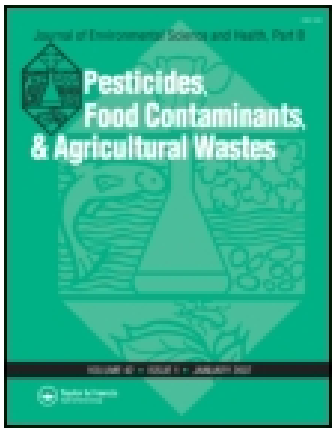

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A sensitive enzyme-linked immunosorbent assay amplified by biotin-streptavidin system for detecting non-steroidal anti-inflammatory drug ketoprofen

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A sensitive enzyme-linked immunosorbent assay amplified by biotin–streptavidin system for detecting non-steroidal anti-inflammatory drug ketoprofen

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A sensitive biotin–streptavidin-amplified enzyme-linked immunosorbent assay (BA-ELISA) method was developed for detecting non-steroidal anti-inflammatory drug ketoprofen. Compared with traditional ELISA method, the sensitivity of proposed immunoassay was enhanced by the biotin–streptavidin system. Under the optimal condition, the median inhibitory concentration (IC_{50}) was 0.25 ng mL^{-1}, with minor cross-reactivity to a number of structural analogs. This developed assay was successfully applied to detect the ketoprofen residues in different fish samples, and good recoveries (72.6–105.5%) were obtained. The results indicated that this immunoassay method could specifically detect trace ketoprofen residues and could be widely used for routine monitoring of food samples.

Keywords: Biotin–streptavidin, ELISA, fish samples, ketoprofen, PPCPs.

Introduction

The environmental problems caused by pharmaceuticals and personal care products (PPCPs) were firstly proposed by Daughton and Ternes.[1] After that, PPCPs have attracted great attention in the scientific community and public. These emerging pollutants basically contain prescription/non-prescription drugs and personal-care products. As a type of PPCPs, non-steroidal anti-inflammatory drugs (NASIDs) have the physiological effect of anti-inflammatory, antipyretic, pain-relieving and treating rheumatic disease, which was widely used in the world for 100 years,[2] especially in aquaculture industry. NASIDs mainly include piroxicam, ibuprofen, diclofenac sodium, aspirin, indometacin, ketoprofen, etc. The widespread application of these drugs in aquaculture may pollute local water environment, harm aquatic organisms and indirectly impact human health. Lin et al.[3] believed that the major polluting agent in some rivers of Taiwan was just NASIDs. Meanwhile, clinical research has proved that NASIDs could lead to some adverse effects in the gastrointestinal tract, cardiocerebral-vascular system and nervous system in the human body. Although increasing concerns on the potential environmental and ecological problems of NASIDs have been raised, only partial NASIDs residual amount in livestock and aquatic products are strictly controlled. Actually, supervision and regulation on NASIDs residue are hard to carry out, because that the concentration of NASIDs remaining in the environment or life body is extremely low and hard to be detected by the existing analytical method. For further environmental study or food detection, more sensitive and specific analytical methods for detecting NASIDs at trace levels are urgently needed.

The exiting analytical methods for detecting NASIDs include biosensor,[4] gas chromatography (GC),[5,6] gas chromatography-mass spectrometry (GC-MS),[7] liquid chromatography,[8,9] liquid chromatography-mass spectrometry (HPLC-MS),[10–12] capillary electrophoresis, etc. However, the instrumental analytical methods are generally labor-intensive, time-consuming, requiring expensive instruments and complex pro-treatment procedures. Compared with instrumental analytical methods, enzyme-linked immunosorbent assay (ELISA) is well adapted for detecting trace pollutant, thanks to its high specificity, sensitivity and high throughput.

For achieving higher sensitivity, conventional ELISA assays have been enhanced with a combination of

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fluorescence, chemiluminescence or biotin–streptavidin system. Especially, the biotin–streptavidin system is an effective technique and has been widely used for sensitivity improvement, because of its high specificity and strong affinity (the affinity constant is 10^{15} L mol^{-1}).[13] Biotin is a relatively small molecule, which can conjugate to protein without significantly altering the biological activity of protein. In BA-ELISA, several biotins conjugate with one immunoglobulin, and then streptavidin-HRP combines with biotins in the following step. The signal intensity can be increased due to more enzyme molecules catalyzing the substrate. Many researches, using biotin–streptavidin system [14–18] for signal amplification, had been reported. However, these researches are mainly used in microbiological, clinical or immunological assays, only a few in the small molecular organic analyses.

In this study, a biotin–streptavidin amplified indirect competitive ELISA had been developed for detecting nonsteroidal anti-inflammatory drug ketoprofen. Immunoassay procedures were optimized, and then their sensitivity and accuracy were compared with those of liquid chromatography. Experimental results had proved that this proposed BA-ELISA had better detection performance and could be successfully applied to detect ketoprofen residues in fish samples. We believed this study could provide a model for sensitive detecting small-size organics by immunoassay and be further used for researches in environmental sciences.

Materials and method

Reagent and apparatus

Ketoprofen (KET) was purchased from J&K Chemical (Shanghai, China). Bovine serum albumin (BSA), egg albumin (OVA), biotinyl-*N*-hydroxysuccinimide ester (BNHS), *N,N*-Dimethylformamide (DMF), *N*-Hydroxysuccinimide (NHS), *N,N′*-dicyclohexylcarbodiimide (DCC), dimethyl slfoxide (DMSO), *n*-butyl amine, acid chloride butyl acetate, hydrogen peroxide, coomassie brilliant blue G250, Tween 20 were purchased from Sinopharm (Shanghai, China). Streptavidin-horseradish peroxidase(SA-HRP) and goat anti-rabbit HRP-IgG were purchased from Sangon Biotech (Shanghai, China).

Polystyrene 96-well microtiter plates were purchased from Sangon Biotech. A Multiskan MK3 ELISA reader (Thermo, USA) was applied for the determination of absorbance in dual wavelength mode (450–650 nm). KET-protein conjugate was characterized by UV-2012 PC spectrophotometer (UNICO, USA). The ultrapure water was prepared by a Milli-Q system (Millipore, Bedford, MA, USA).

Solution

Solutions were as follows: Phosphate-buffered saline (PBS: 137 mmol L^{-1} NaCl, 2.7 mmol L^{-1} KCl, 10 mmol L^{-1} Na_2HPO_4, 2 mmol L^{-1} KH_2PO_4), carbonate buffer solution (CBS: 15 mmol L^{-1} Na_2CO_3, 34.9 mmol L^{-1} $NaHCO_3$), PBST (PBS with 0.05% Tween 20), phosphate-citrate buffer (0.1 mol L^{-1} citric acid, 0.2 mol L^{-1} Na_2HPO_4; pH = 4.3), TMB substrate solution: 0.4 mL, 2.5 g L^{-1} TMB ethanol solution, 10 mL phosphate-citrate buffer and 10 μL of 30% H_2O_2.

Immunogen and coating antigen synthesis

Ketoprofen (KET) is a kind of small molecular organic matter possessing only antigenicity, which could be combined with specific antibody but could not induce immune response in an organism. For successfully obtaining relative polyclonal antibody, first it is necessary to synthesize protein-KET conjugates to form the complete antigen. In this study, activated ester method and mixed anhydride method [19] were employed to covalently couple small molecular hapten to carrier proteins, using terminal carboxyl group on KET molecule. The prepared hapten-protein conjugates KET-BSA and KET-OVA were used as the immunogen and the coating antigen, respectively. The conjugating mechanism is presented in Figure 1.

The obtained protein conjugates were identified by UV-Vis spectrophotometer. The coupling ratios were estimated on the basis of mole absorbance ε [20] and calculated as Eq. (1):

$$\text{The coupling ratio} = \frac{\varepsilon_{\text{conjugate}} - \varepsilon_{\text{protein}}}{\varepsilon_{\text{hapten}}} \qquad (1)$$

Immunization protocol for anti-KET polyclonal antibody

Four male New Zealand white rabbits (2–2.5 kg) were used for obtaining anti-KET polyclonal antibody (pAb-KET). Rabbits had unrestricted access to drinking water and standard laboratory feed. Immunogen 1 mg mL^{-1}, emulsified with Freund's complete adjuvant (1:1, V/V), was hypodermicly injected at multiple sites on the neck. After 3 weeks, each rabbit was encouraged with an additional 0.5 mg immunogen emulsified with Freund's incomplete adjuvant and draw blood 7 days later. The rest of booster injection and drawing blood was expected to continue on a weekly basis. Serum was isolated by centrifugation and stored at 4°C before use. The titer of antiserum was determined by an indirect competitive ELISA. After immunization for 4 months, the antiserum, which had the highest titer, was selected and used in the subsequent study.

Preparation of biotinlyated antibody

Biotinylated polyclonal anti-KET antibody (Bi-pAb-KET) was prepared according to the method described by Zhao et al.[21] with some modifications: 5.0 mg of purified

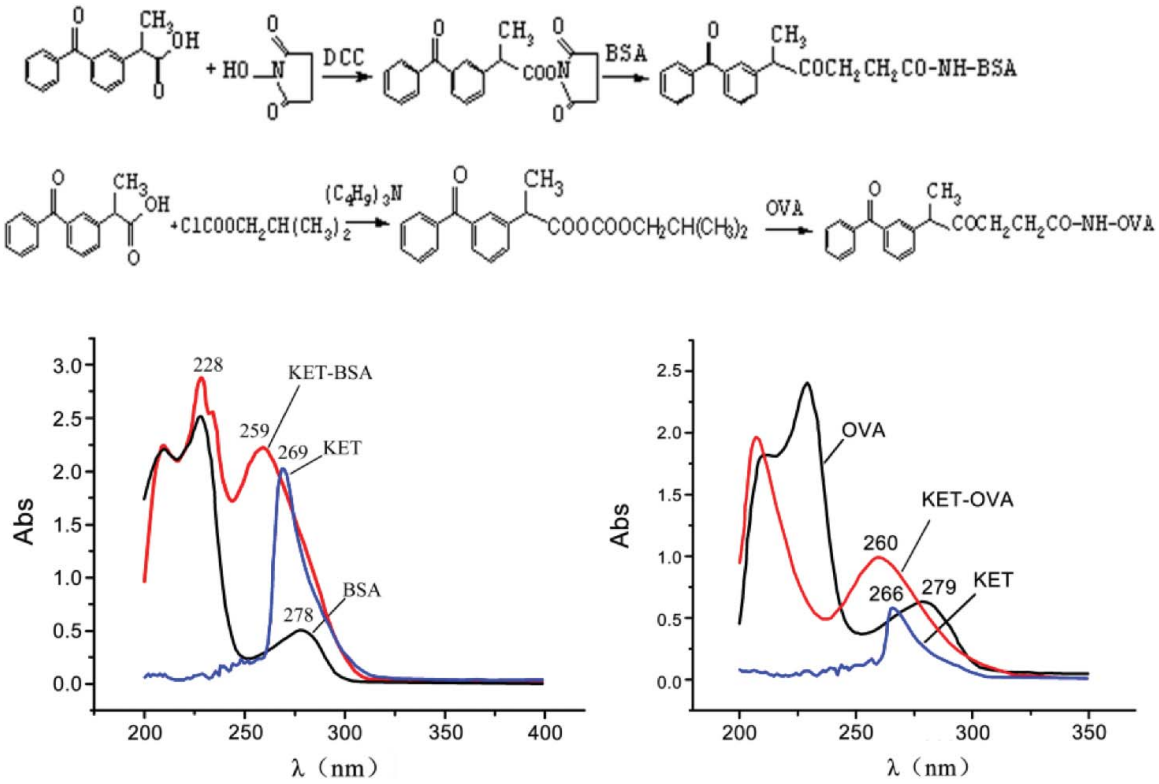


Fig. 1. Synthetic route for ketoprofen immunogen and coating antigen, and their UV-visible spectroscopy.

pAb-KET was dissolved in sodium carbonate buffer (CBS, pH 9.6, 0.1 M) at a concentration of 1.0 mg mL^{-1}. The antibody solution was mixed with 1.0 mg mL^{-1} BNHS in DMSO at the mass ratio of 1:10. The mixture was stirred for 4 h and then dialyzed against PBS for 3 d. The as-obtained biotinylated antibody was stored at 4°C.

Procedures of indirect competitive BA-ELISA method

BA-ELISA determinations were performed in 96-well microplates. A microplate was coated overnight with 100 μL $well^{-1}$ of coating antigen at 4°C. After washing, the unbound active sites were blocked with 200 μL $well^{-1}$ of 0.5% gelatin and later were incubated at 37°Cfor 60 min. After another washing step, the standard or sample (50 μL $well^{-1}$) and Bi-pAb-KET diluent (50 μL $well^{-1}$) were added and the mixture was incubated for 60 min at 37°C. After an additional washing step, 100 μL $well^{-1}$ SA-HRP conjugate (dilution 1:1000) was added and incubated for 60 min. After a final washing step, 100 μL $well^{-1}$ of the TMB substrate solution was added. After sufficient color development (about 15 min), the enzymatic reaction was stopped by adding 50 μL $well^{-1}$ of 2 mol L^{-1} H_2SO_4. The absorbance of each well was read in dual-wavelength mode, with 450 nm as test and 650 nm as reference.

Optimization of the BA-ELISA procedures

Immunoassay is fundamentally dependent on the effective combination of antigen and relative antibody, which is mainly affected by the concentrations of Bi-pAb-KET and the coating antigen, the blocking solution, the incubation time and other assay buffer-related factors such as ionic strength and pH. Therefore, to develop a sensitive immunoassay of KET, these above factors must be optimized.

All determinations were performed in triplicate and the mean absorbance values were calculated. The calibration curves of BA-ELISA were determined by plotting inhibition (%), calculated using Eq. (2), against the logarithm of the standard concentration. The IC_{50}, defined as the concentration of KET giving a 50% inhibition, was used to evaluate the sensitivity of the method. Analogously, the limit of detection (LOD) was evaluated by the IC_{15}.

$$\text{Inhibition}(\%) = \frac{(A_{\max} - A_{\min}) - (A_s - A_{\min})}{A_{\max} - A_{\min}} \times 100\%, \quad (2)$$

where $A_{\max}$ is the absorbance in the absence of KET; $A_{\min}$ is the absorbance of the blank sample; and A_s is the absorbance of KET at the standard concentration.

Cross-reactivity

Different structural analogues of KET were employed to evaluate the cross-reactivity (CR) which reflected the specificity of the optimized immunoassay. The CR values were calculated according to Eq. (3):

$$\mathrm{CR}\ (\%) = \frac{\mathrm{IC_{50}\ of\ ketoprofen}}{\mathrm{IC_{50}\ of\ analogues}} \times 100\% \qquad (3)$$

Sample preparation and evaluation of matrix effect

Like other NASIDs, KET is mainly utilized in aquaculture. Consequently, some fish (*Carassius cuvieri, Pseudosciaena polyactis* and *Trichiurus lepturus*) samples were taken and analyzed, which were purchased from located market.

Before analysis, 1 g of homogenous fish meat sample, without lipid and connective tissue, was ultrasonically extracted twice with 2 g of anhydrous sodium sulfate and 15 mL of ethanol/phosphoric acid (200:1, V/V), followed by centrifugation at 10,000 rpm for 30 min. The supernatants were gently dried under vacuum. And then, the residue was dissolved in 1 mL acetonitrile and 3 mL of n-hexane. After vortex shocking, the supernatant was filtered through a Florisil solid phase extraction column, which was washed with methanol (2 mL min^{-1}) and dried by nitrogen. The residue was dissolved in 1 mL MeOH. The prepared fish sample extract was analyzed by HPLC to confirm the absence of KET before the follow-up studies. Different dilutions (1:1, 1:10 and 1:20) of KET-free extract were spiked with definite amounts of KET to prepare a range of matrix-matched samples. Each sample was subsequently analyzed by BA-ELISA method.

HPLC analysis

In order to verify the results detected by BA-ELISA, the KET concentrations in different samples were detected using HPLC (Agilent LC1100, Palo Alto, CA, USA) with a high pressure pump, a column thermostat (Model 201TP5415), a variable wavelength ultraviolet detector, and a vacuum de-air machine. The temperature of C18 chromatographic column (250 mm × 4.6 mm, 5 μm) was maintained at 35°C. The mobile phase is acetonitrile-20 mmol L^{-1} ammonium acetate buffer solution (85:15 V/V; pH = 3.0 ± 0.05). Sample solution of 20 μL was injected at a flow rate of 1.0 mL min^{-1}. The detection wavelength was 263 nm. And the retention time of KET was 5.6 min.

Results and discussion

Characterization of immunogen, coating antigen and antibody

In this study, the BSA-KET and OVA-KET conjugates were successfully synthesized. The hapten-protein conjugates were characterized by a UV-visible spectrophotometer, and the results were exhibited in Figure 1. Meanwhile, the coupling ratio is determined as Eq. (1).

The maximum absorption peaks of KET hapten and protein were appeared at 269 nm, 278 nm and 279 nm, respectively (Fig. 2). However, the maximum absorption peaks of KET-protein conjugates were appeared at 259 and 260 nm. The displacement of the absorption peaks indicated that the hapten was successfully conjugated to the protein. The coupling ratio was 9.93 for KET-BSA and was 8.65 for KET-OVA.

The as-obtained BSA-KET artificial antigen was injected into rabbits. After immunity for 16 weeks, the highest antibody titer was 1:128,000. The immunogen concentrations reached 5.25 mg mL^{-1}, the coating antigen reached 4.28 mg mL^{-1}, and pAb-KET reached 26.54 mg mL^{-1}, as determined by the Coomassie blue staining.

Suitable operating conditions of the immunoassay method

A sensitive BA-ELISA for quantitatively detecting KET must have the lowest IC_{50} value. Therefore, it is essential to optimize the factors which vastly influence the sensitivity of the established BA-ELISA. First, the optimum concentrations for Bi-pAb-KET and the coating antigen were studied as the primary influencing factor. According to checkerboard titration, the optimal reagent concentration of coated KET-OVA was 4.28 μg mL^{-1} and the Bi-pAb-KET was at 1:3000 dilutions.

The blocking step is required in immunoassay procedures. This step serves to eliminate unoccupied sites on the plates. Otherwise, the unoccupied sites may interact with components such as Bi-pAb-KET or SA-HRP during subsequent steps. An optimum blocking reagent should achieve the lowest background value. In this study, different blocking solutions, such as gelatin (0.1%, 0.5% and 1%), 0.5% OVA and 0.5% milk powder in PBS, were compared. As a result, 0.5% gelatin achieved the lowest background value (0.09). Other blocking agents had similar values: 0.1% gelatin (0.11), 1% gelatin (0.13), 0.5% OVA (0.109) and 0.5% milk powder in PBS (0.106). Therefore, 0.5% gelatin was selected as the blocking agent in the subsequent experiments.

Immunosorbent assay method is based on the combination of coating antigen, absorbed on the surface of the solid phase, and the relative antibodies in the liquid phase. This combination process is strongly affected by the incubation time. The maximal absorbance (A_{0max}) and IC_{50} from the competition curves in different incubation times were tested. Known from Figure 2a, a clear increase in the maximum signal was observed with increasing incubation time. The lowest IC_{50} value was obtained when incubated for 60 min. As a result, the optimal incubation time for antigen and antibody combining was selected to be 60 min.

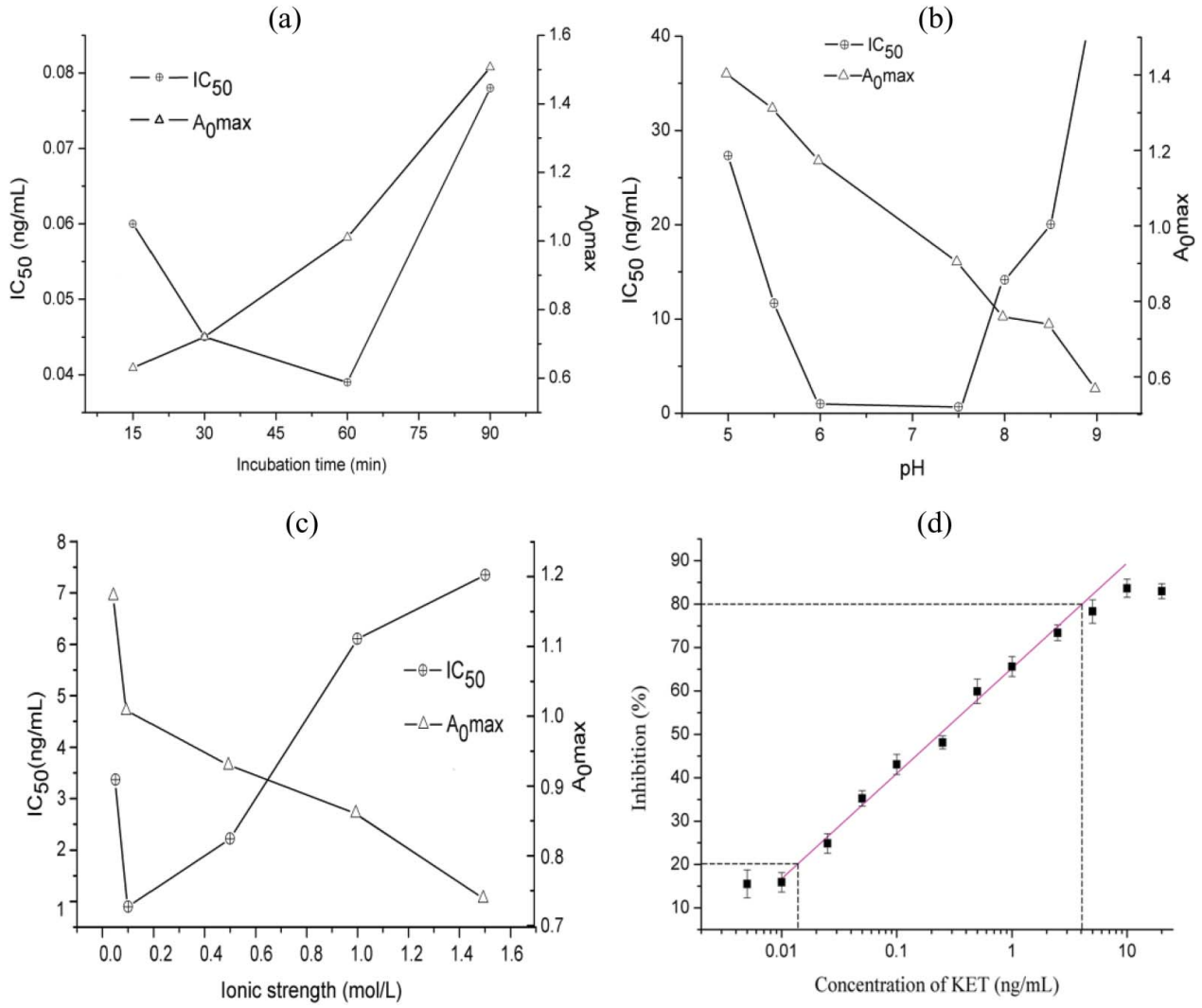


Fig. 2. Suitable operating conditions and working curve for detecting KET concentration by BA-ELISA method; (a) incubation time; (b) pH; (c) ionic strength; (d) plot of inhibition ratio(%) with the concentration of KET.

The binding of antibody and antigen is characterized by the weak intermolecular bonds, and could be affected by the pH. In this study, the pH of the optimum assay buffer was adjusted from 5 to 9. The A_{0max} value and IC_{50} were determined under varying pH; the results are shown in Figure 2b. The A_{0max} value diminished notably with increasing pH, which indirectly demonstrated that the stability of immunoassay was weakened when the condition changed from acidic to alkaline. The best combination of IC_{50} and A_{0max} was available at pH 7.4. So, pH 7.4 was selected in the further optimization assays.

To examine the influence of ionic strength, competitive curves for KET were investigated in media with NaCl concentration ranging from 0 to 1.5 mol L^{-1}, as shown in Figure 2c. In the absence of salts, the A_{0max} of assay was 1.126, and then the value diminished with increasing salt concentration, with the IC_{50} changing from 0.039 to 7.15 ng mL^{-1}. The lowest IC_{50} was available at an ionic strength of 0.1 mol L^{-1}. Hence, a salt concentration of 0.1 mol L^{-1} was chosen for the buffer in the competition assay.

LOD and working range

Under the optimum conditions, the calibration curve was performed as shown in Figure 2d. The linear working range, which is determined as the concentration range that causes 20% to 80% color inhibition,[22] was 0.012 to 4.25 ng mL^{-1}. The limit of detection (LOD) and sensitivity of the assay, represented by IC_{15} and IC_{50} values, were 0.0074 and 0.25 ng mL^{-1}, respectively. A mean bias value of 1.9%, ranging from 1.3% to 2.8%, denoted the accuracy of the measures.

The repeatability of immunoassay method

The intra-assay reproducibility and inter-assay reproducibility were determined to study the precision of the

Table 1. The repeatability of immunoassay method.

Immunoassay methods	*object*	*KET concentration (ng mL^{-1})*				
		0.01	*0.05*	*0.1*	*0.5*	*1*
BA-ELISA	Intra-assay coefficient of variation (%)	8.21	5.26	7.45	4.76	3.93
	Inter-assay coefficient of variation (%)	11.84	8.49	5.38	6.11	4.21

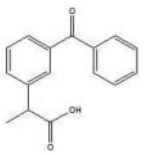
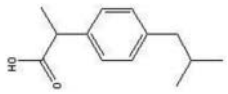
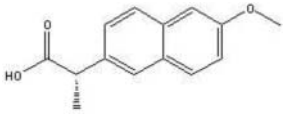
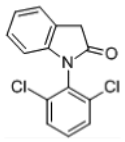
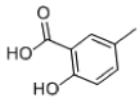
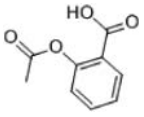
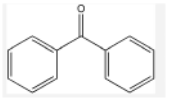
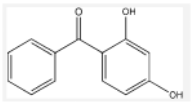
proposed BA-ELISA. Intra-assay reproducibility was estimated after executing over 10 replicates, and inter-assay repeatability was assessed over several weeks. Known from Table 1, the intra-assay coefficients of variation (CV) were <9% and the inter-assay CV was <12%. Although the inter-assay CV is relatively high, it is still acceptable for the immunoassay method, because the colorimetric assays are prone to higher variation when the concentration of analyte is lowest.[23]

Specificity

Assay specificity indicates the ability of the antibody to combine with only the target molecule. Cross-reactivity (CR,%) is an important factor in immunoassay, and is generally utilized to estimate the specificity of antibody. The CR values were calculated according to Eq. (3).

Ibuprofen, naproxen, naproxen, diclofenac, 5-methylsalicylic acid, acetylsalicylic acid, benzophenone and 2,4-dihydroxybenzophenone were selected as KET analogues. The molecular structures of analogues and the CR results

Table 2. The cross-reactivity of antibody with ibuprofen and naproxen.

Analogues	*Chemical structure*	*IC_{50} (ng mL^{-1})*	*Cross-reactivity %*
Ketoprofen		0.25	100
Ibuprofen		5.03×10^3	0.005
Naproxen		1.08×10^2	0.23
Diclofenac		1.32×10^2	0.19
5-Methylsalicylic acid		4.21×10^3	0.006
Acetylsalicylic acid		5.45×10^3	0.005
Benzophenone		3.1	8.1
2,4-Dihydroxybenzophenone		9.26	2.7

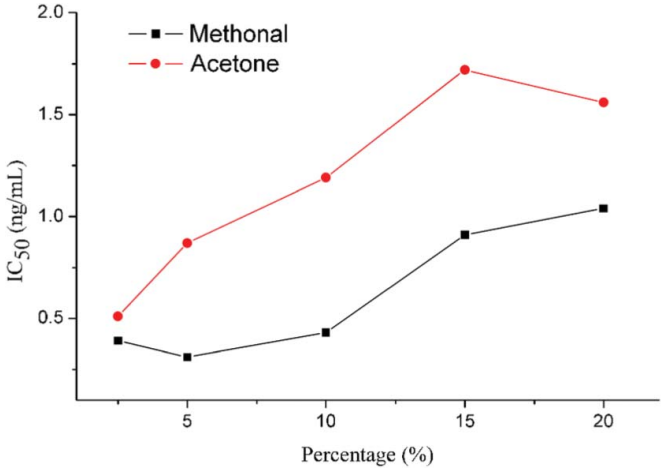


Fig. 3. The matrix effect on the assay performance.

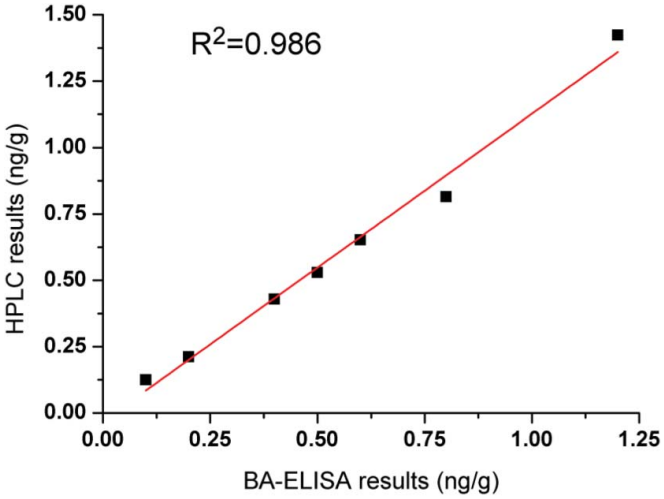


Fig. 4. Comparison of HPLC and ELISA determination in the spiked fish meat samples.

are shown in Table 2. It had proved that the obtained anti-KET antibody exhibited high affinity and was suitable for the specific detection of KET at low levels.

Matrix effects and recovery

Organic solvents, such as methanol and acetone, are normally used to extract KET from practical samples. In order to estimate the influence of different matrices on immunoassay performance, different extracts from *C. cuvieri* were diluted and then spiked with KET at the final concentration ranging from 0.01 to 10 ng mL^{-1}. The prepared samples were analyzed by BA-ELISA, and the IC_{50} results were compared in Figure 3.

In both methonal and acetone trials, sensitivity clearly decreased with increasing percentage of organic solvent. However, when methonal percentage was ranging from 2.5% to 10%, the sensitivity values were much closer to the results without matrix. The matrix effects exhibit that the low amounts of organic solvent negatively affected the sensitivity of immunoassay. Therefore, in the subsequent studies, 5% methanol was selected as the optimal extract dilution of prepared samples.

Table 3. Recoveries of KET from spiked samples.

Sample	*KET spiked level (ng g^{-1})*	*Mean (ng g^{-1})*	*Recovery (%)*	*Coefficient of variability (%)*
C. cuvieri	0.1	0.079	79.7	11.1
	0.5	0.49	99.8	6.4
	1	1.06	105.5	8.3
P. polyactis	0.1	0.097	96.6	8.5
	0.5	0.49	97.2	11.3
	1	1.02	101.7	10.8
T. lepturus	0.1	0.073	72.6	12.4
	0.5	0.42	85.3	5.2
	1	0.96	95.6	9.7

The recovery of samples spiked with target analyte was calculated to assess the analytic performance of immunoassay. Blank samples (determined by HPLC) were spiked with different amounts of KET, and the recoveries were measured. Three kinds of fish meat homogenates, *C. cuvieri, P. polyactis* and *T. lepturus,* were spiked with 0.1, 0.5 and 1 ng KET g^{-1} meat and extracted as above procedures. Extracts were diluted 20 times and then analyzed by BA-ELISA. Each sample was tested three times in duplicate under an optimized procedure to investigate the repeatability. The results are shown in Table 3. The average recovery rates were 79.7% to 105.5% for *C. cuvieri*, 96.6% to 101.7% for *P. polyactis*, and 72.6% to 95.6% for *T. lepturus*. The intra-assay CVs ranged from 6.4% to 11.1%, 8.5% to 11.3% and 5.2% to 12.4%, respectively.

To evaluate the accuracy of the proposed BA-ELISA, the spiked *C. cuvieri* meat samples, containing 0.1–1.5 ng g^{-1} KET, were simultaneously analyzed using BA-ELISA and HPLC for comparison. A good correlation (R^2 = 0.986) was obtained as showed in Figure 4. This result verified that the BA-ELISA was accurate for the analysis of KET residues.

Conclusion

In conclusion, this study provided a highly sensitive and effective BA-ELISA immunoassay for rapid detection of non-steroidal anti-inflammatory drugs—ketoprofen in fish samples. The anti-KET polyclonal antibody, used in the BA-ELISA, had high specificity against ketoprofen, with negligible cross-reactivity to structural analogs. The proposed method had a low limit of detection and high sensitivity. The linear range was from 0.012 to 4.25 ng mL^{-1}. The IC_{15} and IC_{50} were 0.0074 ng mL^{-1} and 0.25 ng mL^{-1}, respectively. The recoveries and coefficients of variation of the established immunoassay in

three kinds of fish samples were acceptable. The spiked samples were tested using BA-ELISA and the HPLC method. These two methods had consistent results. Therefore, it was confirmed that the proposed BA-ELISA immunoassay could make an efficient, sensitive and selective method for KET residue determination.

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