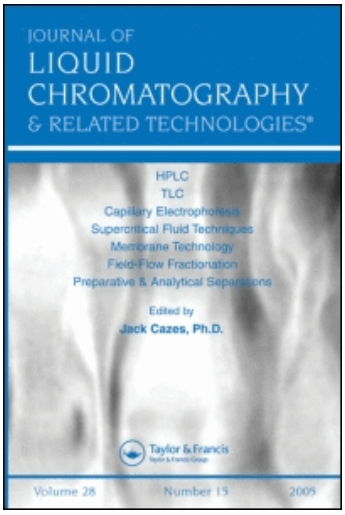

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HPLC Method for the Analysis of Atrazine in Freshwater Bivalves

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ABSTRACT

A method for the analysis of atrazine in freshwater bivalves has been developed. The method involves a simple liquid–liquid extraction procedure followed by high-performance liquid chromatography analysis using a C_{18} column, a methanol–water (60 : 40, v/v) mobile phase, a flow-rate of 1 mL/min, and UV detection at 230 nm. After optimization of the conditions, the method was validated, showing 81.7% atrazine recovery. Performance data for the method such as linearity, precision, and quantitation limit are also reported.

Key Words: Atrazine; HPLC; Bivalves.

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INTRODUCTION

Atrazine, the common name of 2-chloro-4-ethylamino-6-isopropyil-amino-s-triazine (chemical formula: $C_8H_{14}ClN_5$) is among the most important selective herbicides, used for pre- and post-emergence weed control.[1] Atrazine has been classified as a possible human carcinogen[2] and has been banned from European countries, but has been used in other countries such as The United States and Brazil. According to Sindicato Nacional da Indústria de Produtos para Defesa Agrícola (SINDAG), between 1998 and 2000, Brazil spent about seven billion dollars in pesticides for the internal market; herbicides have been used more frequently than insecticides and fungicides.

According to the United States Environmental Protection Agency,[3] the maximum admissible concentration of atrazine in drinking water is 3 μg/L. In contrast, the European Union established hard limits, i.e., 0.1 μg/L for individual pesticides in drinking water.[4] In Brazil, the maximum admissible concentration of atrazine in drinking water is 2 μg/L, according to Portaria 1469 of December 29th, 2000 (Ministério da Saúde).

Atrazine is a potential contaminant due to its chemical properties such as high potential leaching, high persistence in soil, slow hydrolysis, low vapor pressure, low to moderate water solubility, and moderate adsorption onto organic soil, and clay.[1] Part of this herbicide is degraded in the environment by chemical and microbiological processes[5] and part is leached into the soil by rain or irrigation water reaching superficial water.[6] Pereira et al.[7] conducted a study in San Joaquin River, California, and detected trace amounts of atrazine in water at most sites. It has been estimated that the Mississippi River transports about 160 tons of atrazine/year to the Gulf of Mexico.[8,9]

Due to its chemical and physical properties, atrazine has been shown to be relatively stable in the environment, so that its utilization harms the biota. Atrazine toxicity to mussel larvae, fishes, tiger salamanders, and aquatic insects has been reported by several authors.[1,10–13] Studies of the bioaccumulation of atrazine in mussels and fishes have shown accumulation via gills and blood in the liver, brain, intestine, gonads, and gall bladder.[14,15]

Among aquatic organisms, freshwater mollusk bivalves are excellent monitors for bioaccumulation studies since they are sedentary, filter large amounts of water, and live buried in sand.[16] The mollusk bivalve *Anodontites trapesialis* can be found in several rivers in São Paulo state, Brazil, and has already been used by our group for the biological monitoring of organochlorine pesticides.[17,18] At present, we are investigating the use of this mollusk to study the bioaccumulation of atrazine. Thus, the aim of this paper is to describe the results obtained with the development and validation of the method for atrazine analysis in this freshwater bivalve tissue.

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The analysis of atrazine in environmental samples, such as water and soils, has been well documented in the literature. High performance liquid chromatography,[4,19–24] gas chromatography,[19,25–27] and capillary electrophoresis[28,29] have been used in multiresidue methods or in methods directed at the analysis of atrazine and its degradation products. However, there are few methods for the analysis of this herbicide in aquatic organisms.[30,31] There are many challenges for the analysis of pesticides in biological samples, such as high complexity of extraction methods and the clean-up and concentration of the compounds, particularly for samples with high lipid contents. To our knowledge, this is the first validated method reported in the literature for the analysis of atrazine in the mollusk selected for this study.

EXPERIMENTAL

Bivalve Mollusks

Anodontites trapesialis adults were collected from Galo Bravo Lake, Ribeirão Preto, São Paulo state, Brazil, and transported to the Zoology Invertebrate Laboratory, Biology Department, FFCLRP-USP, where they were placed in an aquarium with 20 L of water from a natural source. To obtain a bivalve sample exposed to atrazine, a group of five bivalves (68.9 g) were placed in the aquarium containing 20 L of water fortified with atrazine at the concentration of 1.0 μg/mL. After 24 h of exposure, the bivalves were frozen, the valves were removed and the soft parts were triturated, lyophilized, and stored at ambient temperature (25°C) until the time for analysis. The control group consisted of five bivalves placed in an aquarium containing water with no atrazine.

Chemicals and Reagents

Atrazine (97.7%) was obtained from Novartis. The methanol (Carlo Erba) utilized to prepare atrazine standard solutions and mobile phase, and the dichloromethane (Mallinkrodt) utilized in the extraction of atrazine from biological samples were HPLC-grade. Water was purified with a Milli-Q-Plus system (Millipore). A stock solution of atrazine was prepared in methanol at the concentration of 1 mg/mL and working solutions in the concentration range of 4.0 to 80.0 μg/mL were prepared by appropriate dilutions. The solutions were stable for at least one month when stored at −20°C.

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Instrumentation and Chromatographic Conditions

The Shimadzu HPLC system consisted of two pumps model LC-10AD, a Diode-Array detector, a system controller model SCL-10A, and a Rheodyne 7725i injector with a 20-μL loop. This equipment was controlled by a Shimadzu Class-VP software for data acquisition and treatment. The Shimpack CLC-ODS column (250 × 4.6 mm I.D., 5 μm, Shimadzu) was protected by a Lichrospher C18 guard column (4 × 4 mm I.D., 5 μm, Merck). The mobile phase was methanol : water (60 : 40, v/v), used at a flow-rate of 1 mL/min. Detection was performed at 230 nm.

Sample Preparation and Calibration Curves

One hundred milligrams of lyophilized bivalve tissue were transferred to extraction tubes and, after the addition of 1 mL water, the tubes were vortex mixed for 30 s. After that, 200 μL of 1.5 mol/L NaOH and 4 mL of dichloromethane were added. The tubes were capped and submitted to mechanical shaking for 20 min. After centrifugation for 5 min at 1800*g*, 3 mL of the organic phases were transferred to clean tubes and the solvent was evaporated to dryness. The residues were dissolved in 200 μL mobile phase and 20 μL were analyzed.

Calibration curves were prepared by spiking 100 mg of control bivalve tissue with 25 μL of atrazine solutions at concentrations of 4, 20, 40, and 80 μg/mL, with resulting atrazine concentrations of 0.1, 0.5, 1.0, and 2.0 μg/100 mg bivalve. Plots of atrazine concentrations vs. peak areas were constructed and the linear regression lines were used for the determination of atrazine concentration in the samples.

Method Validation

Recovery

Samples of control bivalves were spiked with atrazine concentrations of 0.1, 0.5, 1.0, and 2.0 μg/100 mg bivalve, submitted to the extraction procedure, and the concentration of these samples were calculated using a calibration curve obtained by direct injection of atrazine solutions in the mobile phase. This calibration curve, in the concentration range of 0.1 to 2.0 μg/mL, was prepared by transferring 25 μL of 4, 20, 40, and 80 μg/mL atrazine solutions to glass tubes. The solvent was evaporated to dryness, and 200 μL of mobile phase was added. After vortex mixing, the solutions were submitted to chromatographic analysis.

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Linearity

The linearity of the method was evaluated using control bivalve samples spiked with atrazine concentrations corresponding to 0.1, 0.5, 1.0, 2.0, 4.0, and 8.0 μg/100 mg control bivalve.

Within-Day and Between-Day Precision

The precision of the assay was determined by analyzing aliquots of a bivalve tissue exposed to 1.0 μg/mL atrazine for 24 h. Within-day precision was determined by analyzing 10 aliquots of bivalve tissue on the same day and between-day precision was determined over a 1-week period ($n = 5$). The precision of the method was calculated as the relative standard deviation (RSD).

Quantitation Limit

Quantitation limit is defined as the lowest concentration, which can be determined with acceptable precision and accuracy (RSD and a systematic error lower than 20%, respectively).[32] In the present study, the quantitation limit was evaluated using samples of 100 mg control bivalve spiked with atrazine solutions at the concentrations of 0.01 and 0.03 μg/100 mg bivalve ($n = 5$).

RESULTS AND DISCUSSION

The UV absorption spectrum for atrazine shows a maximum absorption at 222 nm, and, therefore, 230 nm was chosen as a good wavelength for the analysis of atrazine using methanol–water as the mobile phase, avoiding interference detection. Figure 1 shows typical chromatograms for control bivalves and atrazine-spiked bivalves.

After defining the analytical conditions, the method was validated, in terms of linearity, recovery, precision, and quantitation limit using lyophilized bivalve tissue spiked with known amounts of atrazine. Tables 1 and 2 summarize the data obtained in the validation of the method. A mean recovery of 81.7% with an RSD lower than 10% was obtained in the concentration range of 0.1–2.0 μg atrazine/100 mg bivalve, a value considered acceptable for the extraction of analytes from biological samples.[33] An important factor for the extraction of atrazine from bivalve samples was the addition of 1.5 mol/L NaOH, in order to avoid atrazine protonation and increase the recovery. The calibration curve obtained by least-squares linear regression was linear up to 8.0 μg

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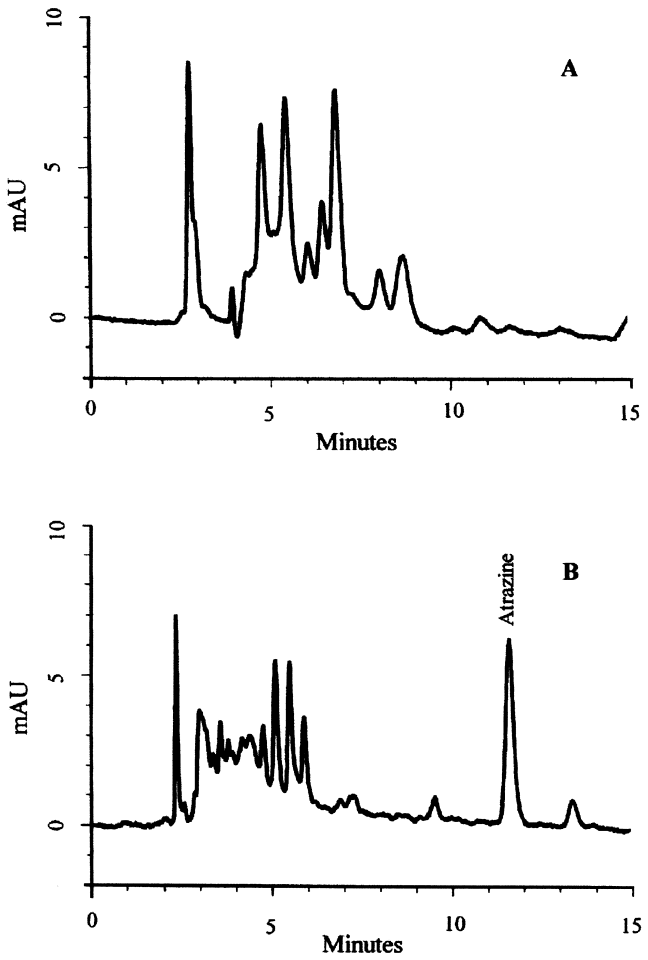

Figure 1. Chromatograms referring to the analysis of atrazine in a control specimen of the bivalve *A. trapesialis* (A) and in a specimen spiked with 0.1 μg atrazine/100 mg weight (B). Mobile phase: methanol–water (60 : 40, v/v); flow-rate = 1 mL/min; $\lambda = 230$ nm; injection volume = 20 μL.

atrazine/100 mg bivalve, with typical equations and correlation coefficients of $y = 10822x + 749.97$ and $r = 0.999$, respectively. The precision of the method was assessed by replicate analysis of bivalve samples exposed to 1.0 μg/mL atrazine in the aquarium for 24 h. The within-day and between-day precision values are reported in Table 2. Relative standard deviation of less than 10%

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Table 1. Recovery of atrazine from tissue of the bivalve species *A. trapesialis*.

Concentration (μg atrazine/100 mg bivalve)	Recovery (%)
0.1	85.2
0.5	87.1
1.0	78.2
2.0	76.5
Mean	81.7
RSD	6.3

Note: RSD, relative standard deviation; $n = 2$ for each concentration.

were obtained for all samples analyzed. It is worth mentioning that acceptable precision was obtained even though an internal standard was not used. The quantitation limit determined from extracted bivalve tissue was 0.03 μg atrazine/100 mg bivalve (Table 2) and the accuracy was less than 20%, in agreement with the literature for validation of liquid chromatographic methods.[32] The results of this investigation suggest that the assay is sensitive enough to be used in environmental analysis of *A. trapesialis* tissue.

CONCLUSION

The method described in the present paper is simple and rapid. The results obtained for recovery, linearity, precision, and quantitation limit show that this

Table 2. Precision and quantitation limit of the method for the analysis of atrazine in the tissue of the bivalve *A. trapesialis*.

	Concentration (μg atrazine/100 mg bivalve)	RSD (%)
Within-day precision ($n = 10$)	0.28	5.3
Between-day precision ($n = 5$)	0.27	4.7
Quantitation limit ($n = 5$)[a]	0.03	5.9

Note: RSD, relative standard deviation; *n*, number of determinations.
[a]Accuracy (% error) = 18.3.

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is an efficient method for the quantitation of atrazine in the tissue of the freshwater bivalve *A. trapesialis*.

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