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THE EFFECT OF COOKING AND COLD STORAGE PROCESSES ON FLORFENICOL RESIDUES IN MUSCLE TISSUES OF STURGEON (*Acipenser gueldenstaedtii*) REARED IN BLACK SEA

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This research was performed to determine the effect of boiling, grilling and cold storage processes on florfenicol residues in muscle tissues of sturgeons. A total of 16 sturgeons, 10 of which have received single dosage of florfenicol at 10 mg kg^{-1} bw day^{-1} level and remaining 6 have not received any florfenicol were used in this study. The analyses were performed by HPLC. The mean recovery rate and repeatability pooled-RSD r% of analytical method for florfenicol analysis of sturgeon muscle were determined as 83.4±1.07% and 17% respectively. The florfenicol levels were 40.30±8.23% in the muscle tissue of boiled fish, 57.80±7.46% in the boiling juice, 101.10±4.01% in the grilled tissue and 78.01±15.40% and 62.36±11.60% in the muscle tissues of fishes, which were stored at -20°C, on the 20th and 50th days respectively. The initial florfenicol level in the fish muscle was significantly reduced *(P<0.05)* by boiling and cold storage processes. A decrease occurred in florfenicol level in boiling process due to the transfer from muscle tissue to boiling juice, and there was no decrease in the florfenicol level as a result of grilling.
Keywords: HPLC, florfenicol, residue, sturgeon, cooking and cold storage

INTRODUCTION

Fish, rich in protein, minerals, and vitamins, is an important food source for human and farm animals (Okocha *et al.,* 2018; Sobral *et al.,* 2018). Nowadays, aquaculture production is becoming increasingly widespread in the world. The most significant problem of aquaculture is bacterial, viral, parasitic and fungal infections that cause great economic losses (Chang, 2017; Sobral *et al.,* 2018). Antibacterial drugs such as florfenicol (FF), tetracycline, oxytetracycline (OTC), erythromycin, sulfadimethoxine + ormetoprim, and sulfamerazine can be used in the control and treatment of fish diseases (Okocha *et al.,* 2018). However, in many countries, drugs such as chloramphenicol (CAP), malachite green, nitrofurans, and fluoroquinolones are forbidden in food animals due to their undesirable effects on human health (Chang, 2017). Florfenicol is a broad-spectrum antibiotic widely used in fish farming (Yanong *et al.,* 2005; Granja *et al.,* 2012) and other animal diseases in the worldwide (Smet *et al.,* 2018). Florfenicol is commonly used as a licensed drug for antibacterial purposes in aquaculture production in Turkey (Türe *et al.,* 2018).

Veterinary drugs used in food animals can cause residues in animal products (meat, milk, eggs, etc.). Florfenicol residues have also been determined in previous studies conducted on fish (Ansari *et al.,* 2014; Barani and Fallah, 2015). Drug residues in food cause a variety of adverse effects such as allergy, suppression of the immune system, teratogenic and carcinogenic effects in humans and the development of resistance against drugs in bacteria (Lozano and Trujillo, 2012; Baynes *et al.,* 2016; Tian *et al.,* 2017). To avoid these effects, the specified legal withdrawal time and maximum residue limit (MRL) for each veterinary drug are compiled (EU, 2010). However, the data on the withdrawal times of all veterinary drugs have not been established (Elbagory *et al.,* 2016). These data are very limited especially for fish and other aquatic organisms. Metabolism of drugs in fish, which are poikilothermic species, can vary significantly according to body temperature and environmental conditions such as water temperature, pH, mineral content, etc. (Aboubakr *et al.,* 2014). The determination of MRL for veterinary drugs is usually performed on unprocessed (raw) animal products. However, animal products are generally used after some processing (Fadwa *et al.,* 2015). Thermal treatment (hot or cold) affects the chemical structure of the drugs (decomposition and ultimately the development of active or inactive products) and its resolution in tissues etc. (Baynes *et al.,* 2016). Thus, the levels of the main compound in the

tissues may vary (Cooper *et al.*, 2011; Heshmati, 2015; Sever and Baydan, 2015). However, there are few studies on the fate of veterinary drug residues in processed animal products (especially in fish) (Lan *et al.*, 2001; Mitrowska *et al.*, 2012). It has been reported that cooking is ineffective on the residues of quinolones such as oxolinic acid and flumequine in fish. Similarly, enrofloxacin and ciprofloxacin residues in flatfish are resistant to high temperature (Heshmati, 2015).

Florfenicol is stable at 25ºC in environmental conditions (under a range of simulated field conditions) (Hayes *et al.*, 2003). However, florfenicol degrades into a metabolite, florfenicol amine (FFA), rapidly in the deep sea sediment environment (Hektoen *et al.*, 1995).

Filazi *et al.* (2015) investigated the effects of storage conditions and cooking methods on florfenicol and FFA residue levels in eggs and they found that the residue level decreased with the duration of storage and was still detected on the 28th day without any significant difference between storage conditions at 20˚C and 4˚C. Although, they detected a significant decrease in residue levels as a result of frying and boiling, the decomposition was insufficient and they claimed that FF and FFA residues are heat-labile. In a study of Franje *et al.* (2010) investigating the heat-stability of amphenicols [FF, thiamphenicol (TAP) and CAP], they found the heat-stability as the highest in water and the lowest in chicken meat. Florfenicol was found more heat stable than TAP and CAP in water whereas in chicken meat, TAP was more stable than FF and CAP.

Sturgeons are among the world's most valuable wildlife resources. These northern hemisphere fishes can be found in large river systems, lakes, coastal waters, and inner seas. All sturgeons and parts or derivatives thereof (e.g. caviar, meat, skin, etc.) that enter international trade require the issuance of CITES permits or certificates (CITES, 2019). A total of 35 countries are presently involved in sturgeon aquaculture for meat and caviar. The most commonly used species is the Siberian sturgeon (*Acipenser baerii*), the Russian sturgeon (*Acipenser gueldenstaedtii*), the sterlet (*Acipenser ruthenus*) and the stellate sturgeon (*Acipenser stellatus*) (Bronzi *et al.*, 2011).

In this study, it was aimed to determine the effect of cooking and cold storage processes on the FF residues in muscle tissue of sturgeon (*Acipenser gueldenstaedtii*) reared in Black Sea water.

MATERIAL AND METHODS

***Samples*:** A total of 16, approximately one-year-old and 150 g in weight sturgeons (*Acipenser gueldenstaedtii*) consisting of 10 sturgeons treated with a single dose (10 mg kg^{-1} bw day^{-1}) of florfenicol intramuscularly, and 6 sturgeon samples, which were not received any florfenicol were used for residue analyses and, for the estimation of recovery rate and repeatability pooled-RSD r% in this study. The muscle samples of 10 fish were taken at 1th, 3th and 6th hours were analyzed by high-performance liquid chromatography (HPLC) to determine the FF residue level. A total of five samples for each of the boiling, grilling and cold storage processes were used in the analyses. Samples were taken from the FF pharmacokinetic study in sturgeon, which growing in the Black Sea, carried out by Veterinary Faculty of Ankara University and Trabzon Central Fisheries Research Institute (CFRI). This study was conducted with the permission of the Local Ethical Committee of the Trabzon CFRI with protocol number 42208298-040-04-02.

***Cooking and cold storage processes*:** Five fish were used for boiling process. The skinless muscle tissues of each fish were mixed separately before the process. For boiling, 1 g of tissue from each sample was put in a volumetric flask and 5 ml deionized water was added. The sample was boiled in electric heater with thermostat (Heating Mantle-Thermal Laboratory Equipment) for 10 min. at 98ºC. Boiling juice and muscle tissue of each sample were put in separate glass tubes and FF extraction analysis was started. For grilling, 1 g of muscle tissue from samples was grilled in a teflon pan without oil on the electric cooktop at level 4 for 5 min. For cold storage process, five fish samples stored at -20ºC on the 0th, 20th, and 50th day of cold storage were tested for FF extraction in order to find the effect of cold storage on FF residue level. An internal standard of 1.5 ppm CAP concentration was added to all samples analyzed to see its wavelength in HPLC.

Method: The calibration curve was prepared by using 100 µg ml^{-1} stock FF standard solutions. Serial dilutions (0, 0.375, 0.75, 1.5, 3, 6 µg ml^{-1}) were prepared by diluting the stock solution with 0.1% acetic acid. The prepared standards were read in a 20 µl volume on an HPLC device and a calibration curve was drawn from the mean values obtained from repeated readings (n=6). Recovery (as µg/g 0.75, 1.5, 3) and repeatability pooled (RSD r%) were estimated as shown in Table 1.

Table 1. Results of recovery and repeatability pooled-RSD r% for FF analysis from fish muscle.

FF concentration (µg/g)	FF recovered from fish sample (µg/g) (mean±se) n:6	Recovery %	Repeatability pooled RSD r%
			17
0.75	0.61±0.063	81.3	
1.5	1.27±0.055	84.6	
3	2.53±0.131	84.4	
Average recovery rate, mean±sem		83.4±1.07	

*sem: standard error of mean

***Analysis of the samples*:** The prepared FF (0.375, 0.75, 1.5, 3 and 6 ppm) and CAP (1.5 ppm) internal standards were read in a 20 µl volume on HPLC and a calibration curve was drawn from the mean values obtained from repeated readings.

After the cooking and cold storage process, 1 g sample (tissue

or water after boiling) put in glass tubes with a screw cap and then 1 ml of 0.1% acetic acid, 4 ml of 0.1 M phosphate buffer (pH: 7) and 4 ml ethyl acetate were added. Samples were mixed in shakers (Heidolph-Unimax 1010) for 10 min. and then centrifuged at 5000 r.p.m. (Hettich Universal 320 R) for another 10 min. After centrifugation, 3 ml clear supernatant from the upper side of each tube was transferred to another tube. The supernatants in the tubes were evaporated at 40°C nitrogen evaporator (VLM EVA). The process was ended after complete evaporation of the supernatant and 1 ml mobile phase (725 ml deionized water, 265 ml acetonitrile, 4 ml 10% acetic acid) was added to the tubes. This mixture is drawn into a 5 ml plastic syringe and filtered through 0.25 µm Cronus filter and put into 20 µl HPLC vials to read (Van de Riet *et al.*, 2003; Anadón *et al.*, 2008).

HPLC conditions; Detector: Photo Diode Array Detector, Flow: Isocratic flow, Mobile phase: 725 ml ultrapure water, 265 ml acetonitrile, 4 ml 10% acetic acid, injection volume: 20 µl, column temperature: 25ºC, C18 column: Phenomenex HperClone ODS (150 mm × 4.60 mm × 5 µm) with guard column (Phenomenex), Wavelength: 223 nm, Flow rate: 0.8 ml / min., duration: 30 min.

***Statistical analysis*:** To compare the difference among data, paired sample t test and analysis of variance in repeated measurements (RM-ANOVA) were applied for continuous variables. Post-hoc comparisons on parameters were performed using the TUKEY procedure. Analyses were conducted using TURCOSA Cloud (http://www.turcosa.com.tr, Turcosa Analytics Ltd Co, Turkey) statistical software. The result of *P* value less than <0.05 was considered as statistically significant.

RESULTS

No pollution was observed in the chromatograms in the selectivity studies (Fig. 1). Recovery and repeatability pooled as RSD r%, results are shown in Table 1. The curves of linearity and recovery results are shown in Fig. 1 and 2 respectively. Chromatograms of standard of 1.5 µg g^{-1} CAP and 3 µg g^{-1} FF, blank muscle tissue sample added 1.5 µg g^{-1} CAP and blank muscle tissue which was added 3 µg g^{-1} FF+ 1.5 µg g^{-1} CAP are given in Fig. 3, 4 and 5 respectively.

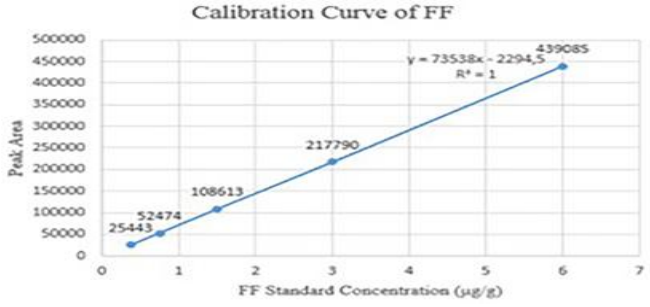

Figure 1. Calibration curve of different concentrations of FF (n= 6).

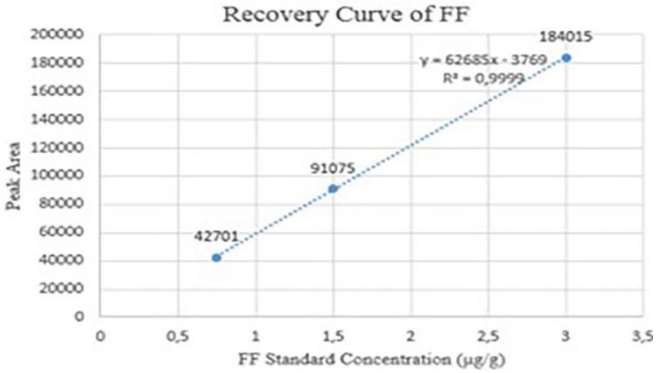

Figure 2. The recovery curve obtained by adding 0.75, 1.5 and 3 µg/g FF to the fish muscle (n= 6).

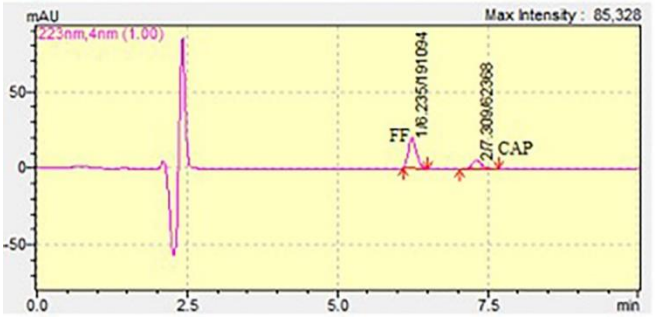

Figure 3. Chromatograms images of standard solutions of FF (3 µg/g) and CAP (1.5 µg/g).

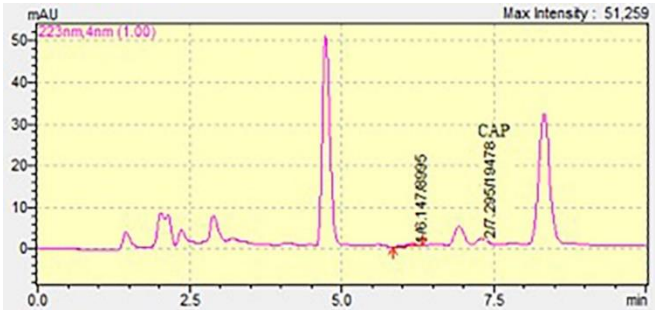

Figure 4. Chromatogram images of blank muscle samples, to which 1.5 µg/g CAP was added.

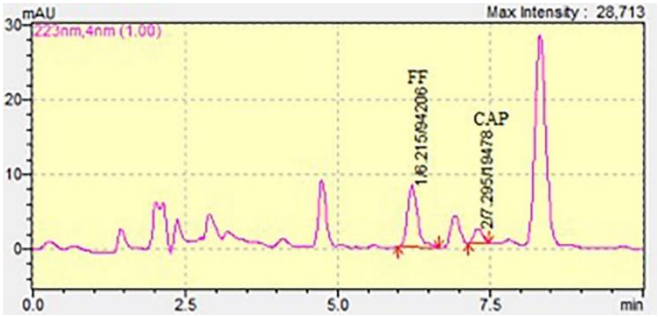

Figure 5. Chromatogram image of blank fish muscle sample, to which 3 µg/g FF and 1.5 µg/g CAP was added.

Table 2. FF levels in fish muscle and boiling juice after the boiling process and rate of change in the level of FF.

Sample No	Untreated fish samples	Fish muscle after boiling		Juice after boiling		Fish muscle + juice after boiling	
n: 5	µg/g	µg/g	%	µg/g	%	µg	%
1	14.68	5.67	38,62	4,33	29,49	10,00	67,76
2	6.22	0.97	15,59	4,21	67,76	5,18	82,44
3	8.89	3.22	36,22	5,91	66,54	9,13	102,14
4	5.15	3.45	66,99	2,88	55,98	6,33	121,78
5	6.41	2.82	43,99	4,44	69,35	7,26	112,46
Mean±sem	8.27±1.71	3.23±0.75	40.30±8.23	4.35±0.48	57.80±7.46	7.58±0.88	97.30±9.86
P	0.014 df:4						

P<0.05

Table 3. FF levels in fish samples after the grilling process and rate of change in the FF levels.

Sample	FF level		
n: 5	untreated fish sample (µg/g)	grilled sample (µg/g)	grilled sample (%)
1	8,43	8,66	102,65
2	8,43	8,43	100,02
3	3,58	4,16	116,40
4	3,58	4,24	118,61
5	3,32	3,35	101,10
Mean±sem	5.47±1.21	5.77±1.14	101.10±4.01
P	0.094 df:4		

P> 0.05

Table 4. FF level, found in cold storage (-20°C) process of fish samples, and change ratio of the levels by days.

Sample	FF level (µg/g) by day			FF change ratio by days%		
n: 5	0	20	50	0-20	20-50	0-50
1	12.77	6.70	4.87	52,47	72,69	38,14
2	5.17	5.84	5.13	112,96	87,84	99,23
3	6.22	3.09	3.30	49,78	106,80	53,05
4	8.89	5.04	3.79	56,69	75,20	42,63
5	8.43	9.97	6.64	118,27	66,60	78,77
Mean±sem	8.30±1.31a	6.13±1.13ab	4.75±0.58b	78.01±15.40	81.82±7.14	62.36±11.60
P	0.048 df:2					

Values with different superscripts differ significantly (P<0.05)

The determined FF levels in the fish muscle samples after boiling, grilling and cold storage are given in Tables 2, 3 and 4 respectively.

DISCUSSION

Recovery and repeatability pooled (RSDr%) results were proved to be capable of determining the residues of FF in the muscle of *Acipenser gueldenstaedtii* by HPLC-DAD. In order to maintain the pharmacological effects of the drugs, they should not be affected by thermal changes during different storage conditions, pellet form preparations or extrusion processes in aquaculture. For this reason, pharmacologically active substances are tested for stability (Hsieh *et al.,* 2011). In a study, it was found that amfenicols were mostly stable in the form of medicated feed for up to 1 month in freezer, FF and CAP were not changed, only TAP was reduced by about 10%, in refrigerator FF, CAP and TAP decreased by 15-25% while it was recorded to be unstable when kept at room temperature in the dark (Pietro *et al.,* 2014).

The FF is exposed to high temperatures (up to about 149°C) during the extrusion process for the production of floating feeds in fish farming. In a study performed for this purpose, no significant degradation of FF concentration was observed after production of both floating and sinking pelleted fish feeds (Merck Animal Health, 2013). In another study, FF was found to be unstable at 80°C -100°C at pH 10 that had been adjusted with phosphate buffer (Elimam *et al.,* 2017). In a study on the heat stability of FF, TAP and CAP, it has been reported that matrix and heat technique are effective on the heat stability of drugs (Franje *et al.,* 2010).

Florfenicol levels (except sample 2, Table 2) found in this study, which was conducted to determine the effect of thermal processes on FF residues, were much higher than legal MRL

(1000 µg/kg) levels, set by EU (EU, 2010), in the raw and cooked samples, taken at the times at which residues can be at the highest level (samples of 1st, 3rd and 6th hours). The mean FF levels of untreated fish muscles were obtained as 8.27±1.71µg/g. After boiling, the mean FF levels in fish muscle significantly decreased to 3.23±0.75µg/g (P=0.014) (P< 0.05) and 4.35±0.48 µg/g in boiling juice. The finding indicates that FF residues were significantly infused into juice (57.80±7.46%) by boiling. The total FF amount in tissue and juice was close to the value of untreated tissue (97.30±9.86%). No significant difference ($P>0.05$) could be detected between the raw (5.47±1.21µg/g) and treated (5.77±1.14µg/g) samples (Table 3) when the samples grilled on the oil-free pan.

The mean FF levels in the freezing fish muscles were obtained as 8.30±1.31µg/g, 6.13±1.13µg/g and 4.75±0.58µg/g for 0th, 20th and 50th days, respectively. Compare to the initial levels (day 0), on the 20th and 50th day of freezing at -20 °C, the residue level were detected as 78.01±15.40% and 62.36±11.60% respectively. The mean differences were found to be statistically significant among the three time points (P=0.048). The mean FF levels obtained on 50th day were found to be statistically lower than 0th day (P<0.05) (Table 4). Similarly, decreases in FF and FFA residue levels in eggs during storage at 20˚C and 4˚C were reported by Filazi *et al.* (2015).

There are very limited researches on veterinary drug residues in fish and the effects of cooking and cold storage or other conservation processes on them. Franje *et al.* (2010) have reported that the main compound is reduced by the heating process applied to amfenicols (include FF), but degradation products having antimicrobial activity can be produced, so that the heating of amphenicol residues in the food cannot always be considered safe. In this study, a decrease in the FF level in boiled fish tissue was determined similar to the results of the studies in different matrices conducted by Franje *et al.* (2010) and Filazi *et al.* (2015). However, this decrease occurred as FF transition to boiling juice in this study. The differences in the rate of decrease in FF level between the studies can be attributed to the differences in the studied matrices.

In a study conducted in Nigeria, in which the fate of tetracycline and CAP residues in fresh and frozen *Clarias gariepinus* and *Oreochromis niloticus* fish species were investigated, and the antibiotic level was found to be higher in the raw fish samples compare to the frozen ones (P<0.05), and also antibiotic levels (tetracycline 2.185 ± 0.412 ppm, CAP 0.837 ± 0.165 ppm) in tilapia and catfish varied according to sources of the fish (MRL set by both local and international safety agencies for tetracycline is 0.2 ppm; for CAP is zero) (Olusola *et al.*, 2012). The effect of boiling, baking, and frying on the stability of OTC residues in shrimp samples was studied and a 30-60% reduction was recorded (Uno *et al.*, 2006). Processes applied to foods cause the degradation of the residual main compound (antibiotics, etc.) and reduced its level whereas it may increase degradation products (Nguyen *et al.*, 2015).

In conclusion, the results of this study have shown that grilling is not caused any decrease in the FF level, boiling reduces the residue level in fish muscle, but FF was transferred boiling juice and the amount of drug residue is decreased with the duration of freezing. However, it cannot be assumed that these residues will always decrease to a safe level in terms of consumer health with boiling and storage processes that cause a decrease in FF residues, especially when the FF residues in fish are quite higher the MRL. Therefore, implementations of drug residues monitoring program by competent food authorities in order to ensure food safety are of importance.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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