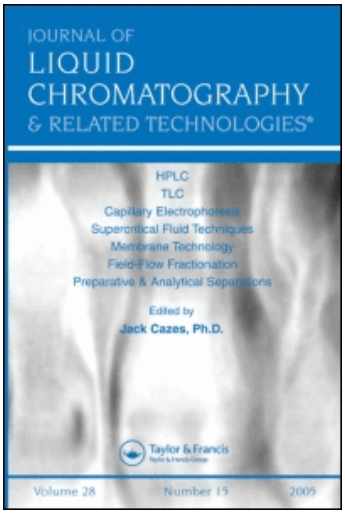

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DETERMINATION OF CHLORAMPHENICOL RESIDUES IN GILTHEAD SEABREAM (*SPARUS AURATA* L.) TISSUES BY HPLC-PDA

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ABSTRACT

A high performance liquid chromatographic method for the determination of chloramphenicol residues in muscle tissue of the cultured fish gilthead seabream (*Sparus aurata* L.) was developed. Chloramphenicol is extracted with ethyl acetate and after centrifugation and solvent evaporation the oily extract is

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partitioned between 3% sodium chloride solution and n-pentane, and chloramphenicol is extracted back into ethyl acetate. After evaporation to near dryness, the residue is dissolved in n-hexane and is cleaned up on a Silica gel SPE mini column. Chloramphenicol was analyzed on a ZORBAX SB - C18 column at a temperature of 50°C, with the mobile phase being methanol:-water 30 + 70 v/v delivered isocratically. Detection was performed using a Photo Diode Array detector monitored at λ_{max} ~278 nm. The mean recovery (R%) achieved was 88.62 ± 9.65% for a range of 10, 25, 50, 100, 200 μg/kg blank fortified samples (n = 4). The limit of detection (LOD) was 1.87 ng corresponding to 5 μg/kg chloramphenicol in muscle and the limit of quantification (LOQ) was 10 μg/kg.

INTRODUCTION

Aquaculture, is the breeding of aquatic organisms which involves many species, but in most cases is the breeding of only one. It has a long tradition and just in the second half of this century, its production has increased to more than 10 million metric tonnes per year, a figure which corresponds to 10% of the fishery production of the whole world. Unique conditions, such as the highly dense population of fish, may contribute to the increase of infectious diseases which can be transmitted to fish at higher rates than if they were free. Drugs with high absorptivity in fish and low persistence in the environment, should be suggested for use in aquaculture. The number of antimicrobials used internationally has been estimated to be about 50. It should be stressed that some of them have been approved for use in cultured fish, for the obvious reason that pharmaceutical industries would have to collect a lot of data before their authorization (1).

It has been proved from several studies, that drug half-lives are longer at lower rather than higher temperatures, and that a metabolic rate increase of 10%, for an elevation in temperature of 1°C, may occur (2). A drug elimination from fish is quite often problematic because pikilothermic animals eliminate these substances slowly in comparison with endothermic. Withdrawal time determination for different fish species and drugs should be defined and fixed in order to reach acceptable residue levels. That is why the use of the veterinary drugs in fish breading may lead to residue problems (3).

Among several drugs used in aquaculture all over the world, chloramphenicol, a broad-spectrum antibiotic predominantly active against the main

pathogenic gram negative bacteria occurring in food producing animals, is used in many countries to combat furunculosis in cultured solmonoids (4). There are indications that chloramphenicol, although it is forbidden (Annex IV Regulation (EEC) No. 2377/90), is used illegally.

Chloramphenicol was first isolated from cultures of *Streptomyces venezuelae* in 1947 and was synthesized in 1949, the first completely synthetic antibiotic of importance to be produced commercially (5). It is rapidly absorbed and distributed fast following oral or parenteral administration to the organs and edible tissues, with maximum blood concentrations being reached 1-5 hours after dosing (6). As far as toxicity, there is an increased incidence of reversible bone marrow depression when plasma concentration exceeds 25 μg/mL. The "grey" syndrome (cardiovascular collapse, respiratory depression, and coma) has been reported in patients with plasma concentrations in the range of 40 to 400 μg/mL (7).

Several methods for chloramphenicol determination in tissues and biological fluids of food producing animals and fish species, such as tilapia, rainbow trout, carp, eel, catfish have been published (8–13). An HPLC-PDA method was developed to be used for the identification of chloramphenicol residues in cultured fish species *Sparus aurata* L., which is very popular and of great importance in our country, the leader in production in the European Union (14).

EXPERIMENTAL

Reagents and Chemicals

HPLC grade methanol, ethyl acetate p.a., n-hexane p.a., and n-pentane p.a. were obtained from LabScan, (U.K.), and sodium chloride HiPerSolv for HPLC from BDH (U.K.). HPLC grade water was from Ultrapure Water RiOs™-Milli-Q® system, Millipore (USA) and SPE Silica Cartridges Sep-Pak Plus were from Waters (USA). Mobile phase filters type HV 0.45 μm and FH 0.5 μm and syringe filters ∅13 mm Millex® GV Hydrophilic PVDF 0.22 μm were from Millipore (USA).

The analytical standard of chloramphenicol (Figure 1) was purchased from Sigma (USA). Fish were anesthetized with quinaldine, which was also obtained from Sigma (USA). Chloramphenicol stock solution was prepared by dissolving 100 mg in 100 mL methanol HPLC grade, and working standard solutions were prepared by dilutions in mobile phase from the stock solution to get concentrations of 0.05, 0.10, 0.20, 0.40, 0.80, 1.5, and 3.0 μg/mL.

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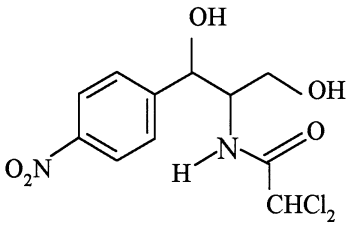

Figure 1. Chemical structure of chloramphenicol.

APPARATUS AND CHROMATOGRAPHIC CONDITION

The apparatuses used were aggregator model PCU Polytron-Aggregate® (Kinematica AG, Switzerland), Vortex mixer, Gennie 2 (Scientific Industries, USA), ultrasonic bath Transsonic 460 (Elma, Germany), reacti-therm heating module (Pierce, U.K.), centrifuge model 102B-K-UT (Runne, Germany), analytical balance model AE240 (Mettler, Switzerland), ultrapure water RiOs™-Milli-Q® (Millipore, USA), SPE manifold (Millipore, USA), automated pipettes (Gilson, France).

The liquid chromatographic system used was the ALLIANCE 2690 MX, Revision 1.21 Separation Module, (Waters) equipped with a UV/vis Photodiode Array Detector 991 (Waters). Injections were performed automatically on a ZORBAX® SB–C18, 5 μm (250 × 4.6 mm) stainless steel column (Hewlett Packard) with a guard column Lichrospher RP–select B (Merck). The flow rate of the mobile phase methanol-water (30 + 70) was 1.2 mL per minute in isocratic mode, the column was kept at a temperature of 50°C and the autosampler's temperature was maintained at 15°C. Chloramphenicol detection was performed by UV/vis Photo Diode Array detector at 278 nm. The control of the LC system, data acquisition, and peak integration were performed by the software Millennium[32] Chromatography Manager (rev.1.21) (Waters, USA). With the above chromatographic conditions, chloramphenicol was eluted at a retention time of Rt=11.25 ± 0.18 min (RSD = 1.6) (n = 10).

Sample Preparation

2 g of homogenized muscle tissue were accurately weighed in a 22 mL glass vial with screw cap and 1 mL of water was added. The test sample was extracted with 10 mL ethyl acetate by aggregation, vortex, and sonication, centrifuged at 3000 rpm for 10 min at 10°C and the supernatant was transferred to another glass

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vial. The residue was extracted once more with 5 mL ethyl acetate and the combined extracts were then evaporated under a gentle stream of nitrogen at 55°C. The remaining oily residue was dissolved in 3 mL of 3% NaCl by vortex and sonication. A partition washing step with 10 mL n-pentane followed, and after centrifugation the upper layer was rejected by aspiration. Chloramphenicol was back extracted with 2×5 mL ethyl acetate and the combined extracts were evaporated to dryness under a gentle stream of nitrogen at 55°C.

Removal of the remaining matrix interfering compounds, and selective enrichment of chloramphenicol, was performed on a disposable extraction silica gel mini column. 2 mL n-hexane were added to the dry residue and after sonication and vortex, the extract was poured onto the silica gel mini column previously prepared for interaction with the sample with 5 mL n-hexane. The vial was rinsed with 2 mL n-hexane and rinsing was poured into the mini column. Rinsing of the mini column for the removal of the undesired matrix compounds was done with 5 mL n-hexane and selective desorbing of chloramphenicol from silica gel was done with 3 mL of HPLC methanol.

The eluate was evaporated to dryness under a gentle stream of nitrogen at 55°C and the residue was redissolved into 1.0 mL of the mobile phase, filtered through 0.22 μm filter into 1 mL the autosampler's glass vial. An aliquot of 100 μL was immediately injected onto the HPLC column.

Quantification

Fortified and incurred seabream tissue samples were quantified by regression analysis using the external standard calibration curve and by plotting peak areas versus chloramphenicol in concentrations from 100 μL injections of working standard solutions.

RESULTS AND DISCUSSION

Chromatography

In this study, for better extraction of chloramphenicol from fish muscle tissue, 1 mL of water was added and chloramphenicol was subsequently extracted with ethyl acetate. In order to remove lipids the oily residue (approx. 0.4 g) was dissolved in 3% NaCl and partitioned with n-pentane. This combination gave better recoveries and more clear extracts in comparison with those of n-hexane.

Three types of disposable extraction mini column were tested for removal of the matrix interfering components and selective enrichment of chloramphe-

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Table 1. Recovery Data and Inter-Assay (Between Day) Variability for Chloramphenicol in Muscle Tissue plus Skin of Gilthead Seabream (*Sparus aurata* L.)

Antimicrobial Compound	Amount Added (μg/kg)	Mean* Amount Found (μg/kg)	Standard Deviation SD	Relative Standard Deviation RSD %	Recovery %
Chloramphenicol	10	9.37	0.98	10.46	93.67
	25	22.77	3.47	15.23	91.08
	50	37.18	2.62	7.05	74.37
	100	95.26	3.9	4.09	95.26

Mean recovery, R% = 88.6 ± 9.64 (RSD = 10.88%)
*Four (4) replicates.

nicol. Florisil (Waters), Silica gel BakerBond (Baker) and Silica gel Sep-Pak Plus (Waters), with best results from Sep-Pak Plus.

For the reconstitution of the final residue we used mobile phase instead of methanol, because peak spreading and shoulder formation on chloramphenicol peak was observed, possibly of the solvent - eluent polarity difference and incomplete mobile phase and solvent mixing (16).

Following the validated chromatographic conditions, the mean retention time of chloramphenicol was 11.25 ± 0.18 min (RSD = 1.6) (n = 10) which was quite better in comparison with that of 25 min (11). Other peaks of drugs often used in aquaculture did not interfere. The mean chloramphenicol recovery was 88.62 ± 9.65 with an acceptable relative standard deviation (RSD = 10.89%). The detection limit was 1.87 ng which corresponded to 5 μg/kg chloramphenicol in muscle (3-fold noise level) and the limit of quantification (LOQ) was 10 μg/kg.

Table 2. Tissue Levels (μg/kg) of Chloramphenicol Residues in Muscle Tissue without Skin of Gilthead Seabream (*Sparus aurata* L.) 32 hours After Injection of 100 μL of a Chloramphenicol Medicated Solution at a Dose of 5 mg/kg

Antimicrobial Compound	Single Dose	Fish	Concentration (ng/kg)	Mean ± sd	RSD %
Chloramphenicol	5 mg/kg	1	60	46.75 ± 10.14	21.7
		2	49		
		3	41		
		4	37		

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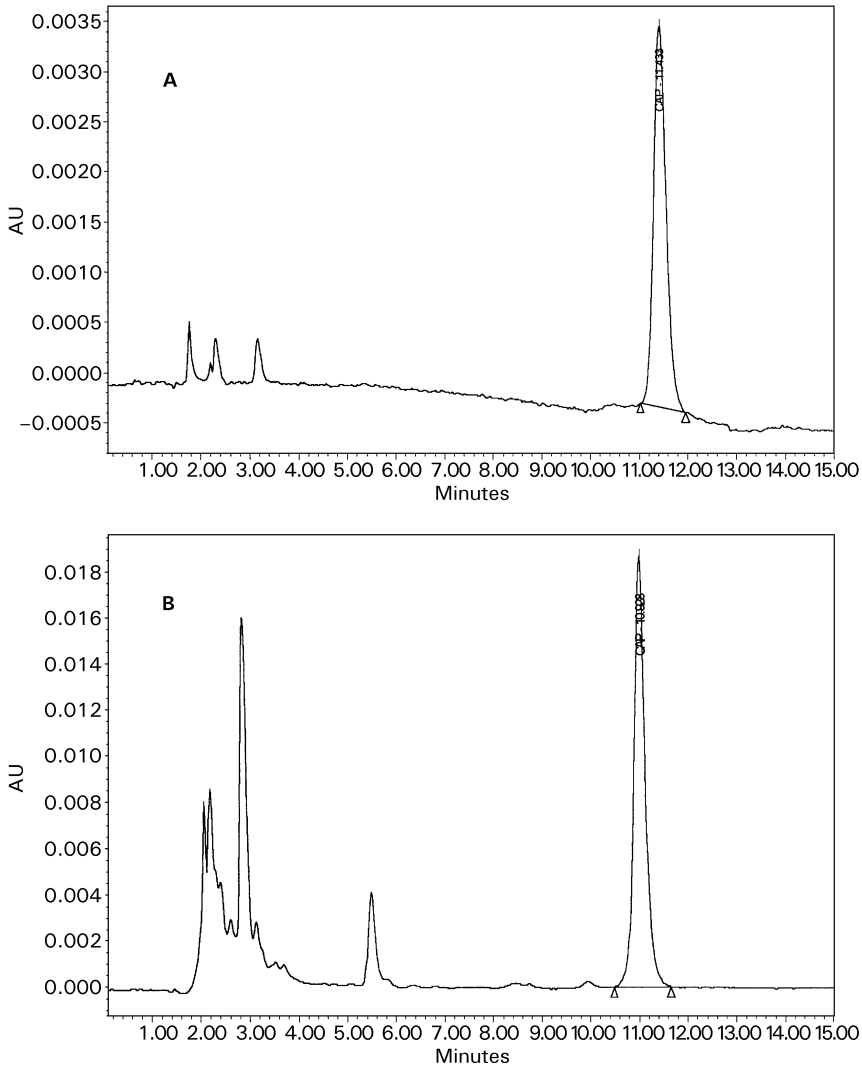

Figure 2. Chromatograms of (A) Standard chloramphenicol 300 ng and (B) a Real incurred muscle tissue sample containing 1815 µg/kg. Chromatographic conditions: column ZORBAX® SB–C18, 5 µm (250 × 4.6 mm); column temperature: 50°C; mobile phase: methanol - water (30 + 70); flow rate: 1.2 mL per minute in isocratic mode; wavelength: 278 nm.

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Method Validation

The analytical method was validated for linearity, accuracy, precision, sensitivity, and specificity. The linearity of chloramphenicol response was calculated using external standard calibration curve with 8 points (zero included) and with standard concentrations of 50 μg/kg, 100 μg/L, 200 μg/L, 400 μg/L, 800 μg/L, 1500 μg/L, and 3000 μg/L. Regression line parameters of the external standard calibration curve were the slope of the line 932.13, the intercept (intersection) with the y-axis = 0 and the regression coefficient = 0.9996. Accuracy has been evaluated on muscle samples fortified at 10 μg/kg, 25 μg/kg, 50 μg/kg, and 100 μg/kg, with 4 replicates for each concentration. Results are shown in Table 1. The mean recovery of the method has been calculated taking into account all the values corresponding to the concentrations (16 samples). Precision: in this study of RSD gave values of 9.8, 13.8, 5.2, and 3.9, respectively, for muscle fortification at 10, 25, 50, and 100 μg/kg with mean value of 10.8%. Sensitivity was determined by calculating chromatographic resolution ($Rs > 1.5$), tailing factor ($Tf = 1.17$), and signal-to-noise ratio ($S/N = 3{:}1$) and specificity by the analysis of solvent and tissue blank samples. No matrix associated interferences at the retention time of chloramphenicol were observed. At this point, PDA contributed with its mathematical spectra comparison gained along the chloramphenicol peak, as purity confirmation. The Limit of Detection (LOD) based on a $S/N = 3{:}1$ was 1.87 ng corresponding to 5 μg/kg and the Limit of Quantification (LOQ) was 10 μg/kg.

Real Incurred Samples

An experiment was also undertaken to obtain real incurred samples and to test the applicability of the described method. Chloramphenicol was administered by injection after fish have been anesthetized by quinaldine. Fish were injected intravascular (caudal vein) with 100 μL of the chloramphenicol medicated solution at a dose of 5 mg/kg in sterile saline. The levels of the observed chloramphenicol residues in muscle tissue, less the skin of the treated seabreams 32 hours after dosing are presented in Table 2. Characteristic chromatograms of a chloramphenicol standard and a real sample are shown in Figure 2. Some unknown peaks observed before parent chloramphenicol peak could be chloramphenicol metabolites, which could not be identified.

In conclusion, the method is suitable to be used with safety and accuracy for the control of chloramphenicol residues in cultured seabreams, and a trained analyst could carry chromatographic analysis of 30 samples per working day.

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