Analysis of Diflubenzuron in Tilapia Filet by HPLC–DAD

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Abstract

A simple and efficient new procedure is presented for the analysis of diflubenzuron (DFB) accumulation in tilapia (*Oreochromis niloticus*) filet. A liquid-chromatography (LC) with diode array detection method with C_{18} solid-phase extraction clean-up was employed. The methods exhibit no significant matrix effect as verified by the recovery efficiency. The limits of detection and quantification were 32 µg/kg and 110 µg/kg, respectively. LC–tandem mass spectrometry analysis confirmed the presence of DFB in filet of tilapia exposed to this pesticide. The method was successfully applied for the analyses of fish captured in three different fee-fishing farms during two seasons and for the analyses of fish from an experimental pond (subjected to Dimilin exposition) and depuration tank during different time intervals.

Introduction

Fish farms can be subject to pesticide and chemical run-off from adjacent agricultural land or industrial sources. In addition, the use of chemotherapeutics among these pesticides is common practice for preventing unwanted organisms, thus promoting productivity increase (1–8). The use of these chemotherapeutic products varies depending on the cultivated species, production system and localization, and this scenario may lead to unacceptable levels of chemical contaminants in the cultured products. The lack of specifically formulated pesticides for aquaculture, particularly in the Brazilian market, introduces the use of products developed for agriculture. Consequently, the information concerning their fate in fish farm ponds is scarce. The use of non-regulated chemotherapeutics and the commercialization of uninspected cultured products may present potential food-safety hazards.

In most nations, aquaculture is considered a new industry, and adequate guidelines and regulations regarding the use of chemicals have not been established (9). The Brazilian aquaculture is responsible for the employment of thousands of people in several regions of the country and economically represents a production of around 258,000 tons/year (10). Despite the economical relevance, there are no legal instruments or specific regulating practices for the use of aquacultural chemotherapeutics. However, there is increasing concern over the contamination of Brazilian aquatic food products with bioaccumulative and potentially harmful chemicals used during the handling of such practices.

The chlorinated diphenyl compound, iflubenzuron (1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea) (DFB), well known by its trade name Dimilin, is an insect growth regulator used to control various insect pests in cotton, field crops, forests, orchards, and public health applications (11). DFB is one of the most used pesticides in Brazilian aquaculture, mainly for the control of crustacean ectoparasites such as *Lerneae sp*. and *Argulus sp.* (8,12).

Tilapias are currently the second most important freshwater aquaculture species in the world (13). According to Alceste and Jory (14), tilapia aquaculture has grown impressively worldwide during the 1990's, and a forecast indicates that the industry will continue to expand significantly in the years to come. Additionally, the dynamic expansion, strong marketing efforts, and increasing popularity, is turning tilapia into a significant substitute for traditional whitefish species. Tilapia is among the most raised fish in Brazil (around 37.8% of total inland aquaculture), mainly in the Southeastern region (10,15), and for this reason *Oreochromis niloticus* (*O. niloticus)* was chosen for this study.

Methods for DFB determination and some metabolites in fish tissues have been previously described (16–19). Most of these protocols for DFB analysis in fish tissues involve several extractions, purification and concentration steps, which makes it laborious and susceptible to random errors, especially when many samples have to be analyzed. The aims of this work are to develop a rapid and simple method for the determination of DFB residues in fish filet using high-performance liquid chromatography techniques coupled with diode-array detection (HPLC–DAD) and determine DFB accumulation in *O. niloticus* subjected to water-borne DFB exposition.

Material and Methods

Chemicals

DFB solid standard was supplied by Ultra Scientific (North Kingstown, RI). HPLC-grade methanol (J.T. Baker, Phillipsburg, NJ) and HPLC-grade trifluoroacetic acid (Mallinckrodt, Paris, France) were used without further purification. Stock solution of DFB (100 mg/L) were prepared by dissolving the compound in methanol and then storing it in a freezer (–20ºC). The anesthetic solution was prepared by diluting an appropriate amount of commercial benzocaine gel (DFL Indústria e Comércio SA, Rio de Janeiro, Brazil) in water.

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Fish sampling

O. niloticus (238.5 ± 89.1 g) contaminated with DFB was obtained through a treatment simulation experiment against ectoparasites using the commercial insecticide DFB (Crompton, Brazil), in accordance with the procedure usually conducted by Brazilian fish farmers (8). The fish were kept in 10,000-L concrete ponds located at the Center for Water Resources and Applied Ecology, School of Engineering of São Carlos (Universidade de São Paulo, Brazil) with the following characteristics: static system, sediment layer of 10 cm, density of 8 fish/m^3, and feeding regime of two times per day with commercial pelletized food. Immediately before the pesticide administration (1.0 g/m^3 of active ingredient) and at different time intervals (5, 24, 48, 120, 168, and 240 h after the pesticide exposition), fish were caught (n = 6) and anesthetized with benzocaine (2%) prior to tissue collection. Sampling procedures were performed as approved by the Animal Care Committee from the Brazilian College of Animal Experimentation (20).

A depuration time experiment using *O. niloticus* (227.1 ± 83.1 g) exposed to pesticide in an experimental pound (starting 10 days after administering DFB) was assessed in a 1,000-L fiberglass tank without sediment, density of 25 fish/m^3, and 50% daily renewal of water in one single operation. Fish sampling (n = 6) was carried out at intervals of seven days.

Furthermore, fish samples were caught from three different aquacultural fee-fishing enterprises from Socorro, Brazil in which the use of pesticide during management practices was identified by previously conducted interviews (8, 21). During two different seasons (June: low season and January: high season) 10 fish (*O. niloticus*) from each enterprise were caught, anesthetized, transported in ice to the laboratory, and the filets were collected and stored (–20°C) until analyses.

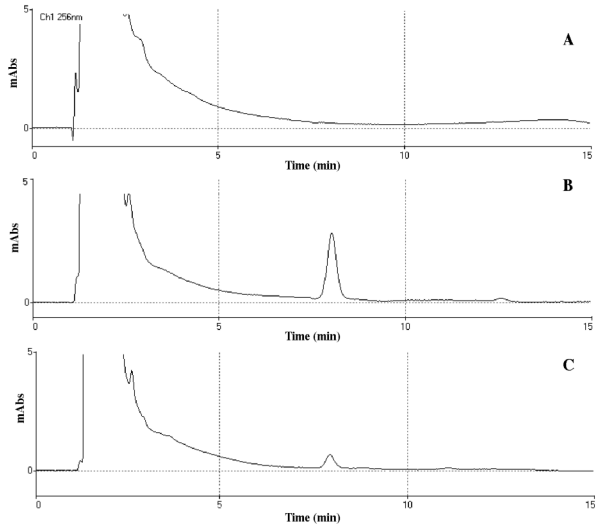

Figure 1. Typical chromatograms of different samples of fish collected before pesticide administration (A); fish filet spiked with DFB (6.8 mg/kg) (B), and fish subjected to waterborne Dimilin exposure (C).

Table I. Mean recovery (R), Minimum and Maximum values (min – max), and Standard Deviation (S) of DFB from Spiked Muscles from *O. niloticus*

DFB (mg/kg)	Replicas	[R (min – max) ± S] %
0.34	n = 5	101.1 (83.5 – 117.7) ± 15.7
0.68	n = 5	96.8 (85.8 – 114.6) ± 9.6
3.40	n = 5	96.9 (91.2 – 103.9) ± 5.0
6.80	n = 5	97.2 (88.4 – 99.9) ± 5.0

Chromatographic conditions

HPLC–DAD analyses were performed using a Shimadzu LC-10AD-VP coupled with a Shimadzu SPD-M10AVP DAD detector (Kyoto, Japan). The analytical column was a Zorbax C_{18} reversed-phase column (150 mm × 4.6 mm i.d., 5 µm). An isocratic elution (methanol–water–TFA, 70–29.9–0.1, v/v/v) with a flow rate of 1 mL/min was employed in the HPLC measurements.

A subsequent confirmative HPLC–ESI-MS analysis was performed to identify DFB in fish subjected to DFB exposition. HPLC–ESI-MS analyses were carried out in a multiple stage ion-trap mass spectrometer (Bruker, Billerica, MA) model Esquire 4000 using a Shimadzu (Japan) HPLC prominence pump model 20AD and a rheodyne manual injector (loop of 20 µL). The chromatographic separation was achieved using a Zorbax C_{18} reverse-phase column (150 mm × 2.1 mm i.d., 5 µm) and an isocratic elution (methanol–water–formic acid, 70:29.9:0.1, v/v/v) at a flow rate of 0.3 mL/min. An electrospray ionization interface was used with the following mass spectrometer parameters: nebulizer pressure, 40.0 psi; drying gas (N_2) flow rate, 9.0 L/min; and drying gas temperature, 350°C. The scan mode was utilized in the scan range of 50–400 *m/z*.

Sample pretreatment

Exactly 5.0 g of filet (free of bones and scales) were homogenized with a mixer (food processor) in a glass beaker with 30 mL of methanol for approximately 1 min. All contents were transferred to a centrifuge tube and centrifuged at 5,000 rpm for 10 minutes at 4°C. An aliquot of 6 mL of the supernatant were collected and filtered using an unconditioned Bakerbond C_{18} cartridge (J.T. Baker). The eluate (20 µL) was injected into the HPLC system.

Results and Discussion

Calibration curve

The calibration curve was obtained by dilution of DFB stock solution in methanol. These were prepared in triplicate by dilution to yield 0.1, 0.5, 1.0, 2.5, 5.0, 10, and 15 mg/L. The standard curve was linear in the investigated range. The equation obtained by regression analysis was: $y = -8180.57 + 45989.88x$ (R = 0.99980).

Limits of detection and quantification

A blank extract was prepared using fish filet from the experimental pond before pesticide administration. The limits of detection (LOD = 32 µg/kg) and quantification (LOQ = 110 µg/kg) were estimated at 3 s and 10 s, respectively, where s is a standard deviation of measured signal-to-noise ratio (n = 20) after blank injection. No significant matrix effect was observed (Figure 1). A preconcentration step (10 times) was included when DFB concentrations in the samples were above the LOQ. Therefore, 5 mL of the extract was dried under N_2 flow in a small beaker and then resolubilized in 0.5 mL of methanol.

Recovery studies

The recovery rates were determined by comparing the analysis results of the spiked *O. niloticus* filets with those of the standard solution. An extract of 5.0 g of tilapia filet (free of spines and scales) was spiked with an appropriate amount of a standard stock solution (Figure 1B) to provide a nominal concentration of 0.34, 0.68, 3.40, and 6.80 mg/kg. Samples were homogenized, centrifuged, and filtered as described previously. All experiments were carried out in quintuplicate.

Good recovery averages and precision (standard deviation) were obtained in the analysis of spiked filets when compared with the results obtained by DiPrima and coworkers (16) analyzing fish (species not informed); Hormazábal and Yndestad (18) analyzing spiked muscles of salmon and rainbow trout; and Lopes (19) analyzing spiked muscles of pacu (*Piaractus mesopotamicus*) (Table I).

However, we must take into account that the DFB spiked in filet does not interact within the fish tissue in the same manner as it would in a real situation of DFB exposition. We can presume that in such cases a more complex interaction could occur. Therefore, we must be attentive to the limitation in this type of recovery studies. Thus, Zitko (22) discusses the absence of certified standard reference material for any of the pesticides of interest to aquaculture, whether in sediment or in biological tissue matrices for the analytical validation procedures. Therefore, impossibility arises and comprehensive analytical methods validated by interlaboratory tests are implemented.

DFB waterborne exposition

A real situation of tilapia exposure to DFB was performed in an experimental fish farm pond, and filets contaminated with DFB were analyzed (Figure 1C). Mean concentration of DFB in *O. niloticus* filet from different conditions are presented in Table II.

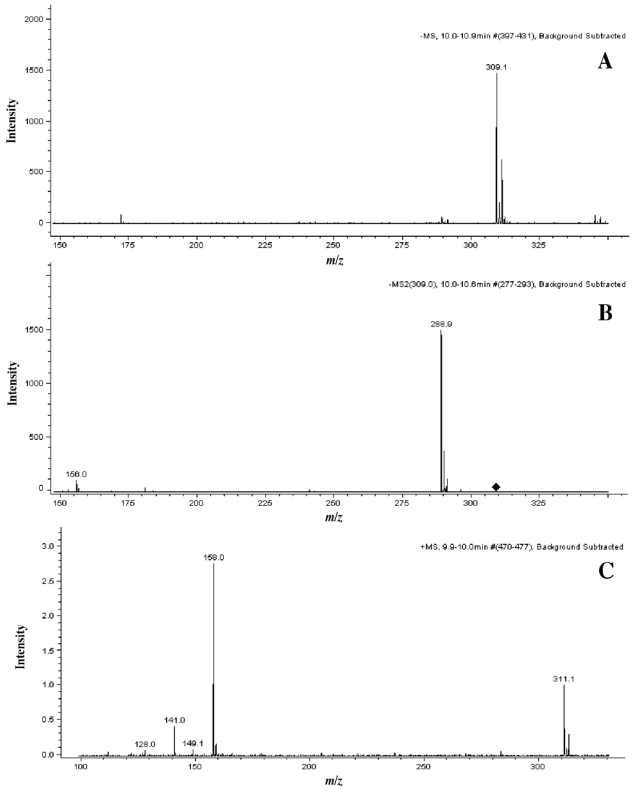

Figure 2. Mass fragmentograms of DFB: ESI(–)-MS, (A); ESI(–)-MS–MS, (B); and ESI(+)-MS (C).

A posterior confirmative HPLC–ESI-MS–MS analysis of DFB in tilapia filet from ponds subjected to DFB administration was performed and their fragmentation was accessed by electrospray ionization-ion trap mass spectrometry in both positive- and negative-ion modes (Figure 2). A detailed study of diflubenzuron fragmentation was demonstrated by Yang and coworkers (23) and will not be discussed here.

The bioaccumulation ratio calculated as DFB concentration in tissue divided by the DFB concentration in water vary from 8.8 fold (minimum value 240 h) to 29.8-fold (maximum value 48 h). Similarly, bioaccumulation ratio of DFB in different fish species were estimated by others authors, ranging from 13 to 160 fold (19, 24–29). However, for the differences between these works, mainly regarding the experimental model, DFB concentration, and species/tissue studied, cautious comparisons must be conducted. Nevertheless, bioaccumulation process seems to be directly related to the exposure concentration in water (25–29). Moreover, the rapid decrease of DFB from tissues was verified by other authors (26,27,30), especially when the fish were transferred to clear water, as verified in tilapias maintained in a depuration tank. In this case, two weeks after the transference to clear water, residues of DFB were no longer detected.

Conclusion

The new proposed HPLC–DAD method is simple, accurate, and rapid. An experienced technician is able to carry out this procedure in less than 20 min to analyze a single sample. Only a small amount of solvent is required, and little residue is pro-

Table II. DFB (mean ± standard deviation) in *O. niloticus* Filet from Different Samples

Sample Category	Sample Subcategory	DFB (mg/kg)
Experimental pond	BPA*	ND†
	5 h	0.86 ± 0.70
	24 h	1.21 ± 0.20
	48 h	1.57 ± 0.57
	120 h	1.34 ± 0.43
	168 h	1.14 ± 0.10
	240 h	0.68 ± 0.08
Depuration tank	7 days	0.022 ± 0.008‡
	14 days	ND
	21 days	ND
Fee fishing A	June 2005	ND
	January 2006	ND
Fee fishing B	June 2005	ND
	January 2006	ND
Fee fishing C	June 2005	ND
	January 2006	ND

* BPA – Before pesticide application.
† ND – Not detected.
‡ Samples preconcentrated.

duced. This method can be considered sufficiently sensitive and reliable for routine analysis of DFB accumulation in *O. niloticus* filet following waterborne exposure to DFB.

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