SPECIAL GUEST EDITOR SECTION

Determination of Oxytetracycline from Salmon Muscle and Skin by Derivative Spectrophotometry

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A method was developed for the identification and quantification of oxytetracycline residues present in salmon muscle and skin using UV-Vis derivative spectrophotometry. With this method, it was possible to reduce the number of steps in the procedure typically required for instrumental analysis of a sample. The spectral variables, order of the derivative, scale factor, smoothing factor, and analytical wavelength were optimized using standard solutions of oxytetracycline dissolved in 900 mg/L oxalic acid in methanol. The matrix effect was significant; therefore, quantification for oxytetracycline residues was carried out using drug-free salmon muscle and skin samples fortified with oxytetracycline. The LOD and LOQ were found to be 271 and 903 µg/kg, respectively. The precision and accuracy of the method were validated using drug-free salmon muscle and skin tissues fortified at three different concentrations (8, 16, and 32 mg/kg) on 3 different days. The recoveries at all fortified concentrations were between 90 and 105%, and RSDs in all cases were less than 6.5%. This method can be used to screen out compliant samples and thereby reduce the number of suspect positive samples that will require further confirmatory analysis.

The tetracyclines (TCs) are antimicrobials widely used in human, animal, and plant medicine to treat a wide range of infections caused by bacteria. Because of their broad spectrum of activity, cost benefits, and lack of significant adverse side effects, TCs account for over 50% of the total antibiotic consumption in veterinary medicine (1). An important member of the TCs is oxytetracycline (OTC), a drug used primarily to treat bacterial diseases of the salmon industry, some of which are very difficult to control and can cause sudden loss of these species with significant economic losses for the producers. In the United States, OTC is approved by the U.S. Food and Drug Administration for the treatment of diseases in fish (2). It is currently used in Chile to treat salmonid rickettsial syndrome, bacterial kidney disease, vibriosis, streptococcosis, and flavobacterium (3, 4) and as a prophylactic (5–7). Due to its wide use in the salmon industry, its residual concentrations in salmon can trigger adverse effects on human health. For these reasons and to protect public health, the United States, European Union (EU), Japan, and Chile have been defined the maximum residue limits (MRLs) as 2000, 100, 200, and 100 µg/kg, respectively, corresponding to the sum of OTC and its 4-epimers (8).

In today´s global economy in which fish is extensively traded, sensitive methods are needed by companies in the industry and regulatory agencies to support the regulation of the use of OTC in salmon production. Presently, most methods available for the determination of these residues in food of animal origin are based on LC with UV (9), fluorescence (10, 11) or MS detection (12, 13). Other LC detection methods, such as chemiluminescence (14, 15) and electrochemical (16), have also been used for the analysis of OTC in honey and shrimp, respectively. However, these methods for drug determination have the disadvantage of requiring sophisticated techniques that use expensive equipment. Without access to any of this sophisticated and expensive instrumentation, we are proposing a method to measure the concentrations of OTC in salmon muscle and skin samples using a simple and readily available UV-Vis spectrophotometer.

Experimental

Apparatus

(**a**) *Spectrophotometer*.—A Shimadzu® UV-1603 spectrophotometer (Kyoto, Japan) with 10 mm quartz cells was used for absorbance and derivative absorption spectra measurement over a range of 190–500 nm versus solvent using slit wide values of 2.0 nm, sampling intervals of 0.2 nm, and scan speed of 480 nm/min. The spectral data were processed by Shimadzu® software kit version 3.7 (No. 206-60570-04).

(**b**) *LC*.—The LC system (Waters Corp., Milford, MA) was equipped with an RP C18 XTerra column (4.6 × 250 mm id 5 µm), a photodiode array (PDA) detector Model 996 set at 350 nm, a quaternary gradient Model 600E pump, a manual Rheodyne® 7125 injector, a workstation computer, and Empower® software for control of the acquisition and analysis tools of the chromatographic data. The mobile phase was

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900 mg/L oxalic acid–acetonitrile, pH 2 to 3 (80 + 20, v/v) at a flow rate of 1.0 mL/min.

(**c**) *Analytical balance.*—Sartorius R200D (Goettingen, Germany).

(**d**) *Centrifuge*.—Eppendorf 5430 (Hamburg, Germany).

(**e**) *pH meter.*—Orion Digital Research Ion-Analyzer® 701 (Beverly, MA).

(**f**) *Ultra-Turrax homogenizer*.—T-25 IKA, Staufen im Breisgau, Germany).

(**g**) *Vortex mixer.*—Multipulse (Vision Scientific Co. Ltd, Daejeon, South Korea).

(**h**) *Filtration system*.—Büchner 1 L filtering flask and vacuum pressure pump 1/6 HP Bell® (Shanghai, China) using Whatman cotton cellulose filter paper No. 5C (GE Healthcare, Piscataway, NJ).

(**i**) *Peristaltic pump*.—Ismatec® X-04917 (Glattbrugg, Switzerland).

Reagents

(**a**) *Acetonitrile and methanol.*—LC grade (Merck, Darmstadt, Germany).

(**b**) *OTC.*—≥ 95% (Sigma Chemical Corp., St. Louis, MO).

(**c**) *Oxalic acid ($H_2C_2O_4$), anhydrous sodium phosphate monobasic (NaH_2PO_4), citric acid ($C_6H_8O_7$), trichloroacetic acid (CCl_3COOH), EDTA, and sodium hydroxide (NaOH).*—Analytical reagent grade (Merck).

(**d**) *Water.*—Purified to 18.2 MΩ cm using a Device Milli-Q system (EMD Millipore Corp., Billerica, MA).

Preparation of Solutions

(**a**) *Standard solutions of OTC.*—Individual stock standard solutions of OTC were prepared at 500 mg/L in methanol in a volumetric flask and were stored at 4°C in brown vials for a maximum period of 1 month. The standard working solutions were prepared by dilutions of the standard stock solution in methanol; these solutions were prepared daily immediately before use.

(**b**) *McIlvaine buffer–EDTA solution (pH 4.0).*—Solution A (a mixture of $NaH_2PO_4/C_6H_8O_7$/EDTA) was prepared by dissolving 8.89 g NaH_2PO_4, in 500 mL deionized water. Solution B was prepared by dissolving 10.5 g $C_6H_8O_7$ in 500 mL deionized water. Then, 500 mL solution A and 300 mL solution B were mixed, and 1.86 g EDTA was added to this solution.

(**c**) *CCl_3COOH solution, 245.1 mg/L.*—Prepared by dissolving 24.51 g in 100 mL deionizer water.

(**d**) *$H_2C_2O_4$ solution, A 900 mg/L.*—Prepared by dissolving 1.26 g in 1000 mL methanol.

Sample Preparation

Salmon obtained from a local watershed were skinned and filleted. Samples were homogenized in a food blender and stored at –20°C until analysis. (*Note*: The entire extraction–cleanup procedure and determination by derivative spectrophotometry should be completed in 2 h).

(**a**) *Sample fortification.*—The calibration curve was prepared from blank salmon samples fortified with 10, 20, 30, 40, and 50 mg/L standard OTC solution in order to prepare 8, 16, 24, 32, and 40 mg/kg tissue equivalent calibration standards, respectively. The fortified samples were allowed to stand 20 min at room temperature protected from light. Three replicate samples were prepared at each of the three fortification levels.

The recoveries of OTC were determined from blank salmon samples fortified at 8, 16, and 32 mg/kg that were allowed to stand 20 min at room temperature and protected from light as was done for the calibration standards. For each fortification level, three replicates of the samples were extracted and the experiment was repeated on 3 different days.

(**b**) *Extraction and cleanup.*—The procedure for OTC determination in salmon muscle and skin was carried out as follows: To 5 g tissue in a 50 mL polypropylene centrifuge tube was added between 10 and 50 mg/L OTC, and it was left to stand in the dark for 20 min at room temperature. The sample was homogenized for 5 min with the Ultra-Turrax T-25. Then, 20 mL McIlvaine buffer–EDTA solution, pH 4.0, was added to the sample, and this was vortex-mixed for 10 min after which 2 mL 245.1 mg/L CCl_3COOH was added, vortex-mixed for 5 min, and centrifuged at 4000 rpm for 15 min. The remaining tissue residue was extracted once more, and the extracts from both extractions were pooled together for subsequent SPE cartridge cleanup. A Sep-Pak C18 cartridge was conditioned with 6 mL methanol and 4 mL water using a peristaltic pump at a flow rate of 4 mL/min. The OTC extract was loaded onto the conditioned SPE cartridge and eluted with 4 mL 900 mg/L $H_2C_2O_4$. A regent blank sample was also prepared using the same conditions, but deionized water was added instead of OTC standard solution.

Spectrophotometric Conditions

The SPE eluate was evaluated by UV-Vis spectrophotometry versus a blank sample to obtain a derivative spectrum. The second derivative spectrum was selected for quantification using smoothing factor, 16000; scale factor, 10000; and analytical wavelength, 393 nm.

Method Validation

The characteristic operational parameters of the method were validated in terms of the following parameters as described below: matrix effect, linearity, LOD, LOQ, confirmation identity, selectivity, accuracy, and precision.

(**a**) *Matrix effect*.—The matrix effects of salmon muscle and skin were evaluated. The study was carried out by comparing the response of the standard solutions with the response of the extracts obtained according to the procedure for the extraction and cleanup of OTC from fortified salmon at different concentrations; this study was performed on the same day and on different days

(**b**) *Linearity.*—Standard solutions ranging in concentration from 10 to 50 mg/L OTC were prepared. The weighed blank salmon muscle samples were fortified with 10, 20, 30, 40, and 50 mg/L in order to prepare 8, 16, 24, 32, and 40 mg/kg tissue equivalents, respectively. The samples were prepared in triplicate at each of the concentrations. The r^2 and lack-of-fit test (P) were used to evaluate the linearity.

(**c**) *LOD and LOQ*.—The LOD and LOQ for the method were determined from the muscle blank in the matrix (n = 20). The LOD was estimated as 3 σ/m and LOQ 10 σ/m, where σ is

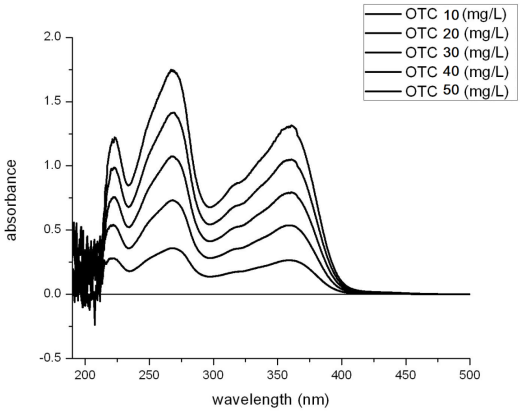

Figure 1. Classical UV-Vis spectra of OTC in $H_2C_2O_4$ in concentrations between 10 and 50 mg/L; spectra for standard solutions of OTC dissolved in 900 mg/L $H_2C_2O_4$.

the SD of the blank and m is slope of the OTC matrix-matched calibration curve.

(**d**) *Selectivity*.—Selectivity was evaluated by analyzing different batches of blank salmon muscle and skin samples.

(**e**) *Accuracy*.—The within-day and between-day accuracy of the method was evaluated by using fortified salmon muscle samples. Three sets of salmon muscle and skin samples fortified with OTC at 8, 16, and 32 mg/kg were prepared, extracted, and analyzed according to the described procedure. Three replicate samples were extracted and analyzed on day 1, and the experiments were repeated over a 3-day period. The concentrations of OTC in the samples were calculated against a matrix-matched calibration curve.

(**f**) *Precision*.—Three sets of samples, each containing at least seven salmon muscle and skin samples fortified with OTC at 8, 16, and 32 mg/kg, were prepared and analyzed by the same operator in 1 day and over a 3 day period. The RSDs for the within-day and between-day analysis were calculated.

Results and Discussion

OTC has good solubility in a variety of polar solvents. A solution of 900 mg/L $H_2C_2O_4$ in methanol was used in this study in order to directly assess the spectral characteristics of the extract obtained from salmon muscle and skin tissue using the described procedure. To accomplish this, standard solutions of OTC ranging in concentration between 10 and 50 mg/L dissolved in 900 mg/L $H_2C_2O_4$ were prepared and their spectra were acquired. The acquired spectra for OTC in oxalic acid shown in Figure 1 have three distinct and characteristic bands centered at 267, 223, and 360 nm.

To assess the matrix effect of salmon muscle and skin tissue on the UV-Vis signal for OTC, the concentration of OTC in the muscle and skin was kept at 50 mg/L and compared with the equivalent standard solution containing OTC at 50 mg/L. As can be seen in Figure 2, it is possible to observe the matrix effect on the UV detector signals in the range between 190 and 350 nm. It can also be observed that in the UV detector signal of the matrix there is a slight shift and a small decrease in the spectral band centered at 360 nm. However, between 380 and 400 nm the spectral band is not altered. As a result, we chose to use the derivative spectrophotometry technique (Figure 2). To ensure that the detector signal observed between 380 and 400 is attributable to only OTC and to avoid matrix interference, the zero order spectra and the first and second derivative spectra of the extracts obtained from the salmon muscle tissue and salmon skin that had been fortified only with 500 µL methanol were compared with a 20 mg/L standard solution OTC (Figure 3).

Selection of Spectral Parameters

The spectral parameters that were selected were derivative order, smoothing factor, scale factor, and analytical wavelength. These parameters were obtained from the classical spectra of the OTC in $H_2C_2O_4$ at concentrations between 10 and 50 mg/L.

Selection of the Derivative Order

The first order derivative spectrum shows a high sensitivity and S/N; however, there could be a decrease in the accuracy of the results due to the matrix effect. Therefore, it was decided to use the second order derivative spectra as it presents characteristic bands of OTC after extraction and purification that appear to be unaffected by the presence of the matrix.

Selection of the Smoothing Factor

Using the second derivative with a scanned range of 310 nm, the smoothing factor tested at values of 2000, 4000, 8000, and 16000. These values are defined by default and correspond to the point numbers, which are in relation with the range of wavelength at which the spectra were scanned according to the mathematical model described by Savitzky and Golay (17).

The derivative spectra obtained using different smoothing factors are shown in Figure 4; they indicate that as the value of the smoothing factor increases the noise decreases, favoring the signal clarity. It is also worth noting that no distortion effects were observed. A smoothing factor of 16000 was therefore selected.

Selection of the Scale Factor

The scale factor corresponds to the level of amplification of the derivative used. For the determination of OTC in this

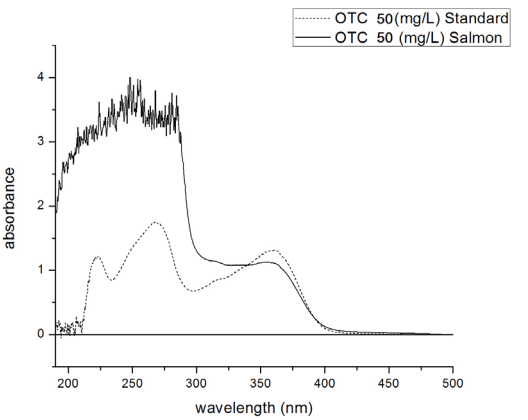

Figure 2. Comparison of the classical spectra of OTC in salmon and in the standard.

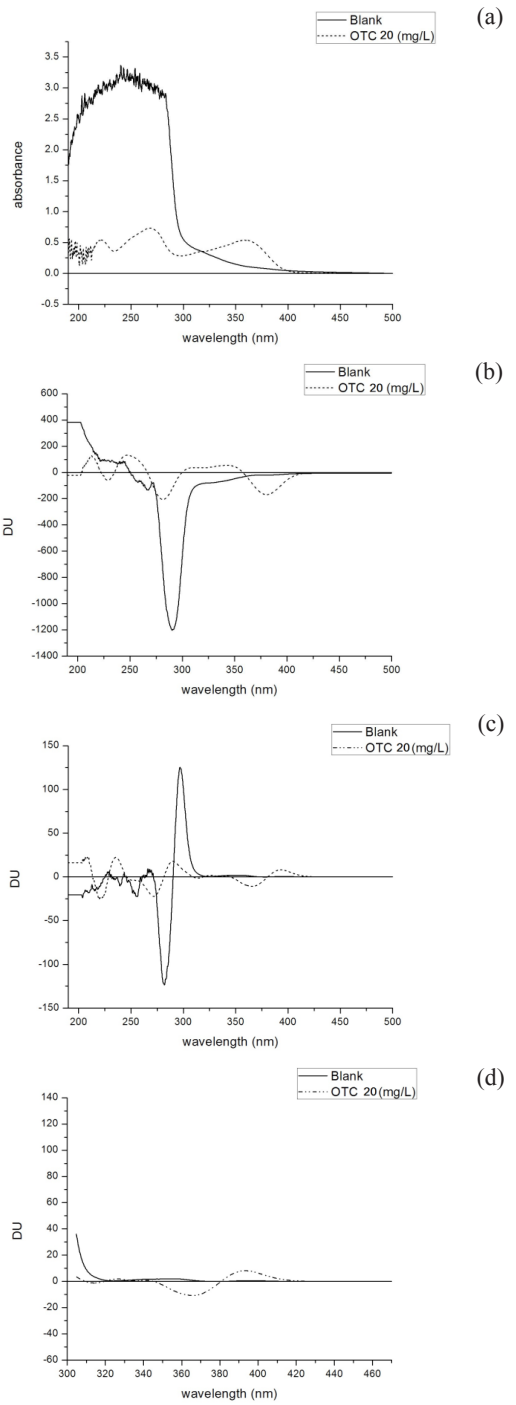

Figure 3. UV-Vis spectra of blank salmon muscle and OTC 20 mg/L standard (a) zero order, (b) first derivative, (c) second derivative, and (d) zoom of second derivative.

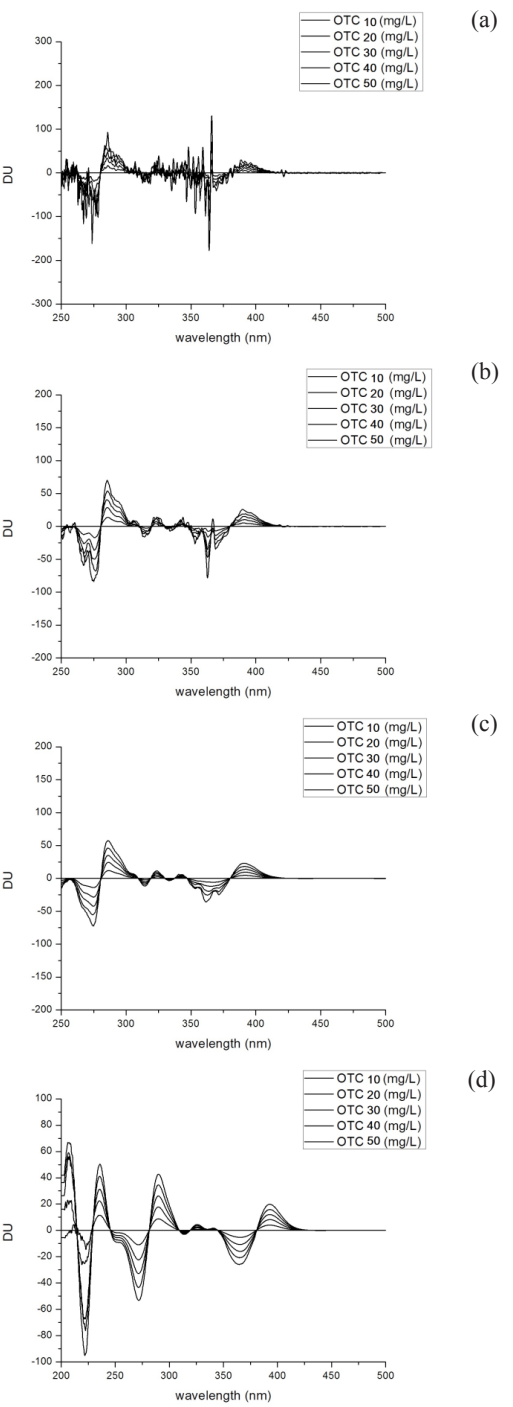

Figure 4. UV-Vis spectra of the second order for OTC standard solutions with different smoothing factors: (a) 2000, (b) 4000, (c) 8000, and (d) 16 000.

study, a scale factor of 10 000 was used because it facilitated the reading of the analytical signal. However, it must be noted that the LOD and LOQ remained unchanged, because the analytical signal was amplified by the same proportion as the background noise. Moreover, the same standard was compared with the same concentration of extract obtained from salmon muscle and skin using the extraction and cleanup procedure in the described method. The results obtained demonstrate that with an 11-fold increase in concentration of OTC in the final extract compared to when it was first extracted from the fish muscle and skin, it is possible to analyze the fish extract directly (Figure 5).

Validation of the Method

In order to validate the method, the second order derivatives spectra in samples of OTC prepared with and without matrix were evaluated at 393 nm, where the matrix does not interfere. The absorption intensity, DU, of the OTC samples prepared with

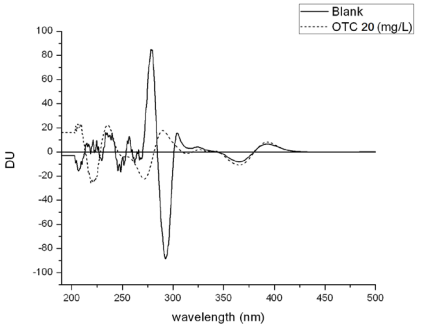

Figure 5. UV-Vis spectra of OTC 20 mg/L standard and second order derivative mode in salmon.

and without matrix were calculated and compared statistically using Student's *t*-test. The calculated *t*-value (t_{exp}) and critical *t* value (t_{crit}) were 10.6 and 2.36, respectively, for seven degrees of freedom at the 95% confidence limit. The responses were statistically different when t_{exp} was not contained between t_{crit} values. For accurate determination of OTC in muscle and skin of salmon, a matrix-matched calibration curve was used.

The optimal analytical wavelength was previously selected at 393 nm where matrix interference was negligible (Figure 6). Further, the spectral variables selected for this determination were derivatives of second order, smoothing factor of 16 000, and scaling factor of 10 000.

Subsequently, muscle and skin samples from drug-free salmon were fortified with OTC by adding aliquots of 80 and 400 µL OTC standard solutions to prepare samples containing OTC concentrations between 10 and 50 mg/L. In Figure 6 a and b, the classic UV-Vis and second derivative spectra of fortified samples are shown. The second derivative spectra for the OTC fortified samples show clearly defined, distinguishable, and measurable signals at the analytical wavelength of 393 nm (Figure 6c).

Linearity.—The linearity of the matrix-matched calibration curve, evaluated by analyzing in triplicate three sets of matrix-matched calibration solutions containing OTC at 8, 16, 24, 32, and 40 mg/kg generated from these standard solutions, can be described by the equation DU = 0.35441 C (concentration, mg/kg) – 0.23. Good linearity was observed within the concentration range from 8 to 40 mg/kg with $r^2 > 0.997$ for the calibration curves generated.

LOD and LOQ.—At least 30 salmon muscle and skin samples were analyzed on 3 consecutive days to estimate the LOD and LOQ, which were calculated as 271 and 903 µg/kg, respectively. In general, the estimated LOQ corresponded to the lowest fortification level.

The LOQ of this method was 903 µg/kg, which is considerably higher than that reported by other investigators for OTC analysis in muscle tissue of fish (8, 18). However, the LOD is contained within the MRL of 2000 µg/kg permitted by the United States.

Selectivity.—Twenty blanks from salmon obtained from three different sources were evaluated following the procedure described in the *Experimental* section. The mean DU was 0.55, which is equivalent to 19.5% of the DU corresponding to the lowest concentration of the calibration curve; in this context, the selectivity of the method is 80%.

Accuracy and precision.—Accuracy and precision were determined by analyzing fortified muscle and skin of salmon

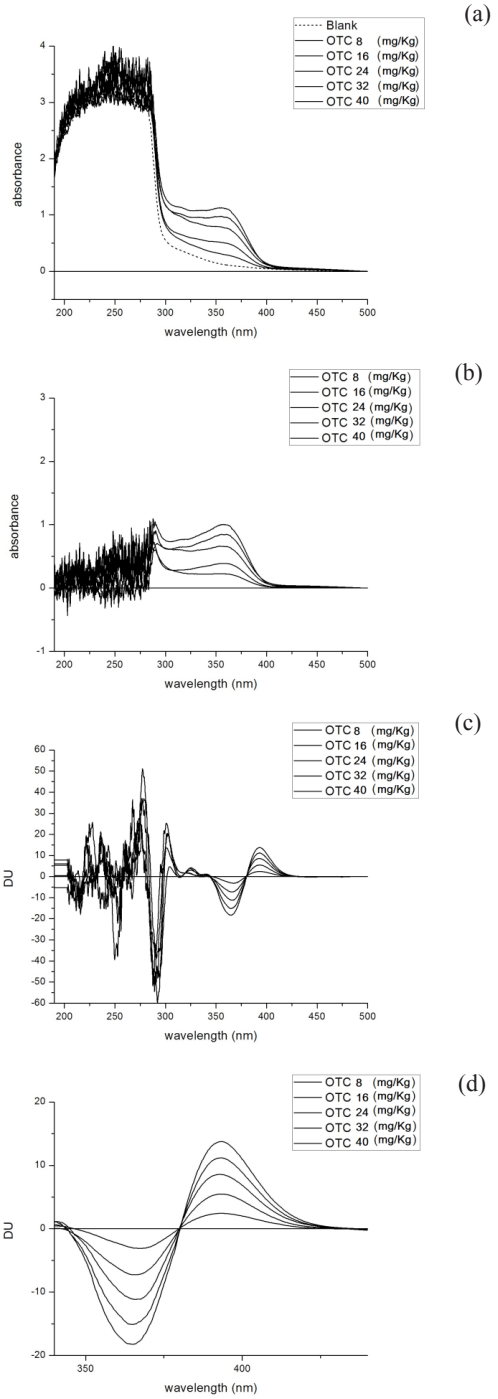

Figure 6. (a) Classical spectra of salmon samples with OTC concentrations of 8, 16, 24, 32, and 40 mg/kg and one OTC blank in salmon; (b) classical spectra with the blank removed; (c) second order spectra of salmon samples with the blank removed; and (d) amplification of second order spectra of salmon samples with OTC concentrations of 8, 16, 24, 32, and 40 mg/kg.

at three different concentration levels (8, 16, and 32 mg/kg) on 3 different days. In all cases, fortified samples were prepared and analyzed according to sample preparation procedure. Recovery (accuracy) and precision at different levels of fortification and days are shown in Table 1. The recoveries at all fortified levels

Table 1. Interassay and intra-assay validation results for five assays

Analyte	Fortification level, mg/kg	Recovery variation, %	Recovery mean, %	RSD, % within day	RSD, % between day
OTC	8	93.4–103.5	97.9	4.5	6.2
OTC	16	97.6–104.9	101.2	3.3	3.6
OTC	32	89.8–104.0	97.9	3.6	3.9

were between 89.8 and 104.9%, and RSDs in all cases were less than 6.5%.

Comparison Between the Proposed Method and a Chromatographic Method

In a separate experiment, five extracts obtained from different samples of salmon muscle tissue and skin fortified with OTC were analyzed by LC-PDA UV and eight extracts by second order derivative spectrophotometry. To compare the spectrophotometric method with the LC method, Student's *t*-test with a 95% threshold level was used. In Table 2, the mean, SD, and calculated *t* (t_{exp}) are shown. Taking into account 10 degrees of freedom and 95% confidence, the t_{exp} is slightly smaller than the tabulated *t* (2.228), indicating that the methods are not significantly different and that the results agree within the experimental error.

The real samples were evaluated using the same procedure. The samples were obtained from different markets. In all cases, the OTC concentrations were minor compared to the LOD value.

It is important to point out that this method only can be applied successfully in salmons that are growing, because the LOQs are below the OTC concentration of these salmon tissues. For salmon in quarantine, this method can only serve for screening.

Conclusions

Derivative UV-Vis spectrophotometry was used for the quantification of OTC residues in salmon muscle and skin tissues. The proposed method is simple and economical, achieving a reduction in working steps compared with methods described in the literature (8–15).

The recoveries at all spiked levels were between 89.8 and 104.9%, and RSDs in all cases were less than 6.5%. LOD and LOQ obtained were 271 and 903 µg/kg, respectively. It is possible to detect and determine OTC in salmon muscle and skin in countries like the United States, where the LOQ is below the MRL allowed (2000 µg/kg).

In the EU, Japan, and Chile it is not possible to determine the OTC in salmon muscle and skin below 903 µg/kg. This value is greater than the MRLs allowed. However, the proposed method would allow their use as a screening tool in order to decrease the samples that must be analyzed by confirmatory methods. This fact allows a decrease in costs for control laboratories in aquaculture. It is important to point out that this method only can be applied successfully to salmon that are growing, because the LOQs are under the OTC concentration of these salmon tissues. Additionally, the simplicity of the method permits at least 20 samples/day to be processed and analyzed by one analyst.

In this paper, an alternative method for determining OTC in a biological matrix is proposed, which is simple and economical in comparison with those reported in the literature that included various steps for the treatment of the sample and more sophisticated equipment for determination. This method may also be used for screening purposes to decrease the number of presumptive positive samples that need to be subjected to further confirmatory analysis.

Table 2. Comparison between the proposed method and a chromatographic method (LC-PDA UV)

Added concn, mg/kg	Found concn (proposed method),[a] mg/kg	Found concn (LC-PDA UV),[b] mg/kg	Student's *t*-value calculated
0.009	Not detected	Not detected	—
0.09	Detected	Detected	—
3.96	3.63 ± 0.7	3.52 ± 1.5	0.6
7.92	6.79 ± 0.33	7.43 ± 0.37	1.05

[a] $n = 8$.

[b] $n = 5$.

Acknowledgments

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