Sensitive time-resolved fluoroimmunoassay for the quantitative detection of okadaic acid

Yuan Qin*†, Jiayu Li†, Jiani Kuang, Sicheng Shen, Jingwen Jiang, Zhi Zhang, Chenhao Zhao, Xiumei Zhou, Biao Huang* and Bingnan Han*

College of Life Sciences and Medicine, Zhejiang Sci-Tech University, Hangzhou, China

Okadaic acid (OA) is a biotoxin from marine microalgae and widely present in shellfish, which severely affects the seafood safety. Therefore, it is essential to establish a highly sensitive OA analysis and detection method. In this study, a new type of immunoassay technology was established on the basis of the competition method using time-resolved fluoroimmunoassay (TRFIA). OA-bovine serum albumin (OA-BSA) coated on a 96-well plate competes with OA standard or samples to bind OA antibodies. A rare-earth ion-labeled secondary antibody, which fluoresces strongly under the effect of the enhancement solution, was then added as a tracer for detection. The established linear range of OA detected by TRFIA was $2.49 \times 10^{-3} - 50$ ng/ml, and the limit of detection was 2.49×10^{-3} ng/ml. The average coefficients of variation from intra-assay and inter-assay batches were 3.34% and 5.87%, respectively, and the recovery rate was 93.04%–111.66%. The OA in shellfish samples was determined by TRFIA and high-performance liquid chromatography (HPLC), and the results showed a good correlation. This study established a TRFIA to detect OA, which has the characteristics of simplicity, sensitivity, precision, and high accuracy, far exceeding the EU or the US standards for the detection of shellfish toxins. It is expected to make proper contribution in marine biotoxin detection.

KEYWORDS

okadaic acid, time-resolved fluoroimmunoassay, quantitative detection, diarrheic shellfish poisoning, phycotoxins, shellfish

Introduction

Under the influence of factors such as climate change, some algae have the ability to produce toxic compounds, and some of them can simultaneously produce multiple algal toxins (Caron et al., 2010; Griffith and Gobler, 2020; Onofrio et al., 2020; Marampouti et al., 2021). This adversely affects the ecosystem. Toxins from harmful algal blooms

(HABs) can poison or even kill shellfish and other marine organisms and can be passed on to humans through the food chain or direct contact, thus causing poisoning and even death in humans (Klijnstra et al., 2021; Hu et al., 2022). About half of all unnatural deaths in marine mammals can be attributed to ingestion of HABs or animals contaminated with them and cause about 60,000 annual poisonings worldwide (Griffith and Gobler, 2020; Klijnstra et al., 2021). Marine biotoxin pollution leads to direct economic losses and limits the development of the ocean farming and seriously affects human health and safety (Ajani et al., 2021; Tian et al., 2021). Since the 20th century, shellfish poisoning incidents have been reported in many countries. According to the symptoms of poisoning, shellfish toxins are divided into paralytic shellfish poisoning (PSP), neurotic shellfish poisoning, amnesic shellfish poisoning (ASP), and diarrheic shellfish poisoning (DSP) (Fraga et al., 2013). Among these shellfish toxins, DSP is the most frequently reported to cause poisoning worldwide (Lloyd et al., 2013).

OA is first discovered and the main kind of DSP (Verma et al., 2020). It was first identified from *Halichondria melanodocia* and *Halichondria okadai* in 1981 and is mainly produced by phytoplankton such as *Prorocentrum lima*, *Prorocentrum concavum*, *Dynophysis acuta*, and *Dynophysis fortii*, etc. (Valdiglesias et al., 2013). Like other algal toxin, OA is mainly accumulate by bivalve mollusks, such as scallop, mussel, and oyster and other shellfish through filter feeding of harmful algae, passed to humans through the food chain, causing human poisoning (Pérez et al., 2019; Yin et al., 2022). OA is colorless and odorless, making it challenging to recognize (Valdiglesias et al., 2013). Its high lipophilicity degree makes OA easy to accumulate in adipose tissue and not easily metabolized and digested (Reguera et al., 2014). The most studied toxicological mechanism of OA is its inhibitory effect on serine/threonine protein phosphatase (Fu et al., 2019). This toxin can cause diarrhea, nausea, and other typical DSP poisoning symptoms and have carcinogenesis and neurotoxicity effects (Valdiglesias et al., 2013; Ferron et al., 2014).

The general detection methods of OA are roughly divided into instrumental analysis (such as HPLC and liquid chromatography mass spectrometry) and immunoassay (such as enzyme-linked immunosorbent assay, namely ELISA and immunochromatographic assay). In recent years, some novel detection methods have also been developed. For example, the colorimetric detection system was established by fixing protein phosphatase 1 (PP1) in a microfluidic chamber modified by Al_2O_3 sol–gel to determine the inhibitory effect of OA on PP1; the Au@Pt/HRP immunoassay for OA detection was established by using the dual catalytic action of Au@Pt NPs and HRP on TMB-H_2O_2 (Castanheira et al., 2021; Tian et al., 2022). Among the detection methods, immunoassays are proven to be

convenient, sensitive, cost-effective, and suitable for high-throughput screening (Wang and Xianyu, 2022).

Time-resolved fluoroimmunoassay (TRFIA) is a kind of immunoassay that has been developed in recent decades. Rare-earth metal ions, Eu^{3+} and Sm^{3+}, are used as labels because of their characteristics of high fluorescence intensity, ability to overcome high-background absorption, and long relaxation time (Wang et al., 2009; Huang et al., 2009; Zhang et al., 2011). TRFIA has been used for the detection of small molecules, such as salbutamol in the environment, aflatoxin b1 in feed, and oxyfluorfen in food, and has demonstrated excellent sensitivity and accuracy (Hu et al., 2018; Fang et al., 2019; Sheng et al., 2020). In this study, an OA detection method was developed using TRFIA on the basis of competitive immunization and applied this TRFIA method on shellfish.

Materials and methods

Materials and instruments

OA-BSA conjugates, OA, and mouse monoclonal antibody to OA were purchased from China National Marine Environment Monitoring Center. Goat anti-mouse IgG was obtained from Huamei Biological Engineering (Luoyang, China). Diethylenetriaminepentaacetic (DTPA) and BSA were acquired from Saiguo Biotechnology (Guangzhou, China). Eu^{3+}-N1-(p-isothiocyanatobenzyl)-diethylenetriamine-N1, N2, N3, and N4-tetraacetic acid (DTTA) were bought from Boshi Biological Technology (Hangzhou, China). Labeling buffer (50 mmol/L Na_2CO_3-$NaHCO_3$, pH 9.0), coating buffer (50 mmol/L Na_2CO_3-$NaHCO_3$, pH 9.6), blocking buffer (0.05% proclin-300, 1% BSA, 0.9% NaCl, 50 mmol/L Tris-HCl, pH 7.8), assay buffer (0.01% Tween-20, 0.05% NaN_3, 50 mmol/L Tris-HCl, 0.9% NaCl, 0.2% BSA, 20 μM DTPA, pH 7.8), washing buffer (0.01% proclin-300, 50 mmol/L Tris-HCl, 0.02% Tween-20, 0.9% NaCl, pH 7.8), and enhancement solution (1 ml Triton X-100, 50 μmol trioctylphosphine oxide, 15 μmol/L β-NTA, pH 3.2) were prepared in our laboratory.

Ultracel-50k ultrafiltration tubes (Millipore, Massachusetts, USA) were used to change the buffer of IgG. A sephadex-G50 column (Cell Biotechnology, Shanghai, China), a peristaltic pump, and an automatic fraction collector were used to separate the reaction product, and 96-well plates were used as reaction vessels. A micro-oscillator and an electric heating incubator were used to determine immune responses. A plate washer was used to wash away materials that did not bind to the 96-well plate. Fluorescence (F) value of the reaction was measured by a time-resolved immunofluorescence analyzer (Darui Biotechnology, Foshan, China). Photodiode Array UV-Vis Detector (Shimadzu, Kyoto, Japan) and HPLC column

(SilGreen, Beijing, China) were used to detect toxin samples and standards.

Preparation for europium (Eu^{3+})−labeled IgG

Eu^{3+}-labeled IgG was prepared as previously described (Chen et al., 2022). The original buffer of IgG was exchanged with labeling buffer with ultrafiltration tube and centrifuged. Afterward, 125 μl of 2 mg/ml IgG was added to 30 μl of 2 mg/ml Eu^{3+}-DTTA and shaken overnight at room temperature. A sephadex-G50 column and an automatic fraction collector were used to separate the reaction product. The eluate was collected in a 2 ml/tube. F at 340 nm was monitored. The solution with the highest fluorescence value was collected as Eu^{3+}-labeled IgG.

Procedure of OA-TRFIA

An indirect competitive TRFIA was established in this study. In brief, 100 μl coated antigen OA-BSA was added to the 96-well plate and incubated overnight at 4°C. The washing buffer was used to wash the plate once and 150 μl/well of blocking buffer was then added for 2 h to seal the non-specific binding sites. After the blocking buffer was discarded, either 50 μl of OA standard or 50 μl of shellfish samples were added to the 96-well plate, followed by the addition of 50 μl of OA antibody to incubate. After the plate was washed twice, 100 μl of Eu^{3+}-labeled IgG was added to the plate to incubate. After the plate was washed six times, 100 μl of enhancement solution was added to each well, followed by shaking for 3 min. The TRFIA analyzer (excitation wavelength and emission wavelength was 340 nm and 615 nm, respectively) was used to measure the F.

Optimization

The factors that have marked influences on sensitivity were optimized. Coated antigen concentration, antibody concentration, Eu^{3+}-labeled IgG dilution ratio, reaction time, and reaction temperature were optimized on the basis of the high F_{max}/IC_{50} values and low IC_{50} values (F_{max}: maximum fluorescence value; in this study, it refers to the fluorescence signal without OA).

Sensitivity

After 10 standards were measured at 0 concentration, the mean and SD of F were calculated. Sensitivity was the concentration corresponding to the mean F minus twice SD, which is also the limit of detection (LOD).

Precision

The intra-assay coefficient of variation was computed through the repeated measurements of a single sample. The inter-assay coefficient of variation was computed through the repeated measurements of a single sample for several days.

Specificity

BSA and other classes of marine toxins were used as potential antigens to assess the method's specificity under optimal operating conditions. Other common shellfish toxins which have the same toxicological mechanism as OA, namely, saxitoxin, domoic acid, and microcystin LR, were selected for this study.

Matrix effect on immunoassay

The matrix effect of the samples was studied. The extracted shellfish samples were diluted one, three, and five times with PBS, and the standard curves were drawn. The matrix effect and sample dilution ratio were determined by comparing the standard curve obtained using the standard solution without the matrix solution.

Recovery

The added recovery rate was used as the evaluation standard for TRFIA accuracy. A standard of known concentration was added to a sample of known concentration. The recovery rate was calculated by measuring the actual and theoretical concentrations.

Sample processing

Scissors and a precision knife were used to remove the soft tissues of shellfish samples (scallop, mussel, and oyster), which were then homogenized with a tissue grinder, added with a certain concentration of OA, and stored at −20°C for subsequent study. The samples were prepared as previously described (Wang et al., 2011). In brief, 200 mg of the shellfish samples were added with 1 ml of 90% methanol, and homogenization was continued for 1–2 min. The supernatant was centrifuged at 6000 r/min for 10 min and then collected. The solution was diluted with PBS and mixed evenly. Moreover, 50 μl of the sample was isolated for detection. Phytoplankton samples were processed according to the method described previously in the literature (Wu et al., 2020).

Detection OA by HPLC

The samples were prepared as previously described (Uchida et al., 2014). In brief, 0.2 g of homogenized shellfish samples were added with 0.9 ml of methanol, vortexed, and sonicated for 10 min. Centrifugation was performed at 8000 r/min for 5 min, and the supernatant was collected. Another 0.9 ml of methanol was added to the residue, and the procedure was repeated. The extract was combined, and the volume was fixed to 2 ml. Afterward, 125 µl of 2.5 mol/L NaOH was added to 1 ml of the extract, mixed and sealed, and heated at 70 °C for 1 h. After the mixture was cooled, the same amount of HCl was added and mixed. The solution was passed through 0.22 µm organic phase microporous membrane and HLB solid phase extraction column, and the volume was fixed to 1 ml. HPLC column (250×10 mm^2, 5 µm), and LC-20A Photodiode Array UV-Vis Detector was used to detect toxin samples and standards. The mobile phase was water and acetonitrile, the flow rate was 2 ml/min, the wavelength was 200 nm, the detection time was 25 min, and the injection volume was 20 µl. The methanol solution was also analyzed. The concentration of the toxin sample was calculated according to chromatographic peak positions and peak areas.

Statistical analysis

The TRFIA results were compared with the HPLC results and the correlation between them was calculated by IBM SPSS Statistics 26 (SPSS Inc., Chicago, IL, USA). Pearson correlation was used to analyze the correlation between variables and was statistically significant at $P < 0.05$.

Results

Optimization of OA-TRFIA

Preparation for Eu^{3+}-labeled IgG

The Eu^{3+}-labeled IgG was prepared as mentioned above. The reaction solution was separated using dextran gel and eluate. The F at 340 nm was monitored. The statistical curves of goat anti-mouse IgG antibody coupled with Eu^{3+}-DTTA showed purified protein peaks and free peaks. The first and second peaks corresponded to the successfully labeled goat anti-mouse IgG and unlabeled free Eu^{3+}-DTTA, respectively, as shown in Figure S1. The solution corresponding to the protein peak was collected as Eu^{3+}-labeled IgG for subsequent studies.

Optimization of concentrations

The structural formula OA is shown in Figure 1A. The schematic diagram of OA-TRFIA is shown in Figure 1B. The concentration of OA-BSA coated antigen was optimized based on the higher F_{max}/IC_{50} values and lower IC_{50} values. Figure 2A shows that under the same conditions, the largest F_{max}/IC_{50} value and relatively low IC_{50} value were obtained when the concentration of OA-BSA was 0.5 µg/ml. Eu^{3+}-labeled IgG was optimized according to sufficient specific binding while avoiding non-specific binding. Figure 2B shows that under the same conditions, a relatively large specific binding and a relatively
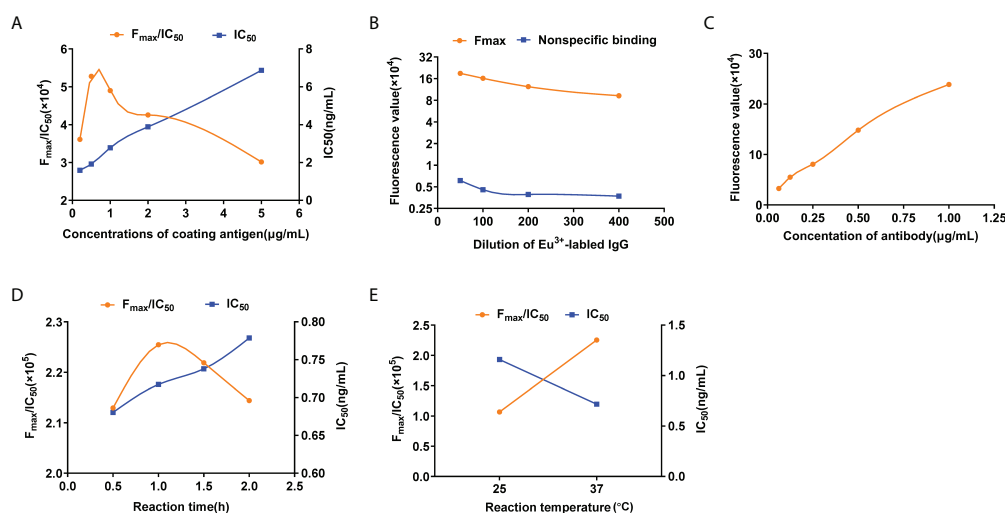

FIGURE 1
(A) Molecular formula of OA. (B) Schematic of TRFIA system for OA detection.
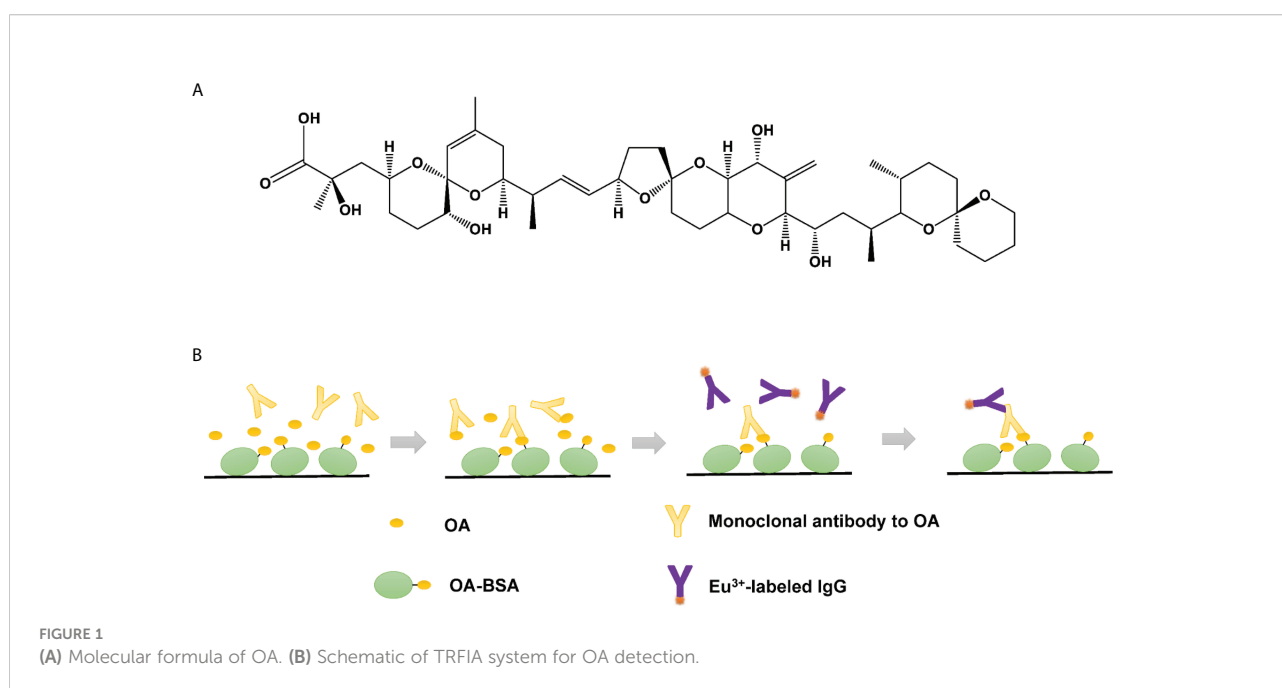
FIGURE 2

Optimization conditions of OA-TRFIA. **(A)** F_{max}/IC_{50} values and IC_{50} values in different coating antigen concentration. **(B)** Maximum fluorescence values and non-specific binding fluorescence values at different Eu3+-labeled antibody concentrations. **(C)** Fluorescence valuesin different antibody concentrations. **(D)** F_{max}/IC_{50} values and IC_{50} values in different incubation times. **(E)** F_{max}/IC_{50} values and IC_{50} values in different incubation temperatures.

small non-specific binding were observed when the dilution ratio of Eu³⁺-labeled IgG was 1:100. OA antibody was optimized at a concentration corresponding to 30%–50% of the F_{max}. Figure 2C shows that when the concentration of OA antibody was 0.25 µg/ml, the corresponding fluorescence value was within 30%–50% of the F_{max}. In summary, the optimum values were 0.5 µg/ml for the concentration of OA-BSA antigen, 0.25 µg/ml the concentration of OA antibody, and 1:100 for the dilution ratio of Eu³⁺-labeled IgG.

Optimization of reaction temperature and time

Incubation time and temperature were also optimized according to the high F_{max}/IC_{50} values and low IC_{50} values. Four reaction times and two reaction temperatures were evaluated. Figures 2D, E show that under the same conditions, the largest F_{max}/IC_{50} value and the smallest IC_{50} value were obtained when the incubation time and the incubation temperature were 1 h and 37°C, respectively. In summary, the optimum incubation temperature was 37°C, and the optimum incubation time was 1 h.

Identification of OA-TRFIA

Sensitivity, precision, and specificity

The standard curve of OA-TRFIA was established under the optimized conditions. As shown in Figure 3, the equation of the standard curve was y=-0.21391-1.08852×x, where Logit Y=ln(y/

1-y), y=F/F₀, and $R^2 = 0.99756$. The results showed that the LOD and IC_{50} of OA were 2.49×10^{-3} ng/ml and 0.72 ng/ml, respectively. The detection range was $2.49 \times 10^{-3} – 50$ ng/ml. The average coefficients of variation intra-assay and inter-assay batches were 3.34% and 5.87%, respectively.

Cross reaction

TRFIA was used to detect saxitoxin, domoic acid, microcystin LR, and BSA to evaluate cross-reaction. As shown in Table 1, no cross reaction occurred.

Application of OA-TRFIA in samples

Matrix effect and recovery

The matrix effect study showed that the matrix effect did not affect TRFIA detection when the shellfish samples were diluted three times, as shown in Figure S2. Therefore, this sample dilution factor was used for subsequent studies. Table 2 shows the test results of diluted shellfish samples. Table 3 shows that the recovery rate of diluted shellfish samples was 93.04%–111.66%, indicating that the established method had high accuracy.

Validation of OA-TRFIA with HPLC

After OA was artificially added to the samples of mussels, scallops, and oysters, its content in the samples was determined by HPLC. As shown in Figure 4 ($R^2 = 0.97$, *P*<0.01), the TRFIA
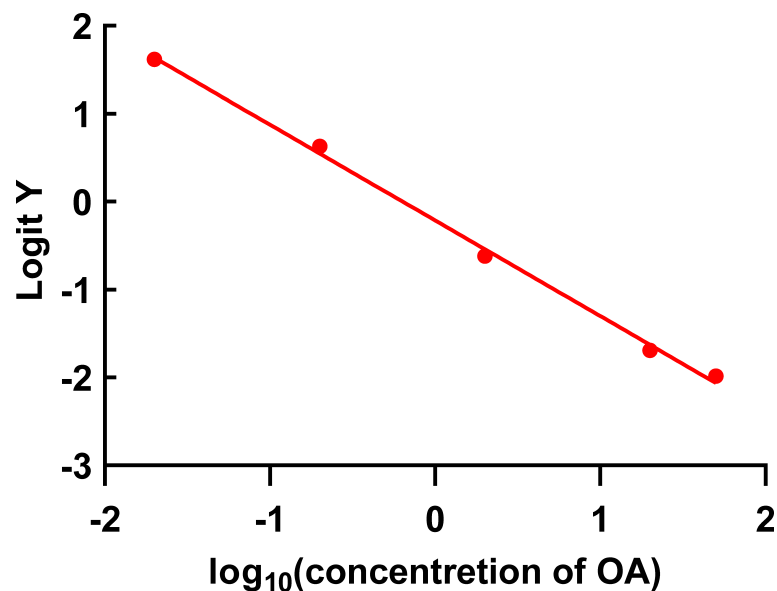
FIGURE 3
Standard curve of OA-TRFIA (the concentrations of OA were 0, 0.02, 0.2, 2, 20, and 50 ng/ml).

TABLE 1 Cross-reactivity (CR) with other marine toxins by TRFIA.

Compound	CR%
Saxitoxin	<0.05
Domoic acid	<0.05
Microcystin LR	<0.05
BSA	<0.05

established in this study has high accuracy. Moreover, phytoplankton samples were also used to detected OA by OA-TRFIA and HPLC, as shown in Table S1.

Discussion and conclusion

As an important toxin from marine microalgae, OA has been detected worldwide (Lv et al., 2021). Different algal toxins are

TABLE 2 TRFIA detection of shellfish samples.

Sample category	Sample	Logit Y	Corresponds to the concentration (ng/ml)
Blank sample	——	1.91	——
Scallop	Sample 1	-0.33	1.15
	Sample 2	-0.72	2.51
	Sample 3	-1.63	17.42
	Sample 4	-1.80	25.88
Mussel	Sample 1	-0.28	1.03
	Sample 2	-0.98	4.18
	Sample 3	-1.39	10.16
	Sample 4	-1.86	29.99
Oyster	Sample 1	-0.07	0.69
	Sample 2	-0.20	0.89
	Sample 3	-1.40	10.23
	Sample 4	-1.66	18.63

TABLE 3 Recoveries of samples spiked with OA by TRFIA.

Samples	Spiked concentration (ng/ml)	TRFIA	
		Mean recovery ± SD (%, n = 3)	RSD (%)
1	1.00	93.04 ± 3.02	3.25
2	5.00	101.99 ± 7.93	7.77
3	10.00	104.35 ± 8.11	7.77
4	50.00	111.66 ± 7.43	6.66

RSD, relative standard deviation.

affected differently by nutrients due to their different structures. The production of carbon-rich toxins such as OA is often promoted by limiting nitrogen or phosphorus (Accoroni et al., 2018). It is mainly transmitted to humans through the consumption of seafood, and shellfish have accumulated the highest levels of this toxin due to the filter-feeding of harmful algae (Campos et al., 2020). The research to the effects of OA and other diarrheic shellfish poisoning (DSP) on aquatic animals is insufficient, and no documented mortality of bivalves associated with DSP exposure so far (Campos et al., 2020). However, there has been a growing number of publications focusing on the effects of OA on bivalves. The results of genomics and proteomics showed that OA affects the gene expression of shellfish cells, leading to cytoskeletal instability, apoptosis, stress/immune responses, and cellular energy metabolism abnormality (Madamanchi et al., 2001; Manfrin et al., 2010; Huang et al., 2015; Dou et al., 2020). It can be seen that OA affects the growth, development, and reproduction of shellfish,

and seriously affects human consumption of shellfish and other seafood. Therefore, a detection method for the rapid and sensitive detection of OA in shellfish must be developed.

In this study, we established an indirect competition TRFIA for OA in shellfish. Goat anti-mouse IgG, i.e., secondary antibody, was labeled with europium for low detection limit and high sensitivity (Esteve-Turrillas et al., 2010; Sesay et al., 2013; Li et al., 2014; Sheng et al., 2020). Compared with labeling the primary antibody, labeling the secondary antibody avoids the interference of the marker to the binding site of the antigen on the first antibody. Furthermore, the secondary antibody, labeled with polyclonal antibody, can act as an amplified signal.

Under the optimized conditions, the detection range was 2.49×10^{-3} – 50 ng/ml, which is equivalent to 3.74×10^{-5} – 0.75 mg toxins per kilogram of shellfish, a value far beyond the EU or the US shellfish toxin detection limit of 0.16 mg/kg (Farrell et al., 2018). The IC_{50} was 0.72 ng/ml, the LOD was 2.49×10^{-3} ng/ml, the inter-batch and intra-batch precision values were 3.34% and
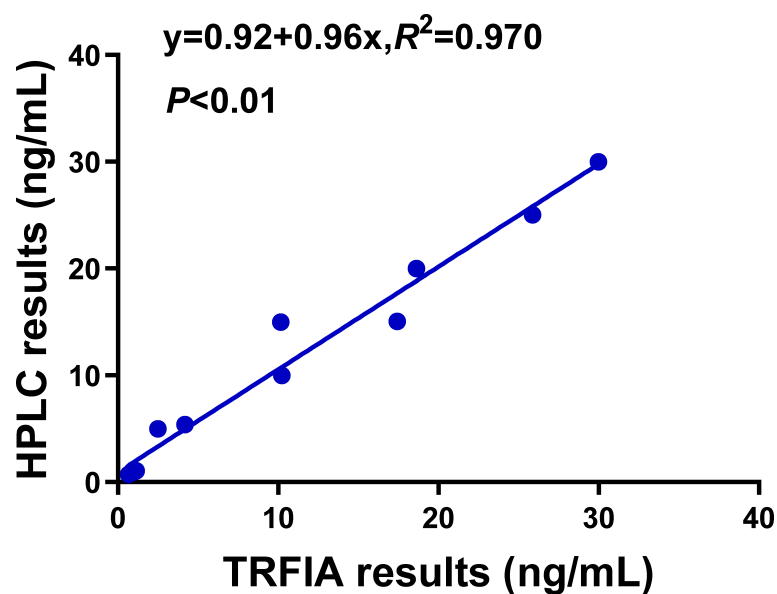

FIGURE 4
Correlation between TRFIA and HPLC for the concentrations of OA in shellfish samples.

5.87%, respectively, and the recovery was 93.04%–111.66%. Compared with those of the previously reported indirect competition ELISA (detection limit 12 pg/ml, detection range 20–750 pg/ml), the detection limit and detection range were improved in TRFIA, which reflects its superiority (Wang et al., 2017). The unique fluorescence signal of rare-earth ions, the decay time different from that of conventional ions and the amplification of fluorescence signal by the enhancement solution enhance the sensitivity and detection range of the proposed method.

The OA-TRFIA established in this study neither cross-reacted with saxitoxin in PSP and domoic acid in ASP, which also has neurotoxicity, nor cross-reacted with microcystin LR, which has the same toxic mechanism.

The results also showed that the established method could be applied in the detection of actual shellfish samples, and the results were verified by HPLC. Compared with instrumental analysis, OA-TRFIA uses fewer organic solvents, which reduce the potential danger in the experimental process and reduce the requirements for operators and operating instruments, eliminates the need for complex sample pretreatment, reduces costs, and simplifies the operation. Moreover, besides europium (Eu^{3+}), samarium (Sm^{3+}) could also be used for labeling another detection antibody. Based on the results of this study, a dual-labeled TRFIA method can be established to detect OA and another toxin molecule, simultaneously, in a future study.

In conclusion, we developed OA-TRFIA, an ultrasensitive, rapid, and economic method for the wholesale screening and supervising of OA in shellfish samples. This method is expected to make proper contribution in marine biotoxin detection.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

YQ, JL, SS, JJ, and JK performed the experiments. JL, JJ, JK, ZZ, CZ, and XZ analyzed the data and made graphs. YQ and JL wrote the manuscript. YQ, BH, and BNH were responsible for project management and revised the manuscript. All authors contributed to the article and approved the submitted version.

Funding

Acknowledgments

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.961751/full#supplementary-material

References

Accoroni, S., Ceci, M., Tartaglione, L., Romagnoli, T., Campanelli, A., Marini, M., et al. (2018). Role of temperature and nutrients on the growth and toxin production of prorocentrum hoffmannianum (Dinophyceae) from the Florida keys. *Harmful. Algae.* 80, 140–148. doi: 10.1016/j.hal.2018.11.005

Ajani, P. A., Sarowar, C., Turnbull, A., Farrell, H., Zammit, A., Helleren, S., et al. (2021). A comparative analysis of methods (lc-ms/ms, lc-ms and rapid test kits) for the determination of diarrhetic shellfish toxins in oysters, mussels and pipis. *Toxins. (Basel).* 13 (8), 563. doi: 10.3390/toxins13080563

Campos, A., Freitas, M., de Almeida, A. M., Martins, J. C., Domínguez-Pérez, D., Osório, H., et al. (2020). OMICs approaches in diarrhetic shellfish toxins research. *Toxins. (Basel).* 12 (8), 493. doi: 10.3390/toxins12080493

Caron, D. A., Garneau, M. E., Seubert, E., Howard, M. D., Darjany, L., Schnetzer, A., et al. (2010). Harmful algae and their potential impacts on desalination operations off southern California. *Water Res.* 44 (2), 385–416. doi: 10.1016/j.watres.2009.06.051

Castanheira, A., Dos Santos, M. B., Rodriguez-Lorenzo, L., Queirós, R., and Espiña, B. (2021). A novel microfluidic system for the sensitive and cost-effective detection of okadaic acid in mussels. *Analyst* 146 (8), 2638–2645. doi: 10.1039/d0an02092c

Chen, X., Zhou, K., Xiang, Z., Zhou, X., Wang, Y., Hong, J., et al. (2022). Establishment and clinical application of time-resolved immunofluorescence assay of lipoprotein-associated phospholipase A2. *Anal. Biochem.*, 648, 114674. doi: 10.1016/j.ab.2022.114674

Dou, M., Jiao, Y. H., Zheng, J. W., Zhang, G., Li, H. Y., Liu, J. S., et al. (2020). *De novo* transcriptome analysis of the mussel perna viridis after exposure to the toxic dinoflagellate prorocentrum lima. *Ecotoxicol. Environ. Saf.* 192, 110265. doi: 10.1016/j.ecoenv.2020.110265

Esteve-Turrillas, F. A., Parra, J., Abad-Fuentes, A., Agulló, C., Abad-Somovilla, A., and Mercader, J. V. (2010). Hapten synthesis, monoclonal antibody generation, and development of competitive immunoassays for the analysis of picoxystrobin in beer. *Anal. Chim. Acta* 682 (1-2), 93–103. doi: 10.1016/j.aca.2010.09.042

Fang, S., Zhang, Y., Liu, X., Qiu, J., Liu, Z., and Kong, F. (2019). Development of a highly sensitive time-resolved fluoroimmunoassay for the determination of trace salbutamol in environmental samples. *Sci. Total. Environ.* 679, 359–364. doi: 10.1016/j.scitotenv.2019.05.057

Farrell, H., Ajani, P., Murray, S., Baker, P., Webster, G., Brett, S., et al. (2018). Diarrhetic shellfish toxin monitoring in commercial wild harvest bivalve shellfish in New South Wales, Australia. *Toxins. (Basel).* 10 (11), 446. doi: 10.3390/toxins10110446

Ferron, P. J., Hogeveen, K., Fessard, V., and Le Hégarat, L. (2014). Comparative analysis of the cytotoxic effects of okadaic acid-group toxins on human intestinal cell lines. *Mar. Drugs* 12 (8), 4616–4634. doi: 10.3390/md12084616

Fraga, M., Vilariño, N., Louzao, M. C., Rodríguez, P., Campbell, K., Elliott, C. T., et al. (2013). Multidetection of paralytic, diarrheic, and amnesic shellfish toxins by an inhibition immunoassay using a microsphere-flow cytometry system. *Anal. Chem.* 85 (16), 7794–7802. doi: 10.1021/ac401146m

Fu, L. L., Zhao, X. Y., Ji, L. D., and Xu, J. (2019). Okadaic acid (OA): Toxicity, detection and detoxification. *Toxicon* 160, 1–7. doi: 10.1016/j.toxicon.2018.12.007

Griffith, A. W., and Gobler, C. J. (2020). Harmful algal blooms: A climate change co-stressor in marine and freshwater ecosystems. *Harmful. Algae.* 91, 101590. doi: 10.1016/j.hal.2019.03.008

Huang, B., Xiao, H., Zhang, J., Zhang, L., Yang, H., Zhang, Y., et al. (2009). Dual-label time-resolved fluoroimmunoassay for simultaneous detection of aflatoxin B1 and ochratoxin a. *Arch. Toxicol.* 83 (6), 619–624. doi: 10.1007/s00204-009-0410-6

Huang, L., Zou, Y., Weng, H. W., Li, H. Y., Liu, J. S., and Yang, W. D. (2015). Proteomic profile in perna viridis after exposed to prorocentrum lima, a dinoflagellate producing dsp toxins. *Environ. Pollut.* 196, 350–357. doi: 10.1016/j.envpol.2014.10.019

Hu, L., Wang, R., Wang, M., Xu, Y., Wang, C., Liu, Y., et al. (2022). Research progress of photocatalysis for algae killing and inhibition: A review. *Environ. Sci. Pollut. Res. Int.* 29, 47902–47914. doi: 10.1007/s11356-022-20645-9

Hu, X., Yao, J., Wang, F., Yin, M., Sun, Y., Hu, M., et al. (2018). Eu(3+) -labeled IgG-based time-resolved fluoroimmunoassay for highly sensitive detection of aflatoxin B(1) in feed. *J. Sci. Food Agric.* 98 (2), 674–680. doi: 10.1002/jsfa.8514

Klijnstra, M. D., Faassen, E. J., and Gerssen, A. (2021). A generic lc-hrms screening method for marine and freshwater phycotoxins in fish, shellfish, water, and supplements. *Toxins. (Basel).* 13 (11), 823. doi: 10.3390/toxins13110823

Li, M., Sheng, E., Yuan, Y., Liu, X., Hua, X., and Wang, M. (2014). Sensitive time-resolved fluoroimmunoassay for quantitative determination of clothianidin in agricultural samples. *Environ. Sci. Pollut. Res. Int.* 21 (9), 5803–5809. doi: 10.1007/s11356-014-2506-7

Lloyd, J. K., Duchin, J. S., Borchert, J., Quintana, H. F., and Robertson, A. (2013). Diarrhetic shellfish poisoning, Washington, USA, 2011. *Emerg. Infect. Dis.* 19 (8), 1314–1316. doi: 10.3201/eid1908.121824

Lv, J. J., Yuan, K. K., Lu, M. Y., He, Z. B., Li, H. Y., and Yang, W. D. (2021). Responses of JNK signaling pathway to the toxic dinoflagellate prorocentrum lima in the mussel perna viridis. *Ecotoxicol. Environ. Saf.* 227, 112905. doi: 10.1016/j.ecoenv.2021.112905

Madamanchi, N. R., Li, S., Patterson, C., and Runge, M. S. (2001). Reactive oxygen species regulate heat-shock protein 70 *via* the JAK/STAT pathway. *Arterioscler. Thromb. Vasc. Biol.* 21 (3), 321–326. doi: 10.1161/01.atv.21.3.321

Manfrin, C., Dreos, R., Battistella, S., Beran, A., Gerdol, M., Varotto, L., et al. (2010). Mediterranean Mussel gene expression profile induced by okadaic acid exposure. *Environ. Sci. Technol.* 44 (21), 8276–8283. doi: 10.1021/es102213f

Marampouti, C., Buma, A. G. J., and de Boer, M. K. (2021). Mediterranean Alien harmful algal blooms: Origins and impacts. *Environ. Sci. Pollut. Res. Int.* 28 (4), 3837–3851. doi: 10.1007/s11356-020-10383-1

Onofrio, M. D., Mallet, C. R., Place, A. R., and Smith, J. L. (2020). A screening tool for the direct analysis of marine and freshwater phycotoxins in organic spatt extracts from the Chesapeake Bay. *Toxins. (Basel).* 12 (5), 322. doi: 10.3390/toxins12050322

Pérez, L., Escudero, I., Cabado, A. G., Molinero-Abad, B., and Arcos-Martínez, M. J. (2019). Study of ceramic membrane behavior for okadaic acid and heavy-metal determination in filtered seawater. *J. Environ. Manage.* 232, 564–573. doi: 10.1016/j.jenvman.2018.11.077

Reguera, B., Riobó, P., Rodríguez, F., Díaz, P. A., Pizarro, G., Paz, B., et al. (2014). Dinophysis toxins: Causative organisms, distribution and fate in shellfish. *Mar. Drugs* 12 (1), 394–461. doi: 10.3390/md12010394

Sesay, A. M., Micheli, L., Tervo, P., Palleschi, G., and Virtanen, V. (2013). Development of a competitive immunoassay for the determination of cortisol in human saliva. *Anal. Biochem.* 434 (2), 308–314. doi: 10.1016/j.ab.2012.12.008

Sheng, E. Z., Tan, Y. T., Lu, Y. X., Xiao, Y., and Li, Z. X. (2020). Sensitive time-resolved fluorescence immunoassay for quantitative determination of oxyfluorfen in food and environmental samples. *Front. Chem.* 8. doi: 10.3389/fchem.2020.621925

Tian, Y., Du, L., Zhu, P., Chen, Y., Chen, W., Wu, C., et al. (2021). Recent progress in micro/nano biosensors for shellfish toxin detection. *Biosens. Bioelectron.* 176, 112899. doi: 10.1016/j.bios.2020.112899

Tian, Y., Yuan, L., Zhang, M., He, Y., and Lin, X. (2022). Sensitive detection of the okadaic acid marine toxin in shellfish by Au@Pt NPs/horseradish peroxidase dual catalysis immunoassay. *Anal. Methods* 14 (12), 1261–1267. doi: 10.1039/d1ay01973b

Uchida, H., Watanabe, R., Matsushima, R., Uchida, N., Nagai, H., Kamio, M., et al. (2014). A convenient HPLC method for detection of okadaic acid analogs as 9-anthrylmethyl esters with automated sample cleanup by column switching. *J. AOAC. Int.* 97 (2), 391–397. doi: 10.5740/jaoacint.sgeuchida

Valdiglesias, V., Prego-Faraldo, M. V., Pásaro, E., Méndez, J., and Laffon, B. (2013). Okadaic acid: More than a diarrheic toxin. *Mar. Drugs* 11 (11), 4328–4349. doi: 10.3390/md11114328

Verma, M., Chaudhary, M., Singh, A., Kaur, N., and Singh, N. (2020). Naphthalimide-gold-based nanocomposite for the ratiometric detection of okadaic acid in shellfish. *J. Mater. Chem. B.* 8 (36), 8405–8413. doi: 10.1039/d0tb01195a

Wang, K., Huang, B., Zhang, J., Zhou, B., Gao, L., Zhu, L., et al. (2009). A novel and sensitive method for the detection of deoxynivalenol in food by time-resolved fluoroimmunoassay. *Toxicol. Mech. Methods* 19 (9), 559–564. doi: 10.3109/15376510903380720

Wang, L., Sang, Y. X., and Wang, X. H. (2011). Enzyme-linked immunosorbent assay for okadaic acid: investigation of analytical conditions and sample matrix on assay performance. *J. AOAC. Int.* 94 (5), 1531–1539. doi: 10.5740/jaoacint.10-412

Wang, Y., and Xianyu, Y. (2022). Nanobody and nanozyme-enabled immunoassays with enhanced specificity and sensitivity. *Small. Methods* 6 (4), e2101576. doi: 10.1002/smtd.202101576

Wang, R., Zeng, L., Yang, H., Zhong, Y., Wang, J., Ling, S., et al. (2017). Detection of okadaic acid (OA) using ELISA and colloidal gold immunoassay based on monoclonal antibody. *J. Hazard. Mater.* 339, 154–160. doi: 10.1016/j.jhazmat.2017.06.030

Wu, D., Chen, J., Wang, J., He, X., Xin, M., and Wang, B. (2020). Monitoring and warning of lipophilic marine algal toxins in mariculture zone based on toxin profiles of phytoplankton. *Ecotoxicol. Environ. Saf.* 197, 110647. doi: 10.1016/j.ecoenv.2020.110647

Yin, M., Wang, W., Wei, J., Chen, X., Chen, Q., Chen, X., et al. (2022). Novel dual-emissive fluorescent immunoassay for synchronous monitoring of okadaic acid and saxitoxin in shellfish. *Food Chem.* 368, 130856. doi: 10.1016/j.foodchem.2021.130856

Zhang, J., Gao, L., Zhou, B., Zhu, L., Zhang, Y., and Huang, B. (2011). Simultaneous detection of deoxynivalenol and zearalenone by dual-label time-resolved fluorescence immunoassay. *J. Sci. Food Agric.* 91 (2), 193–197. doi: 10.1002/jsfa.4151