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Persistence of Azadirachtin Residues on Olives after Field Treatment

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In this work an HPLC method for the determination of azadirachtin residues on olives was developed, and the field degradation kinetics of the pesticide was studied. In field trials the active ingredient (a.i.) decay had a half-life time of 0.8 days, which was too short to show a good efficacy of treatment. The mechanism of disappearance of the pesticide studied with model systems showed that it was unrelated to evaporation, thermodegradation, and co-distillation, but it was related to photodegradation. The high photodegradation rate of commercial formulations calls for the need to test different formulates in order to increase the persistence of the residue and thus the pesticide's efficacy.

KEYWORDS: HPLC; analysis; azadirachtin; residues; olives; photodegradation

INTRODUCTION

Azadirachtin is a limonoid of the tetranortriterpenoid type (**Figure 1**) extracted from the oil obtained from seeds of the neem tree (*Azadirachta indica*). The neem tree is original to the Union of Myanmar (Burma). It is widespread in India, and to a lesser extent in Africa and Asia. Neem extracts show an insecticidal activity and antifeedant and repellent properties. Neem extracts and pure azadirachtin are used to control Lepidoptera and Diptera both by contact and ingestion. This active ingredient (a.i.) seems to be selective, easily degradable, and nonmutagenic. Moreover, it has a low toxicity against nontarget and beneficial organisms and causes less disruptance to ecosystems than conventional insecticides (*1*). The azadirachtin content in the seed extracts and in commercial formulates was determined by HPLC (*2*−*4*), by enzyme-linked immunosorbent assay (*5*), and by supercritical liquid chromatography (*6*). Analytical methods for determining azadirachtin residues on leaves and soils are reported (*5*), but residues on fruits have not been described. In Italy this insecticide is registered for many crops with a maximum residue level (MRL) of 0.5 mg/kg and a pre-harvest interval (PHI) of 3 days for all crops. In this work an HPLC method for the determination of azadirachtin residues on olives was developed, and the field degradation kinetics of azadirachtin was studied. This paper also reports the results of the disappearance mechanism of this insecticide obtained on a model system.

MATERIALS AND METHODS

Field Trials. A field trial was carried out in an olive grove at Elmas, in the neighborhood of Cagliari, Italy. The grove was planted in 1960 with a tree spacing interval of 7 × 7 m (200 plants/ha); the cultivar was Tonda di Cagliari. A random-block design with four replications was used, and each block contained three trees in a single row. Treatments were carried out with an F-320 portable motorized sprayer (Fox Motori, Reggio Emilia, Italy). The commercial formulation was Oikos (azadirachtin A 3.2%, Sipcam, Milan, Italy), and was used at the doses recommended by the manufacturer (3.8 L/ha). Four treatments were carried out every 14 days, from August 8 to September 19, 2001. Sampling for residue control was carried out at 1, 2, 3, and 7 days after the last treatment. Random 2-kg samples were collected from each block (ca. 0.7 kg per plant).

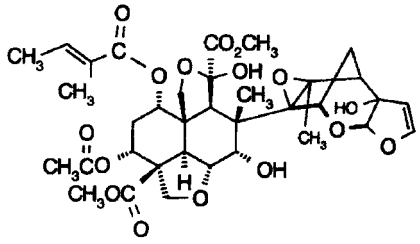

Figure 1. Structure of azadirachtin.

The weather conditions were continuously recorded with an SM 3800 automatic weather station (SIAP, Bologna, Italy). Rainfall was continuously recorded with an AD-2 automatic weather station (Silimet, Modena, Italy).

No rainfall was recorded after the last treatment and during the experiments. Maximum and minimum average temperatures were 26.5 and 18.7 °C, respectively.

Chemicals and Materials. Acetonitrile, acetone, and methanol were HPLC grade (Merck, Milan, Italy); water was distilled and filtered through a Milli-Q apparatus before use (Millipore, Milan, Italy). Azadirachtin (95−98% purity) was purchased from Sigma Aldrich (Steinheim, Germany), Na_2SO_4 was analytical grade. Stock standard solutions of the pesticides (ca. 200 mg/kg) were prepared in methanol. Working standard solutions of the pesticides were prepared by diluting with the mobile phase (acetonitrile/water; 15:85, v/v). Membranes of

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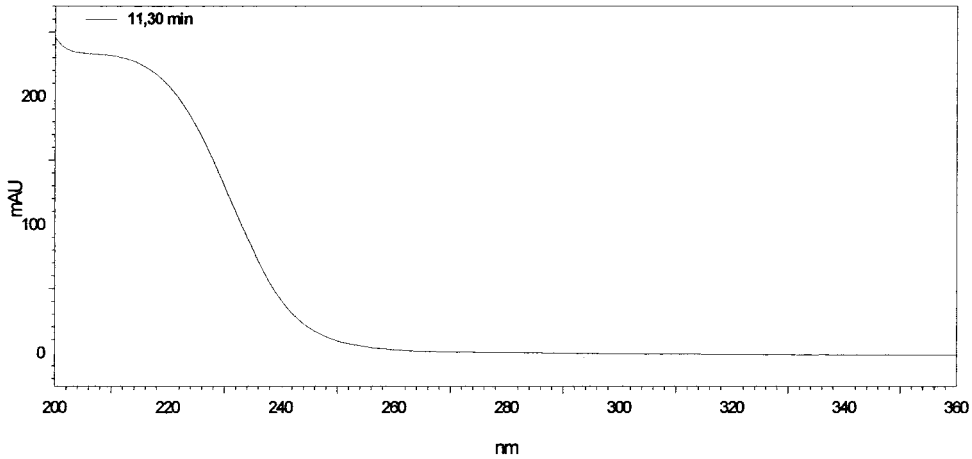

Figure 2. UV spectrum of azadirachtin.

regenerated cellulose (0.45 μm, 25-mm diam) were purchased from Schleicher & Schuell (Dassel, Germany).

Apparatus and Chromatography. *HPLC Analyses.* An Agilent Technologies (Waldbronn, Germany) model 1100 liquid chromatograph was used, fitted with a diode array detector (DAD) model UV6000LP (Thermo Quest, San Josè, CA). A Spherisorb S5 ODS2 (250 × 4.6 mm, 5 m) column was employed.

The gradient profile for the separation of azadirachtin was as follows: initial 15:85 (v/v) acetonitrile/water, reaching 85:15 (v/v) in 15 min. Before performing each injection, the LC system had to be stabilized for 10 min with acetonitrile/water (15:85, v/v). The sample injection volume was 100 mL with a flow rate of 1 mL/min. The analysis was performed by setting the detector at the wavelength of 215 nm depending on the maximum absorbance spectrum (**Figure 2**).

Extraction Procedure from Olives. After sampling, a 25-g aliquot of whole olives was weighed in a screw-capped flask with 10 g of Na_2SO_4 and 50 mL of acetonitrile. The mixture was stirred in a shaker (Stuart Scientific) for 1 min; then 2 mL of the organic extract was dried under a nitrogen flux and redissolved with 1 mL of acetonitrile/water (15:85, v/v), and finally injected into the HPLC for analysis.

Recovery Assays. Samples of untreated whole olives were fortified with the appropriate amount of the standard solutions to reach concentrations of 0.02, 0.05, 0.10, 0.50, and 1.0 mg/kg. The samples were allowed to settle for 30 min prior to extraction. They were then processed according to the extraction procedure reported above. Four replicates for each concentration were analyzed.

Extraction of the Waxes from the Fruits. The extraction of the epicuticular waxes from the olives was carried out as described by McDonald et al. (*7*). Untreated olives of predetermined weight and volume were dipped in chloroform for 1 min; the total quantity of wax was calculated by evaporating 10 mL of chloroform extract to dryness.

Model Systems. *Test A.* The a.i. dissolved in acetone (100 μL) was applied on a membrane of regenerated cellulose. After evaporation of the solvent, the membrane was placed in a 10-mL vial with a screw-closed cap. A control vial was kept at room temperature in the dark, while another was placed in a heater at 50 °C for 24 h. The vial was then moved to the freezer at −20 °C where it was left for 5 h to allow the a.i. in the gaseous state to condense on the vial walls. After removing the vial from the freezer, the membrane was immediately placed in a 20-mL vial containing 5 mL of the extraction solvent. After the vial was shaken, the content was analyzed to determine the amount of the a.i. in the membrane. The amount of a.i. evaporated from the membrane was calculated from the presence of residues in the vial walls, while the thermodegraded pesticide was estimated from the difference between the residue in the control vial and the sum of residues present on the filter and the walls of the vial.

Test B. The a.i. dissolved in acetone (100 μL) was placed on a regenerated cellulose membrane. After evaporation of the solvent, the membrane was placed on the top of a 10-mL vial (containing 5 mL of distilled water) with a screw-closed cap with a hole in the middle. A vial without water, used as a control, was placed in the dark at room temperature, while the vial containing water was placed in a heater at 50 °C for 24 h. During evaporation the water passed through the filter and entrained the pesticide residue on the membrane by codistillation. This water amount was then determined by weight loss. From the amount of pesticide residue on the filter after this experiment, we determined the loss of pesticide by codistillation. For a suitable evaluation of codistillation, the possible losses due to evaporation and thermodegradation determined by test A had to be taken into account.

Sunlight Photodegradation Experiments. Aliquots of azadirachtin were poured into Petri dishes (5 cm diam), and the solvent was allowed to evaporate at room temperature. Each day the dishes were exposed to direct sunlight from 9 a.m. to 5 p.m., and were removed for analysis at prefixed intervals. Controls were stored in the dark at room temperature. The residue in the dish was dissolved with 5 mL of the mobile phase and injected for analysis.

Photodegradation experiments and A and B tests were also carried out in the presence of epicuticular waxes extracted from the olives at the same concentration as in the olives (72 $\mu g/cm^2$) and using commercial formulates. Each experiment was replicated 4 times.

Statistical Analysis. Variance analysis (ANOVA) and comparisons between average values were performed with the Duncan test at $P < 0.05$.

RESULTS AND DISCUSSION

Chromatography. The adopted acetonitrile/water gradient elution allowed a chromatographic separation of azadirachtin. The run time was 15 min and the retention time of azadirachtin in the chromatographic condition described above was 11.3 min. As no interfering peaks were present in the olive chromatograms when the extraction was carried out immediately after sampling (**Figure 3**), no cleanup was necessary.

Linearity. Standard calibration curves of azadirachtin were constructed by plotting concentrations against peak areas. Good linearity was achieved for azadirachtin between 0.02 and 2.5 mg/kg with a correlation coefficient of 0.9997.

Method Validation. The recovery listed in **Table 1** ranged from 84 to 110%, with coefficients of variation between 4 and 14%. Four replicates of each fortification level were analyzed. Because of the sensitivity of the detector the limit of determination reached for the olives was 0.02 mg/kg. These low detection limits were obtained thanks to the high sensitivity of the detector, which was due to a cell path length of 50 mm. Moreover, with the DAD, it was possible to know the peak purity and confirm the a.i. by overlapping the sample spectra with those of the standards.

Residues on Olives. The azadirachtin is a nonsystemic insecticide and therefore should not penetrate into the fruit. We have verified this by analyzing separately the surface extract

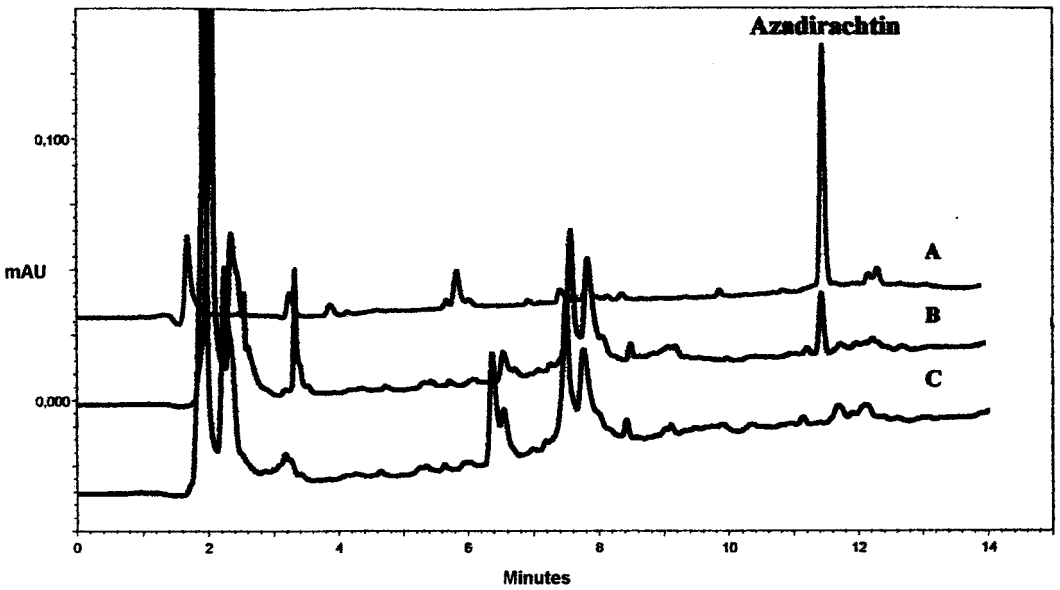

Figure 3. Olive chromatograms: (A) blank fortified at 1.0 mg/kg; (B) sample; (C) olives blank.

Table 1. Recoveries of Azadirachtin from Olives

fortification (mg/kg)	% ± SD
1.00	97 ± 12
0.50	84 ± 12
0.10	110 ± 4
0.02	106 ± 8

Table 2. Evolution of Azadirachtin Residues (mg/kg ± SD) in Olives after Treatment

days after treatment[a]	residues (mg/kg) ± SD
–0	<0.02
0	0.35 ± 0.09
1	0.28 ± 0.11
2	0.10 ± 0.09
3	0.03 ± 0.00
7	<0.02

[a] –0 = before treatment.

and the homogenate whole fruit extract. Because the latter showed many interferents, the analyses were carried out on the surface extract. The azadirachtin residues determined on the olives before and after the last treatment are reported in **Table 2**. No residues were found on olives before the last treatment. The residue just after the treatment was 0.35 mg/kg, which is lower than the maximum residue level fixed for olives in Italy (0.5 mg/kg). Moreover, after 3 days the olive residues were 0.03 mg/kg, indicating a 90% decrease, and after a week the a.i. was not detectable. The degradation rate calculated as a first-order kinetics shows a half-life time ($t_{1/2}$) of 0.8 days ($r = 0.9650$). The high rate of degradation of this a.i. suggests that no accumulation effect of the residue is caused by repeated treatment. No azadirachtin residues on the olives at harvest means no residues in the olive oil.

Residue Decrease Mechanism in Model Systems. In general, pesticides deposited on fruit rapidly penetrate inside the epicuticular waxes and the cuticle (*8*). When the water contained in the fruit passes through these two layers, it may remove pesticide molecules (codistillation). In addition, heat can cause evaporation and degradation of pesticides on the fruit surface. These three factors (codistillation, evaporation, and thermodegradation) are mainly responsible for the disappearance of contact pesticides. **Table 3** shows the data from the two tests from which codistillation, evaporation, and thermodegradation can be evaluated.

Table 3. Azadirachtin Residues during Tests in Model Systems

	waxes	control (c) (μg/cm^2)	vial (v) (μg/cm^2)	filter (f) (μg/cm^2)	difference $c-(v+f)$ (μg/cm^2)
		test A			
active ingredient	without	0.97 ± 0.04	< 0.01	0.77 ± 0.08	0.20
	with	1.00 ± 0.03	< 0.01	0.96 ± 0.03	n.s.[a]
formulation	without	0.89 ± 0.02	< 0.01	0.78 ± 0.06	n.s.
	with	0.93 ± 0.02	< 0.01	0.89 ± 0.06	n.s.
		test B			
					($c-f$)
active ingredient	without	0.97 ± 0.06		0.59 ± 0.05	0.38
	with	0.91 ± 0.12		0.92 ± 0.07	n.s
formulation	without	0.94 ± 0.02		0.86 ± 0.03	n.s
	with	0.94 ± 0.01		0.85 ± 0.03	n.s.

[a] n.s. = nonsignificant.

Table 4. Half-Lives ($t_{1/2}$) and Correlation Coefficients (r) of Azadirachtin after Exposure to Direct Sunlight

	waxes	$t_{1/2}$ (hours)	r
active ingredient	without	13.2	–0.977
	with	9.6	–0.992
commercial formulation	without	2.7	–0.991
	with	2.8	–0.994

Test A shows that azadirachtin has no tendency to evaporate and thermodegrade whether as an a.i. or in commercial formulations. Test B shows that 38% of the a.i. without waxes codistillated. When the insecticide was propagated in the epicuticular waxes, codistillation was absent. No codistillation was observed using the commercial formulate either with or without the epicuticular waxes.

During the photodegradation tests, azadirachtin was exposed to direct sunlight with and without fruit waxes. According to our calculations, the degradation of the a.i. follows first-order kinetics; the half-lives ($t_{1/2}$) and correlation coefficients (concentration/time) are reported in **Table 4**.

In the experiment carried out using the a.i. the calculated $t_{1/2}$ was 13.2 h, whereas in the commercial formulate the decay rate

was 5 times higher. This shows that additives in the formulation accelerated the photodegradation of azadirachtin (formulate additives acted as catalysts in the photodegradation of azadirachtin).

Epicuticular waxes do not affect the photodegradation of commercial formulates. Moreover, the decay rate of the a.i was increased by 30%.

CONCLUSIONS

The developed method for the determination of azadirachtin residues on olives showed a sensitivity of 0.02 mg/kg. No cleanup was required. In field trials the a.i. decay had a half-life time of 0.8 days, which was too short, thus diminishing substantially its effectiveness.

The data relating to the disappearance of azadirachtin in model systems points out that photodegradation is the main causal factor. This means that propagation of the a.i. through the epicuticular waxes does not protect it from solar radiation. Moreover, the higher photodegradation rate observed in the commercial formulate points out the need to test different formulates in order to increase the persistence of residues and therefore also its efficacy.

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