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Rotenone Residues on Olives and in Olive Oil

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The disappearance of rotenone on olives under field conditions was studied. The field data showed that rotenone residues on olives decreased with a half-life ($t_{1/2}$) of 4.0 days. After pre-harvest time (10 days) the residues were higher than the maximum residue level fixed in Italy (0.04 mg/kg). Experiments with model systems showed that the mechanism of disappearance of rotenone is not related to evaporation, thermodegradation, or co-distillation, but only to photodegradation. When the olives were processed for oil, the residues in the oil were higher than the residues on the olives by a factor of 2.4−4.8.

KEYWORDS: Rotenone; residues; disappearance; olives; olive oil

INTRODUCTION

In recent years the trade of organic products in Europe, North America, and Japan showed high growth rates that are rarely found in food markets (*1*). One reason for this success is the prohibition of synthetic pesticides in organic agriculture. To control pests in organic farming, only natural pesticides can be used, and these include rotenone. Rotenone (**Figure 1**) is a nonsystemic botanical insecticide obtained from leguminous plants such us *Derris elliptica*, *Lonchocarpus nicou*, and *Tephrosia vogelii* (*2*). Rotenone has been used for hundreds of years in Asia and South America to stupefy fish in rivers. The compound is named after Roten, who was the first researcher to study this pesticide in Japan at the beginning of the past century. It is used to control aphids, thrips, suckers, and other insects on fruits and vegetables, but shows a short persistence because it decomposes in the presence of light. It is a nonselective insecticide.

Rotenone is a contact insecticide with a secondary acaricidic action. Rotenone inhibits Site I respiration within the electron-transport chain (*3*). Rotenone shows a pyrethrin-like behavior but with a stronger action and a higher persistence (*4*). It is considered highly toxic for man and for warm-blooded animals (estimated lethal dose for humans: 300−500 mg/kg) (*3*). However, rotenone and rotenoid-containing plants are reported to have anticancer activity in rats and mice (*5*). Novel studies showed that rats chronically treated with rotenone develop neuropathological and behavioral symptoms of Parkinsonism (*6*).

Although rotenone has been a commercial product for many years, there are very few reported studies of its residues in food.

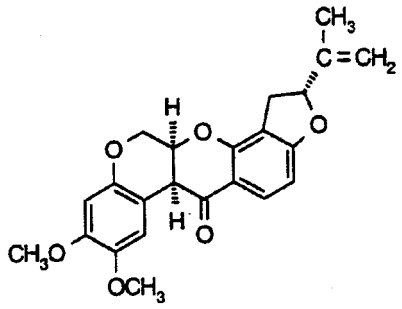

Figure 1. Rotenone structure.

In 1980 Newsome and Shields (*7*) studied residues on lettuce and tomato, and in 2000 Moore et al. (*8*) reported the evaluation of rotenone residues on baby food. The effectiveness of rotenone against the main parasite of olive trees, *Bactrocera oleae*, has recently been shown (*9*). To our knowledge no studies on the residues of rotenone on olives have been reported. To study these aspects we have carried out an experimental trial aimed at evaluating the persistence of rotenone residues on olives under field conditions. The olives were processed into oil and the amount of residues passed into the oil was evaluated. The main factors affecting the persistence of pesticide residues are: fruit growth, evaporation, co-distillation, thermodegradation, and photodegradation. In this investigation experimental trials have been carried out in model systems to determine which of these possible mechanisms was responsible for the disappearance of the active ingredient (a.i.).

MATERIALS AND METHODS

Field Trials. The trial was carried out in an olive grove located at Uta, near Cagliari, Italy. 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 on September

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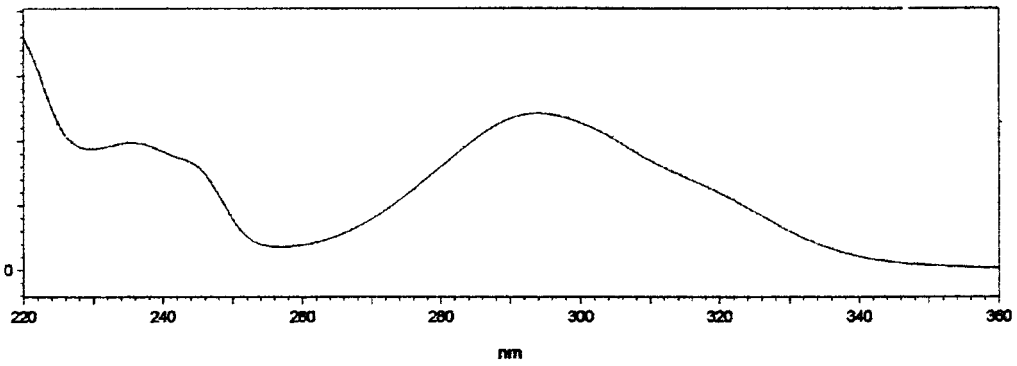

Figure 2. Rotenone spectrum.

14 and 26, 2001, with an F-320 portable motorized sprayer (Fox Motori, Reggio Emilia, Italy). The commercial formulation Rotena (6.2% a.i.; Serbios, Milan, Italy) was used at the doses recommended by the manufacturer (300 g/hL; 10 hL/ha). 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). After the last treatment, it did not rain during the entire experiment. Maximum and minimum average temperatures were 24.5 and 18.1 °C, respectively. Olive samples (1 kg) were collected before and after the last treatment and subsequently at 2, 5, 9, and 12 days.

Chemicals and Materials. Acetonitrile was HPLC grade; acetone and chloroform were solvents for analysis (Merck, Milan, Italy); water was distilled and filtered through a Milli-Q apparatus (Millipore, Milan, Italy) before use. Rotenone (95−98% purity) was purchased from Sigma Aldrich (Steinheim, Germany), Na_2SO_4 was analytical grade. Stock standard solutions of the pesticide (ca 500 mg/kg) were prepared in acetone. Working standard solutions for HPLC determinations were prepared by diluting with the mobile phase (acetonitrile/water; 50:50, v/v).

Apparatus and Chromatography. *HPLC Determinations.* An Agilent Technologies (Waldbronn, Germany) model 1100 liquid chromatograph was used, fitted with a diode array detector (DAD), UV6000LP (Termo Quest, San Josè, CA). A Spherisorb S5 ODS2 (250 × 4.6 mm, 5 μ) column was employed. The gradient profile for the separation of rotenone was as follows: initial mobile phase acetonitrile/water (50:50; v/v), reaching 85:15 (v/v) in 10 min. Before each injection, the LC system had to be stabilized for 10 min with an acetonitrile/water mobile phase (50:50; v/v). The injection volume was 100 μL and the flow rate was 1 mL/min. The analysis was performed at the wavelength of 295 nm according to a maximum reported in the spectrum (**Figure 2**).

Extraction Procedure from Olives and Olive Oil. *Olives*. A 25-g portion of whole olives was weighed into a screw-capped flask with 10 g of Na_2SO_4, and 50 mL of acetonitrile was added. The mixture was agitated in a shaker (Stuart Scientific) for 30 min. The organic extract was allowed to separate and 2.5 mL was evaporated to dryness under nitrogen stream. The residues were taken up with 1 mL of the mobile phase and injected in HPLC for analysis.

Olive Oil. One g of olive oil was weighed into a 30-mL screw-capped tube, and 100 μL of a 10% (w/v) oxalic acid water solution and 10 mL of acetonitrile were added. The tube was agitated in vortex for 10 min. The acetonitrile layer was allowed to separate, and 2 mL was poured into a 10-mL beaker and allowed to evaporate to dryness under nitrogen stream. The residue was taken up with 0.4 mL of the mobile phase and injected in HPLC for analysis.

During the dryness process under nitrogen stream in both extraction procedures, when some liquid drops were still present in the beaker it was necessary to stop the nitrogen flow to avoid loss of residues and allow them to evaporate naturally.

Recovery Assays. Samples of untreated olives and olive oil were fortified with the appropriate amount of the standard solutions to reach concentrations of 0.02, 0.10, 0.50, and 2.0 mg/kg. The fortified samples were allowed to settle for 30 min prior to extraction. They were later processed according to the above extraction procedure. Four replicates of each concentration were analyzed.

Extraction of the Waxes from the Fruits. The extraction of the epicuticular waxes from olives was carried out as described by McDonald (*5*). Untreated olives of predetermined weight and volume were dipped in chloroform for 1 min; the total quantity of wax was calculated by evaporating to dryness 10 mL of chloroform extract (on olives 72 $\mu g/cm^2$).

Model Systems. *Test A*. The a.i. dissolved in acetone (100 μL) was placed 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. The vial was then removed from the freezer and opened, and the membrane was immediately placed in a vial containing the extraction solvent (acetonitrile). The vial content was analyzed to determine the amount of residue in the membrane. After adding 5 mL of the extraction solvent in the vial and shaking, the content was submitted to analysis. The presence of residues in the vial walls allows establishment of the amount of a.i. evaporated from the membrane; and 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 we can estimate the thermodegraded pesticide.

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. The water amount was then determined by weight loss during evaporation. During evaporation the water passes through the filter and may entrain the pesticide residue on the membrane by codistillation. From the amount of pesticide residue present on the filter after this experiment, we can determine the loss of pesticide by codistillation. For a suitable evaluation of codistillation, the possible losses due to evaporation and thermodegradation determined by test A must be taken into account.

Sunlight Photodegradation Experiments. An aliquot of rotenone was poured into Petri dishes (5 cm diameter), and the solvent was allowed to evaporate at room temperature. The dishes were exposed to direct sunlight and removed at prefixed intervals (0, 1, 2, 4, 8, 16, 24, 32, and 40 h) for analysis. 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 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) was carried out, and the comparison between average values was performed with the Duncan test at $P < 0.05$.

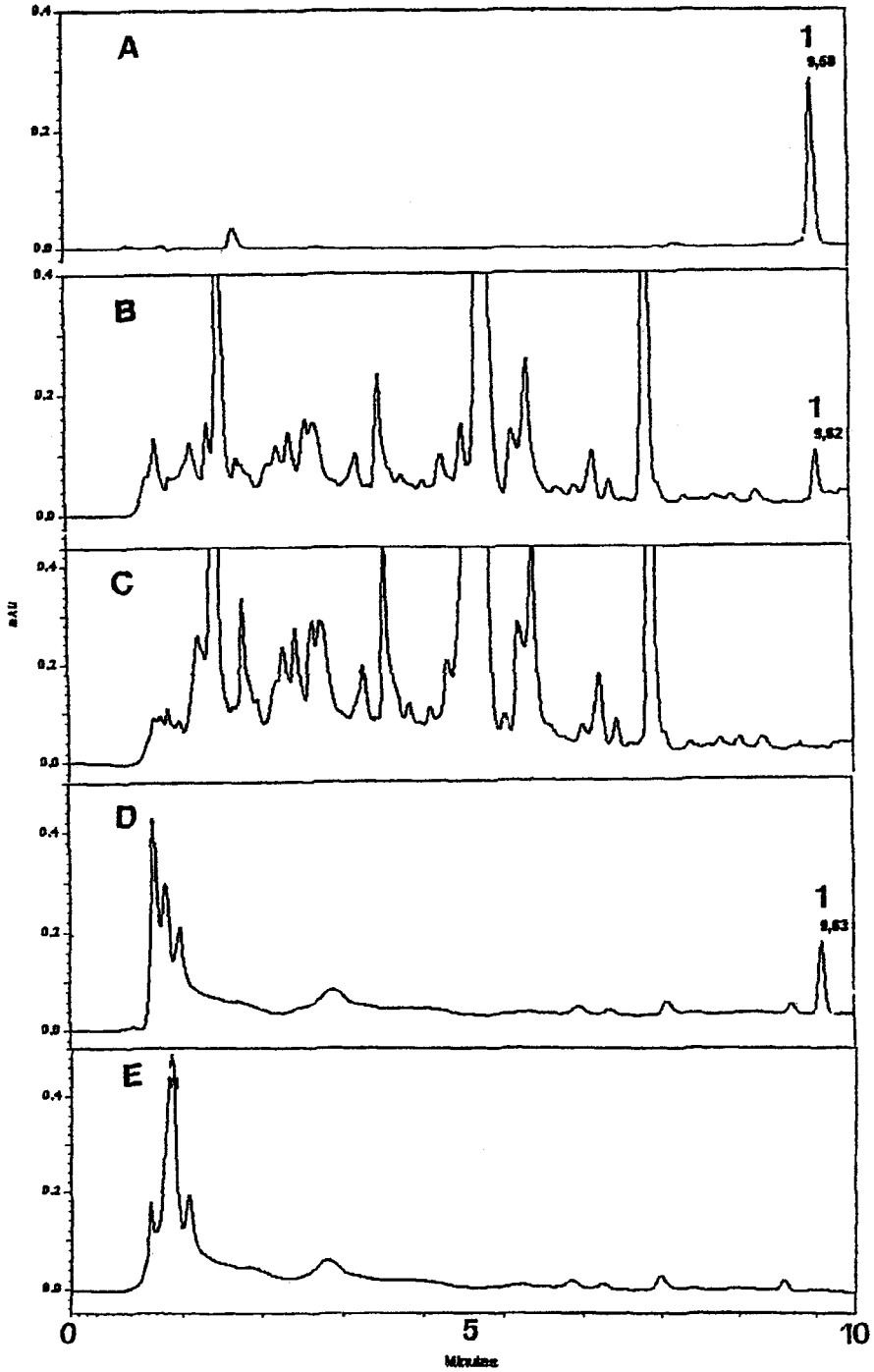

Figure 3. HPLC chromatograms of rotenone (peak 1) in standard solution at 1.0 mg/kg (A), in an olive oil sample (B), in an olive oil control (C), on an olive sample (D), and on an olive control (E)

RESULTS AND DISCUSSION

Analytical Method. *Residue Determination*. Analytical determination of this a.i. is commonly carried out by HPLC (*7*, *8*, *11*, *12*) and GC−MS (*13*) techniques. With GC−MS the determination of rotenone residues in olive oil was not possible, because the matrix effect became remarkable with a marked decrease in the intensity of the rotenone signal. Therefore, we used an HPLC method with an acetonitrile/water gradient to avoid the purification process, as interfering peaks were not present in the extract (**Figure 3**). Standard calibration curves of rotenone were constructed by plotting concentrations against peak areas. A good linearity was achieved between 0.02 and 2.00 mg/kg with a correlation coefficient of 0.9997.

The recovery data were in the 75−104% range, with coefficients of variation between 1 and 8%. The determination limit according to Thier and Zeumer (*14*) was 0.02 mg/kg. Low determination limits (0.02 mg/kg) were achieved because of the sensitivity of the detector, which has a cell path length of 50 mm. The DAD enable determination of the peak purity and confirm the a.i. by overlapping the sample spectra with those of the standards.

Olives and Olive Oil Residues. Rotenone is recorded in Italy on many crops, such as olives, with a maximum residue limit (MRL) of 0.04 mg/kg with a pre-harvest time of 10 days. Because the average weight of the olives was constant during the experiment, no dilution effect occurred. Rotenone residues

Table 1. Rotenone Residues (mg/kg ± SD) on Olives after Treatment and in Olive Oil

days after treatment	olives	olive oil
0	1.14 ± 0.14	
12	0.07 ± 0.02	
0	0.99 ± 0.04	
2	0.52 ± 0.13	1.89 ± 0.18
5	0.44 ± 0.12	1.05 ± 0.12
9	0.19 ± 0.04	0.51 ±0.05
12	0.11 ± 0.02	0.53 ± 0.18

Table 2. Rotenone Residues (mg/kg ± SD) 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)[a]
		Test A			
active ingredient	without	0.85 ± 0.04	< 0.01	0.61 ± 0.03	0.24
	with	0.80 ± 0.05	< 0.01	0.73 ± 0.03	n.s.
formulation	without	1.29 ± 0.05	< 0.01	1.11 ± 0.13	n.s.
	with	1.22 ± 0.08	< 0.01	1.22 ± 0.06	n.s.
		Test B			$(c-f)$
active ingredient	without	0.84 ± 0.03		0.66 ± 0.01	0.18
	with	0.81 ± 0.05		0.71 ± 0.10	n.s
formulation	without	1.32 ± 0.02		1.17 ± 0.15	n.s
	with	1.29 ± 0.09		1.24 ± 0.07	n.s.

[a] n.s. = non-significant.

in olives was 0.07 mg/kg before the last treatment. This shows that the rate of decay was not high enough to cause the rotenone distributed on olives after the first treatment to disappear completely.

After the last treatment, the residue was 0.99 mg/kg (**Table 1**). The residue decreased progressively in time and after 12 days it was 0.11 mg/kg, i.e., about three times higher than the legal limit of 0.04 mg/kg. The decay rate, calculated as a first-order kinetics ($r = 0.9877$), shows a half-life ($t_{1/2}$) of 4.0 days. The rotenone half-life on olives was higher than those on lettuce and tomatoes (*7*), where the half-life was between 0.9 and 3.6 days, respectively, depending on the formulate used. Some samples of olives were processed for oil with a yield of 14−16%. The residues in the oil were higher than those on the olives by a factor of 2.4−4.8. Taking into account the fact that the yield in oil ranged between 14 and 16%, it can be concluded that, on average, about half of the residues passed from the olives to the oil. To meet the fixed MRL, the pre-harvest time must be increased; which, based on the data of this experiment, should be of 20 days. This time would allow residue decrease to very low levels on olives and, consequently, in olive oil.

Test A shows that rotenone does not have a tendency to evaporate or thermodegrade (**Table 2**). **Test B** shows that during water evaporation rotenone was not co-distilled. The only factor that causes a rapid decrease of rotenone residues was solar radiation. Moreover, during photodegradation without waxes, the half-life calculated with a first-order kinetic was 11.2 min (**Table 3**). Epicuticular waxes do not affect photodegration. Using formulates containing the a.i., there was a reduction of a factor of 2 in the degradation rate. In this case, also, epicuticular waxes did not affect the degradation kinetics. Chromatograms obtained during photodegradation (**Figure 4**) show the formation of three main degradation products.

Based on photodecomposition literature data (*15*) these three compounds could be rotenolone, dehydrorotenone, and rotenonone. Rotenolone was determined by Newsome and Shields

Table 3. Half-Lives ($t_{1/2}$) and Correlation Coefficients (*r*) of Rotenone after Exposure to Direct Sunlight

	waxes	$t_{1/2}$ (min)	*r*
active ingredient	without	11.2	− 0.9998
	with	12	− 0.9855
formulation	without	23.4	− 0.9965
	with	25.3	− 0.9838

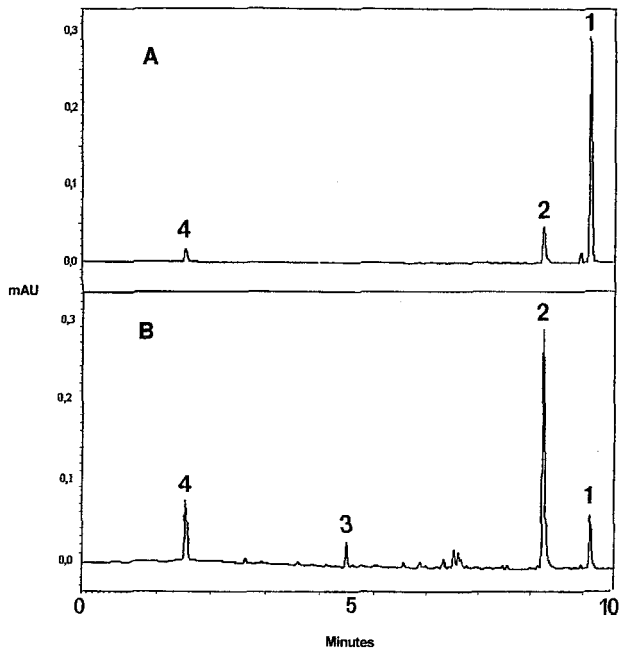

Figure 4. HPLC chromatograms of rotenone (peak 1) after 1 h of sunlight exposure (A) and after 6 h of sunlight exposure (B).

(*7*) on lettuce and tomato, but its content was less than $^1/_{10}$ that of rotenone. We are at present carrying out studies to define the structures of these photodegradation products.

CONCLUSIONS

Field data obtained in these trials demonstrate that rotenone on olives decays more slowly than rotenone on other crops, such as lettuce and tomato. The low maximum residue level (MRL) fixed in Italy (0.04 mg/kg) makes it very difficult to obtain olives with lower residues at the pre-harvest interval. When the olives are processed, the residues in the oil were higher than those on the olives by a factor of 2.4−4.8.

The model systems show that the pesticide decay mechanism is not due to evaporation, thermodegradation, or co-distillation, but is due to photodegradation. Moreover, the considerable rate of rotenone decay in model systems in sunlight was on the order of minutes, while in the field it was on the order of days. A tentative explanation is provided by the hypothesis that rotenone deposited on the olives after treatment rapidly propagates inside the epicuticular wax and in the cuticle (*16*). In this case, we can suppose that penetration of the pesticide in the cuticle protects rotenone from the sunlight degradation.

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