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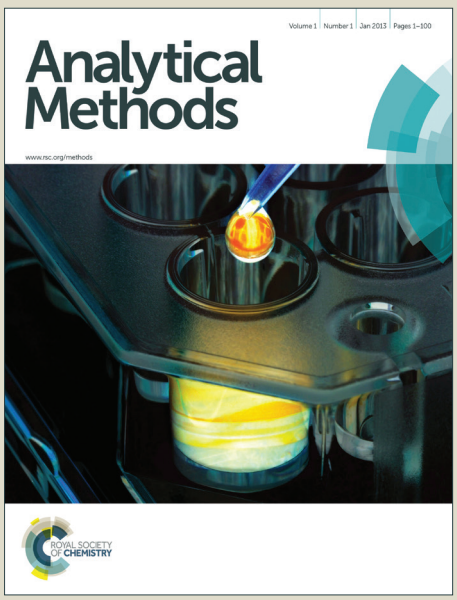

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Determination of quinoxaline antibiotics in fish feed by enzyme-linked immunosorbent assay using monoclonal antibody

Juan Peng, Dezhao Kong, Liqiang Liu, Shanshan Song, Hua Kuang, Chuanlai Xu*

Olaquindox (OLA), mequindox (MEQ), and quincetone (QCT) are widely-used synthetic antibiotics of the quinoxaline-1,4-dioxide family. However, no studies have focused on the detection of sum of OLA, MEQ, and QCT by mAb-based enzyme-linked immunosorbent assay (ELISA). In this study, a specific mAb 2F3 against OLA, MEQ, and QCT was successfully prepared. Furthermore, using the mAb 2F3, an indirect competitive ELISA (icELISA) was developed using MQCA (quinoxaline marker) coupled to OVA as the heterologous coating antigen. Under optimized assay conditions, the IC_{50} values were 1.03, 1.54, and 1.73 ng/ml for OLA, MEQ, and QCT, respectively. The recoveries ranged from 82.1% to 96.3%, and no cross-reactivity with other compounds was detected except for carbadox (0.9%). The developed icELISA was rapid and reliable for the determination of sum of OLA, MEQ, and QCT in fish feed.

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Introduction

Quinoxaline, which consists of a quinoxaline ring moiety bound to one or two acyclic chains, is a synthetic non-nutritive feed additive.[1-3] Olaquindox (N-(2-hydroxyethyl)-3-methyl-2-quinoxaline-carboxamide-1,4-dioxide; OLA), quinocetone (3-methyl-2-quinoxalinbenzenevinylketo-1,4-dioxide; QCT), cyadox (CYA), carbadox (CBX), and mequindox (MEQ), which belong to the quinoxaline family, are antibiotics and growth promoters used in aquaculture and animal husbandry.[4, 5] MQCA (3-methyl-quinoxaline-2-carboxylic acid) is a biomarker of OLA, QCT, and MEQ, [6, 7] while QCA (quinoxaline-2-carboxylic acid) is synthesized in vivo from CBX.[8-10] The chemical structures of these compounds are shown in Fig. 1.

In 1998, OLA and CBX were banned in the poultry and aquaculture industries due to potential carcinogenic, teratogenic, and mutagenic effects.[8, 11, 12] Due to low toxicity effects, MEQ and QCT, two antibiotic derivatives of the quinoxaline-1,4-dioxide family, have been widely used in China as OLA and CBX alternatives.[10] However, recent studies have suggested that these additives may pose considerable health hazards when used at high concentrations and for prolonged periods of time.[13, 14] Consequently, it is necessary to monitor MEQ and QCT levels in feed.

Analytical methods that have been used to detect quinoxaline antibiotics include chromatography-tandem mass spectrometry (LC-MS/MS) and high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS).[15-19] These methods are widely used in drug residue analyses due to their accurate analyte quantification and capacity to simultaneously analyze multiple samples. However, these methods require extensive sample preparation, skilled technicians, and

expensive equipment; therefore, they are unsuitable for routine screening analyses.[9, 20, 21] On the other hand, the enzyme-linked immunosorbent assay (ELISA) is simple, inexpensive, and sensitive. [22]

Immunoassays have been developed for OLA residue detection.[3, 23-26] However, few researchers have used monoclonal antibody based-ELISA for QCT and MEQ detection. The simultaneous detection of multi-residue quinoxaline antibiotics has been reported. In 2013, Le et al.[10] developed an ELISA for the simultaneous measurement of five quinoxaline-1, 4-dioxides (OLA, QCT, CBX, MEQ, and CYA) in porcine and chicken feeds using QCT modified with a para-amino benzoic acid. The results revealed that the half maximal inhibitory concentration (IC_{50}) were 26.4, 8.6, 17.3, 24.5, and 13.1 ng/ml for OLA, QCT, CBX, MEQ, and CYA, respectively. Cheng et al.[27] developed an ELISA to simultaneous measure OLA, QCT, CBX, MEQ, and QCA levels in swine liver using olaquindox succinic anhydride derivatives as antigens; the IC_{50} values were 1.34, 2.5, 0.38, 0.36, and 1.11 ng/ml, respectively. However, these methods were developed based on polyclonal antibodies. Few studies have focused on immunoassays based on highly specific and sensitive monoclonal antibodies against OLA, QCT, and MEQ.

The objectives of this study were to produce a monoclonal antibody that can simultaneously recognize OLA, QCT, and MEQ and develop an indirect competitive ELISA (icELISA) for the detection of these three quinoxaline antibiotics in fish feed. Research findings have revealed that using partial structures of target analytes as heterologous coating antigens may improve the sensitivity of the assay by 20× compared to homologous coating antigens.[28] Therefore, several haptens were designed as coating antigens to assess the optimal coating for icELISA.

Materials and Methods

Chemicals and materials

Standards of OLA, QCT, MEQ, MQCA, and QCA were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Horseradish peroxidase-conjugated goat anti-mouse IgG, gelatin, 3,3′,5,5′-Tetramethylbenzidine (TMB), and Freund's complete and incomplete adjuvants were purchased from Sigma-Aldrich (St. Louis, MO, USA). Keyhole Limpet Hemocyanin (KLH) and ovalbumin (OVA) were acquired from Sunshine Biotechnology Co., Ltd. (Nanjing, China). All other reagents and chemicals were obtained from the National Pharmaceutical Group Chemical Reagent Co., Ltd. (Beijing, China). Female BABL/c mice were obtained from the Shanghai Laboratory Animal Center (Shanghai, China). Fish feed was kindly donated by Tongwei Co., Ltd. (Sichuan, China). Vacuum rotatory evaporator was bought from Shanghai shenshun technology Co., Ltd. (Shanghai, China).

Solutions and buffers

The following solutions and buffers were used in this study, (1) 0.05 M carbonate buffer (CB), pH 9.6, as coating buffer; (2) 0.05 M CB with 0.2% w/v gelatin as coating buffer; (3) 0.01 M phosphate-buffered saline (PBS) containing 0.05% v/v Tween 20 and 0.1% w/v gelatin as antibody dilution buffer; (4) 0.01 M PBS, pH 6.5, as standard dilution buffer; (5) PBS with 0.05% Tween 20 as washing buffer; (6) 2 ml of 0.06% w/v TMB in glycol with 10 ml of 0.1 M citrate phosphate buffer, pH 5.0, containing 1.8 µl of 30% hydrogen peroxide as substrate; (7) 2 M H_2SO_4 as stop solution; and (8) methanol:acetonitrile:water (35/35/30, v/v/v) as sample extract solution.

Synthesis of immunogen

OLA was first converted to the derivative of OLA, called OLA-HS, by the succinic anhydride method with slight modifications. Briefly, 26.30 g of OLA and 13.00 g of succinic anhydride were dissolved in 20 ml anhydrous pyridine and heated to 65℃ for 24 h. A large number of yellow precipitates were formed following the dropwise addition of 20 ml double deionized water under constant stirring. The precipitates were extracted and purified three times with ethyl acetate and evaporated to dryness in a vacuum rotatory evaporator.

Purified OLA-HS (3.63 mg), EDC (7.64 mg), and NHS (4.60 mg) were dissolved in 250 μl of N,N'-dimethylformamide (DMF) and stirred for 12 h at room temperature.[29] The activated mixtures were added dropwise to KLH in 0.05 M CB and stirred for 12 h. The immunogen was obtained following dialysis in PBS (0.01 M, pH 7.4) for 3 d.

Synthesis of coating antigen

Several haptens were designed for coating antigen. Hapten 1 consisted of OLA-HS, and hapten 2 consisted of MQCA. For the preparation of hapten 3, 1 mM MQCA was activated with 1.1 mM isobutyl chloroformate and 1.1 mM tributylamine at 4°C for 1 h, added dropwise to 1 mM aminocaproic acid (ACA) dissolved in 0.05 M CB, and stirred for 6 h at 4°C. MQCA-ACA (hapten 3) was obtained following purification (Sundia Med Technology Co., Ltd., Shanghai, China).[30] Hapten 4 was prepared by a similar method to that of hapten 3, except that aminobutyric acid (ABA) was used instead of aminocaproic acid (ACA). Hapten 5 consisted of QCA.

Haptens 1 through 5 were attached to OVA using the modified active ester method previously described. The coating antigen was prepared following dialysis in 0.01 M

PBS. The immunogen and coating antigens were both characterized by spectrophotometry.

Production of mAb

Ten female BALB/c mice (8 weeks of age) were administered subcutaneous injections in the back (100 μg/mouse emulsified in Freund's complete adjuvant/mouse). Five booster immunizations were performed on a monthly basis (50 μg/mouse in Freund's incomplete adjuvant). Seven days after the sixth injection, antiserum was collected from the caudal vein of each mouse. The mouse exhibiting the highest affinity and inhibition ratio by icELISA was administered a seventh injection intraperitoneally (25 μg). The spleen of the injected mouse was fused with SP2/0 myeloma cells and cultured in 96-well plates. The hybridoma cells were cultured in HAT and HT solutions. Eight days post-culture, supernatants were screened by icELISA for secretion of mAb against OLA, MEQ, and QCT. Selected hybridoma cells with a high inhibition against OLA, MEQ, and QCT were subcloned three times by limiting dilution. Paraffin-primed BALB/c mice were used for ascite production. Antibodies were purified by ammonium sulfate precipitation.[31]

ELISA procedure

icELISA was performed as described elsewhere.[32] Briefly, microtiter plates were incubated with coating antigen (100 μl/well) diluted in coating buffer for 2 h at 37°C. After rinsing with washing buffer, the plates were blocked with block solution (200 μl/well) for 2 h at 37°C. After washing, 50 μl of standard solution and 50 μl of diluted mAb were added to each well and incubated for 30 min at 37°C. After washing, 100 μl of goat-anti-mouse IgG-HRP diluted in antibody dilution buffer was added, and the

plates were incubated for 1 h at 37°C. Subsequently, the plates were washed four times, and 100 µl of substrate solution was added. Following incubation for 15 min at 37°C, 50 µl of stop solution was added. Optical density (OD) was measured at 450 nm in a microplate reader.

ELISA optimization

The effects of different ionic strengths and pH values were evaluated to develop a highly sensitive and specific icELISA. Briefly, serial dilutions of OLA standards were added dropwise to coated plates. Substrate solution was added following a 30-min incubation with HRP-labeled goat anti-mouse immunoglobulin. OD was measured in a microplate reader following the addition of stop solution.

Cross-reactivity of ELISA

Carbadox, another olaquindox structure analog, MQCA, QCA and four other veterinary drugs (chloramphenicol, streptomycin, tetracycline, and erythromycin) were used to determine the cross-reactivity (CR) of the developed assay. CR values were calculated by comparing IC_{50} of OLA with those of other competing compounds in ic-ELISA using the following equation.[32]

$$CR = [(IC_{50} \text{ of OLA}) / (IC_{50} \text{ of other drugs})] \times 100\%$$

Sample preparation

The samples were prepared as described elsewhere.[10] Briefly, 2 g of ground feed was transferred into 15-ml tubes and vigorously stirred for 1 min with 10 ml extract solution. Following centrifugation at 8,000 RPM (the speed of centrifuge, revolutions per minute) for 5 min, the resulting supernatant (1 ml) was dried with nitrogen at

40°C, dissolved in 2 ml PBS, and extracted with 2 ml hexane. Following a 3-min equilibration period, the hexane layer was removed and the PBS layer was kept for further experiments.

To assess compound recovery, samples were spiked with 2, 10, and 50 ng/g of mixed standards (OLA, QCT, and MEQ). The target analytes were extracted from the matrix and determined with the developed icELISA. The accuracy of the method was evaluated by the recovery values.

Results and Discussion

Hapten and mAb

The production of antibodies with high affinity is mainly attributed to the design of a suitable hapten for immunization; therefore, the structure of the target molecule should be maintained.[33] In this study, OLA-HS (hapten 1) was selected as the hapten for immunization. The introduction of HS as the spacer arm fully exposed the quinoxaline ring, which is a common structure in quinoxaline antibiotics (OLA, QCT, MEQ, and CBX) and quinoxaline metabolites (MQCA and QCA). Consequently, the antibody produced in this study had high cross-reactivity for OLA, QCT, and MEQ. Initially, we attempted to use OLA-HS coupled to OVA as coating antigen; however, the sensitivity was unsatisfactory, with IC_{50} of 32.5, 36.2, and 54.1 ng/ml for OLA, QCT and MEQ, respectively. To improve the sensitivity of the assay, four haptens with a similar structure to that of OLA were designed as coating antigens. MQCA coupled to OVA was used as the coating antigen (hapten 2-OVA), with IC_{50} of 5.3, 8.7, and 9.2 ng/ml for OLA, QCT, and MEQ, respectively. With hapten 2-OVA there was a 6× increase in assay sensitivity relative to that of hapten 1-OVA. Additionally, aminocaproic acid (ACA) and aminobutyric acid (ABA) were introduced as spacer

arms of MQCA as hapten 3 and hapten 4, respectively, to further enhance heterology while preserving the quinoxaline ring of MQCA. As shown in Table 1, assay sensitivity improved with the introduction of the spacer arms. Notably, a 6-carbon length spacer arm (hapten 3-OVA, IC_{50}= 1.03, 1.54, and 1.73 ng/ml for OLA, QCT, and MEQ) matched a 4-carbon length spacer arm (hapten 4-OVA, IC_{50}= 2.8, 3.1, and 3.4 ng/ml for OLA, QCT, and MEQ). However, the antibody had no affinity when QCA coupled to OVA was used as coating antigen (hapten 5-OVA), probably because of the absence of a methyl group. Therefore, we selected OLA-HS coupled to KLH as the immunogen for the production of antibodies and MQCA-ACA (hapten 3) coupled to OVA for ELISA heterologous coating. Figure 2 depicts the synthesis of immunogen and coating antigen.

ELISA optimization

IC_{50}, maximal absorbance (A_{max}), and standard curve linearity are used to evaluate ELISA performance. A_{max}/IC50 ratio is an estimate of ELISA sensitivity; high ratios are indicative of high ELISA sensitivity.[34] As shown in Table 2, the optimal conditions for OLA icELISA included 0.01 M borate standard dilution buffer (pH 8.2) with 1.6% NaCl. These conditions were applied for the development of icELISA for QCT and MEQ.

ELISA sensitivity and specificity

To assess the sensitivity of the developed icELISA, three calibration curves for OLA, MEQ, and QCT (concentration range: 0–100 ng/ml) were generated. The IC_{50} values were 1.03, 1.54, and 1.73 ng/ml for OLA, MEQ, and QCT, respectively, based on a four-parameter logistic equation (Fig. 3).

The specificity of the assay was evaluated by measuring CR with CBX, MQCA, QCA, and four veterinary drugs. As shown in Table 1, mAb recognized MEQ and QCT (CR: 67 and 60%, respectively), which might be attributed to structural similarities between these compounds and OLA. CR of carbadox was 0.9%, which could be attributed to the lack of a methyl group. On the other hand, mAb had no CR with MQCA, QCA, or other antibiotics (chloramphenicol, streptomycin, tetracycline, and erythromycin), due to differences in structure (Table 3).

Recovery

Spiked samples were analyzed by the developed icELISA. As shown in Table 4, the recovery values were 82.1–90.1% for OLA, 84.2–96.3% for QCT, and 88.3–93.2% for MEQ. These findings were similar to those reported by Wu et al.[16] using LC-MS-MS (83–108%). Therefore, the developed method is reliable in real sample analyses.

Conclusions

In this study, mAb against three quinoxaline antibiotics was prepared using OLA-HS coupled to KLH as the immunogen. Moreover, five coating antigens were developed and the effects of the corresponding hapten structures were investigated. The results revealed that hapten 3-OVA was the optimal coating antigen based on improved sensitivity compared to that of hapten 1-OVA, due to the presence of an overlapped quinoxaline ring and a 6-carbon spacer arm. Using this novel coating (hapten 3-OVA), a highly sensitive icELISA was developed for detection sum of OLA, QCT, and MEQ in fish feed with IC_{50} values of 1.03, 1.54, and 1.73 ng/ml, respectively. CR of carbadox was low (0.9%); no CR was detected with other antibiotics. The recovery

values were 82.1–96.3% in fish feed. Therefore, our developed icELISA represents a feasible and convenient tool for simultaneously screening quinoxaline antibiotics in fish feed.

Acknowledgements

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Captions:

Fig. 1 Structures of four quinoxaline 1,4-di-N-oxide derivatives and their marker residues

Fig. 2 Synthesis route of immunogen antigen and coating antigen

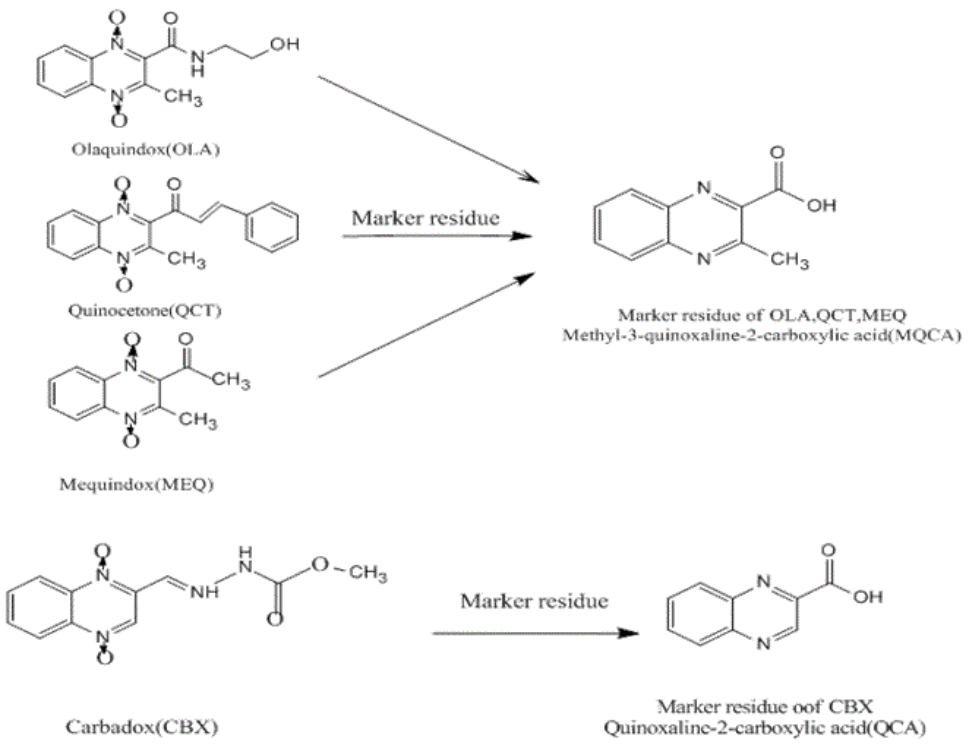
Fig. 3 Standard curves from the optimal ELISA for OLA, MEQ and QCT.

Table 1 IC_{50} of the antibody against three quinoxaline antibiotics using different coating antigens

Table 2 Optimization of parameters for ELISA performance

Table 3 Cross-reactivity of three quinoxaline 1, 4-di-*N*-oxide derivatives and other compounds

Table 4 Recovery for OLA, MEQ and QCT spiked in fish feed



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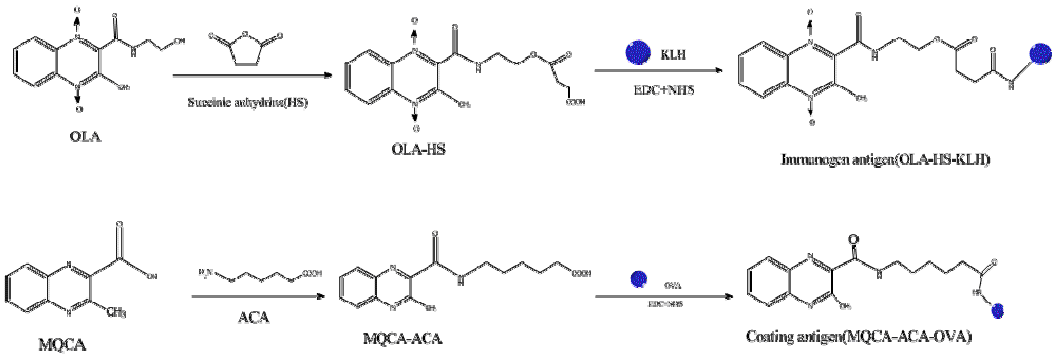

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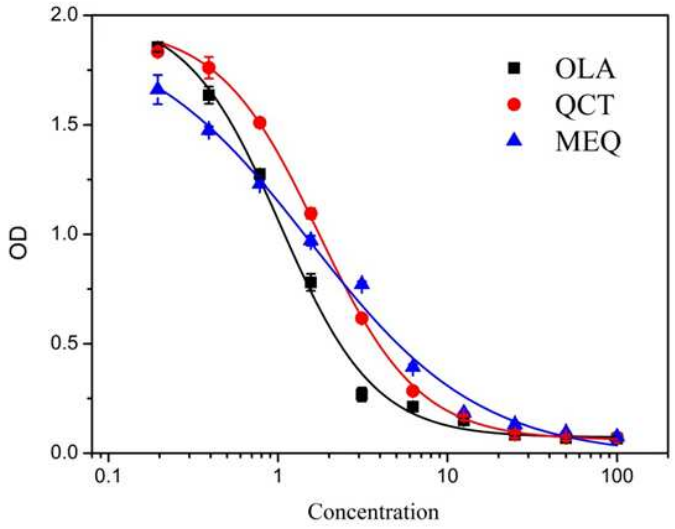

Fig. 3 Standard curves from the optimal ELISA for OLA, MEQ and QCT.

Table 1 IC_{50} of the antibody against three quinoxaline antibiotics using different coating antigens

		Hapten 1-OVA	Hapten 2-OVA	Hapten 3-OVA	Hapten 4-OVA	Hapten 5-OVA
IC_{50} (ng/ml)	OLA	32.5	5.3	1.03	2.8	—
	QCT	36.2	8.7	1.54	3.1	—
	MEQ	54.1	9.2	1.73	3.4	—

Table 2 Optimization of parameters for ELISA performance

Parameters		Amax	IC50（ng/ml）	Amax/ IC50
Concentration of NaCL	12.8%	0.912	1.35	0.68
	6.4%	1.306	1.45	0.90
	3.2%	1.552	1.50	1.03
	1.6%	1.844	1.09	1.69
	0.8%	1.884	1.9	0.99
	0.4%	1.929	1.73	1.12
PH	9.6	1.312	1.65	0.80
	8.2	1.986	1.12	1.77
	7.4	1.977	1.20	1.65
	6.5	1.405	1.88	0.75
	4.7	1.593	1.90	0.84

Table 3 Cross-reactivity of three quinoxaline 1, 4-di-*N*-oxide derivatives and other compounds

Compound	IC_{50} (ng/ml)	Cross-reactivity (%)	Compound	IC_{50} (ng/ml)	Cross-reactivity (%)
OLA	1.03	100	chloramphenicol	>10000	<0.01
MEQ	1.54	67	streptomycin	>10000	<0.01
QCT	1.73	60	tetracycline	>10000	<0.01
CBX	110.3	0.9	erythromycin	>10000	<0.01
MQCA	>10000	<0.01	QCA	>10000	<0.01

Table 4 Recovery for OLA, MEQ and QCT spiked in fish feed

Sample	Compound	Spiked level (μg/kg)	Average recovery (%)
Fish feed	OLA	2	82.1
		10	88.8
		50	92.1
	MEQ	2	84.2
		10	96.3
		50	93.1
	QCT	2	88.3
		10	91.6
		50	93.2