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Screening of Tetracycline Residues in Fish Muscles by CCD Camera-Based Solid-Surface Fluorescence

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A methodology for the screening of tetracyclines (TCs), including tetracycline (TC), oxytetracycline (OTC), and chlorotetracycline (CTC), in different fish muscle matrices has been proposed. This method was based on in situ fluorescent derivation of TCs, transferring weakly fluorescing TCs to highly fluorescent species, on alkaline-activated solid silica gel G plates (SGGPs). By coupling solid-surface fluorescence (SSF) with charge-coupled device (CCD) camera imaging, a CCD camera-based SSF (CCD-SSF) methodology has been developed. Calibration curve, repeatability, selectivity, limit of detection (LOD), and limit of quantification (LOQ) have been explored for evaluating the performance of the method itself. Linear calibration curves were obtained over a range of 0.20−1.0 ng/spot for all three TCs. The LODs, defined as 3σ, for TC, OTC, and CTC were 0.14, 0.15, and 0.16 ng/spot, respectively. The trueness of method was validated by HPLC, and no significant difference between CCD-SSF and HPLC was found, on a basis of 95% confidence level. By spiked recovery studies, a linear calibration curve ranging from 20 to 300 μg/kg of TC in fish muscle samples with a correlation coefficient (R^2) equal to 0.994 was obtained. The total average recovery for TC in fish muscle samples from six different fish matrices, fortified with TC at 50, 100, and 200 μg/kg levels, was 75.7% with average relative standard deviations (RSDs) ranging from 2.0 to 7.7%. RSDs ranged from 2.5 to 5.8% and from 5.2 to 7.6% for in-day and interday repeatability, respectively. The detection and quantification limits in fish muscle matrices were 16 and 53 μg/kg of TCs, respectively. The newly developed CCD-SSF method has been applied to the screening of the TC residues in fish muscle samples. The method has been demonstrated to bear some advantages, such as its simplicity, high throughput, low cost, use of fewer pollutants, and reasonable sensitivity.

KEYWORDS: Solid-surface fluorescence; CCD camera; screening; tetracyclines; fish muscle matrices

1. INTRODUCTION

Tetracyclines (TCs) are broad-spectrum antibiotics showing activity against Gram-positive and Gram-negative bacteria, including some anaerobes, by inhibiting the synthesis of bacterial proteins by binding to the 30S bacterial ribosome and preventing the access of aminoacyl *t*RNA to the acceptor site on the *m*RNA−ribosome complex (*1*). TCs have been widely used in the prevention and treatment of infectious diseases and as food additives for growth promotion, as well, in fish farming. The intensive use of these antibiotics has raised questions about the impact of veterinary medicines on organisms in the environment and on human health. Numerous studies have suggested a link between antibacterial use in agriculture and antibacterial-resistant infections, and there has been evidence that antibacterial resistance from agriculture can be transferred to humans (*2*). To protect public health, the U.S. Food and Drug Administration (FDA) has set a 2 μg/g oxytetracycline (OTC) tolerance in fish muscle and a 21-day withdrawal period (*3*). The European Community (EC) proposed maximum residue limits (MRLs) of 100 μg/kg for muscle, 300 μg/kg for liver, and 600 μg/kg for kidney for all food-producing animals (*4*). The Ministry of Agriculture of the People's Republic of China has set a MRL of 100 μg/kg TCs for fishery drug residues (*5*). It is important to develop sensitive analytical methods to monitor the food supply to ensure that any antibiotic residues present are below the set tolerance level, thus promoting food safety and consumer confidence.

Several techniques have been employed to assay TCs. Among them high-performance liquid chromatography (HPLC) has been by far the most widely used technique with UV (*6*−*8*), fluorescence (*9*−*11*), or mass spectrometric (*12*, *13*) detectors. Other methods include thin-layer chromatography (*14*), capillary electrophoresis (*15*, *16*), ion chromatography (*17*), and chemiluminescence (*18*, *19*). Microbiological inhibition tests have been used for the screening stage (*20*). These methods still suffer from one or more disadvantages, including high price, requirement of special equipment, low sensitivity, or time required, to name only a few. For example, the official methods of analysis for TCs have been based mostly on chromatography methodology, but the large amounts of volatile organic solvents used in

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the mobile phase are undesirable for most analysts. Microbiological analysis, the most sensitive technique for the residue analysis of TCs in food products, requires a long period of incubation and lacks precision and specificity. Analyses of TC residues in animal tissue matrices generally require multistep sample preparation. To simplify this process, a methodology that combined sorbent extraction and solid matrix time-resolved luminescence (TRL) was developed by Chen et al. (*21*). The method was illustrated by OTC screening in catfish muscle. Extraction and enrichment were accomplished by immersing small C18 sorbent strips into tissue homogenates for 20 min, followed by a 3 min rinse in water and a 2 min dip in a reagent solution. After desiccation, TRL was measured directly on the sorbent surface. Tissue particulates no longer interfered via attenuation or scattering, rendering centrifugation and filtration unnecessary. A bioluminescent sensor strain incorporating bacterial luciferase reporter genes was also used in the detection of traces of TCs in rainbow trout (*22*). The biosensing strain contained a plasmid incorporating the bacterial luciferase operon of Photorhabdus luminesced under the control of the tetracycline responsive element from transposon Tn10 (*23*). By using the biosensing technique, there was need for neither centrifugation of homogenized tissue nor use of organic solvents.

Although there have been numerous methodologies described for the determination of TCs, the qualitative and quantitative methodology and its application for TC determination in fish samples have been limited. Due to increasing concern about the impact of TC-promoted aquaculture products on human health, fast and high-throughput qualitative and quantitative methods suitable for various needs are desirable for routine analyses of TCs in different matrices.

Solid-phase fluorescence (SPF) or solid-surface fluorescence (SSF) are techniques based on the retention, preconcentration, or just spotting of the analyte on an appropriate solid support and with direct spectrometric measurements on the solid phase. Resins (*24*), silica gel (*25*), nylon (*26*), and filter paper (*27*) have been used as the solid supports in different studies. When spotted onto a solid substrate, a tiny amount of a liquid sample would subsequently dry into a small solid residue, and the analytes in the solid residue would be concentrated prior to analysis; the background arising from the sample substrate would be further reduced as well (*28*). The advantage of SSF lies in the higher emission efficiency on a solid surface in comparison with those in the solution, due to the excited molecules being isolated and collision-restricted, and thus enables very sensitive determinations of SSF. Charge-coupled devices (CCDs) are two-dimensional detectors containing an array of sensors that can image an area in seconds. By coupling CCD detection, which has achieved fast and efficient TLC analysis (*29*), with SSF, the entire solid substrate can be imaged in a single exposure, and this yields rapid, high sample throughput measurement.

Currently, our interest is focusing on developing methodologies for highly sensitively quantitative analyses and fast screening of TCs from fish matrices. The aim of a screening test is to "filter" samples to distinguish a few potentially violative samples and subject them to a quantitative method for confirmation with a more exact instrument method (*30*).

Fluorescence methodologies offer the advantage of wide linear dynamic ranges, low detection limits, and good selectivity. Unfortunately, the intrinsic fluorescence of TCs is unfavorable to their determination, which is attributed to a low quantum yield. Most fluorometric methods including chromatographic methods with fluorescence detection for the determination of TCs have been based on their reaction with di- or trivalent metal ions to form highly fluorescent chelates (*31*), such as aluminum(III), copper(II), and magnesium(II), and some lanthanide ions, especially those of europium, samarium, and terbium. These methods are generally tedious and time-consuming.

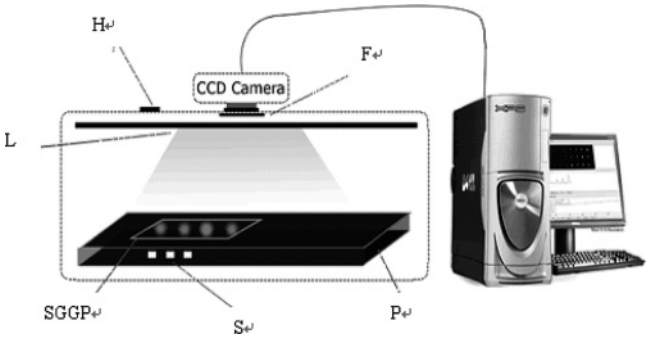

Figure 1. Schematic diagram of CCD-SSF: L, excitation Light (255, 315, and 365 nm); SGGP, silica gel G plate (10 cm × 5 cm); F, filter; H, view hole; S, switch; P, sample platform (with an irradiation area of 30 cm × 45 cm). The excitation source, silica gel G plates, sample platform, and filter are set in a darkened chamber.

Herein we propose a simple and rapid method, named CCD camera-based SSF (CCD-SSF), for the determination of TCs from fish muscle matrices. This method was based on the in situ fluorescent derivation of TCs on alkaline-activated solid gel G plates (SGGPs). The in situ fluorescence derivation was carried out by spotting TCs onto preactivated nonfluorescent SGGPs, and the TCs were converted into highly fluorescent species due to alkaline degradation. The formed SSF was acquisitioned by a CCD camera, and the CCD image was processed with self-programmed software, Chem-Image-Processor (CIP). The method of quantitative analysis and the screening test method as well were validated by spiked recoveries studies.

2. EXPERIMENTAL PROCEDURES

Reagents and Solution. Tetracycline (TC), oxytetracycline (OTC), chlorotetracycline (CTC), ciprofloxacin, sulfamethoxazole, chloramphenicol, furazolidone, streptomycin, and mebendazole were purchased from the Institute of Medicine Bioproduct Identification of China (Beijing, China). Bovine serum albumin (BSA) and L-tryptophan were of biological reagent grade. All other chemicals were of analytical reagent grade, and solvents were of HPLC grade. TC stock solution (100 mg/L) was prepared by dissolving the hydrochloride salt of TCs (0.050 g) in 500 mL of water and kept in a refrigerator (4 °C). Further dilutions with water were made for appropriate concentrations before use. Doubly distilled water was used throughout.

Prepare of SGGPs. SGGPs (10 cm × 5 cm, Qingdao Ocean Chemical Industry Factory, China) were used as the solid support of the SSF. The SGGPs were treated prior to use by dipping the plates in a 0.05 mol/L of NaOH solution for <3 s to ensure an even coating. The coating plates were dried in air for an hour, followed by 2 h of activation in the oven. The activated plates were stored in a desiccator.

CCD-SSF System. A CCD camera-based SSF detection system has been set up (**Figure 1**). The SGGP was illuminated with a UV lamp with a maximum excitation band at 365 nm (ZF-2, Qilin Medical Instrument Factory, China). The fluorescence generated by the in situ fluorescent derivation of TCs on SGGPs was acquired by a CCD camera (Canon Power Shot A 75). The shutter assembly of the camera also provided mounting for a 400 nm cutoff filter (L-42, Shimadzu) to reject any unwanted light from the UV. The CCD camera used the "M" screening mode, and the shutter speed was 15 s.

Image Processing. With CIP, the fluorescent spots (image taken by the CCD camera) were transformed into either peak height or peak area or numerical value of brightness (**Figure 2**).

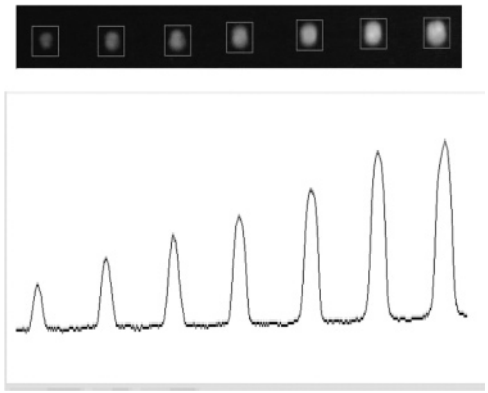

Figure 2. CCD-SSF image and corresponding intensity curves: (upper panel) CCD-SSF image; (lower panel) relative fluorescence intensity curve, for each spot. TC was used to illuminate the data processing. Concentrations were 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 ng/spot.

Fish Tissue Samples. Marine fishes, yellow croaker, silvery pomfret, and besugo, and freshwater fishes, tilapia, weever, grass carp, crucian, common carp and silver carp, were purchased from a local market. After removal of bone and skin, the muscle of each fish was minced, collected in a small sealed plastic bag, and kept frozen in a refrigerator before use.

Extraction and Cleanup Procedure. For the determination of TCs in fish muscle, a previous extraction and cleanup step was needed. With their multiple proton-donating groups, TCs showed a strong propensity to chelate multivalent cations in biological matrices; thus, extraction with EDTA−Mcllvaine buffer, a mild acidic solvent containing EDTA, was accepted as a universal extraction method for TC analysis. This method was also used in this study.

The thawed samples were fortified to the desired level of TCs. To 1.0 g of fortified sample was added 4.0 mL of pH 4.1 EDTA−Mcllvaine buffer solution. The fortified sample was then homogenized until completely homogeneous. The homogenate was transferred to a 7 mL centrifuge tube and was centrifuged at 14000 rpm for 5 min; the supernatants were decanted into a disposable centrifuge tube. Two hundred microliters of trichloroacetic acid (5%) was added to the centrifuge tube and mixed, and the mixture was centrifuged at 14000 rpm for 3 min. Another 200 μL of trichloroacetic acid (5%) was added, and the mixture was centrifuged at 14000 rpm for another 3 min followed by filtering through filter paper. The filtrate was passed through a STRATA-X (33 μm particle size, Phenomenex) SPE cartridge, previously activated with 1.5 mL of methanol and 1.5 mL of methanol 5% in water. TCs were eluted from the cartridge with 0.2 mL of methanol. The eluate was then used for the following measurement.

HPLC Conditions. A standard HPLC method was used for the validation of the newly proposed method by comparison, in terms of accuracy, recovery, and precision. A HPLC (Shimadzu LC-10AT, Japan) was used in the process of validation. The analytical column was a Diamonsil DOS-C18 reversed phase column, 4.6 × 250 mm, 5 μm i.d., Dikma Technologies. A Shimadzu SPD-10AVP UV detector was used for detection at 355 nm. The mobile phase used for analysis was methyl alcohol/acetonitrile/0.01 M oxalic acid (25:10:65 v/v/v), which was filtered through a 0.45 μm filter under vacuum. The flow rate of the mobile phase was 1.0 mL/min, and the volume injected was10 μL.

CCD-SSF Procedure. A micropipet (1 μL) was used to spot the standard solution or the extraction and cleanup solution of samples fortified with different level of TCs onto the surface of the preactivated SGGP (forming a spot of less than 1 mm × 1 mm). After 5 min of air-drying in the dark, the SGGP was put into the darkened chamber of the CCD-SSF setup and illuminated with a 365 nm UV source. Emission light from the TC derivatives was acquired by CCD, and the CCD-SSF image was transferred into relative fluorescence intensity by the CIP software. To achieve a high-quality CCD-SSF image and thus a highly sensitive measurement, a quintuple-spotting method was used, that is, spotting a small value of sample onto the same spot five times instead of spotting a larger volume of sample onto the SGGP, which was prone to result in the diffusion of the sample spot and debase the quality of the CCD-SSF image.

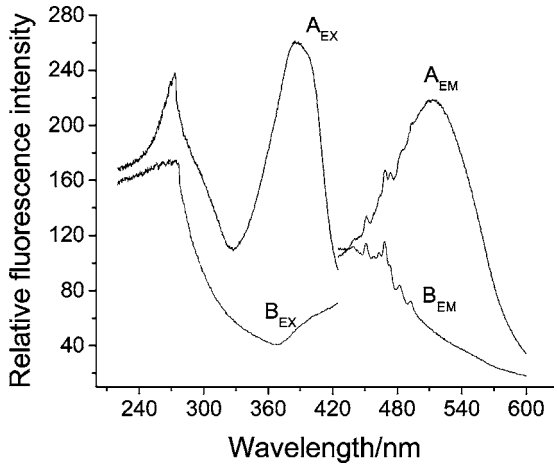

Figure 3. Excitation (EX) and emission (EM) spectra of TC on SGGP: (A) 1.0 μg/mL of TC; (B) blank. The spectra were acquired on a Shimadzu RF-5301 photofluorometer, with a solid sample setup, and the excitation and emission band-passes were set as 10 and 5 nm, respectively.

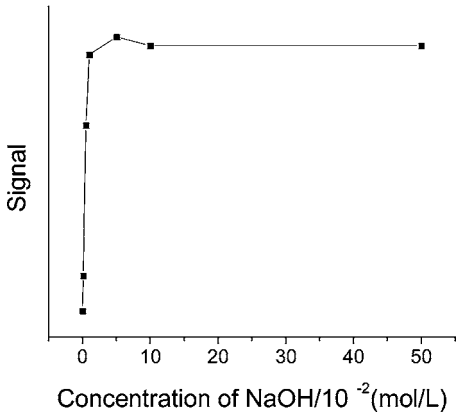

Figure 4. Dependence of CCD-SSF signal on the concentration of NaOH solution (5.0 ng/spot of TC).

3. RESULTS AND DISCUSSION

3.1. Procedure Development and Optimization. *Fluorescent Derivation.* TC is a weakly fluorescing compound, but it was transferred into its highly fluorescent derivative in alkaline medium (*32*). When spotted onto an alkaline-treated SGGP, the TC spot showed a yellow-green image, which could be observed even with the naked eye. The SSF spectra showed a maximum excitation wavelength and an emission wavelength at 380 and 510 nm, respectively (**Figure 3**). A lamp with a maximum excitation band at 365 nm was used as the excitation source, and a 400 cutoff filter was used for eliminating the excitation light from the lamp and collecting the emission from the fluorescent derivatives. OTC and CTC evidenced the same fluorescent derivation behavior. TC was used as a model for the exploration and validation of the procedure.

Procedure Optimization. To achieve in situ fluorescence derivation of TC, the pretreatment of the SGGPs by a NaOH solution was needed. To get an optimal procedure for the pretreatment of SGGPs, the concentration of NaOH solution, the dipping time, the air-dry time, and the activation time in an oven were explored. **Figure 4** demonstrates the dependency of the SSF signal on the concentration of NaOH solution. By

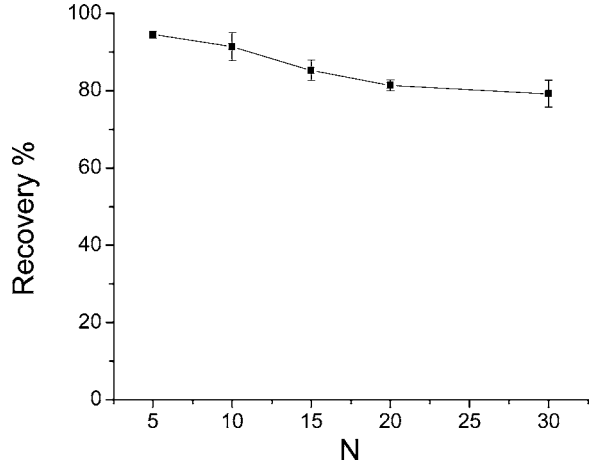

Figure 5. Efficiency of in situ enrichment by multiple spotting. *N* stands for the times of spotting sample solution onto a single spot and also represents the multiple enrichment in theory. The recovery stands for the percent ratio of the response from a multiple-spotting spot to the response from a single-spotting spot of the same mass of TC.

robustness test, a procedure of dipping in 0.05 mol/L of NaOH solution (pH 12.7), for <3 s, followed by 1 h of air-drying and 2 h of activation in an oven was set for the pretreatment of SGGPs. The preactivated SGGPs were stored in a desiccator ready for use.

TC was sensitive to light. The fluorescence intensity decreased gradually on exposure to room light. To avoid light degradation, following spotting of the sample in room light, the SGGPs were left in a darkened chamber to air-dry. Exploring the stability of the derivatives of TC gave a result showing that the decrease in intensity was 2.7% in 30 min. To achieve a better result, the stand-by time following the sample spotting should be kept consistent to minimize the effect of room light.

Multiple Spotting. In conventional methods, a total 10−50 times enrichment including extraction and concentration of the eluate might be needed in dealing with real fish samples. The concentration of the eluate was usually achieved by drying the eluate in a flow of nitrogen (nitrogen-drying process) following extraction. The concentrating step usually took ≥0.5 h. In our study, the multiple-spotting method was used for the enrichment. With the multiple-spotting method the in situ enrichment was achieved and thus no nitrogen-drying process was needed. The lower recoveries in higher N (**Figure 5**) were attributed to the eroding of the SGGPs due to the heavy load of solution.

Solid Substrate and Background Depression. A method based on solid surface room temperature fluorescence for the determination of TC on filter paper has been reported (*33*). In the present work, we selected nonfluorescent SGGPs as the substrate of the solid-surface fluorescence for the sake of reducing the background contribution from the substrate efficiently. The advantage of the SGGP also lay in its excellent mechanical strength of the glass-backed sorbent layer in comparison to those of filter paper, allowing it to be soaked in NaOH solution with pH as high as 12.7 and activated in the oven for 2 h. The SGGPs used in this study were commercial products usually used for TLC, and the quality of these SGGPs was known to vary significantly from manufacturer to manufacturer and from batch to batch. To achieve a reasonable repeatability as well as reproducibility, an internal background depression approach was used. That is, eight blank blocks were randomly chosen in the SSF image panel around a sample image spot, and the brightness of these blank blocks was averaged and set as the background value, I_0. On the other hand, the brightness of the sample spot in the CCD-SSF image was set to be I; thus, $I_s = I - I_0$ represents the emission from the fluorescence derivative of TC, OTC, or CTC. A plot of I_s against the mass of TCs loaded on each spot yielded the calibration curve of the method itself. A plot of I_s against the concentration of TCs in fish muscle sample yielded the calibration curve of the quantitative methodology for fishery drug residue analysis.

Table 1. Effect of Interferents on Determination of TC by CCD-SSF Method (2.0 μg/mL of TC)

interferent	max tolerated concn (μg/mL)	interferent	max tolerated concn (μg/mL)
Na^+	6.0×10^3	glucose	4.0×10^3
K^+	6.0×10^3	BSA	2.0×10^3
Mg^{2+}	2.0×10^3	L-tryptophan	1.0×10^3
Ca^{2+}	3.0×10^3	β-cyclodextrin	2.0×10^2
Cu^{2+}	6.0	ciprofloxacin	6.0
Al^{3+}	2.0×10^2	sulfamethoxazole	2.0×10^2
SO_4^{2-}	6.0×10^3	chloramphenicol	1.6×10^2
PO_4^{3-}	6.0×10^3	furazolidone	1.6×10^2
CO_3^{2-}	6.0×10^3	streptomycin	2.0×10^2
starch	3.0×10^3	mebendazole	2.0×10^2

Interference Test. Various substances that might exist in fish muscle and other drugs that might be used in aquaculture were tested for the sake of evaluating the selectivity of the proposed methods. Different levels of interferents were added into a 2.0 μg/mL TC standard solution and the responses measured. The criteria for interference were fixed at a ±10% variation of the established level of TC, and the experimental results are shown in **Table 1**. The results indicated that only Cu^{2+} and ciprofloxacin interfere with the detection of TC, and both gave higher results. In the case of determination of TCs in fish muscle, EDTA−McIlvaine buffer would be used in the process of extraction; the interference from presenting Cu^{2+} would be eliminated by forming chelates of EDTA. Ciprofloxacin is a forbidden drug in aquaculture. In practice, after a screening test, positive results would be followed by further confirmation; thus, the positive result produced from the presence of ciprofloxacin would give a clue to the violation of regulation by using ciprofloxacin.

Limit of Detection (LOD), Limit of Quantification (LOQ), and Repeatability. Following the optimized procedure, the response of the CCD-SSF was quantitatively related to the mass of TCs loaded on each spot. A satisfactory calibration curve was observed using the linear least-squares regression procedure for all three TCs when the mass of TCs loaded on each spot was <1.0 ng. As the mass loaded on each spot increased, the calibration curve gradually leveled off toward the mass axis, and nonlinearity regression was obtained for all three TCs over a range of 0.2−40 ng/spot versus the CCD-SSF response (**Table 2**). The LOD and LOQ were calculated according to $3S_0/S$ and $10S_0/S$, respectively, where S_0 represents the standard deviation of the blank signal ($n = 11$) and S represents the slope of the calibration curve. With quintuple repetition spotting, this LOD corresponds to a solution LOD of 0.030−0.032 μg/mL of TCs, and the LOQ corresponds to a solution LOQ of 0.10−0.11 μg/mL of TCs, respectively, and a linearity calibration curve of 0.040−0.20 μg/mL of TC was obtained (**Figure 6**).

Interday and in-day repeatability and interplate and intraplate repeatability as well were tested. Three group experiments, named the in-day/in-plate experiment, and in-day/interplate experiment, and the interday/interplate experiment, each consisted of six samples ranging from 0.20 to 10 ng/spot; six

Table 2. Calibration Curves, Limits of Detection, and Correlation Coefficients

	tetracycline (TC)	oxytetracycline (OTC)	chlorotetracycline (CTC)
calibration curve 0.20–40 ng/spot	$I_s = -1.3 \times 10^5 \exp(-0.11x) + 1.4 \times 10^5$	$I_s = -1.2 \times 10^5 \exp(-0.095x) + 1.3 \times 10^5$	$I_s = -1.5 \times 10^5 \exp(-0.098x) + 1.6 \times 10^5$
calibration curve 0.20–1.0 ng/spot	$I_s = 2.4 \times 10^4 x - 3.9 \times 10^2$	$I_s = 2.4 \times 10^4 x - 6.1 \times 10^2$	$I_s = 2.2 \times 10^4 x + 1.7 \times 10^3$
correlation coefficient R	0.999	0.998	0.999
S_0 ($n = 11$)	1.1×10^3	1.2×10^3	1.2×10^3
LOD (ng/spot)	0.14	0.15	0.16
LOQ (ng/spot)	0.47	0.50	0.53
LOD[a] (μg/mL)	0.030	0.030	0.032
LOQ[a] (μg/mL)	0.10	0.10	0.11

[a] With quintuple-spotting method.

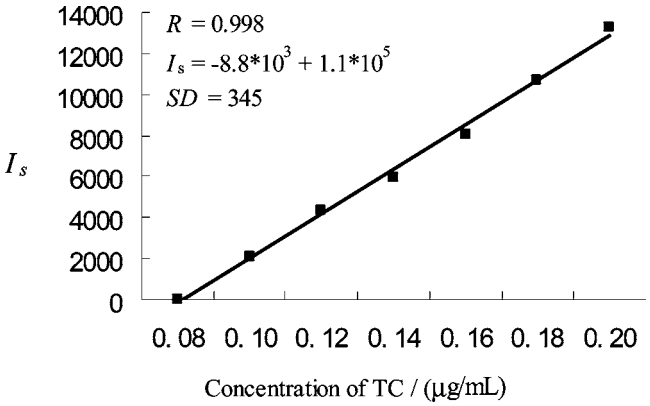

Figure 6. Calibration curve for lower concentration of TC with a quintuple-spotting method.

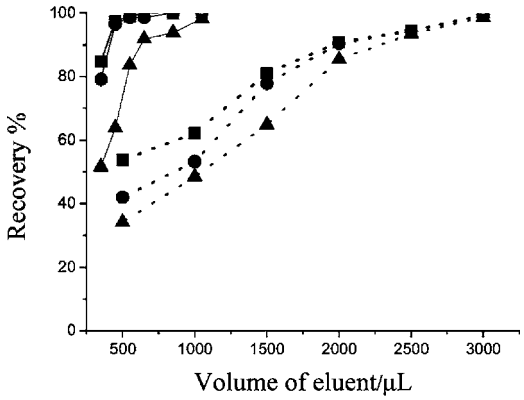

Figure 7. Comparison of the elution efficiency for STRATA-X (solid lines) and C18 (dotted lines). Mass of TC: (■) 100 ng; (●) 200 ng; (▲) 500 ng.

replicate measurements for each sample were carried out. The results showed that the average relative standard deviation (RSD %) for in-day/in-plate, in-day/interplate, and interday/interplate experiments were 2.9, 5.6, and5.6%, respectively.

Test of Significance. The official method most used for the determination of TCs in aquaculture is HPLC (*5*). Standard solution containing 0.80 μg/mL of TC was measured by HPLC and CCD-SSF, with 11 replicate measurements for each, respectively. Recoveries of 0.82 and 0.79 μg/mL of TC were obtained for CCD-SSF and HPLC, respectively. Standard deviations of 0.042 and 0.025 μg/mL of TC were obtained for CCD-SSF and HPLC, respectively. The *F* test and Student's *t* test at the 95% confidence level demonstrated that there was no significant difference between the CCD-SSF and the accepted HPLC method, on the basis of either standard deviation (representative of repeatability) or accuracy (trueness).

3.2. Methodology for Screening of TCs in Fish Muscle. *Extraction Optimization.* Two commercial SPE cartridges, that is, C18 (200 mg) and STRATA-X (30 mg) cartridges, were tested for a better performance in facilitating the extraction of TCs with a low concentration in fish muscle matrices. Generally, the mechanism of TCs interacting with sorbent includes hydrophobic interactions, hydrogen bonding, chelation, and cation exchange. Although C18 cartridges have been widely used in the literature to preconcentrate TCs, STRATA-X has proved to provide some advantages over C18 in our work.

Five milliliters of TC standard solutions containing 100, 200, and 500 ng of TC was loaded onto C18 and STRATA-X cartridges, respectively. Methanol was used in eluting the extract. All tests were carried out at a flow rate of 1.0 mL/min. The recoveries were tested by changing the volume of methanol over the range of 100−3000 μL. In the case of STRATA-X, the recovery reached 98% in both 100 and 200 ng loads when 200 μL of methanol was used; 800 μL of methanol was needed, however, in 500 ng loads. In the case of C18, 3000 μL of eluent was needed for all three load levels (**Figure 7**). With the STRATA-X cartridge, a better repeatability, <4% (RSD), was also obtained. In the following experiments, 200 μL was used for the elution.

STRATA-X, with a modified styrene−divinylbenzene polymer, does not contain a silanol backbone, thus avoiding the problem of TCs interacting too strongly with the silanols of a silica-based cartridge, as usually occurs in C18, which would debase the elution efficiency. STRATA-X particles (33 μm) also give a higher surface area than those of C18, and this provides high capacity and high extraction efficiency.

Representative TCs. In practice, when aquaculture products are wanted to screen for TCs, it is difficult to obtain information on the usage of individual TCs, TC, OTC, or CTC, and this makes the choice of standard reference material problematic. According to the early experiment, we knew that three of the TCs gave similar responses in this CCS-SSF method. Could we use one to stand for all three? This idea has been validated, and the results are shown in **Table 3**. The average response from individual TCs and the total average response from all three TCs were calculated and compared. The results showed that although OTC gave a lower response and CTC gave a higher one, there were no significant differences either between the average response from TC and those of OTC, between the average response from TC and those of CTC, or between the average response from each of the TCs and the total average response of TCs, on the basis of a 99% confidence level. TC has proved to be a suitable representative of all three TCs (**Figure 8**).

Calibration Curve in Fish Muscle Matrices. To evaluate the performance of the screening of TCs in fish muscle matrices,

Table 3. Comparison between the Individual Average Response from Each of the TCs and the Total Average Response of TCs[a]

	I_s (five replicate measurements)				relative error (RE, %) $(\langle I_s\rangle - \langle I_{TCs}\rangle)/\langle I_{TCs}\rangle$		
mass (ng/spot)	$\langle I_{TCs}\rangle$	$\langle I_{TC}\rangle$	$\langle I_{OTC}\rangle$	$\langle I_{CTC}\rangle$	RE_{TC}	RE_{OTC}	RE_{CTC}
0.20	4132	4062	3850	4358	–1.7	–6.8	5.5
0.40	9698	9803	9127	10164	1.1	–5.9	4.8
0.60	14740	14776	14206	15238	0.24	–3.6	3.4
0.80	18805	18608	18007	19800	–1.0	–4.2	5.3
1.0	23789	23598	22885	24884	–0.8	–3.8	4.6

[a] The angled brackets indicate average value.

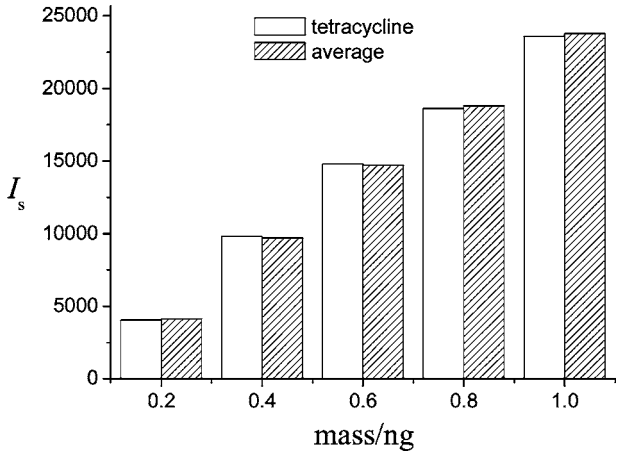

Figure 8. Comparison between the average response of TC and the total average response of TCs.

the calibration curve was constructed by using crucian as a model fish. Linearity was observed over a range of 20−300 μg/kg in fish muscle with a $R^2 = 0.997$. The minimum detectable concentration (3σ) and the minimum determination concentration (10σ) were 16 and 53 μg/kg of TC, respectively, in fish muscle. This method has proved to meet the requirement of official methods.

Repeatability. The repeatability test was carried out by using fortified fish muscle samples from tilapia, weeever, crucian, silver carp, silver pomfret and besugo. The concentration was fortified at 100 μg/kg of TC, corresponding to the MRL. In-day repeatability tests were carried out within the same day with three batches, and interday repeatability tests were carried out on three different days with 54 replicates for each. The results are listed in **Table 4**. Satisfactory repeatability was obtained: the RSD lay in a range of 2.5−5.8% for in-day and 5.2−7.6% for interday, respectively, in all tested fish muscle matrices. This result meets the criteria set by the Ministry of Agriculture of China and the decision 2002/657/EC, which requires a <10% RSD for in-day relative standard deviation and a <15% RSD for interday relative standard deviation.

Spiked Recovery Studies. One gram of muscle was sampled from each of six kinds of freshwater and seawater fishes, tilapia, weeever, crucian, silver carp, silver pomfret and besugo, respectively, and the samples were fortified, at three final concentrations of 50, 100, and 200 μg/kg of TC, for each fish sample, respectively, corresponding to 0.5, 1, and 2 times the MRL. Overall, 6 blank and 18 fortified fish muscle samples were used for spiked recovery studies and overall 60 measurements were carried out in parallel. Several appropriate standard solutions were spotted onto each SGGP in parallel, and the responses were used for the calculation of recoveries. The results demonstrated that there was no TC found in control samples. The average recoveries were 64.5 and 61.7% for fortified marine fish and freshwater fish muscle samples, respectively, and the total average recovery for all fortified fish muscle sample was 63.1%. The RSD was 8.7 and 8.6% for fortified freshwater fish and marine fish muscle samples, respectively. The total RSD was 9.0% for all fortified fish muscle sample. There is no significant difference between freshwater fish and marine fish with regard to either recovery or precision on the basis of a 95% confidence level. Considering that the cleanup sample solutions were reconstructed in methanol after the sample preparation procedure and that the differences in solvents between the standards and cleanup samples might affect derivation yield and fluorescence intensity, a correction was carried out by using methanol instead of water as the solvent of standard solution of TCs. The results showed that the fluorescence intensities of TC derivatives in methanol were some what lower than that in water; the average correction coefficient was 0.833 (intensity in methanol/intensity in water). Thus, a total average recovery of 75.7% for all fortified fish muscle samples would be actually obtained.

Comparison with HPLC. One gram of muscle was sampled from each of nine kinds of freshwater and marine fishes, tilapia, weeever, crucian, silver carp, grass carp, common carp, silver carp, silver pomfret and besugo, respectively, and the fish muscle samples were fortified at a concentration of 100 μg/kg of TC, corresponding to the MRL. Overall, nine blank and nine fortified fish muscle samples were analyzed in parallel by CCD-SSF and HPLC. The recoveries were calculated relative to the standard solutions in water. There was no TC found in control samples by either CCD-SSF or HPLC. The average concentrations found in fortified fish muscle sample were 61.5−68.8 and 82.6−93.1 μg/kg by CCD-SSF and HPLC **(Table 5)**, respectively. The total average recoveries in all fish matrices were 65.0 and 88.5%, and the standard deviations were 2.4 and 3.2 μg/kg for CCD-SSF and HPLC, respectively. The lower recovery in CCD-SSF has been attributed to the effect of fish muscle matrices and the differences in solvents between the standards and cleanup samples. On correction for the solvent effect, the average concentration found in fortified fish muscle samples by the CCD-SSF method would be 73.8−82.6 μg/kg. Although the recovery of this method was lower than those of HPLC, this method has its own advantages, such as simplicity, high throughput, and low cost.

Decision Limit (CC_α) and Detection Capability (CC_β). CC_α and CC_β were determined in accordance with the 2002/657/EC European Decision (*34*).

In the case of TCs, which had an established maximum permitted limit, CC_α was calculated by analyzing 20 aliquots of fish muscle, all fortified with TC at the maximum permitted limit (100 μg/kg). The concentration at the maximum permitted limit, plus 1.64 times the corresponding standard deviation, gave a CC_α ($\alpha = 5\%$) of 107 μg/kg. Then, CC_β was established by analyzing 20 aliquots of fish muscle, all fortified with TC at the calculated CC_α level. The concentration at the CC_α, plus 1.64 times the corresponding standard deviation, gave a CC_β ($\beta = 5\%$) of 114 μg/kg.

Screening of TCs in Fish Muscle. Although the CC_α and CC_β might be used in a screening test, the CC_α−CC_β approach gave consideration in both α-type error or false-positive rate and β-type error or false-negative rate equally, both setting to 5% probability. In the case of assessing a maximum permitted concentration level, as in this work, assessing the MRL of fishery drugs in aquaculture product, public concern lies in the β-type error. The probability of β-type error should be decreased.

Table 4. Results of Repeatability Studies (Fish Muscle Sample Fortified at 100 μg/kg)

fish	sample no./ items	TC found in fish muscle matrices (μg/kg) first day	second day	third day	interday average	interday RSD (%)
tilapia	1	64.2	68.4	55.6	61.7	6.7
	2	60.5	64.5	56.4		
	3	62.5	63.7	59.6		
	in-day average	62.4	65.5	57.2		
	in-day SD	1.8	2.5	2.1		
	in-day RSD (%)	3.0	3.9	3.7		
weever	1	63.4	56.3	67.8	61.2	7.6
	2	58.3	58.6	61.6		
	3	61.3	55.4	68.5		
	in-day average	61.0	56.7	66.0		
	in-day SD	2.6	1.6	3.8		
	in-day RSD (%)	4.2	2.9	5.8		
crucian	1	67.2	68.6	61.5	66.4	5.7
	2	65.4	71.5	65.7		
	3	70.9	66.8	60.4		
	in-day average	67.8	69.0	62.5		
	in-day SD	2.8	2.4	2.8		
	in-day RSD (%)	4.2	3.4	4.5		
silvery pomfret	1	59.2	62.5	64.2	64.1	4.9
	2	62.8	67.8	66.2		
	3	65.4	68.3	60.5		
	in-day average	62.5	66.2	63.6		
	in-day SD	3.1	3.2	4.5		
	in-day RSD (%)	5.0	4.9	4.5		
yellow croaker	1	59.5	66.2	68.2	64.9	6.5
	2	64.3	62.8	71.3		
	3	58.6	64.3	68.5		
	in-day average	60.8	64.4	69.3		
	in-day SD	3.1	1.7	1.7		
	in-day RSD (%)	5.0	2.6	2.5		
besugo	1	72.3	59.5	63.5	64.3	7.3
	2	67.9	62.5	58.7		
	3	69.4	64.3	60.2		
	in-day average	69.8	62.1	60.8		
	in-day SD	2.2	2.4	2.5		
	in-day RSD (%)	3.2	3.9	4.0		

Table 5. Spiked Recovery Studies Using CCD-SSF and HPLC

fish sample	TC added (μg/kg)	CCD-SSF TC found (μg/kg)	CCD-SSF recovery (%)	HPLC TC found (μg/kg)	HPLC recovery (%)
tilapia	0				
	100	63.2	63.2	84.5	84.5
crucian	0				
	100	66.9	66.9	91.2	91.2
grass carp	0				
	100	64.7	64.7	89.5	89.5
yellow croaker	0				
	100	66.5	66.5	89.6	89.6
silvery pomfret	0				
	100	62.8	62.8	85.2	85.2
besugo	0				
	100	67.3	67.3	91.6	91.6
weever	0				
	100	61.5	61.5	84.6	84.6
common carp	0				
	100	68.8	68.8	93.1	93.1
silver carp	0				
	100	63.7	63.7	87.6	87.6
mean		65.0	65.0	88.5	88.5
SD (μg/kg)		2.4	2.4	3.2	3.2
RSD (%)		3.7	3.7	3.6	3.6

With this consideration in mind, a threshold for screening of TCs in fish was established. The samples below the threshold were assessed as negative, whereas those above were presumed to be positive and regarded as violative. Samples from six different fish muscle matrices, tilapia, weever, crucian, silvery pomfret, yellow croaker and besugo, were fortified at the MRL level. Three batch experiments were carried out in parallel. The results are shown in **Figure 9** (top). Data points from control samples are also included in this figure (bottom). Statistical treatment yielded the means ($\bar{x}$ and $\bar{x}_0$) and standard deviations (σ and σ_0) of the response from both control and fortified fish muscle samples; each point averaged three replicates. Herein σ and σ_0 were used in a way equal to S and S_0. Strictly, σ and σ_0 would be smaller than S and S_0. Because these two groups of data were well separated, screening was possible on the basis of a threshold value drawn in between. Statistically for any random population, the probability at which the response from fish muscles fortified with TC at the MRL lay above $\bar{x}_{100} - 3\sigma_{100}$ is 99.87%. When a screening threshold was set at $\bar{x}_{100} - 3\sigma_{100}$, the β-type error or false-negative rate would be decreased to a 0.13% level, in theory, whereas the α-type error or false-positive rate increased to a higher level.

False-Positive/-Negative Rates and Sensitivity/Specificity Rates. The false-positive/-negative rate and the sensitivity/specificity rate were defined as in ref *35*.

To evaluate false-positive/-negative rates and sensitivity/specificity rates, 50 fish muscle samples fortified around the MRL (0−200 μg/kg) were prepared for a screening of TCs (with a threshold set at $\bar{x}_{100} - 3\sigma_{100}$), where 24 were negative and 26 were positive. The results showed that zero false negatives and 3 false positives were found and gave a 0% false-negative rate,

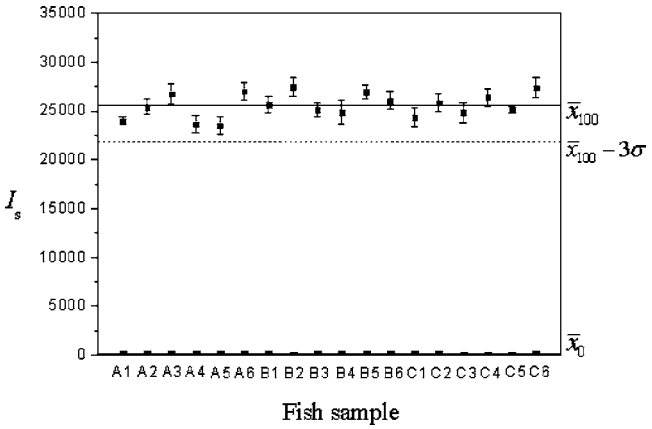

Figure 9. Establishing threshold level for TCs in fish muscle. The data points at the bottom were the response produced from control samples, whereas the data points at the top were the response from the fortified samples at 100 μg/kg of TC. Each dot represented the mean of three replicates. Statistical treatment yielded the means ($\bar{x}$) and standard deviations (σ) for these two groups. The value of $\bar{x}_{100} - 3\sigma_{100}$ was chosen as the screening threshold; 1, 2, 3, 4, 5, and 6 represented tilapia, weever, crucian, silvery pomfret, yellow croaker, and besugo respectively. A, B, and C represented three different batches.

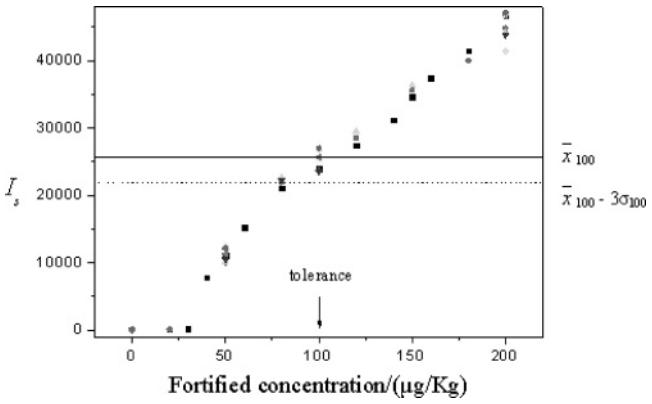

Figure 10. Screening result of TC blind fortified samples. The data points were the results of the screening for 50 blank fish samples, each fortified at a randomly selected level in the range of 0–200 μg/kg. Of 50 samples, there were 0 false negatives and 3 false positives found.

an 11.1% false-positive rate, a 100% sensitivity rate, and an 88.9% specificity rate, respectively **(Figure 10)**.

3.3. Conclusion. In conclusion, a simple, rapid screening method for TC residues in fish muscle samples has been developed and validated in accordance with 2002/657/EC. This methodology was based on the in situ fluorescence derivation of TCs on alkaline-activated SGGPs. This method would be performed in high throughout by using spotting of the previously cleaned up samples onto SGGPs, where the produced SSF response would be easily acquired by a CCD camera. The fluorescence image would be transferred to either brightness values or peak heights for plotting against the concentration of TCs and yielding the calibration curve for quantification. With a screening threshold set at $\bar{x}_{100} - 3\sigma_{100}$, satisfactory false-negativefalse-positive rates and sensitivity/specificity rates were obtained.

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