

# Capillary Electrophoresis-Based Immunoassay for the Determination of Brevetoxin-B in Shellfish using Electrochemical Detection

Xiaowei Zhang<sup>1</sup> and Zhaoxiang Zhang<sup>2\*</sup>

<sup>1</sup>School of Science, Qingdao Technological University, Qingdao 266033, China, and <sup>2</sup>College of Chemistry and Molecular Engineering, Qingdao University of Science & Technology, Qingdao 266042, China

\*Author to whom correspondence should be addressed. Email: zhangzhx02@ustc.edu

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**Neurotoxic shellfish poisoning is a significant food-borne disease caused by potent cyclic polyether toxins (brevetoxins) that accumulate in the flesh of shellfish. Here we report a new procedure for brevetoxin analysis by capillary electrophoresis-based immunoassay (CE–IA) with electrochemical (EC) detection. In this method, after the competitive immunoreaction in liquid phase, the horseradish peroxidase (HRP)-labeled antigen and the bound enzyme-labeled complex were separated and then the system of HRP catalyzing H<sub>2</sub>O<sub>2</sub>–o-aminophenol reaction was adopted. The limits of detection (signal-to-noise = 3) was determined to be 0.1 ng/mL. Intra-day relative standard deviations (RSD, *n* = 5) for migration time and peak area were 3.6 and 4.5%, respectively. Inter-day RSD was 6.9 and 7.8% for migration time and peak area, respectively. The CE–IA with EC detection provides a sensitive analytical approach, not previously available, for the determination of brevetoxin-B in shellfish samples.**

## Introduction

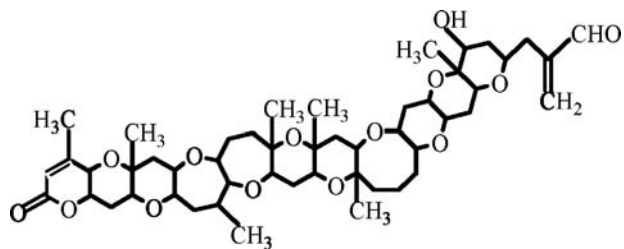
Neurologic shellfish poisoning (NSP) is a human intoxication caused by the ingestion of a variety of bivalve molluscs such as shellfish, mussels, oysters and scallops. A toxin of NSP, known as brevetoxin, is produced by *Karenia brevis* and accumulates in various kinds of molluscs through the food chain (1). The *Karenia brevis* produces two types of brevetoxins (BTXs, also known as PbTx) based on their backbone structure, with the A type composed of 10 fused ether rings and the B type composed of 11 fused ether rings (2). Brevetoxin-A (also known as PbTx-1) and brevetoxin-B (also known as PbTx-2) are the primary brevetoxins produced in algae. These brevetoxins have similar characteristics: heat and acid stability, inactivation by ordinary cooking methods. The symptoms caused by these toxins also present some similarities. A major problem in avoiding the disease is that molluscs contaminated with brevetoxins look, smell and taste normal, and the unwitting diner could easily consume a unsafe meal if measures are not taken beforehand to test the molluscs. The threat to human health resulting from consumption of brevetoxin-contaminated shellfish has led to the implementation of shellfish monitoring programs in many countries.

A common method routinely used for the detection of brevetoxins in monitoring programs is mouse bioassay based on the response of a living organism to the toxic effects elicited by this suite of algal toxins and metabolites (3). However, test conditions, including animal strain and sex, salt concentration and sample treatment, considerably affect the test results. The second category of approaches to brevetoxin analysis includes

biochemical methods such as enzyme-linked immunosorbent assay (ELISA) (4). This method grants high sensitivity and high selectivity, despite some cross-reactivity. An instrumental method commonly used for determining brevetoxins is high-performance liquid chromatography (5, 6). This method offers good sensitivity and can separate and detect a series of toxins in a single run.

Combining the effective separation power of capillary electrophoresis (CE) and the ligand specificity of immunoassay (IA), CE–IA has proved to be a powerful technique for the separation and analysis of biological samples, because CE has the ability to separate antigen-antibody complexes from free antigens and antibodies in both competitive and noncompetitive formats (7–10). With CE–IA, the assay time is faster because the immunoreaction occurs in solution, which allows rapid reaction by liquid phase kinetics. It also shows some advantages such as high detection sensitivity, low reagent consumption and more simple operation than conventional immunoassays. Most of the CE–IA methods rely on a laser induced fluorescence (LIF) detector for a wide range of compounds with high sensitivity (11–16). However, major disadvantages of LIF include the high background due to the noise from Rayleigh and Raman scattering and the fluorescent impurities in the solvent. Although ultraviolet (UV) detection can be used for characterization of separation of immunocomplexes, the major disadvantage of the UV detector is the lack of sensitivity. Cheng's group reported a CE–IA method based on chemiluminescence detection with horseradish peroxidase (HRP) catalyzing the luminol–H<sub>2</sub>O<sub>2</sub>–p-iodophenol reaction (17). Electrochemical (EC) detection provides excellent sensitivity for the small dimensions associated with CE, while offering a high degree of selectivity toward electroactive species (18). According to the preceding report, CE–IA with EC detection that combines the high separation efficiency of CE, the high specificity of IA, and the high sensitivity of EC detection has been proven to be a promising technique (19) and has the potential to be an effective method for the determination of brevetoxin-B in various sample matrixes. However, brevetoxin-B detection using CE–IA with EC detection has not been studied so far.

In this paper, we use brevetoxin-B (Figure 1) as a model to set up a new method for the determination of toxin. The CE–IA with EC detection was developed based on the competitive immunoreaction between HRP-labeled brevetoxin-B (Ag<sup>+</sup>) and free brevetoxin-B (Ag) with a limited amount of antibrevetoxin-B antibody (Ab). The immunoreactive samples after incubation can be directly subjected to CE separation. The



**Figure 1.** Chemical structure of brevetoxin-B.

enzymatic product, 2-aminophenoxazine-3-one (AP), produced from the oxidation of  $\text{H}_2\text{O}_2$ -o-aminophenol (OAP) with  $\text{H}_2\text{O}_2$  catalyzed by HRP, was amperometrically detected at a Pt electrode at the outlet of the reaction capillary. The detection of brevetoxin-B using the two methods (both CE-IA with EC detection and ELISA, which used the same immunogen) was also compared. Shellfish, a nutritious food source, are featured globally in different cuisines, but are highly sensitive to the quality of their marine environment. Therefore, the developed method was applied for the analysis of brevetoxin-B in shellfish samples.

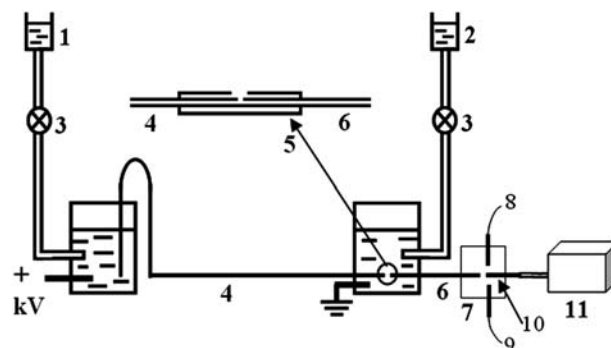
## Experimental

### Reagents and solutions

The brevetoxin-B kit was purchased from Abraxis (San Diego, CA), consisting of Ag standards, a solution containing  $\text{Ag}^*$  and a solution containing Ab. The kit was stored at  $4^\circ\text{C}$ . The running buffer consisted of 1.0 mM  $\text{H}_2\text{O}_2$  and 10 mM Britton-Robinson buffer (BR buffer; the mixture of 0.58 mL phosphoric acid, 0.58 mL glacial acetic acid and 0.62 g boric acid powder was dissolved and diluted to 1 L with double-distilled water, and adjusted to pH 5.0 with 0.2M NaOH). A 10-mM stock solution of OAP was prepared by dissolving an appropriate amount of OAP in water. Polyvinylpyrrolidone (PVP,  $M_r = 1,300,000$ ) was purchased from Shanghai Chemical Plant (Shanghai, China). It was used as a capillary inner wall dynamic coating reagent to decrease the adsorption of enzyme on the capillary inner wall (20). All buffer reagents and other chemicals were of analytical grade and supplied by local standard reagent suppliers, unless otherwise stated. All solutions were prepared with double-distilled water and filtered through 0.22- $\mu\text{m}$  cellulose acetate membrane filters (Shanghai Yadong Resin, Shanghai, China) before use.

### Instrumentation

The basic design of the CE-IA with EC detection system for brevetoxin-B is shown in Figure 2. The CE separation with EC detection was performed on a model MPI-A CE setup (Remax Electronics, Xi'an, China), equipped with a high-voltage power supplier (0–30 kV) for electronic sampling and separation and an EC potentiostat (0–2.5 V) for EC detection. The anodic high-voltage end of the separation system was isolated in a Plexiglas box fitted with an interlock for operator safety. Fused-silica capillaries (50  $\mu\text{m}$  i.d., 375  $\mu\text{m}$  o.d.) of 20 and 5 cm were used as separation and reaction capillary, respectively. The EC detection was carried out with a three-electrode system consisting of a Pt working electrode, an Ag/AgCl reference electrode and a Pt auxiliary electrode. The working



**Figure 2.** Schematic diagram of CE-IA with EC detection system for brevetoxin-B. 1, running buffer reservoir; 2, OAP substrate reservoir; 3, switch valve; 4, separation capillary; 5, interface; 6, reaction capillary; 7, three-electrode detection cell; 8, reference electrode; 9, auxiliary electrode; 10, working electrode; 11, EC detector.

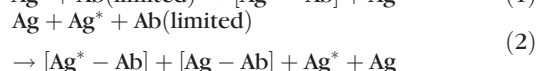
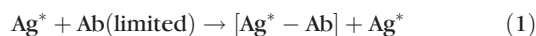
electrode was cleaned by ultrasonication in ethanol and double-distilled water for 5 min before use. The EC detection cell was filled with 10 mM of BR buffer solution. The interface of the two capillaries and the EC detection system has been described elsewhere (21). The reaction capillary was coaxial along the separation capillary and working electrode. The running buffer reservoir and OAP substrate reservoir were fixed at the same height.

### Preparation of shellfish samples

Brevetoxin-B free shellfish samples, predetermined by a commercial ELISA kit for the evaluation of shellfish tissue matrix effects, were purchased fresh from the market on the same day an experiment was to be performed. Shellfish samples contaminated by brevetoxin-B were collected from Zhangzi Island (Dalian, China). Extraction of the brevetoxin-B from shellfish samples was similar to that previously described (22), with slight modifications. Shellfish samples were removed from their shells and drained, and shellfish meat was homogenized with a blender. Two grams of the homogenized sample was weighed, and 12 mL of 100% methanol was added and mixed using a vortex mixer for 3 min at full speed, and centrifuged at 3,000 rpm for 10 min at room temperature. The supernatant was saved, and the pellet was extracted again with 8 mL of 100% methanol. The supernatants were pooled together and evaporated to dryness with nitrogen at  $40^\circ\text{C}$ , and the residue was resuspended in 15 mL of 100% MeOH. The supernatant was filtered through a 0.22- $\mu\text{m}$  cellulose filter into a storage bottle and sealed tightly. The crude extract was stored at  $4^\circ\text{C}$  for future use.

### Preparation of immunoreactive samples

The immunoreaction protocol was competitive format, and the immunoreaction was conducted as follows:



where  $\text{Ag}^*$  is HRP-labeled brevetoxin-B with a fixed amount, Ab is antibrevetoxin-B with a limited and fixed amount, and Ag is brevetoxin-B standard or in shellfish samples.

A volume of 10  $\mu\text{L}$  different concentrations of brevetoxin-B standard or shellfish sample extracts was mixed with 10  $\mu\text{L}$  of  $\text{Ag}^+$  (1:100 dilution) and 10  $\mu\text{L}$  of Ab in a 200  $\mu\text{L}$  microcentrifuge tube, and then diluted with 10 mM pH 5.0 BR buffer to 50  $\mu\text{L}$ . During incubation at 37°C for 30 min, the brevetoxin-B standard or brevetoxin-B in shellfish samples ( $\text{Ag}$ ) competed with the  $\text{Ag}^+$  to react with the limited amount of Ab. After incubation, the mixture was analyzed by CE separation with EC detection.

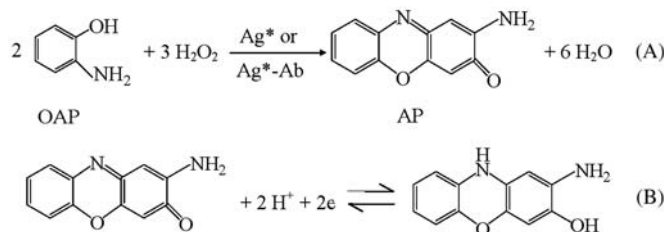
### CE separation with EC detection procedure

The capillary was flushed daily in the order of  $\text{H}_2\text{O}$  (1 min), 1.0M NaOH (15 min),  $\text{H}_2\text{O}$  (1 min) and conditioned with running buffer for 10 min by pressure. Between two runs, the capillary was conditioned with running buffer for 6 min. After the EC signal reached a constant value, the incubated solution was injected into the positive end of the separation capillary with an injection voltage of 10 kV and an injection time of 10 s. With the sample exchanged with separation buffer, the  $\text{Ag}^+$  and the formed immunocomplexes ( $\text{Ag}^+-\text{Ab}$ ) were separated according to mobility difference with 15 kV separation voltage. At the interface, the enzyme substrates OAP were introduced into the reaction capillary by gravity. The HRP, labeled on  $\text{Ag}^+$  and  $\text{Ag}^+-\text{Ab}$ , successively entered the reaction capillary and catalyze the reaction between OAP and  $\text{H}_2\text{O}_2$  to AP and  $\text{H}_2\text{O}$ . Then, the enzymatic product, AP, was detected at the Pt working electrode at the outlet of the reaction capillary with the detection potential of  $-0.35$  V. The reaction scheme is shown in Figure 3. Two peaks will appear in the electropherograms, corresponding to  $\text{Ag}^+$  and  $\text{Ag}^+-\text{Ab}$  immunocomplex. According to competitive reaction, with the increase of the  $\text{Ag}$  concentration, the peak of formed  $\text{Ag}^+-\text{Ab}$  complex decreased and the peak of the unbound  $\text{Ag}^+$  correspondingly increased. The concentrations for free  $\text{Ag}^+$  and compound  $\text{Ag}^+-\text{Ab}$  were calculated by peak areas, and the peak areas of the unbound  $\text{Ag}^+$  were used for quantification in this work.

## Results and Discussion

### Optimization of CE-IA with EC detection conditions

The incubation time of  $\text{Ag}^+$  and Ab was studied (Figure S1 in Supplementary Data) and the optimal incubation time was 30 min. This contrasts with a solid phase IA, such as an ELISA, where only free  $\text{Ag}$ s are in solution, while the immunocomplexes are in a solid phase; the thermodynamics of solutions cannot be applied to the system, which therefore needs a relatively longer time to reach equilibrium.

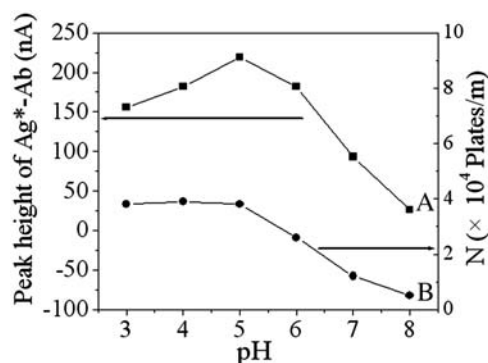


**Figure 3.** Reaction schemes of: HRP labeled on  $\text{Ag}^+$  or  $\text{Ag}^+-\text{Ab}$  catalyzing enzyme substrates OAP and  $\text{H}_2\text{O}_2$  to AP and  $\text{H}_2\text{O}$  (A); AP reduced in EC detection cell (B).

After incubation, the immunoreactive sample was directly subjected to CE separation. To optimize the CE separation conditions, buffer solutions at different concentrations, including sodium phosphate, sodium borate, sodium acetate, sodium citrate, sodium tartrate and BR were examined for separation and detection of the immunoreactive samples. The results showed that BR buffer solution was more suitable because the peak heights and peak-to-peak resolution (R) of free  $\text{Ag}^+$  and immunocomplexes  $\text{Ag}^+-\text{Ab}$  excel over those of other buffer solutions. The relationships of peak heights and R to the concentration of BR buffer (varied from 5 to 100 mM) were tested. The best peak height and resolution was obtained using 10 mM BR buffer solution.

The pH of buffer solution is also an important parameter in the CE-IA. For liquid-phase CE-IA, the immunoassay is performed in solution, and the immunological species are separated via CE. The effect of pH values (ranging from 3.0 to 8.0) on the separation of free  $\text{Ag}^+$  and immunocomplexes  $\text{Ag}^+-\text{Ab}$  was investigated. As shown in Figure 4, pH 5.0 produced the highest peak value and optimal separation efficiency (theoretical plate number, N) for the immunocomplex. When the pH was above 6.0, the peak height and separation efficiency of immunocomplex  $\text{Ag}^+-\text{Ab}$  obviously decreased and the peak broadened. The pH change may induce conformational changes in the formation of the immunocomplex  $\text{Ag}^+-\text{Ab}$ , therefore, the values of peak height, separation efficiency and shape of immunocomplex peak was altered. The optimal pH value of the BR buffer solution was chosen as 5.0.

In initial experiments, a 10-mM BR buffer (pH 5.0) was used as a separation buffer for the separation of free  $\text{Ag}^+$  and immunocomplexes  $\text{Ag}^+-\text{Ab}$ . A broadened and destroyed peak was observed because of the adsorption of analytes onto the capillary inner wall. To improve separation efficiencies by eliminating analyte adsorption, a buffer solution containing PVP was used. The relationships of separation efficiency, resolution and migration time to the concentration of PVP (0.5–3.0%, w/v) in the separation buffer were tested (Figure S2 in Supplementary Data). The separation efficiency (theoretical plate number, N) was increased with the PVP concentration increasing up to 1.0%, then the separation efficiency slightly decreased with further increase of PVP concentration. The resolution and migration time increased with increasing of PVP concentration. Considering the separation efficiency, resolution and migration time, 1.0% PVP was selected in subsequent studies. Using a



**Figure 4.** Effect of buffer pH on peak height (A) and separation efficiency (B) of immunocomplex  $\text{Ag}^+-\text{Ab}$  by CE separation and EC detection.



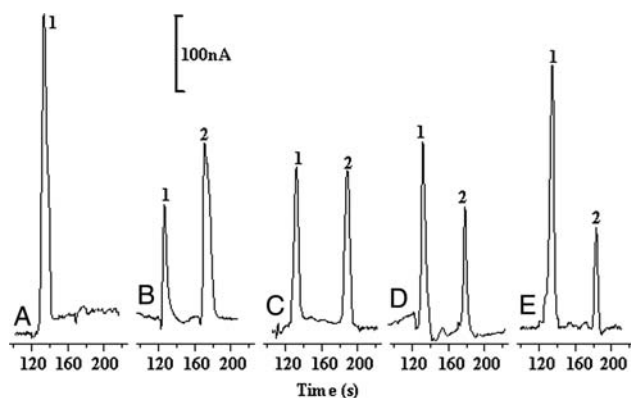
neutral unretained probe mesityl oxide as electroosmotic flow (EOF) marker, the EOF was measured to the magnitude of  $1.56 \times 10^{-4} \text{ cm}^2/\text{V}/\text{s}$  with 10 mM BR buffer (pH 5.0) containing 1.0 % PVP.

With EC detection, the effect of detection potential ( $E_d$ ) on peak currents was studied, ranging from  $-0.70$  to  $-0.20$  V. When the  $E_d$  decreased, the peak currents increased with the larger noise. When the  $E_d$  was  $-0.35$  V, the signal-to-noise (S/N) ratio was the highest.

### CE-based competitive IA for brevetoxin-B detection

We used the competitive model [Equation (2)] in which  $\text{Ag}^*$  was first mixed with different concentrations of Ag, which then competed for binding with the limited and fixed amount of Ab to form immunological complexes. The amount of  $\text{Ag}^*-\text{Ab}$  will be inversely proportional to the amount of immunocomplex formed by the antibody and unlabeled brevetoxin-B ( $\text{Ag}-\text{Ab}$ ). As shown in Figure 5, as the amount of brevetoxin-B increased, more HRP labeled brevetoxin-B was replaced and released from the immunocomplex through competitive reaction. Compared with a control (without the addition of brevetoxin-B, Figure 5B), the peak area of immunocomplex  $\text{Ag}^*-\text{Ab}$  gradually decreased, and the peak area of free  $\text{Ag}^*$  correspondingly increased (Figures 5B–5E).

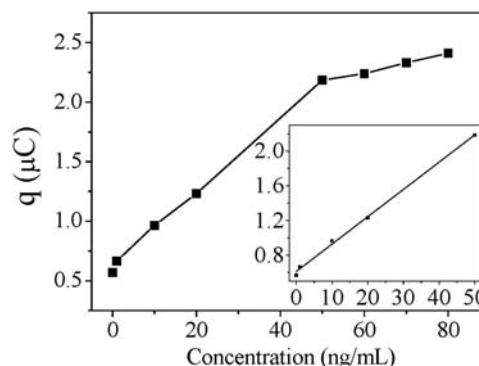
Different concentrations of brevetoxin-B (from 0.01 to 100 ng/mL) were analyzed under the optimized CE–IA conditions. The calibration curve for brevetoxin-B (Figure 6) was acquired by plotting the peak area of free  $\text{Ag}^*$  against the concentration of brevetoxin-B. The linear range and limit of detection (LOD) for brevetoxin-B were 1.0 ~ 50.0 ng/mL ( $r = 0.9988$ ) and 0.1 ng/mL, respectively, with the following equation of linear regression:  $y = 0.61x + 0.031$  (where  $y$  is peak area,  $\mu\text{C}$ , and  $x$  is the concentration of brevetoxin-B, ng/mL). Intra-day relative standard deviations (RSD,  $n = 5$ ) for migration time and peak area was less than 3.6 and 4.5%, respectively. Inter-day RSDs ( $n = 5$ ) were 6.9 and 7.8% for migration time and peak area, respectively.



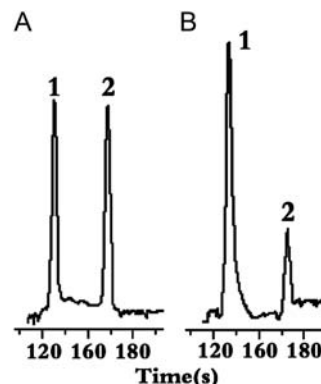
**Figure 5.** CE–IA with an EC detector for brevetoxin-B detection: only free  $\text{Ag}^*$  without brevetoxin-B Ag and antibrevetoxin-B Ab (A); control free  $\text{Ag}^*$  (1:100 dilution) binding with  $0.5 \mu\text{g}$  of antibrevetoxin-B Ab without the addition of brevetoxin-B (B);  $0.1 \text{ ng/mL}$  brevetoxin-B and free  $\text{Ag}^*$  (1:100 dilution) competed for binding with  $0.5 \mu\text{g}$  of antibrevetoxin-B Ab (C);  $1.0 \text{ ng/mL}$  brevetoxin-B and free  $\text{Ag}^*$  (1:100 dilution) competed for binding with  $0.5 \mu\text{g}$  of antibrevetoxin-B Ab (D);  $5.0 \text{ ng/mL}$  brevetoxin-B and free  $\text{Ag}^*$  (1:100 dilution) competed for binding with  $0.5 \mu\text{g}$  of antibrevetoxin-B Ab (E). Peak identification: 1,  $\text{Ag}^*$ ; 2,  $\text{Ag}^*-\text{Ab}$ .

### Analysis of brevetoxin-B in shellfish sample

In the application of the proposed CE–IA with EC detection, brevetoxin-B was analyzed in seven shellfish samples under the optimum conditions. Figure 7 shows the electropherograms of blank shellfish sample (Figure 7A shows the sample free of shellfish toxins, predetermined by commercial ELISA kits) and contaminated shellfish samples (Figure 7B). According to Figures 7A and Figure 5B, the sample matrix from the blank shellfish sample did not interfere with the analyte peaks under experimental conditions because the high selectivity of immunoreaction. Compared with Figure 7A, the increase of peak 1 and decrease of peak 2 are shown in Figure 7B. This demonstrates that the shellfish sample contained brevetoxin-B, which competed with the  $\text{Ag}^*$  to react with the limited amount of Ab according to Equation (2), resulting in the increase of the unbound  $\text{Ag}^*$  peak and decrease of the  $\text{Ag}^*-\text{Ab}$  complex peak. To check the validity and reliability of the proposed method, the contents of shellfish toxins in the seven shellfish samples were determined by this method and the routine ELISA. The 3,3',5,5'-tetramethylbenzidine (TMB) spectrophotometric ELISA assay was performed following the manufacturer's procedure. The results of the seven samples determined by the two methods are listed in Table I. The contents of brevetoxin-B in the seven shellfish samples determined with the proposed method were in good agreement with the results of the



**Figure 6.** Calibration curve of brevetoxin-B by CE–IA with EC detection.



**Figure 7.** Electropherograms of immunoreactive samples: blank (A); contaminated shellfish sample (B). Other conditions and peak identification are the same as in Figure 4.

**Table 1**

Comparison of Concentration Results ( $\mu\text{g}/\text{Kg}$ ) of the Proposed Method with the Routine ELISA Method for the Determination of Brevetoxin-B in Different Shellfish Samples ( $n = 3$ )

Sample	Proposed method	ELISA method
Oyster	$31.2 \pm 0.5$	$33.7 \pm 0.7$
Scallop	$9.3 \pm 0.4$	< LOD
Mussel	$83.7 \pm 0.6$	$85.1 \pm 0.9$
Small clam	$164.5 \pm 0.7$	$165.9 \pm 0.3$
Edible whelk	$52.9 \pm 0.6$	$54.3 \pm 0.4$
Cockle	$117.4 \pm 0.8$	$120.8 \pm 0.5$
Periwinkle	$65.1 \pm 0.6$	$68.4 \pm 0.3$

routine ELISA method, indicating the high feasibility and validity of proposed method. The recoveries of brevetoxin-B with the proposed method, tested by spiking standard antigen into the shellfish samples, were  $95.2 \pm 0.8\%$ . The RSDs ( $n = 5$ ) of the reproducibility were in the range of 3.1–7.5%. The results demonstrate that the proposed method is very suitable for the determination of brevetoxin-B in shellfish samples.

## Conclusions

This work establishes CE-IA with EC detection as a powerful tool for the analysis of brevetoxin-B in shellfish samples. The linear range and LOD for brevetoxin-B were 1.0 ~ 50.0 and 0.1 ng/mL, respectively. Quantitative measurements of brevetoxin-B in shellfish samples have been demonstrated with simple pretreatment. Compared to conventional methods such as ELISA, CE-IA with EC detection is more sensitive, uses less Ab, and more quickly reaches equilibrium. This analytical method can be used as a powerful tool for the determination of trace brevetoxin-B in shellfish samples. This work also shows that the CE-IA with EC detection is a potential technique for studying trace brevetoxin-B in all kinds of bivalve molluscs samples, such as mussels, clams, cockles, scallops, and oysters.

## Acknowledgments

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