Sensitive and reliable micro-plate chemiluminescence enzyme immunoassay for okadaic acid in shellfish

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Analytical Methods</th>
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</thead>
<tbody>
<tr>
<td>Manuscript ID:</td>
<td>AY-COM-05-2014-001063.R1</td>
</tr>
<tr>
<td>Article Type:</td>
<td>Communication</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>16-Jun-2014</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Lin, Chao; Key Laboratory for Zoonosis Research, Ministry of Education, Institute of Zoonosis, Jilin University, Liu, Zeng-Shan; Laboratory for Zoonosis Research, Ministry of Education, Institute of Zoonosis, Jilin University, Changchun 130062, P. R. China, Wang, Dong-Xu; Jilin Provincial Key Laboratory of Animal Embryo Engineering, College of Animal Science, Jilin University, Changchun 130062, P. R. China, Ren, Hong-Lin; Laboratory for Zoonosis Research, Ministry of Education, Institute of Zoonosis, Jilin University, Changchun 130062, P. R. China, Li, Yan-Song; Laboratory for Zoonosis Research, Ministry of Education, Institute of Zoonosis, Jilin University, Changchun 130062, P. R. China, Hu, Pan; Laboratory for Zoonosis Research, Ministry of Education, Institute of Zoonosis, Jilin University, Changchun 130062, P. R. China, Zhou, Yu; Laboratory for Zoonosis Research, Ministry of Education, Institute of Zoonosis, Jilin University, Changchun 130062, P. R. China, Guo, Yi-Ping; Dalian Zhongxin Testing Technology, LLC, Dalian 116045, P. R. China, Meng, Xian-Mei; Jilin Business and Technology College, Changchun 130062, P. R. China, Lu, Shi-Ying; Laboratory for Zoonosis Research, Ministry of Education, Institute of Zoonosis, Jilin University, Changchun 130062, P. R. China,</td>
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</tbody>
</table>
Sensitive and reliable micro-plate chemiluminescence enzyme immunoassay for okadaic acid in shellfish

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Abstract

Background: In this study, a highly sensitive and reliable analytical micro-plate chemiluminescence enzyme immunoassay (CLEIA) based on a monoclonal antibody (McAb) against okadaic acid (OA) was developed and validated for the detection of okadaic acid from shellfish matrix.

Methods: A competitive immunocomplex was formed through the binding of an immobilised antigen, OA in analyzed samples and the McAb against OA. The conjugate OA-BSA was immobilised physically on a polystyrene micro-plate well as
a solid phase antigen. Subsequently, free toxins in the analyzed samples competed
with the solid phase antigen to bind the McAb against OA. The assay conditions,
including the composition and pH of the coating solution, the dilution ratios and
amounts of the McAb and the HRP-labelled goat anti-mouse IgG antibody, the time
of the antibody-coating, incubation and chemiluminescence reactions and other
relevant variables were studied and optimised.

Results: The optimised system allowed OA determination in a linear working range
from 0.0098-10 µg kg⁻¹ (R=0.99), and the calibration curve obtained for OA revealed
a detection limit of 0.0098 µg kg⁻¹. Importantly, the CLEIA was approximately
10-fold sensitive than an ELISA using the same antibody. In addition, the intra- and
inter-assay RSDs were both less than 10.0%. Moreover, this method was successfully
applied to the evaluation of OA in seashell, with recoveries of 97.2%, 111.2% and
104.7%, respectively, for low-, medium- and high-concentration samples.

Conclusions: Good recoveries were obtained from spiked food samples, and the
results correlated well with those obtained using conventional indirect competition
ELISA, indicating the potential utilisation of the CLEIA as a preliminary screening
tool for analyzing OA contamination in shellfish.

Key words: Okadaic acid (OA); micro-plate chemiluminescence enzyme
immunoassay (CLEIA); monoclonal antibody (McAb); seafood

I. Introduction

The suite of marine toxins in DSP (diarrheic shellfish poisoning) can be detected in
various species of filter-feeding bivalve molluscs, such as oysters, mussels, scallops
and clams¹⁻⁵. Okadaic acid (OA), as the major disease-causing toxin in DSP, is
considered to pose the greatest risk to human health. Studies carried out on animals
have also demonstrated the carcinogenic, mutagenic and immune toxic effects of OA.
Consuming contaminated shellfish can cause diarrhea, nausea, vomiting and
abdominal pain, in addition to other characteristic DSP symptoms⁶⁻⁸. The toxins are
stable at high temperatures and have long-term carcinogenicity⁹. In the European
Union, Commission Regulation (EC) No. 853/2004 established a maximum permitted level of 160 µg of OA equivalents kg$^{-1}$ in bivalve molluscs$^{10,11}$. The regulatory limit of China corresponded to European Union. Due to the potential toxicological risk of DSP to public health and seafood industry, and thus it is necessary to develop practical, reliable and sensitive detection methods. Currently, several assays for DSP in biological samples have been proposed, including mouse bioassays, liquid chromatography coupled to fluorescent (LC-FLD) or mass spectrometric (LC-MS) detection, enzyme-linked immunosorbent assays, colloidal gold immunochromatographic assays, and phosphatase inhibition assays$^{12-16}$. Mouse bioassays produce a positive result only at high levels of total DSP in shellfish, while LC-MS/MS requires expensive equipment and skilled analysts, and colloidal gold probe-based immunochromatographic assays cannot be used quantitatively. Although the ELISA method is specific, sensitive and inexpensive, the colorimetric enzyme activity can be affected by the composition of the medium and the operating conditions. Furthermore, the chromogenic substrate is hazardous to health. To prevent matrix influences, samples should be diluted. However, the insufficient sensitivity of ELISA for OA limits its diagnostic usefulness. CLEIA has the advantages of both the specificity of immunoassays and the sensitivity of chemiluminescence. Over the last decade, CLEIAs have been widely used in clinical diagnostic testing. LuQiu Fang developed a micro-plate chemiluminescence enzyme immunoassay for aflatoxin B1 in agricultural products, and Ryo Tanaka evaluated the analytical and diagnostic accuracy of chemiluminescence enzyme immunoassays (CLEIA) for anti-CCP (autoantibodies against cyclic citrullinated peptide) antibodies and compared it with that of ELISA$^{17,18}$. Quan Wang first reported a chemiluminescent ELISA for diarrhetic shellfish poisoning toxins in shellfish$^{19}$. Subsequently, Marina M. Vdovenko applied a novel chemiluminescent enzyme-linked immunosorbent assay method for OA$^{20}$. However, the most serious drawback of prototype A was a lack of sensitivity, and prototype B had a narrow working range of 0.03-0.2 ng mL$^{-1}$. In this work, we present a sensitive and reliable CLEIA for the quantitative detection of OA and evaluate its feasibility using clinical samples.
2. Materials and methods

2.1. Apparatus

The BHP9504 micro-plate luminescence analyser was from Beijing Hamamatsu Technology Co., Ltd. (Beijing, China). Pipettes from Eppendorf Co., Ltd. (Germany) were used in all experiments.

2.2. Reagents

Okadaic acid (OA), domoic acid (DA), nodularin (NOD), and microcystin-LR (MC-LR) standard samples were purchased from ALEXIS® Biochemicals. Dinophysistoxin (DTX-1) was obtained from Wako Pure Chemicals Industries, Ltd. Saxitoxin (STX), gonyatoxin-1 and brevetoxin-2 (BTX-2) were obtained from ZEN-Biotechnology Co. Ltd. Tetrodotoxin (TTX) was obtained from Sigma. The monoclonal antibody against OA (McAb-OA) was produced by our laboratory, and the HRP-labelled goat anti-mouse IgG antibody was purchased from Dingguo Biotechnology Development Center (Beijing). Methanol (analytical purity) was purchased from Beijing Chemical Reagent Co. Ltd. (Beijing, China).

N-hydroxysuccinimide, N,N-dicyclohexylcarbodiimide (DCC) and N,N-dimethylformamide (N,N-DMF) were purchased from Sigma. Opaque high-binding plates for the chemiluminescence measurements were purchased from three different suppliers: Yijiamei Experiment Equipment Co. Ltd. (Fujian, China, termed plate A); GenoMintel Bioscience & Technology Development Co., Ltd. (Shanghai, China, termed plate B) and JET Bio-filtration Products, Co. Ltd. (Guangzhou, China, termed plate C). The substrate solutions were purchased from Sigma.

2.4. Buffers and calibration standards
A 50 mmol L\(^{-1}\) carbonate buffer (pH 9.6) was used as the OA-BSA coating solution. The blocking buffer was 50 mmol L\(^{-1}\) phosphate solution (PBS, pH 7.4) containing 10% foetal bovine serum and 0.01% thiomersalate. For the standard solution, 50 mmol L\(^{-1}\) PBS (pH 7.4) with 10% foetal bovine serum, 0.05% Tween-20 and 0.01% thiomersalate were used. The washing solution was 10 mmol L\(^{-1}\) PBS (pH 7.4) with 0.05% Tween-20 (PBS-T).

For calibration, serial dilutions of the standards with the standard solution matrix were prepared at concentrations of 0.0098 ng mL\(^{-1}\), 0.039 ng mL\(^{-1}\), 0.156 ng mL\(^{-1}\), 0.625 ng mL\(^{-1}\), 2.5 ng mL\(^{-1}\) and 10 ng mL\(^{-1}\), respectively.

### 2.5. Preparation of the coating antigen

The conjugate OA-BSA was prepared using a modification of previous methods\(^21\). A 60 μL aliquot of N,N-DMF containing 0.5 mg OA, 0.08 mg N-hydroxsuccinimide and 0.15 mg DCC was incubated for 2 h at room temperature and added to 2.0 mg BSA in 50 μL of 0.1 mol L\(^{-1}\) NaHCO\(_3\). The reaction was allowed to continue for another 2 h at room temperature. The unreacted reagents were removed by centrifugal ultrafiltration. The conjugates were dissolved in the appropriate volume of 10 mmol L\(^{-1}\) sodium phosphate-buffered saline (PBS, pH 7.4) with a final concentration of 1 g L\(^{-1}\) and then stored at -20 °C.

### 2.6. Coating of OA-BSA on the micro-plate

The micro-plates were coated with 100 μL (50 ng mL\(^{-1}\)) of OA-BSA per well diluted in 10 mM sodium phosphate-buffered saline (pH 7.4). The plate was allowed to stand sealed at 4 °C overnight. After removing all fluid from each well in the plate, 200 μL of blocking buffer was added to each well, and the plate was incubated at 37 °C for 2 h in order to block the unbound active sites.

### 2.7. Chemiluminescence enzyme immunoassay for OA

A 50 μL volume of OA calibration standard or shellfish sample was added to each well of the OA-BSA coated micro-plates. Simultaneously, 50 μL McAb-OA was
added (1:200,000 ratio). After incubation at 37 °C for 1 h for the competition reaction, the microplate was washed five times with PBST washing solution. A 100 μL volume of diluted goat anti-mouse IgG antibody labelled with HRP was added. After incubation at 37 °C for 1 h for the competition reaction, the microplate was washed as described above. Finally, 100 μL of the chemiluminescence (CL) substrate solution was added to each well and stirred. The relative light units (RLUs) were measured by a BHP9504 micro-plate luminescence analyser. Standard curve was obtained by inhibition rate against the logarithm natural of the analyte concentration and fitted to the equation of Y-lnX. The inhibition rate was calculated as: Inhibition Rate = (A₀−A) /A₀×100%. The A₀ and A were OD₄₉₂ values of control and analyzed sample, respectively. The schematic of the detection of OA with CLEIA is illustrated in Fig. 1.

An indirect competitive chemiluminescence enzyme immunoassay was developed using as-prepared OA-BSA labels based on a micro-plate. The monoclonal antibody against OA was captured with a specific binding antigen. Goat anti-mouse IgG antibody labeled with HRP was used as a secondary antibody combined with the McAb. The HRP complex catalyses the conversion of the chemiluminescent substrate into a sensitized reagent in the vicinity of the molecule of interest after the substrate solution is added to the microplate.

Fig. 1 Schematic representation of the micro-plate chemiluminescence enzyme immunoassay for OA.
2.8. Sample extraction and spiking\textsuperscript{22,23}

Shellfish samples or known non-toxic samples by the HPLC-MS/MS experiment to be spiked for use were extracted as follows: the ground shellfish meat and digestive glands (1 g) or the samples spiked with OA standard at concentrations of 0.5, 2.5, and 5 µg kg\textsuperscript{-1} were extracted with 2 mL 80\% (v/v) aqueous methanol. The supernatants of the homogenised samples obtained by centrifugation were mixed with 2-fold volume methane dichloride. Immediately following, 60\% (v/v) aqueous methanol was used to suspend the organic phase after being dried in a water bath at 40°C (the boiling point of methane dichloride is 39.8 °C). Finally, sodium phosphate-buffered saline (0.01 M, pH 7.4) was added to recover the original volume for analysis.

3. Results and discussion

3.1. Effects of micro-plate

The polystyrene micro-plates showed low background fluorescence and high surface binding force. The RLU was affected by high surface binding of micro-plate in the CLEIA for OA. Three types of microplates were used to study the fluence of RLUs. As shown in Fig. 2A, the RLU from plate A was higher than that from plate B or plate C under the same reaction conditions. The RLU increased with increasing reaction time. Thus, plate A was adopted for all subsequent studies because of its superior surface binding compared with that of the other plates.
Fig. 2 (A) Effect of the type of chemiluminescent plate. Three types of micro-plates, termed A, B, and C, were used to perform the assay, and the effect of the plate type on the RLU was measured. With increasing reaction time, the RLU decreased gradually. For the same incubation time, plate A presented the highest RLU among the three plates. (B) Effect of the pH of the coating solution. Buffers including 50 mmol L$^{-1}$ carbonate buffer (pH 9.6), 50 mmol L$^{-1}$ phosphate buffer (pH 8.0) and 100 mmol L$^{-1}$ Tris-HCl (pH 7.2) were used to study the effects of the coating solution on the RLU. The results revealed that the 50 mmol L$^{-1}$ carbonate buffer (pH 9.6) provided a higher RLU with increasing incubation time. (C) Effect of the dilution ratios of OA-BSA and McAb. The mean block titration was used to determine the optimal concentration of OA-BSA and McAb. (D) Effect of the dilution ratio of goat anti-mouse IgG.

3.2. Optimisation of coating solution

To evaluate the effect of the composition and concentration of the coating solution,
OA-BSA was diluted to pH 9.6, pH 8.0 and pH 7.2 with 50 mmol L\(^{-1}\) carbonate buffer, 50 mmol L\(^{-1}\) phosphate buffer and 100 mmol L\(^{-1}\) Tris-HCl, respectively. The pH value of the coating solution can affect the intermolecular bonds of the antigen binding to the antibody. Fig. 2B showed the effects of pH on the RLU of the CLEIA assay. The RLU increased with increasing pH from 7.2 to 9.6. The highest RLU was obtained when the reaction time was 5 min and the OA-BSA was diluted with 50 mmol L\(^{-1}\) carbonate buffer (pH 9.6).

3.3. Effects of dilution ratios of OA-BSA and McAb against OA

The dilution ratios of the McAb-OA and OA-BSA dramatically impacted the RLU. As shown in Fig. 2C, the RLU increased with increasing McAb-OA concentration and decreased with the increasing dilution ratio of OA-BSA. Considering the dependence of the sensitivity, reliability, and kinetic range of the assay on the volume of OA-BSA, the most suitable dilution ratio of the McAb was 1:200,000, and that of OA-BSA was 50 ngmL\(^{-1}\).

3.4 Effects of the dilution of the HRP-labelled goat anti-mouse IgG antibody

In general, the dilution of HRP can limit assay sensitivity because HRP participates in the chromogenic reaction. The RLU declined with increasing dilution ratio of the goat anti-mouse IgG antibody labelled with HRP (Fig. 2D). The highest RLU was obtained when the dilution ratio of the HRP-labelled goat anti-mouse IgG antibody was 1:2,000, but the lowest RLUs/s\(s_0\) was obtained when the dilution ratio of the HRP-labelled goat anti-mouse IgG antibody was 1:5,000. Therefore, in further work, 1:5,000 was chosen as the most suitable dilution ratio for the goat anti-mouse IgG.

3.5. Effect of coating and blocking time
The coating and blocking time played an important role on the sensitivity of the CLEIA for OA. The coating conditions affect the amount of antibody binding on the microplate (Fig. 3A). Excessive antibody enrichment in wells might increase steric hindrance and reduce the binding opportunities between the antigen and antibody. In addition, the use of a blocking solution can reduce the number of non-specific binding sites. As shown in Fig. 3B, the RLU increased with blocking time in the range of 30-200 min. However, the RLU decreased when the blocking time was greater than 120 min. We speculated that the thick blocking layer increased the steric effect. Considering the assay sensitivity, the optimal coating time at 4 °C was determined to be 24 h, and the optimal blocking time was 120 min. We consider that large time was token on blocking because the ultrasensitive CLEIA was susceptible to non-specific binding interference. Extension of the blocking time was benefit for minimizing nonspecific binding, thus providing low negative background. It is crucial to obtain reliable data in analysis of contamination samples. In further work, to study CLEIA kit for OA, we consider that the microplates after blocking could be sealed in 4 °C for analysis actual samples.
3.6. Optimisation of the incubation time

OA-BSA (50 ng mL\(^{-1}\)), McAb-OA (1:200,000) and HRP-labelled goat anti-mouse IgG (1:5,000) diluted with 50 mmol L\(^{-1}\) phosphate buffer (pH 8.0) were used to study the effect of the incubation time on the RLU at 37 °C. The results shown in Fig. 4(D) indicated that the RLU increased linearly with increasing incubation time. The effect of the incubation time on the McAb-OA standard curve was shown in Table 1. The RLU\(_{S0}\) increased with increasing incubation time. An excellent correlation coefficient...
and high sensitivity were obtained when the incubation time was 60 or 90 min. To
decrease the detection time and improve the efficiency, 60 min was selected as the
incubation time.

![Graph](image1.png)

Fig. 4 (A) Effect of the incubation time. OA-BSA (50), McAb-OA (1:200,000) and goat anti-mouse
IgG antibody labeled with HRP (1:5,000) diluted in 50 mmol L⁻¹ phosphate buffer (pH 8.0) were used
to study the effect of incubation time on the RLU at 37 °C.

(B) Dose–response curve for OA. Each sample was diluted progressively from 0.0098 to 10 µg kg⁻¹
with dilution buffer.

Table 1. Effect of the incubation time

<table>
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<tr>
<th>Incubation time (min)</th>
<th>RLU₅₀</th>
<th>RLU₅₀/₀.₀₀₉₅₀</th>
<th>RLU₅₀/₅₅₀</th>
<th>Correlation coefficient (r)</th>
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<td>-0.9999</td>
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<td>0.8523</td>
<td>Logit(Y)=−0.1310−1.9203 Log(x)</td>
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</table>

*RLU₅₀ represents the RLU when the free-OA samples were analysed. RLU₅₀/₀.₀₀₉₅₀ and RLU₅₀/₅₅₀ represent
the RLU_s when 0.009 ng mL⁻¹ and 5 ng mL⁻¹ OA sample were analysed, respectively.

3.7. Optimisation of the CL reaction time

The reaction time was optimised in order to maximise the sensitivity of the CLEIA
assay. OA-BSA diluted to 50 ng mL⁻¹, HRP-labelled goat anti-mouse IgG antibody
diluted 1:5,000 and McAb-OA diluted 1:200,000 were utilised to study the effects of
the CL reaction time on the RLU. The results indicated that the RLU decreased with increasing reaction time in the range of 5-60 min, as shown in Table 2. When the reaction time was 10 min, the linear range, sensitivity and correlation were suitable. Therefore, a reaction time of 10 min was selected for further experiments.

<table>
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<th>Reaction time (min)</th>
<th>RLU_{S50}</th>
<th>RLU_{S0.009/S50}</th>
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<td></td>
<td></td>
<td></td>
<td>Log(x)</td>
<td></td>
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<tr>
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<td></td>
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<td>Logit(Y)=-0.0397-1.0854</td>
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<td></td>
<td></td>
<td>Log(x)</td>
<td></td>
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</table>

*RLU_{S50} represents the RLU when the free-OA samples were analysed. RLU_{S0.009} and RLU_{S5} represent the RLU when 0.009 ng mL\(^{-1}\) and 5 ng mL\(^{-1}\) OA samples were analysed, respectively.

3.8. Methodology evaluation

3.8.1. Standard curve and sensitivity

Under the optimal conditions, a dose-response curve for OA was established with a linear range of 0.0098-10.0 ng g\(^{-1}\). The linear equation was Y = 59.74+12.99 Ln (X), R=0.99. The detection limit was defined as the minimum dose that could be distinguished from zero. The minimum detected concentration of OA was 0.0098 ng g\(^{-1}\), which was lower than that of ELISA (developed by our colleagues, with a sensitivity of 0.3 ng mL\(^{-1}\)).

3.8.2. Assessment of the assay precision

The intra- and inter-assay precisions, calculated by measuring the OA concentration in three different samples, were determined. Good precisions were obtained. The
intra-assay coefficients of variation (CV) varied from 6.9% to 8.9% (n = 10). The inter-assay CVs varied from 7.5% to 9.1% and were less than 10.0% (n = 10).

3.8.3. Assessment of the assay specificity

The cross reactivity was determined using the optimised CLEIA system. Eight marine toxins were selected for cross-reactive experiments to evaluate the specificity of the McAb against OA by performing competitive assays, including DA, NOD, MC-LR, DTX-1, STX, GTX-1, TTX and BTX-2. There was no cross-reactivity with other marine toxins except for DTX-1, which also belongs to the DSP toxin family and has a similar structure to that of OA. The cross-reactive rate for DTX-1 was approximately 100%, similar to that found for the corresponding ELISA (data not shown). The TEF (toxic equivalency factor) values of both OA and DTX-1 are 1.24. Thus, the 100% cross-reactivity of the assay with DTX-1 does not hinder the ability of the assay to determine the safety level of shellfish. The cross-reactivity could not be evaluated for DTX-2 or DTX-3 due to their unavailability in China. Thus, the cross-reactivity of the assay with other OA homologues, including DTX-2, DTX-3 and okadaic acid esters, requires further study.

3.8.3. Recovery

The proposed method was used to detect OA in seashell samples, and the accuracy was studied by recovery experiments. Ground shellfish meat and digestive glands (1 g) were spiked with OA standard at concentrations of 0.5, 2.5 and 5 µg kg\(^{-1}\) before the extraction procedure. The recovery experiment was repeated five times, and the average recoveries of the low, middle and high concentration samples were 97.2%, 111.2% and 104.7%, respectively.

Table 3. Recoveries of the CLEIA (n = 5)

<table>
<thead>
<tr>
<th>Spiked sample</th>
<th>Spiked amount (µg kg(^{-1}))</th>
<th>Determined amounts (µg kg(^{-1}))</th>
<th>Recovery (%)</th>
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<td>0.483±0.30</td>
<td>96.5%</td>
</tr>
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<td></td>
<td>2.5</td>
<td>2.77±0.16</td>
<td>112.4%</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.235±0.45</td>
<td>104.7%</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.485±0.20</td>
<td>97.4%</td>
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<tr>
<td>2</td>
<td>2.5</td>
<td>2.84±0.31</td>
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<tr>
<td></td>
<td>5</td>
<td>5.245±0.38</td>
<td>104.9%</td>
</tr>
</tbody>
</table>
3.8.4. Analysis of shellfish samples

The proposed CLEIA was applied to evaluate OA in shellfish samples purchased from local markets. The samples were prepared as described in section 2.8. Before analysing the samples using the proposed method, all samples were diluted 20-fold with 0.01 M PBS. The results indicated that the average concentrations of OA in the 5 samples, *Penaeus vannamei*, *Meretrix petechialis*, *Periglypta puerpera*, *Mactra chinensis* and *Scapharca broughtonii* were 3.54, 1.93, 9.56, 24.68 and 48.56 µg kg⁻¹, respectively. All studied samples of shellfish products displayed an edible safety level below 160 µg kg⁻¹, the maximum acceptable level of OA in the European Union. However, some shellfish were found to be contaminated with OA or its analogues and were thus potentially harmful to the health and safety of consumers.

4. Conclusion

A sensitive, and reliable CLEIA was developed for the measurement of OA in seashells. The determination range was 0.0098-10.0 µg kg⁻¹. The sensitivity was 0.0098 µg kg⁻¹, which was an order of magnitude better than that of ELISA. Furthermore, the assay was successfully implemented for the determination of OA in seafood, and the results indicated that the average concentrations of OA in the 5 samples, *Penaeus vannamei*, *Meretrix petechialis*, *Periglypta puerpera*, *Mactra chinensis* and *Scapharca broughtonii*, were 3.54, 1.93, 9.56, 24.68 and 48.56 µg kg⁻¹, respectively. All analyzed samples were safe to eat, with OA levels below 160 µg kg⁻¹, the maximum acceptable level of OA in the European Union. Above all, the proposed assay provided noticeable advantages over ELISA and will facilitate the analysis of a massive number of samples using this safe assay in the market.

References


