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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

1. 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 clams[1-5]. 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 symptoms[6-8]. The toxins are stable at high temperatures and have long-term carcinogenicity[9]. 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.

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4 86 *2. Materials and methods*
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6 87 *2.1. Apparatus*
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8 88 The BHP9504 micro-plate luminescence analyser was from Beijing Hamamatsu
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10 89 Technology Co., Ltd. (Beijing, China). Pipettes from Eppendorf Co., Ltd. were used
11
12 90 in all experiments (Germany).
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14 91 *2.2. Reagents*
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16 92 Okadaic acid (OA), domoic acid (DA), nodularin (NOD), and microcystin-LR
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19 93 (MC-LR) standard samples were purchased from ALEXIS® Biochemicals.
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21
22 94 Dinophysistoxin (DTX-1) was obtained from Wako Pure Chemicals Industries, Ltd.
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25 95 Saxitoxin (STX), gonyatoxin-1 and brevetoxin-2 (BTX-2) were obtained from ZEN-U
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27 96 Biotechnology Co. Ltd. Tetrodotoxin (TTX) was obtained from Sigma. The
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30 97 monoclonal antibody against OA (McAb-OA) was produced by our laboratory, and
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33 98 the HRP-labelled goat anti-mouse IgG antibody was purchased from Dingguo
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35 99 Biotechnology Development Center (Beijing). Methanol (analytical purity) was
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38 100 purchased from Beijing Chemical Reagent Co. Ltd. (Beijing, China).
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41 101 N-hydroxysuccinimide, N,N-dicyclohexylcarbodiimide (DCC) and
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43 102 N,N-dimethylformamide (N,N-DMF) were purchased from Sigma. Opaque
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46 103 high-binding plates for the chemiluminescence measurements were purchased from
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48
49 104 three different suppliers: Yijiamei Experiment Equipment Co. Ltd. (Fujian, China,
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51 105 termed plate A); GenoMintel Bioscience & Technology Development Co., Ltd.
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54 106 (Shanghai, China, termed plate B) and JET Bio-filtration Products, Co. Ltd.
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57 107 (Guangzhou, China, termed plate C). The substrate solutions were purchased from
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59 108 Sigma.
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109 *2.4. Buffers and calibration standards*

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4 110 A 50 mmol L^{-1} carbonate buffer (pH 9.6) was used as the OA-BSA coating solution.
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6 111 The blocking buffer was 50 mmol L^{-1} phosphate solution (PBS, pH 7.4) containing
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8 112 10% foetal bovine serum and 0.01% thiomersalate. For the standard solution, 50
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10 113 mmol L^{-1} PBS (pH 7.4) with 10% foetal bovine serum, 0.05% Tween-20 and 0.01%
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12 114 thiomersalate were used. The washing solution was 10 mmol L^{-1} PBS (pH 7.4) with
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14 115 0.05% Tween-20 (PBST).
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16 116 For calibration, serial dilutions of the standards with the standard solution matrix were
17
18 117 prepared at concentrations of 0.0098 ng mL^{-1}, 0.039 ng mL^{-1}, 0.156 ng mL^{-1}, 0.625 ng
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20 118 mL^{-1}, 2.5 ng mL^{-1} and 10 ng mL^{-1}, respectively.
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22 119 *2.5. Preparation of the coating antigen*
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25 120 The conjugate OA-BSA was prepared using a modification of previous methods[21]. A
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28 121 60 μL aliquot of N,N-DMF containing 0.5 mg OA, 0.08 mg N-hydroxysuccinimide
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31 122 and 0.15 mg DCC was incubated for 2 h at room temperature and added to 2.0 mg
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33 123 BSA in 50 μL of 0.1 mol L^{-1} $NaHCO_3$. The reaction was allowed to continue for
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35
36 124 another 2 h at room temperature. The unreacted reagents were removed by centrifugal
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38
39 125 ultrafiltration. The conjugates were dissolved in the appropriate volume of 10 mmol
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41 126 L^{-1} sodium phosphate-buffered saline (PBS, pH 7.4) with a final concentration of 1 g
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44 127 L^{-1} and then stored at -20 ℃.
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46 128 *2.6. Coating of OA-BSA on the micro-plate*
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48 129 The micro-plates were coated with 100 μL (50 ng mL^{-1}) of OA-BSA per well diluted
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50 130 in 10 mM sodium phosphate-buffered saline (pH 7.4). The plate was allowed to stand
51
52 131 sealed at 4 ℃ overnight. After removing all fluid from each well in the plate, 200 μL
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54 132 of blocking buffer was added to each well, and the plate was incubated at 37 ℃ for 2
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56 133 h in order to block the unbound active sites.
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58 134 *2.7. Chemiluminescence enzyme immunoassay for OA*
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60 135 A 50 μL volume of OA calibration standard or shellfish sample was added to each
136 well of the OA-BSA coated micro-plates. Simultaneously, 50 μL McAb-OA was

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4 137 added (1:200,000 ratio). After incubation at 37 ℃ for 1 h for the competition reaction,
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6 138 the microplate was washed five times with PBST washing solution. A 100 μL volume
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8 139 of diluted goat anti-mouse IgG antibody labelled with HRP was added. After
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10 140 incubation at 37 ℃ for 1 h for the competition reaction, the microplate was washed as
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12 141 described above. Finally, 100 μL of the chemiluminescence (CL) substrate solution
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14 142 was added to each well and stirred. The relative light units (RLUs) were measured by
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16 143 a BHP9504 micro-plate luminescence analyser. Standard curve was obtained by
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18 144 inhibition rate against the logarithm natural of the analyte concentration and fitted to
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20 145 the equation of Y-lnX. The inhibition rate was calculated as: Inhibition Rate＝ (A_0—
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22 146 A) /A_0×100%. The A_0 and A were OD_{492} values of control and analyzed sample,
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24 147 respectively. The schematic of the detection of OA with CLEIA is illustrated in Fig. 1.
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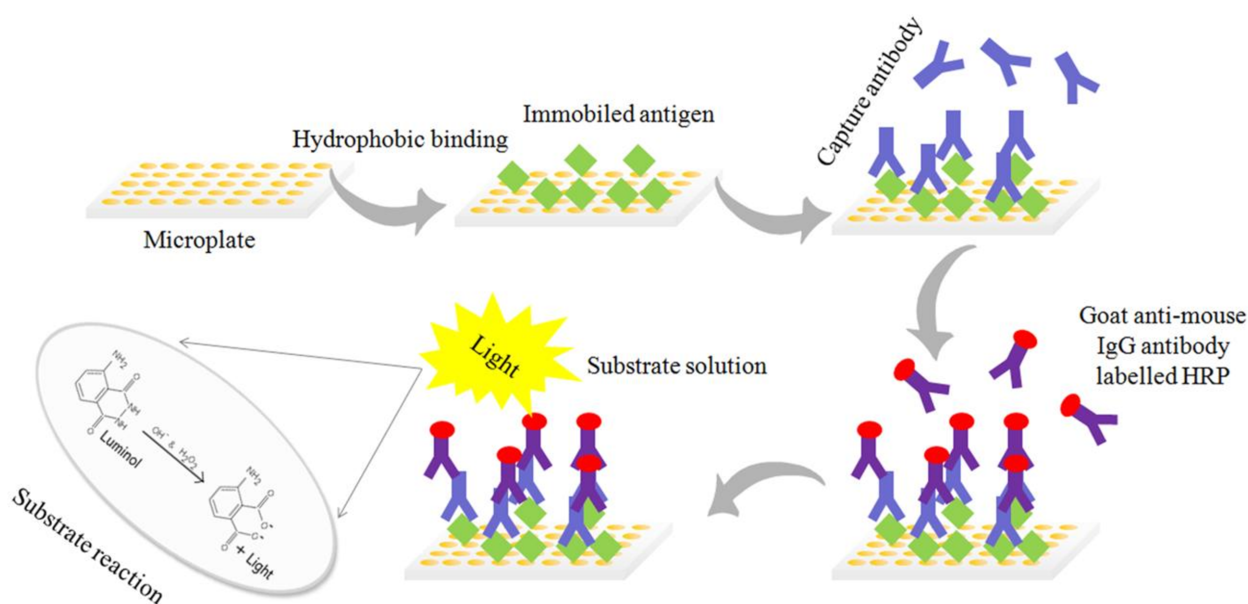

47 148
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49 149 Fig. 1 Schematic representation of the micro-plate chemiluminescence enzyme immunoassay for OA.
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51 150 An indirect competitive chemiluminescence enzyme immunoassay was developed using as-prepared
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53 151 OA-BSA labels based on a micro-plate. The monoclonal antibody against OA was captured with a
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55 152 specific binding antigen. Goat anti-mouse IgG antibody labeled with HRP was used as a secondary
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57 153 antibody combined with the McAb. The HRP complex catalyses the conversion of the
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59 154 chemiluminescent substrate into a sensitized reagent in the vicinity of the molecule of interest after the
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155 substrate solution is added to the microplate.

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4 156 *2.8. Sample extraction and spiking*[22, 23]
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6 157 Shellfish samples or known non-toxic samples by the HPLC-MS/MS experiment to
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8 158 be spiked for use were extracted as follows: the ground shellfish meat and digestive
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10 159 glands (1 g) or the samples spiked with OA standard at concentrations of 0.5, 2.5, and
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12 160 5μg kg^{-1} were extracted with 2 mL 80% (v/v) aqueous methanol. The supernatants of
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14 161 the homogenised samples obtained by centrifugation were mixed with 2-fold volum
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16 162 methane dichloride. Immediately following, 60% (v/v) aqueous methanol was used to
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18 163 suspend the organic phase after being dried in a water bath at 40 ℃ (the boiling point
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20 164 of methane dichloride is 39.8 ℃). Finally, sodium phosphate-buffered saline (0.01 M,
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22 165 pH 7.4) was added to recover the original volume for analysis.
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24 166 *3. Results and discussion*
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27 167 *3.1. Effects of micro-plate*
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30 168 The polystyrene micro-plates showed low background fluorescence and high surface
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33 169 binding force. The RLU was affected by high surface binding of micro-plate in the
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35 170 CLEIA for OA. Three types of microplates were used to study the fluence of RLUs.
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38 171 As shown in Fig. 2A, the RLU from plate A was higher than that from plate B or plate
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41 172 C under the same reaction conditions. The RLU increased with increasing reaction
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43 173 time. Thus, plate A was adopted for all subsequent studies because of its superior
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46 174 surface binding compared with that of the other plates.
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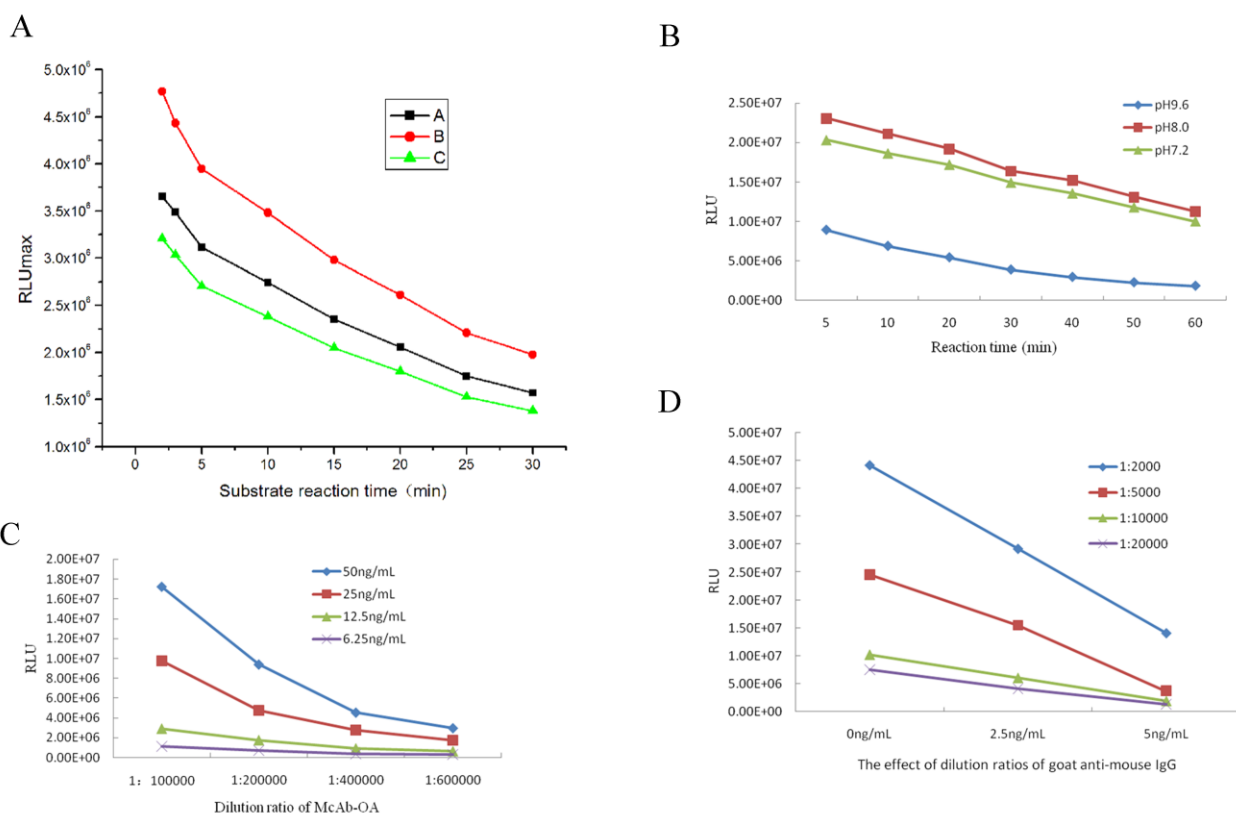

Fig. 2 (A) Effect of the type of chemiluminescent plate. Three types of micro-plates, termed A, B, and C, were used to perform the assay, and the effect of the plate type on the RLU was measured. With increasing reaction time, the RLU decreased gradually. For the same incubation time, plate A presented 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,

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

189 OA-BSA was diluted to pH 9.6, pH 8.0 and pH 7.2 with 50 mmol L^{-1} carbonate buffer,
190 50 mmol L^{-1} phosphate buffer and 100 mmol L^{-1} Tris-HCl, respectively. The pH value
191 of the coating solution can affect the intermolecular bonds of the antigen binding to
192 the antibody. Fig. 2B showed the effects of pH on the RLU of the CLEIA assay. The
193 RLU increased with increasing pH from 7.2 to 9.6. The highest RLU was obtained
194 when the reaction time was 5 min and the OA-BSA was diluted with 50 mmol L^{-1}
195 carbonate buffer (pH 9.6).

196 *3.3. Effects of dilution ratios of OA-BSA and McAb against OA*

197 The dilution ratios of the McAb-OA and OA-BSA dramatically impacted the RLU. As
198 shown in Fig. 2C, the RLU increased with increasing McAb-OA concentration and
199 decreased with the increasing dilution ratio of OA-BSA. Considering the dependence
200 of the sensitivity, reliability, and kinetic range of the assay on the volume of OA-BSA,
201 the most suitable dilution ratio of the McAb was 1:200,000, and that of OA-BSA was
202 50 $ngmL^{-1}$.

203 *3.4 Effects of the dilution of the HRP-labelled goat anti-mouse IgG antibody*

204 In general, the dilution of HRP can limit assay sensitivity because HRP participates in
205 the chromogenic reaction. The RLU declined with increasing dilution ratio of the goat
206 anti-mouse IgG antibody labelled with HRP (Fig. 2D). The highest RLU was obtained
207 when the dilution ratio of the HRP-labelled goat anti-mouse IgG antibody was 1:2,000,
208 but the lowest $RLUs_5/s_0$ was obtained when the dilution ratio of the HRP-labelled goat
209 anti-mouse IgG antibody was 1:5,000. Therefore, in further work, 1:5,000 was chosen
210 as the most suitable dilution ratio for the goat anti-mouse IgG.

211 *3.5. Effect of coating and blocking time*

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4 212 The coating and blocking time played an important role on the sensitivity of the
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6 213 CLEIA for OA. The coating conditions affect the amount of antibody binding on the
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8 214 microplate (Fig. 3A). Excessive antibody enrichment in wells might increase steric
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10 215 hindrance and reduce the binding opportunities between the antigen and antibody. In
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12 216 addition, the use of a blocking solution can reduce the number of non-specific binding
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14 217 sites. As shown in Fig. 3B, the RLU increased with blocking time in the range of
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16 218 30-200 min. However, the RLU decreased when the blocking time was greater than
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18 219 120 min. We speculated that the thick blocking layer increased the steric effect.
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20 220 Considering the assay sensitivity, the optimal coating time at 4 ℃ was determined to
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22 221 be 24 h, and the optimal blocking time was 120 min. we consider that large time was
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24 222 token on blocking because the ultrasensitive CLEIA was susceptible to non-specific
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26 223 binding interference. Extension of the blocking time was benefit for minimizing
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28 224 nonspecific binding, thus providing low negative background. It is crucial to obtain
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30 225 reliable data in analysis of contamination samples. In further work, to study CLEIA
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32 226 kit for OA, we consider that the microplates after blocking could be sealed in 4 ℃ for
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34 227 analysis actual samples.
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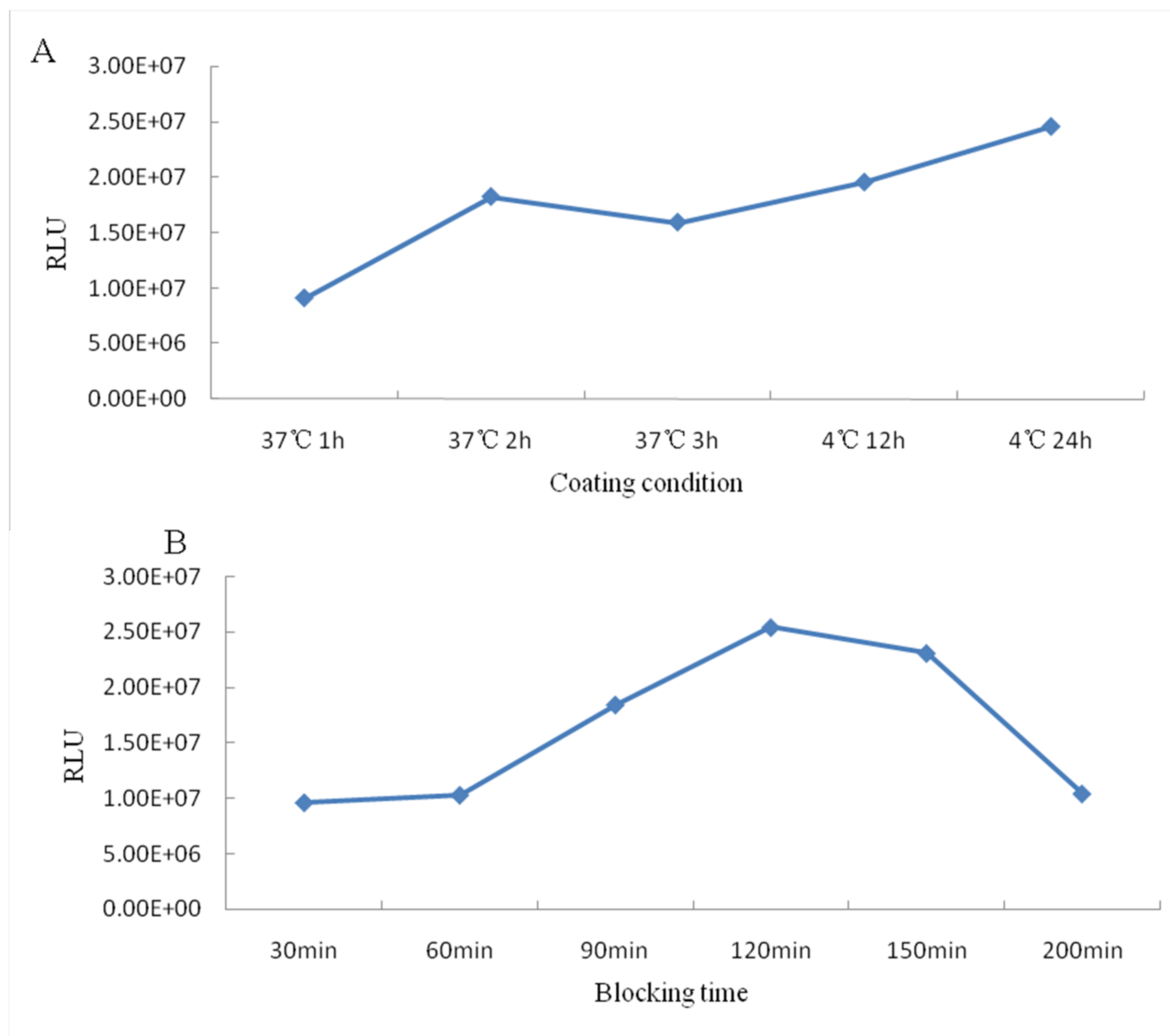

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229 Fig. 3 Effect of coating and blocking time. (A) Coating conditions of 37 ℃ for 1 h, 37 ℃ for 2 h,
230 37 ℃ for 3 h, 4 ℃ for 12 h and 37 ℃ for 24 h were examined for their influence on the RLU. (B)
231 Blocking times of 30-200 min with 1% BSA blocking solution were tested.

232

233 *3.6. Optimisation of the incubation time*

234 OA-BSA (50 ng mL^{-1}), McAb-OA (1:200,000) and HRP-labelled goat anti-mouse
235 IgG (1:5,000) diluted with 50 mmol L^{-1} phosphate buffer (pH 8.0) were used to study
236 the effect of the incubation time on the RLU at 37 ℃. The results shown in Fig. 4(D)
237 indicated that the RLU increased linearly with increasing incubation time. The effect
238 of the incubation time on the McAb-OA standard curve was shown in Table 1. The
239 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.

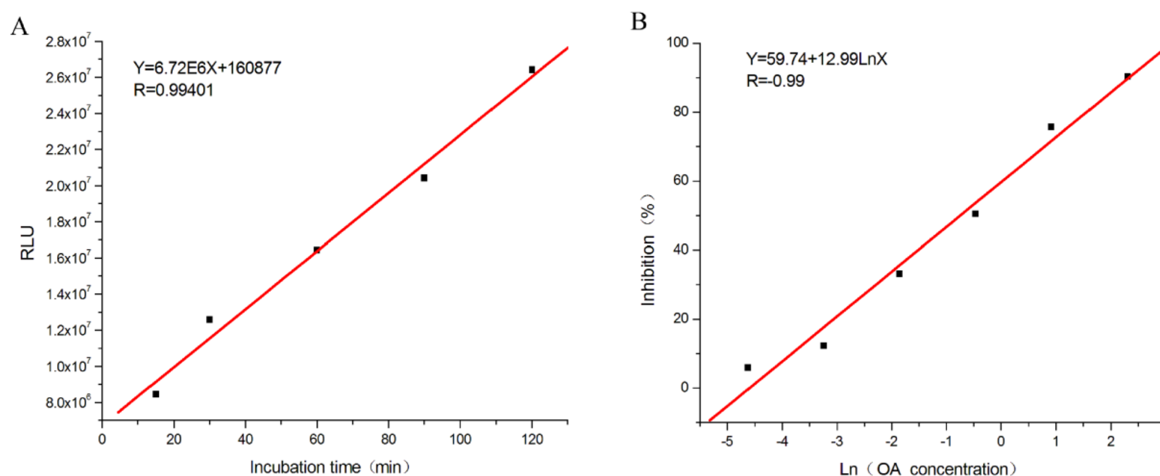

Fig. 4 (A) Effect of the incubation time. OA-BSA (50), McAb-OA (1:200,000) and goat anti-mouse IgG antibody labeled with HRP (1:5,000) diluted in 50 mmol L^{-1} phosphate buffer (pH 8.0) were used to study the effect of incubation time on the RLU at 37 ℃.
(B) Dose–response curve for OA. Each sample was diluted progressively from 0.0098 to 10 μg kg^{-1} with dilution buffer.

Table 1. Effect of the incubation time

Incubation time (min)	RLU_{S0}	$RLU_{S0.009/S0}$	$RLU_{S5/S0}$	Correlation coefficient (r)	Linear equation
15	5356130	0.9123	0.3109	0.8934	Logit(Y)=-0.0410-1.1775 Log(x)
30	22907450	0.9067	0.2725	-0.9921	Logit(Y)=-0.0670-1.0585 Log(x)
60	29345796	0.9345	0.2421	-0.1000	Logit(Y)=-0.0016-1.0474 Log(x)
90	37510730	0.9321	0.2811	-0.9999	Logit(Y)=-0.0050-1.2884 Log(x)
120	46789831	0.8875	0.3452	0.8523	Logit(Y)=-0.1310-1.9203 Log(x)

*RLU_{S0} represents the RLU when the free-OA samples were analysed. $RLU_{S0.009}$ and RLU_{S5} represent the RLUs 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

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4 257 the CL reaction time on the RLU. The results indicated that the RLU decreased with
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6 258 increasing reaction time in the range of 5-60 min, as shown in Table 2. When the
7
8 259 reaction time was 10 min, the linear range, sensitivity and correlation were suitable.
9
10 260 Therefore, a reaction time of 10 min was selected for further experiments.
11
12 261 Table 2. Effect of the CL reaction time

Reaction time (min)	RLU_{S0}	$RLU_{S0.009/S0}$	$RLU_{S5/S0}$	Correlation coefficient （r）	Linear equation
5	34124054	0.9123	0.3109	0.8934	Logit(Y)=-0.0312-1.0943 Log(x)
10	22907450	0.9467	0.2725	-0.9921	Logit(Y)=-0.0267-0.8932 Log(x)
20	19345734	0.9345	0.2421	-0.9806	Logit(Y)=-0.0091-1.0287 Log(x)
30	11513731	0.8921	0.2811	-0.9075	Logit(Y)=-0.0049-1.1086 Log(x)
40	7781831	0.8875	0.3452	0.8523	Logit(Y)=-0.0212-0.0875 Log(x)
60	4566777	0.8709	0.3209	0.8643	Logit(Y)=-0.0397-1.0854 Log(x)

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

264

265 *3.8. Methodology evaluation*

266 *3.8.1. Standard curve and sensitivity*

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

273 *3.8.2. Assessment of the assay precision*

274 The intra- and inter-assay precisions, calculated by measuring the OA concentration
275 in three different samples, were determined. Good precisions were obtained. The

1 2 3
4 276 intra-assay coefficients of variation (CV) varied from 6.9% to 8.9% (n = 10). The
5 6 277 inter-assay CVs varied from 7.5% to 9.1% and were less than 10.0% (n = 10).
7 8 278 *3.8.3. Assessment of the assay specificity*
9 10 279 The cross reactivity was determined using the optimised CLEIA system. Eight marine
11 12 280 toxins were selected for cross-reactive experiments to evaluate the specificity of the
13 14 281 McAb against OA by performing competitive assays, including DA, NOD, MC-LR,
15 16 282 DTX-1, STX, GTX-1, TTX and BTX-2. There was no cross-reactivity with other
17 18 283 marine toxins except for DTX-1, which also belongs to the DSP toxin family and has
19 20 284 a similar structure to that of OA. The cross-reactive rate for DTX-1 was
21 22 285 approximately 100%, similar to that found for the corresponding ELISA (data not
23 24 286 shown). The TEF (toxic equivalency factor) values of both OA and DTX-1 are 1[24].
25 26 287 Thus, the 100% cross-reactivity of the assay with DTX-1 does not hinder the ability of
27 28 288 the assay to determine the safety level of shellfish. The cross-reactivity could not be
29 30 289 evaluated for DTX-2 or DTX-3 due to their unavailability in China. Thus, the
31 32 290 cross-reactivity of the assay with other OA homologues, including DTX-2, DTX-3
33 34 291 and okadaic acid esters, requires further study.
35 36 292 *3.8.3. Recovery*
37 38 39 293 The proposed method was used to detect OA in seashell samples, and the accuracy
40 41 294 was studied by recovery experiments. Ground shellfish meat and digestive glands (1 g)
42 43 295 were spiked with OA standard at concentrations of 0.5, 2.5 and 5 μg kg^{-1} before the
44 45 296 extraction procedure. The recovery experiment was repeated five times, and the
46 47 297 average recoveries of the low, middle and high concentration samples were 97.2%,
48 49 298 111.2% and 104.7%, respectively.
50 51 299 Table 3. Recoveries of the CLEIA (n = 5)

Spiked sample	Spiked amount (μg kg^{-1})	Determined amounts (μg kg^{-1})	Recovery (%)
	0.5	0.483±0.30	96.5%
1	2.5	2.77±0.16	112.4%
	5	5.235±0.45	104.7%
	0.5	0.485±0.20	97.4%
2	2.5	2.84±0.31	113.6%
	5	5.245±0.38	104.9%

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	0.5	0.488±0.25	97.5%
3	2.5	2.75±0.18	112.6%
	5	5.225±0.49	104.5%

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301 *3.8.4. Analysis of shellfish samples*

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

312 *4. Conclusion*

313 A sensitive,and reliable CLEIA was developed for the measurement of OA in
314 seashells. The determination range was 0.0098-10.0 μg kg^{-1}. The sensitivity was
315 0.0098 μg kg^{-1}, which was an order of magnitude better than that of ELISA.
316 Furthermore, the assay was successfully implemented for the determination of OA in
317 seafood, and the results indicated that the average concentrations of OA in the 5
318 samples, *Penaeus vannamei*, *Meretrix pethechialis*, *Periglypta puerperal, Mactra*
319 *chinensis* and *Scapharca broughtonii*, were 3.54, 1.93, 9.56, 24.68 and 48.56 μg kg^{-1},
320 respectively. All analyzed samples were safe to eat, with OA levels below 160 μg kg^{-1},
321 the maximum acceptable level of OA in the European Union. Above all, the proposed
322 assay provided noticeable advantages over ELISA and will facilitate the analysis of a
323 massive number of samples using this safe assay in the market.

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