DETERMINATION OF BIOPESTICIDE AZADIRACHTIN IN SAMPLES OF FISH AND IN SAMPLES OF WATER OF FISH PONDS, USING CROMATOGRAPHY LIQUID OF HIGH PERFORMANCE

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ABSTRACT

The development of a new analytic method for the extraction and determination of the biopesticide present azadirachtin in real samples of fish and in samples of water of fish ponds, it allows the injection of the fish extracts and water of the pomds, in a column cromatographic ISRP-C18 (250mm x 2mm DI), without previous treatment of the sample. The recoveries of azadirachtin in fortified fish samples were higher than 90% with inferior standard deviation at 14.6%. The detection limits and quantification for the azadirachtin were of 0.02 and 0.12μgL^{-1}, respectively. In 86% of the 42 samples of fish azadirachtin masses among 0.14 to 2.317μgL^{-1} was detected.

KEY WORDS: HPLC; Azadirachtin; fish; biopesticides

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INTRODUCTION

The azadirachtin is obtained from *Azadirachta indica*, a member of the *Meliaceae* family, which includes famous wood trees such as mahogany, cedar, chinaberry, muskwood and trichilia. It is iriginally from Southeast Asia and is cultivated in many countries in Asia, all African countries, in Australia and South and Central America. For centuries it has been used in Asia, mainly in India, as a medicinal plant. It has many uses such as antiseptic, curative and vermifuge, in the preparation of medicinal soaps, creams and tooth paste. The tree gives shadow and has a prime wood for furniture, construction, door frames and doors, boxes, firewood and charcoal.

Its use as pesticide became well known in the last 30 years as its main compound was isolated – the azadirachtin. The natural inseticides in *Azadirachta indica* are biodegradable, thus they do not leave toxic residues nor do they contaminate the environment. They have a repellent as well as anti-alimentar propertis, regulate growth and are inseticide, besides possessing ascaricide, fungicide and nematicide characteristics. By its nature, the extracts of *Azadirachta indica* are worldwide approved for use in organic cultures (IAPAR).

PROBLEMS IN FISH FARMING

Modern psciculture is classified in lucrative production, preservantion of the environment and social development. Fish culture depend mainly on the ecosystems they are inserted in. These should remain even to attain the assurance of the activity (VALENTI, 2000). The top economical and ecological problems are associated to the patogens and parasites that arbor the cultivated species. In fish culture they arise as important constraints to the productivity since they provoke retard in the growth and high mortality rates of fishes (RANZANI-PAIVA et al., 1997).

Among the main fish parasite the mongenoides have special note in fish culture. They are ectoparazites of the platelmint family characterized by an fixation apparatus situated in the posterior part of its body. Adults have a stretched, eggy or circular form measuring 1 to 3 milimeters. Damage to fishes is related to the specie of the parasite, with the local of infestation, the number of individuals and the type of feeding (PAVANELLI et al., 1999).

The use of pesticides can result in high levels of residues in the fishes since the restriction periods are not respected (RODRIGUES et al., 1997). Besides that the excessive use and high does of these inceticides may result in fish mortality even in their younger phase. In fish culture, the methyl-parathion, an inceticide of the organophosphorate chemical group, is most used in the control of aquatic predators in production ponds and the preparation of hatcheries for the reception of fish larvas aiming to maximize the production of ROTIFEROS (SENHORINI et al., 1991; PAVANELLI et al., 1998).

Among the products that can be highly efficient in the control of pathogens with a less negative result in the environment is the biopesticide azadirachtin.

CONSIDERATIONS ON THE ANALYSIS OF AZADIRACHTIN

The various analytic methods reported in the literature show the determination of the presence of Azadirachtin in many vegetal samples by means of high performance liquid chromatographic (SCHAAF et al., 2000; SUNDARAM; CURRY, 1993).

Sundaram y Curry (1993) determined the presence of Azadiracthin in Neem Oil and other formulations, such the complex environmental matrix, using the high performance liquid chromatographic technique. These authors have also determined the concentration of Azadirachtin in samples of leaves of conifers, soil from forests and river water, using the same technique. The Azadirachtin was extracted from solid matrix using aqueous methanol followed by the re-extraction with hexane and dichlormethane. After evaporation of the dichlormethane the residue was diluted with ethil acetate purified in a micro-column of florisil and determined in a high performance liquid chromatographic system equipped with a chromatographic column C_{18} and a detector of absorption in the UV region using a water and acetonitrile gradient as mobile phase (SUNDARAM; CURRY, 1995).

Schaaf et al. developed an analytical method, quick and sensitive, to determine the presence of Azadirachtin and tri-terpenoids in *Azadiracta indica* using the high performance liquid chromatographic technique linked to a mass spectrometer. The method shows a detection limit of 1.0 $mg.L^{-1}$ (SCHAAF et al., 2000).

Preparation of the fish samples for the determination in Gas Chromatography and High Performance Liquid Chromatography

From the analytical point of view the preparation of samples of fish tissues is limited by the low polarity of tissues due to the presence of lipides, cholesterol, esthers, momo and triglicerids and phospholipides (HERNANDEZ et al., 1998) which makes difficult the extraction and purification of the sample.

BEGUM et al., (1994) studied the bioaccumulation of dimehoate in liver an muscle of *Clarias batrachus*. For this purpose, 2 g of tissue was mixed with 4 g of anidre sodium sulphate. Then, the mixture received 60 mL of chloride methilen and its was stirred for 5 minutes. The extract was filtered and again it was added chloride methilen with was evaporated in a rotaevaporator. After that, it was added to the residue *n*-hexane and acetonitrile (1:1 v/v) and this mixture was mechanically stirred for 30 minutes. It was then transferred to a separation funnel and the acetonitrile phase was collected. The residue obtained after the evaporation of the acetonitrile was diluted in 0.5 mL of acetone and injected in a gas chromatograph.

According to Abbas e Hayton (1996) the preparation of tissue samples of fishes (brain, white muscle, liver, kidney and plasma) for the chromatographic quantification of parthion and paraoxon residues can be done by the following method: extraction of the tissue sample with 3 mL of issoctane with 200 mL if methanol and concentrated NaCl for protein denaturation. The extract was centifugated for 3 minutes at 5ºC at 3000 rpm. The upper phase was collected and evaporated. Residues were reconstituted in 0.1 and 1.0 mL of issoctane and aliquots of 1,0 mL were injecte3d in the gas chromatrograph. Hernandez et al. (1998) used different types of sea water organisms (*Skeletonema costatum, Artemia salina, Aphanius iberus e Gambusia affinis)* to analyze residues of oragnophoshorates. They liophilized 2 g of tissue which was homogeinized with 30 g of anidere sodium sulphate and 1 g of celite. The extraction was done with 150 mL of acetonitrile:acetone (90:10v/v) in high speed in ultraturrax for 3 minutes. Then, the solution was filtered, pre-concentrated and the final residue was dissolved in 2 mL *n*-hexano and injected in the gas chromatograph.

The high performance liquid chromatography (HPLC) has also been used to analyze toxic residues in water environment as well as to analyze residues of treatment of fish parasites. Bergwerff et al. (2004) used HPLC to analyze residues of malaquita green in

Anguila anguilla. Takino et al. (2003) analyzed the residues of chloranphenicol in fishes. HPLC was used by Haug and Hals (2000) for the determination of oxitetracyclin in *Salvelinus alpinus*, by Dawson et al. (2003) for cloramine T in *Oncorhynchus mykiss* and by Ingelse et al (2001) for organophosphorates in samples of water by direct injection.

In the present study it was developed an analytical method to determine the presence of azadirachtin in samples of water from fish ponds and in fish tissues, using a HPLC system equipped with a ISRP-C_{18}-HSA chromatographic column.

MATERIALS AND METHODS

Reagents

Acetonitrile and methanol were obtained from Carlo Erba (Milan, Italy); the standard sample for azadirachtin was purchased from Aldrich – Sigma (Milwaukee, USA); the monobasic sodium phosphate was obtained from Merck (E. Merck, Darmstadt, Germany). The deonized water was provided by a Milli-Q purification system from Millipore (Millipore, Bedford, MA, USA).

Fish sample

Forty-two samples of fishes were collected, six samples for control, from the fish ponds at the campus of the UNESO at Jaboticabal. These samples were maintained under refrigeration (-8.0ºC) untill the analysis.

Water sample

Water samples were collected form six ponds with concentrations of azadirachtin ranging from 40.0 to 240.0 μgL^{-1}. These samples were kept under refrigeration (-8.0ºC) untill the analysis.

METHODS

Instrumentation

The chromatographic experiments were carried out in an isocratic high performance liquid chromatographic system, brand Varian, equipped with a model 2510 reciproque pump; a UV detector with varied wave-length, model 2550, with wave-length adjusted to 217 nm and an integrator from Spectra-Physics, model SP 4400 chromajet, purchased from Varian Associates, Inc. (Sunnyvale, CA, USA). The sample concentration and the standard solutions were injected in a ISRP-C_{18} (250mm x 2mm DI) column with a manual inlet valve for injection (Rheodyne 7125, Cotati, CA, USA) connected to a loop of 500µL.

Chromatographic conditions

The ISRP-C_{18} (250mm x 2mm DI) chromatographic column used was prepared according to Menezes and Felix (1998). The determination of the azadirachtin was done in room temperature, with mobile phase flux adjusted to 0.5 mL/minute^{-1}. The mobile phase included a mixture of an aqueous solution of monobasic sodium phosphate 0.05 mol.L^{-1} and acetonitrile (63: 37v/v).

Preparation of the analytic curve

The standard solutions of azadirachtin, in concentrations ranging from 15 to 60µg.L^{-1} were used to construct the analytic curve. They were prepared by dilution of a standard solution of azadirachtin 50x10^{5}µg.L^{-1}, obtained by diluting 0.005 g of azadirachtin in 10.0mL of methanol. The solution was diluted 100 times in methanol, resulting in a final concentration of 50x10^{3}µg.L^{-1}.

Evaluation of the time of recuperation of azadirachtin using methanol

Three whole samples of whole pacu with circa 2.0 g were weighted. The samples were ground to a homogene paste. It was

added 50µL a solution of $50x10^3 \mu g.L^{-1}$ of azadirachtin. The macerate was transferred to a flask and 10 mL of methanol were added obtaining a final concentration of $250 \mu gL^{-1}$ of azadirachtin. The flasks were stirred in a stirring table for 30, 60 and 90 minutes. Then, the samples were centrifugated for 10 minutes at 6000 rpm. A volume of 500µL of the floating phase in the HPLC system was injected.

Evaluation of the recuperation of the azadirachtin

Three whole samples of pacu weighing circa 2.0 g, were ground until a homogene mass was obtained. Then, 40, 60 and 80µL of a solution with $50x10^3 \mu g.L^{-1}$ of azadirachtin were added. The macerated matter was transferred to a flask to which was added 10mL of methanol obtaining azadirachtin concentrations of 200.0, 300.0 and $400.0 \mu gL^{-1}$. The flasks were stirred for 90 minutes in a stirring table. Then, the samples were centrifugated for 10 minutes at 6000 rpm. 500µL of the floating solution were injected in the HPLC system.

Determination of azadirachtin in the water used in the fish ponds of pacus (*Piractus mesopotanicus*)

Water samples were collected form six pond. The concentrations of azadirachtin ranged from 40.0 to 240.0 μgL^{-1}. These samples were diluted in the rate 1;10 aiming to avoid interfering peaks from metabolits present in the samples. Thus, 500µL of the sample was injected in the HPLC system.

Determination of azadirachtin in the fish samples

The pacu samples were wighted and submitted to maceration in a mortar until a homogeneous mass was obtained. The macerated was tranfered to a flask and 10.0 mL of ethanol was added. The flasks were stirred for 90 minutes in a stirring table. Then, the sam-

ples were centrifugated for 10 minutes at 6000 rpm. Then, it were injected 500µL of the 1:10 diluted extract in the HPLC system.

RESULTS AND DISCUSSION

Use of ISRP-C18 column in the determination of azadirachtin

Many extraction techniques such as liquid-liquid, soxlet, pressurized liquid chromatography (PLE; DIONEX-ASE®) to accelerate the extraction with solvent have been used to extract compounds from fish tissues (SIMONICH et al. 2000). However, these techniques are expensive and provide a diminished selectivity. Interfering compounds of biological tissues, similar to lipides, including cholesterol, are co-extracted. Thus, after the extraction the separation of the analite of interest of interfering proteins and lipids requires more time than the process of extraction of the focused compound (OSEMWENGIE; STEINBERGH, 2003).

This study evaluates the use of the chromatographic column ISRP-C18 (*internal surface reverse phase*) to perform the separation and the determination of the azadirachtin present in fish tissues.

FIGURE 1 shows the chromatograms of the standard solution of azadirachtin: (A) 60µgL^{-1}, (B) chromatogram obtained after injection of 500µL of the control sample, and (C) chromatogram obtained after the injection of 500µL of a real sample of fish.

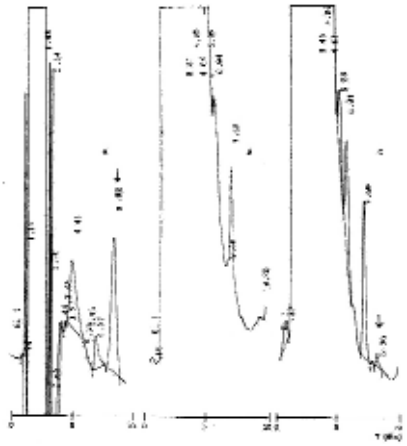

FIGURE 1 – Chromatograms obtained after injection of 500µL of a standard solution with 250µgL^{-1} of azadirachtin (chromatogram A), 500µL of the control sample (chromatogram B) and 500µL of the extract of a real sample of fish.

In chromatogram C it was observed that the azadirachtin was separated from the metabolites without overlapping of interfering peaks.

Evaluation of the time of extraction using methanol as solvent

It is felt that more that 100 structures related to triterpenoids were isolated in various neem true parts. The azarirachtin is the more oxygenated and the most polar among the triterpenoid already isolated from this plant (JOHNSON et al, 1996). Taking into consideration this physical-chemical property it was evaluated the use of ethanol as extraction solvent. According to result in TABLE 1 it was observed that after stirring for 90 minutes the extraction of 95% of the focused analite was obtained.

TABLE 1 – Determination of the time of recuperation of the sample.

Time (in minutes)	Recuperation (%)	DPR (%)
30	27,7	3.25
60	58,7	1.22
90	81,5	1.26

Evaluation of the recuperation of the azadirachtin in fish tissues

The selected method showed good results in the evaluation of azadirachtin in the analysis of fish tissues. This can be due to the limits of detection and quantification as wll as to the adequate recuperation values (TABLE 2) showing that the presence of proteins, contained in the extract, did not interfered in the recuperation of azadirachtin.

TABLE 2 – Evaluation of the recuperation of the azadirachtin in fish tissues.

Enhancement level (μg L^{-1})	Recuperation (%)	DPR (%)
0,20	90,0	14,6
0,30	95,5	7,2
0,40	98,4	14,4

APPLICATION OF THE ANALYTICAL METHOD

Determination of the azadirachtin in real samples of fishes

Once optimized the proposed analytical method, it was proceeded to the determine the levels of azadiracthin in real samples of fishes. The results were obtained by an analytical curve with equation: $y = – 953.64 + 95468.3x$, with a linear coefficient using $r^2 = 0.99877$.

According to TABLE 3 it was observed that in 86% of the analyzed fish samples it was determined the levels of azardirachtin ranging from 0.14 to 1.03μg g^{-1} of fish.

In the remainder samples (14%) no fractions of azadirachtin were detected.

Determination of azadirachtin in samples of water from pacu ponds (*Piaractus mesopotamicus)*

The use of the IS ISRP-C_{18} chromatographic column allowed the direct injection of 500μL of the real sample of water from fish ponds without previous treatment to the determination of azadirachtin. Resuslts can be seen in TABLE 4.

It should be stressed that the results were determined after 24h of treatment with azadirachtin, in which the biopesticide was added to obtain concentrations of 40.0, 80.0, 120.0, 180.0, 200.0 and 240.0 x $10^3 \mu g L^{-1}$.

By analyzing the results in TABLE 4 it becomes evident that the levels of concentration of azaridachtin after 24h of addition are far below the added concentrations. It is possible to infer that part of the concentration was absorbed by fishes and part is degraded as reported by SUNDARAM et al. (1995).

TABLE 3 – Determination of azadirachtin in real samples of fishes.

Sample number	Fish mass (g)	Concentration of azadirachtin (µg g-1)
1	2.12	ND
2	2.577	0,36
3	1.688	0,50
4	2.17	ND
5	1.70	1,03
6	3.024	0,19
7	2.55	0,20
8	1.465	0,27
9	1.62	0,21
10	1.78	0,20
11	1.871	ND
12	2.278	0,25
13	2.663	0,19
14	2.317	0,22
15	2.663	0,15
16	1.058	0,47
17	1.506	0,21
18	2.309	ND
19	2.042	0,28
20	2.108	ND
21	2.435	0.30
22	2.720	0.21
23	2.882	0.23
24	1.829	0.22
25	1.986	0.22
26	2.246	0.25
27	2.176	0.37
28	2.313	0.50
29	2.712	0.14
30	3.492	0.48
31	2.255	0.20
32	2.406	0.53
33	2.055	0.38
34	1.712	0.49
35	2.296	0.44
36	2.155	0.62
37	2.690	ND
38	2.158	ND
39	2.317	ND
40	2.696	0.18
41	2.663	ND
42	2.014	ND

TABLE 4 – Determination of azadirachtin in water samples from fish ponds.

Sample number	Concentration of added azadirachtin 103(_gL-1)	Concentração de azadirachtin após 24 horas (ÌgL-1)
1	40	0.11
2	80	0.43
3	120	1.09
4	160	0.51
5	200	1.86
6	240	2.73

CONCLUSION

The proposed method is simple, quick and the ISRP -C_{18} column can be used for the determination of the concentration of the biopesticide azadirachtin present in real samples of fishes or in water from fish ponds.

It should be emphasized that the use of methanol as extraction solvent was quite efficient for the extraction of the azarirachtin present in fish tissues.

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