis; defines as acceptable when scoring more than 50; and the rest denote as an inadequate green analysis [24].

Results and Discussion

Optimization of instrumental parameters: Emission spectral bandwidths

Figure 2(a) illustrated the effect of emission spectral bandwidths on the detection of STX. Due to the limitation of the instrumentation, the highest emission

spectral bandwidth was 20 nm. Therefore, the optimization of this parameter was conducted at 5, 10, and 20 nm. It was observed that the fluorescence intensity increased with the increasing emission spectral bandwidths [30]. The optimum emission spectral bandwidth was 20 nm, which showed the highest fluorescence intensity of 83.064 a.u. with RSD value at 0.47% while 5 nm spectral bandwidths showed the lowest fluorescence intensity (8.038 a.u.) with RSD value at 0.98%. This phenomenon can be explained by the fact that as spectral bandwidths expanded, so did the light intensity entering the monochromators [30]. Similar findings have been reported in previous studies. For instance, a study by Masilamani et al. [31] had examined the effect emission slit widths (3, 5, and 10 nm) on the fluorescence spectra of acetone. The finding showed a significant increase in fluorescence intensity with wider emission slit widths, exhibiting similar trends to the present study. Besides, Harcher et al. [32] investigated emission slit widths ranging from 1.5 nm to 20 nm and their influence on the fluorescence intensity of quinine. Similarly, the quinine fluorescence intensity drastically increased with the emission slit widths, however, the fluorescence peak resolution was significantly reduced. Thus, the selected 20 nm bandwidth balances maximum signal intensity with sufficient peak distinction, ensuring accurate fluorescence measurement. A similar study by Alfarhan and Hamza [33] explored the effect of slit widths to the fluorescence intensity of different bisphenols compounds. A similar trend was reported that wider emission slit widths had led to higher fluorescence intensity but compromised peak resolution. Other than that, Alfarhani et al. [34] also demonstrated the effect of slit widths to the hydroxy polycyclic aromatic hydrocarbons fluorescence intensities. Similarly, the fluorescence intensity of each compound drastically increased with the emission slit widths.

Optimization of instrumental parameters: Excitation spectral bandwidths

The effect of excitation spectral bandwidths of the monochromators of the instrumentation to the fluorescence intensity was displayed in **Figure 2(b)**. It was observed that the fluorescence intensities increased drastically with increasing emission spectral bandwidths. For instance, the excitation spectral bandwidth at 20 nm showed the highest fluorescence intensity (729.605 a.u.) with RSD value at 0.42% and 5 nm showed the lowest fluorescence intensity (83.064 a.u.) with RSD value at 0.47%. This phenomenon can be explained by the spectral bandwidths that are directly proportional to the slit widths of the monochromators. A wider slit width allows greater amount of light intensity entering the monochromators [35]. Based on Beer's law relationship, the increment of the incident light increased the fluorescence intensity [36]. A nonlinear intensity-spectral bandwidths relation leading to an intensity plateau (≥1000 a.u) of the excitation peak at higher excitation spectral bandwidths (10 and 20 nm) and could not be accurately quantified (Figure S1). This result is also reported in several studies. For example, Alfarhan and Hamza [33] had investigated the effect of excitation slit widths to the bisphenol compounds fluorescence intensity. During the detection of bisphenol S compound, the fluorescence intensities plateaus were observed in both excitation and emission peaks when used larger slit widths (≥10 nm) with a Shimadzu branded fluorescence spectrometer (Shimadzu RF-5301pc). The chosen 5 nm excitation spectral bandwidth prevents signal saturation, as higher bandwidths (>10 nm) led to intensity plateauing in preliminary tests. This ensured consistent excitation energy without excessive background noise, maintaining method reproducibility. Additionally, narrower excitation bandwidths which improved spectral selectivity, reducing interference from potential matrix components. A similar study by Alfarhani et al. [34], whom used the same branded instrumentation, also reported similar issues when analyzing hydroxy polycyclic aromatic hydrocarbons compounds. This limitation might affect the accuracy of their fluorescence intensities. Therefore, excitation spectral bandwidth at 5 nm was chosen and to be used in further optimization.

Optimization of instrumental parameters: Excitation wavelength of oxidized saxitoxin

Figure 2(c) demonstrated the effect of excitation wavelengths of oxidized STX on the fluorescence intensity. It was observed that the optimum excitation wavelength was 334 nm which showed the highest fluorescence intensity (83.064 a.u.) with RSD value at 0.47%. Interestingly, the fluorescent intensities steadily decreased when decreasing the excitation wavelengths. Similarly, the decrement of the fluorescence intensities was observed when increasing the excitation wavelengths. This result was aligned with the findings of Müller et al. [29], who reported with the same excitation wavelength of 334 nm under similar experiment condition. In contrast, AOAC 2005.06 standard method for HPLC/FLD, developed by Lawrence et al. [22], reported with a slightly lower excitation wavelength of 330 nm. This minor shifting is particularly relevant in methods designed for multi-analyte detection. Since different derivatives exhibit varying excitation and emission properties, a compromise excitation wavelength of 330 nm might have been selected to ensure reliable quantification of multiple saxitoxins in complex samples.

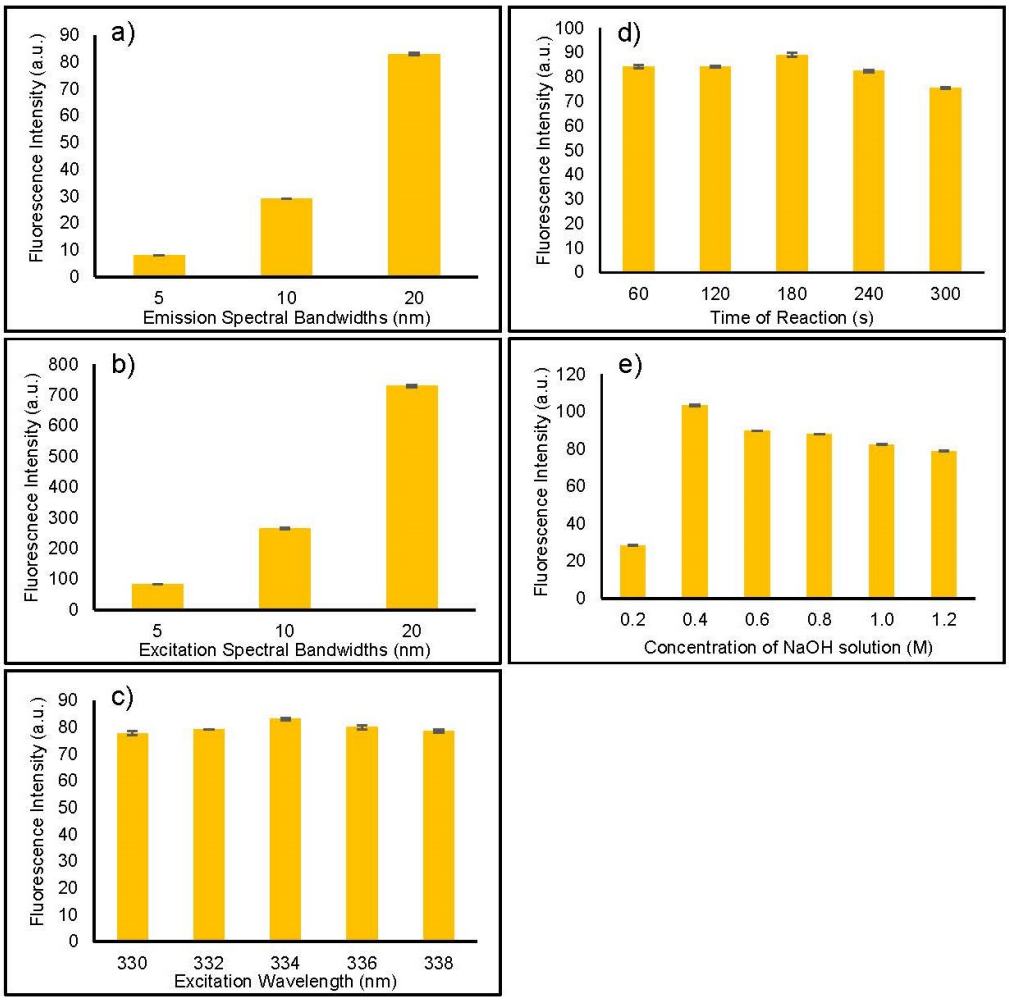

Figure 2. Bar chart of instrument optimization for a) different emission spectral bandwidths (other parameters: excitation spectral bandwidth=5 nm; excitation wavelength of oxidized STX=334 nm, time of reaction=120 s; concentration of NaOH solution=1.0 M), b) different excitation spectral bandwidths (other parameters: emission spectral bandwidths=20 nm; excitation wavelength of oxidized STX=334 nm; time of reaction=120 s; concentration of NaOH solution=1.0 M), c) different excitation wavelength of oxidized STX (other parameters: excitation spectral bandwidth=20 nm; excitation spectral bandwidths=5 nm; time of reaction=120 s; concentration of NaOH solution=1.0 M); Bar chart of derivatization optimization for d) different time of reaction (other parameters: emission spectral bandwidths=20 nm; excitation spectral bandwidths=5 nm; excitation wavelength of oxidized STX=334 nm; concentration of NaOH solution=1.0 M), e) and different concentration of NaOH solution (other parameters: emission spectral bandwidths=20 nm; excitation spectral bandwidths=5 nm; excitation wavelength of oxidized STX=334 nm; time of reaction=180 s)

Optimization of saxitoxin derivatization parameters: Time of reaction

The effect of reaction time during the peroxide oxidation process of STX to the fluorescence intensity was illustrated in **Figure 2(d)**. The optimum time of reaction for the peroxide oxidation process was 180 s which showed the highest fluorescence intensity of 89.062 a.u. with RSD value at 0.77%. It was observed that the fluorescence intensities drastically decreased when increased the time of reaction. This result aligned with the work by Vale [37], who analyzed STX and its derivatives using HPLC/FLD. Around 5 out of 8 late eluted analyte peaks disappeared when increasing the time of peroxide reaction. The findings were expected as STX and its derivatives were very unstable at alkaline conditions, thus, longer time reaction might cause the decomposition of the oxidized STX and decrease the detected fluorescence signals [38]. Other than that, the fluorescence intensities also decreased over time of reaction, which might be due to the incompleteness of the oxidation reaction [38]. Reaction time is crucial in peroxide oxidation, as prolonged durations (>180 s) resulted in fluorescence decay, likely due to analyte degradation.

This suggested that only a limited amount of STX was oxidized due to insufficient oxidation duration. Furthermore, the formation of partially oxidized STX intermediates did not contribute to fluorescence, resulting in lower fluorescence signal.

Optimization of saxitoxin derivatization parameters: Concentration NaOH solution

The effect of concentrations of NaOH solution on the fluorescence efficiency of oxidized STX was investigated, as shown in **Figure 2(e)**. The optimum concentration of NaOH for the STX oxidation process was 0.4 M with the highest fluorescence intensity at 103.402 a.u. (RSD value at 0.26%). It was observed that the fluorescence intensities were steadily decreased when increasing the concentration of NaOH solution. The selected 0.4 M NaOH maintained an optimal pH (~12.5) for efficient STX oxidation, ensuring a high fluorescence response. Higher concentrations (>1.0 M) led to reduced fluorescence, likely due to excessive alkalinity disrupting oxidation efficiency or causing partial degradation of the oxidized product. This balance preserves method sensitivity and minimizes unwanted side reactions.

This trend could be attributed to the influence of pH on oxidation reaction. For instance, a study by Gago-Martínez et al. [39] demonstrated that the optimum pH for STX peroxide oxidation was around 12.2-12.8, otherwise, the fluorescence intensities were reduced. STX is known to be more stable to chemical reaction, including oxidation in mildly acidic to neutral conditions, and excessive alkalinity may hinder the oxidation process by altering the reaction kinetics or promoting side reactions that lead to fluorescence quenching. Besides, higher NaOH concentration may also degrade the oxidized STX product, reducing fluorescence intensities. Therefore, the STX oxidation reaction was hindered under lower pH conditions which caused lower fluorescence intensity.

Method validation

The analytical parameters of the developed method such as linearity, LOD, LOQ, precision and accuracy were validated under optimized conditions (emission spectral bandwidth at 20 nm, excitation spectral bandwidth at 5 nm, excitation wavelength of oxidized STX at 334 nm, time of reaction at 180 s and concentration of NaOH solution at 0.4 M) to ensure the quality and reliability of the method. A five-point calibration curve of STX standard solution (40, 50, 100, 150, and 200 µg/L) showed a strong linear relationship (R^2=0.9979) between the fluorescence intensity and the concentration with linear equation of y=0.3321x + 5.0021. The LOD and LOQ were calculated as 11.92 and 36.11 µg/L, respectively, based on the Equation 1 and Equation 2.

The precision values for 3 different concentrations of STX in terms of intra-day and inter-day fall within the acceptable limits (typically <15%). The RSD values for intra-day study at 50, 100 and150 µg/L were 1.10%, 0.76% and 0.35%, respectively. A similar trend was observed on the RSD values for inter-day study at the three concentrations, which were 2.17%, 2.81% and 0.51%, respectively. Interestingly, it was observed that the lower the concentration, the higher the RSD value. At lower concentration, the fluorescence signal was weaker relatively to the noise and other interferences such as background interference, instrument absolute error, and random fluctuations. Therefore, this would increase the variability relative to the mean signal, resulting in a higher RSD value. Hence, this finding indicated that the analysis in terms of intra-day and inter-day tends to be more stable at higher concentration of STX.

The accuracy results for three different concentrations of STX fall within the acceptable limits (typically between 80-120%). The RR values for three different STX concentrations at 50, 100 and 150 µg/L were 103%, 102% and 101%, respectively. It was observed that the experimental values were more accurate at higher STX concentration. This phenomenon could be attributed to the impact of sample dilution. When samples are diluted at lower concentrations, there is a potential for increased variability during the sample preparation phase. However, this issue was alleviated when working with higher concentrations, resulting in a higher likelihood that the RR value approaching 100%. Robustness and uncertainty were not explicitly tested, as this study focused on rapid screening rather than confirmatory analysis.

Comparative studies

In the realm of STX detection methods, various techniques are evaluated based on their duration and sensitivity. According to **Table 1**, the current study stands out for its remarkable rapidity. This method boasts an exceptionally short detection duration of approximately 4 min, making it the fastest among the other techniques (in terms of the detection of a single analyte). Our current study maintained a reasonable sensitivity with an LOD of 11.92 µg/L. This balance of speed and sensitivity makes it a promising option for applications requiring quick and reliable STX detection for rapid screening and/or further quantitative analysis. In comparison, conventional methods like MBA required longer analysis duration which was approximately 1 h, mainly for the quantification of STX based on the mouse death time. Besides that, MBA also has lower sensitivity with LOD of 175.00 µg/L [14]. Similarly, RBA required more time despite the reasonable sensitivity (LOD of 8.75 µg/L). Understandably, the tedious preparation and incubation of the rat brain membrane homogenate

extended the duration which was approximately 1.5 h [15]. For the chromatographic method, HPLC/FLD, which is the golden standard method, and LC-MS/MS are denoted as highly sensitive by achieving LOD as low as 10.00 µg/L and 0.02 µg/L, respectively, with reasonable analytical duration which were approximately 23 and 8 min, respectively [16, 19, 20]. ELISAs also reported as higher sensitivity with low LOD (3.17 µg/L). However, the MIP immobilization process delayed the whole analytical duration which is approximately around 12.5 h [18]. Lastly, CVA showed higher sensitivity with a lower LOD (0.34 µg/L). Nonetheless, the neuroblastoma-2a cell incubation and toxin exposure process required approximately 24 and 1 h, respectively, which escalated the total analysis duration [17].

Analytical eco-score evaluation

Table 2 shows the Analytical Eco-scale evaluation of the developed peroxide oxidation derivatization coupled with fluorescence spectrophotometry method for the detection of saxitoxin. The calculation covers the sample preparation and instrumental detection for a single sample. A total score of 91 was obtained which indicated an excellent green analytical method. Based on **Table 1**, it scored the highest analytical Eco-scale among the compared analytical methods where MBA scored 85; LC-MS/MS scored 81; RBA scored 78; CVA scored 77; HPLC/FLD scored 69 and ELISA scored 53 (Analytical Eco-scale evaluations for the mentioned conventional methods can be referred to in Supplementary Information). This is because it used eco-friendlier and low volume of reagents with a total PP of 8 for the sample preparation of saxitoxin (derivatization). In comparison, other conventional methods except for MBA (PP=8), utilized more hazardous solvent in higher volume in the sample preparation steps, resulting in higher total PP (11-42). Besides, this work contributed to low electricity requirement for the fluorescence spectrophotometer, contributing to a total instrument PP of 0. In contrast, the standard methods such as HPLC/FLD and LC/MS/MS, where the instrument parts required higher energy per sample (PP=2). Furthermore, this micro-volume usage work encompassed a minimal production of waste, with a PP of 1. In comparison to HPLC/FLD and LC/MS/MS, which employed high volume of mobile phase for flushing process and analysis, yielding high PP of 5. For ELISA, the pre-washing steps for the MIP-coated wells increased the production of organic solvent waste (PP=3).

Table 1. Comparative studies of STX detection method

Analysis Method	Analysis Duration (Approximate)	Sensitivity (LOD=3σ/S)	Analytical Eco-scale total score (Ranking)	References
MBA	1 h	LOD = 350.00 µg/kg (equivalent 175.00 µg/L)	85 (Excellent)	[14]
LC-MS/MS	8 min	LOD = 0.10 µg/kg (equivalent 0.02 µg/L)	81 (Excellent)	[16]
RBA	1.5 h	LOD = 17.49 µg/kg (equivalent 8.75 µg/L)	78 (Excellent)	[15]
Molecularly imprinted polymer-based ELISAs	12.5 h	LOD = 3.17 µg/L	53 (Acceptable)	[18]
HPLC/FLD	23 min	LOD = 20.00 µg/kg (equivalent 10.00 µg/L)	69 (Acceptable)	[19, 20]
CVA	24 h	LOD = 0.34 µg/L	77 (Excellent)	[17]
Peroxide oxidation derivatization coupled with fluorescence spectrophotometry method	4 min	LOD = 11.92 µg/L	91 (Excellent)	Current study

Table 2. Analytical Eco-scale calculations for the work of current study

Reagent				
	Amount (PP)	**Signal Word (PP)**	**Number of Pictogram (PP)**	**Total PP**
Water	<10 mL (1)	None (0)	0 (0)	1×0×0=0
Sodium hydroxide	<10 g (1)	Danger (2)	1 (1) 	1×2×1=2
Glacial acetic acid	<10 mL (1)	Danger (2)	2 (2) 	1×2×2=4
Hydrogen peroxide	<10 mL (1)	Danger (2)	1 (1) 	1×2×1=2
Total PP of reagent				**8**

Instrument		
	Energy used per sample (kWh) (PP)	**Total PP**
Fluorescence spectrophotometer	≤0.1 (0)	0
Vortex mixer	≤0.1 (0)	0
Total PP of instrument		**0**

Other Parameters		
		Total PP
Occupational hazard	Analytical process hermetization (0)	0
Waste	<1 mL (1)	1
Total PP of other parameters		**1**

Calculation	
Overall Total PPs	8+0+1=**9**
Analytical Eco-Scale total score	100−9=**91**
Rating	**Excellent**

Conclusion

This study successfully demonstrated a rapid, selective, and environmentally friendly fluorescence-based method for saxitoxin (STX) detection using peroxide oxidation derivatization. The optimized conditions ensure high fluorescence signal stability, reproducibility, and sensitivity, making it a promising alternative for routine screening of STX in seafood contamination monitoring. Compared to conventional methods, this approach offered a faster analysis time, lower reagent consumption, and a reduced environmental footprint, as supported by the Analytical Eco-Scale assessment. Beyond these findings, future research should focus on expanding the method to multi-analyte detection, refining green chemistry strategies for derivatization, and integrating automation to enhance high-throughput screening capabilities. Additionally, validation across diverse seafood matrices and inter-laboratory studies will be essential to further establish its robustness and applicability in real-world monitoring programs.

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