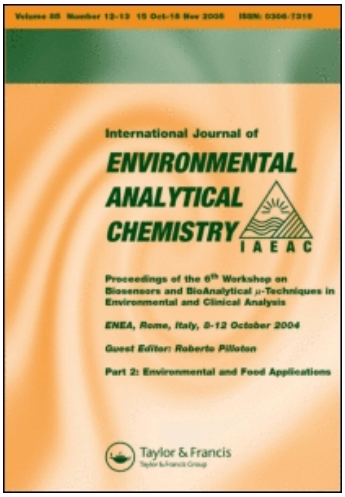

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Monitoring of pesticide residues in olive oil samples: results and remarks between 1999 and 2002

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MONITORING OF PESTICIDE RESIDUES IN OLIVE OIL SAMPLES: RESULTS AND REMARKS BETWEEN 1999 AND 2002

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The occurrence of organophosphorus pesticide residues and their metabolites in virgin olive oil samples was investigated. Forty-eight samples of virgin olive oil were collected directly from olive mills at various locations on the island of Corfu (Greece) where the application of insecticides was performed by spraying from the air and/or from the ground during the three harvest periods (1999–2002). Samples for residues analysis were extracted by liquid–liquid partitioning with solvents of different polarity. The target compounds were determined in the final extract by gas chromatography using flame photometric and nitrogen phosphorus detection. In the case of positive samples the findings were confirmed using columns of different polarity or by GC-MS. The most common pesticide residues found were fenthion and its oxidative metabolites. Concentrations of total fenthion in the positive olive oil samples were below the FAO/WHO Codex Alimentarius maximum residue limit (MRL). Only three samples contained total fenthion residues that exceeded the Codex MRL.

Keywords: Olive oil; Organophosphorus pesticides; Fenthion; Fenthion metabolites

INTRODUCTION

Because of its nutritional and biological characteristics olive oil is one of the most important components of the Mediterranean diet [1]. Greece is among the leading olive oil producing countries internationally. The mean consumption of olive oil is about 20 kg/person per year [2].

The use of pesticides has become necessary for the protection of olive trees against several pests and diseases, mainly the olive fly *Bactocera* (*Dacus*) *oleae*. Organophosphorus insecticides, widely used in olive groves, are applied by spraying the trees from the ground and/or from the air. Nowadays, because of the great concern over possible environmental pollution, aerial application is rarely used.

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Olive trees in Corfu originate from the Venetian period and cover 40% of the total area of the island. Olive cultivation and the special care needed for the production of a high-quality olive oil are very difficult in Corfu owing to the special morphology of a large part of the island with several slopes and rocky ground. During the years 1997 and 1998 when pesticides were only applied from the ground the crop was spoilt by *Dacus*. From 1999, a combined application system (air and ground) of the insecticide fenthion was used in the island. The organophosphorus insecticide fenthion (EPA Toxicity class II) is a lipophilic compound belonging to the class of phosphorothioates [3]. Fenthion is easily degraded in the aquatic environment [4,5] with a half-life of about 14 days and for that reason it has been removed from the National Pesticide Survey (NPS) list of EPA [6,7]. Its transformation product, fenthion-sulfoxide, has not yet been considered as a toxic compound in the environment.

Fenthion is slowly degraded in olives with a half-life of about 35 days and its metabolites have been detected in samples [8,9]. The main route of fenthion metabolism in plants is by oxidation to the sulfoxide, which has a higher biological activity than the parent compound. Subsequent oxidation of the sulfoxide to sulfone, a compound with a lower biological activity, is slow in plants [7]. An additional route of bioactivation is through oxidative enzymatic desulfuration to form fenoxon [7]. These metabolites tend to partition into the olive oil. The presence of their residues in olive oil has been reported by several researchers [10–14]. According to the Codex Alimentarius, the Maximum Residue Limit for fenthion including its metabolites in olive oil is 1 mg/kg.

The objective of this study was to assess olive oil quality relative to the potential pesticide contamination in samples originating from a selected agricultural area, where aerial and/or ground application of fenthion was performed. In the target compounds studied, the insecticides dimethoate, diazinon, methidathion, chlorpyrifos and parathion-ethyl were also included because of their importance in olive culture and the limitations of European Union (EU) directives.

EXPERIMENTAL

Sampling

Forty-eight virgin olive oil samples were collected directly from olive mills during oil extraction from different production areas of the island of Corfu in the period 1999–2002 (three harvest periods). The harvest period in Corfu is relatively extended, from early December to the end of April. Specifically, 13 samples from the first harvest period 1999–2000, 19 samples from the second period 2000–2001 (including one sample from Paxoi, a small island close to Corfu where no spraying was applied) and 16 samples from the third period 2001–2002 (including one sample from Paxoi and five samples from Erikousa, a small island to the north of Corfu, where ground application was performed) have been analyzed. Olive oil samples were collected in dark glass bottles transported to the laboratory and kept at 4°C until analysis.

According to the information received from the local authorities, crop fields (130 000 acres) were sprayed from the air with Lebaycid (50 EC, BAYER, 50% fenthion) during July to October, (11 700 kg of Lebaycid/per year was applied in olive crop fields). A total area of 111 000 acres was treated from the ground using 20 978 kg of Lebaycid annually.

Chemicals and Materials

Pesticide grade *n*-hexane, acetonitrile and acetone were obtained from Labscan (Dublin, Ireland). Water was HPLC grade, a product of Riedel-de-Haen (Seelze, Germany). Certified standard solutions of pesticides were purchased from Dr. Ehrenstorfer (Augsburg, Germany) as a mixture of fenthion and its metabolites (fenthion-sulfoxide and fenthion-sulfone), dimethoate, diazinon, methidathion, chlorpyrifos and parathion-ethyl in acetone, at a concentration of 200 μg/mL. Pesticide mixtures were stored in the dark at −20°C. Working standard solutions in acetone, containing 8 μg/mL (ppm) of each analyte were used for spiking samples. Six multicomponent calibration solutions (from 0.01 to 1.3 μg/mL) were prepared from the previous solution by dilution with acetone. All solutions were stored frozen in the dark at −20°C until use. Calibration standards have been prepared in blank extracts of olive oil for comparison purposes.

Extraction and Cleanup

The samples of olive oil were allowed to reach the room temperature and were mixed well before the test portion was taken for analysis. The extraction method employed was based on liquid–liquid partitioning with solvents of different polarity [12,13]. A 10-g (±0.2 g) sample of oil was mixed with 50 mL of *n*-hexane saturated with acetonitrile. The above mixture was transferred to a separation funnel containing 100 mL of acetonitrile saturated with *n*-hexane. 1 mL of water was added and the whole was shaken gently for 1 min and left for 15 min to allow the phases to separate. The lower acetonitrile phase was run into a second separating funnel containing 25 mL of *n*-hexane saturated with acetonitrile and the above procedure for phase separation repeated. A second 50-mL volume of acetonitrile saturated with *n*-hexane was added to the first separating funnel, followed by 0.5 mL of water, and the extraction procedure was repeated. The combined acetonitrile phases were rotary evaporated to 1 mL. Traces of acetonitrile were removed by rinsing twice with 5 mL of acetone, and the evaporation was repeated to dryness. The residue was redissolved with 2 mL of acetone in a grade A volumetric flask and underwent chromatographic analysis.

GC Analysis

All analyses were performed by capillary GC using the following gas chromatographs: First, Carlo Erba Model Mega 2 gas capillary chromatograph with a split/splitless injection port, coupled to a flame-photometric detector FPD (model 50), equipped with a 526-nm interference filter (phosphorus mode), (Fisons Instruments, Rodano, Italy). A volume of 1 μL of sample or standard solution was injected on a splitless injector with a purge-off time of 90 s by using an A200S autosampler (Fisons Instruments, Rodano, Italy). Separations were conducted on a DB-1701 fused-silica column, 30 m × 0.53 mm i.d., 1 μm film thickness, (J&W Scientific, Folsom, CA, USA) with helium as a carrier gas at a flow rate of 6 mL/min. A 3 m × 0.53 mm i.d. uncoated retention gap was coupled to the front of the analytical column via a press fit connector.

The GC-FPD operating conditions were: injector temperature: 220°C; detector temperature, 260°C; initial oven temperature, 100°C for 1 min, ramped at 35°C/min

to 140°C, then up to 220°C at 5°C/min and finally ramped at 10°C/min to 255°C and held for 25 min.

Quantification was carried out both using standard solutions in acetone and in matrix blank extract since the gas chromatographic response for many pesticides is known to be matrix-dependent [14,15].

Second, a Hewlett-Packard 5890 Series II gas chromatograph equipped with a nitrogen-phosphorus detector, NPD, split/splitless injection port and an autosampler model HP-7673 (Palo Alto, CA, USA). The target compounds were separated with a CP-SIL 13CB fused-silica capillary column, 50 m × 0.32 mm i.d., 0.4 μm film thickness (Chrompack, USA).

The oven temperature program was 1 min at 100°C; 30°C/min to 150°C and held for 2 min; 3°C/min to 205°C; 2°C/min to 260°C and held for 1 min. The helium flow rate was maintained at 1.2 mL/min. The detector temperature was 280°C while the injector was operated in the splitless mode (250°C, 60 s, 1 μL).

GC-MS Analysis

Analyses were run on a HP 6890 Series gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) interfaced to a HP 5973 mass selective detector. Analytes were separated with a DB-5MS capillary column, 30 m × 0.25 mm i.d., 0.25 μm film thickness (J&W Scientific, Folsom, CA, USA).

A split/splitless injector was used in the splitless mode, the injection volume was 2 μL and the injection temperature was set at 220°C. The helium carrier gas flow rate was maintained at 1.0 mL/min. The oven temperature program was 4 min at 50°C; 5°C/min to 150°C and held for 1 min; 5°C/min to 280°C, and held for 10 min. The transfer line temperature was set at 275°C and the ion source temperature was fixed at 220°C. Typical MSD operating conditions were optimized by the autotuning software. Electron impact (EI) mass spectra were obtained at 70 eV and monitored from 50 to 450 amu.

Method Validation

During the in-house validation process, the sensitivity and linearity of the response of both FPD and NPD detectors to the analytes were examined by the injection of standard solutions prepared in both blank matrix extract and acetone solvent. The concentration range of the injected solutions studied was from 0.01 to 1.3 μg/mL. Positive olive oil samples that exceeded the linear range were re-analyzed after dilution with blank matrix extract. The accuracy and precision of the method were assessed by the determination of twelve replicate recoveries at two fortification levels, 0.2 mg/kg oil and at LOQ level. The repeatability of the method was estimated by analysis of four spiked samples in the same day while the reproducibility was assessed by analysis of four spiked samples each day for three days.

RESULTS AND DISCUSSION

The possible contamination of virgin olive oil produced in the island of Corfu by fenthion and its degradation products as a result of its aerial and/or ground application,

was studied during the crop years 1999–2002. The chemical structures of fenthion and its metabolites with the m/z ratios of their most abundant ions are given in Fig. 1.

Chromatographic Analysis

Chromatographic conditions were optimized for good resolution of the target pesticides. Gas chromatographic analysis with the FPD using the DB-1701 column exhibits better baseline resolution and fewer peaks than the NPD using the CP-Sil 13CB column. Quantification of the organophosphorus compounds was carried out using the FPD response. Figures 2 and 3 present the GC-FPD and GC-NPD chromatograms of an extract of an olive oil sample containing residues of fenthion and its metabolites, originating from the region of Vistonas/Corfu.

GC-MS analysis was performed in cases of positive olive oil samples for confirmation purposes. In order to increase the sensitivity of the analysis, four diagnostic ions were selected in the SIM (selected ion monitoring) mode for each target compound (Fig. 1). The presence of these diagnostic ions at the correct retention time (± 10 s) and in the correct abundance ratio ($\pm 25\%$) was used as identification criteria according to the Guidelines for the monitoring of pesticides residues in the European Union [16]. Selected-ion ($m/z = 109$, 125, 279, 294) chromatograms corresponding to the peak of

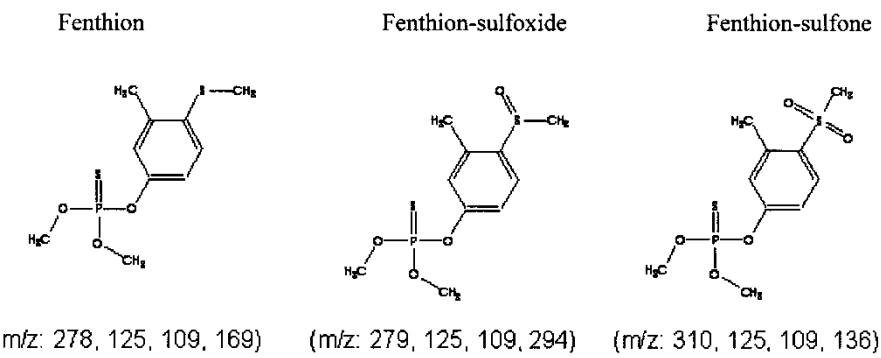

FIGURE 1 Chemical structures of fenthion and its degradation products with the m/z ratios of their respective ions.

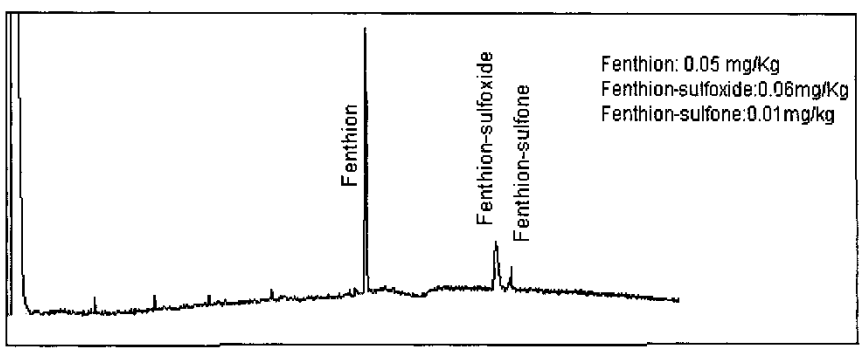

FIGURE 2 GC-FPD chromatogram of olive oil sample collected from the region of Vistonas/Corfu.

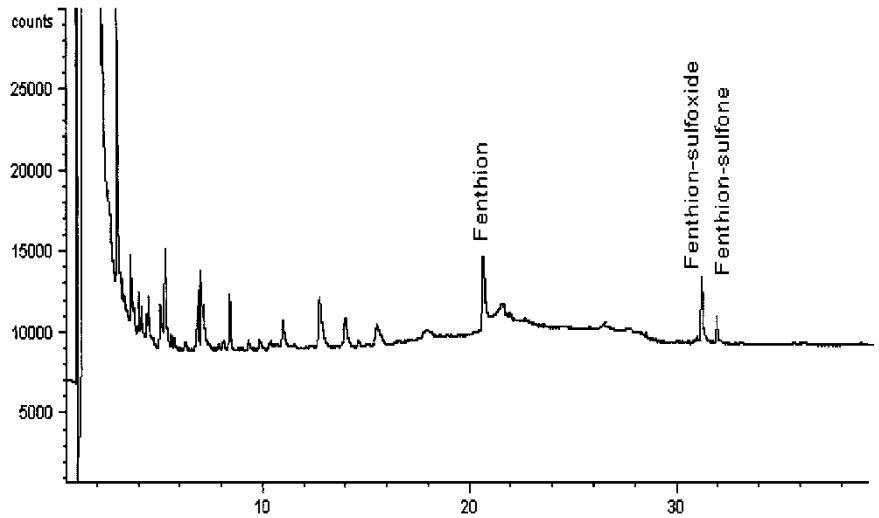

FIGURE 3 GC-NPD chromatogram of olive oil sample collected from the region of Vistonas/Corfu.

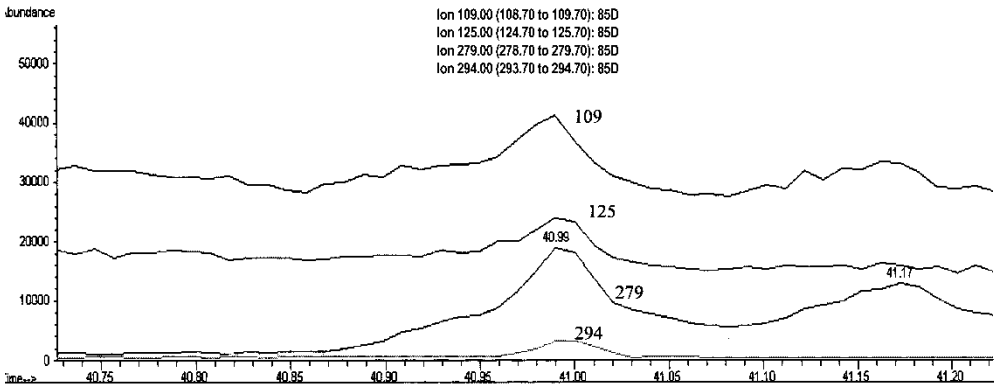

FIGURE 4 GC-MS chromatogram (SIM) of olive oil sample collected from the region of Leykimmi/Corfu. Ion traces of m/z 109, 125, 279, 294 for fenthion sulfoxide (0.2 mg/kg).

fenthion-sulfoxide (Fig. 1) obtained from an olive oil sample collected from the region of Leukimmi/Corfu are presented in Fig. 4.

Validation

The linearity in the response of the FPD was studied with standard solutions prepared in both blank matrix extract and pure solvent at six calibration levels. In both cases, a linear correlation was observed for all the compounds in the studied range. Determination coefficients (the square of the correlation coefficients) found were higher than 0.98 in all cases. The precision (% RSD in the range 6–17%) and the trueness (recovery factors, % R, in the range 70–110%) for the fortification level of 0.2 mg/kg oil of the analytical method for the target compounds have been estimated (Table I). The significance of matrix effect was tested for all compounds with a paired

TABLE I Validation data for the analytical method used for the determination of the organophosphorus pesticide residues in olive oil samples

Pesticide	*Recovery* (% *R*)	*Precision* (% RSD)	*LOQ*[a] (mg/kg)
Diazinon	75	6	0.003
Dimethoate	109	15	0.007
Chlorpyrifos	72	9	0.003
Fenthion	78	7.5	0.002
Parathion-ethyl	81	6.7	0.003
Methidathion	92	12.5	0.007
Fenthion-sulfoxide	89	17	0.004
Fenthion-sulfone	97	14	0.005

[a]Recovery values at the LOQ level were in the range 74–120% as indicated.

t-test [17,18]. The test did not indicate a significant matrix effect for fenthion (tabulated value for $t_{v=5,0.05}$ is 2.57 and t_{calc} 2.25) and the other compounds as previously reported [13] while it did for fenthion-sulfoxide and fenthion-sulfone ($t_{calc} = 3.12$ and $t_{calc} = 2.98$ respectively). An internal quality control procedure was established according to the EN-45001/ISO-17025 requirements since the method has been accredited by UKAS (United Kingdom Accreditation Service). The procedure consisted in incorporating in each batch of samples the analysis of a blank sample and a spiked sample as well as the analysis of calibration solutions. Results were considered when the analysis of the blank sample showed that no contamination had occurred, the recovery factors were between 70 and 120% and the linear regression equations exhibit determination coefficients higher than 0.98.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The instrumental detection limit (LOD) for each pesticide was determined as the analyte concentration that gave a S/N ratio of 3, as calculated by the instrument software and verified by analyzing pesticide mixtures at these concentration levels in blank oil extracts. The instrumental LOD values estimated were in the range 0.003–0.01 μg/mL for the target analytes. The method LOQ values, shown in Table I, ranging from 0.002 to 0.007 mg/kg oil, were validated by analysis of samples spiked at the respective concentrations achieving recoveries in the acceptable range, 74–120%.

Monitoring Results

During the first harvest period 1999–2000, all samples analyzed contained detectable residues of fenthion and metabolites at concentrations significantly lower than MRL. 54% of the samples contained residues at concentrations below 0.10 mg/kg and 38% of the samples were in the range 0.1–0.35 mg/kg. Samples analyzed during the second period 2000–2001 provided a percentage of 28% with no detectable residues, while 67% of the samples contained fenthion and metabolites at low concentration levels ranging from 0.02 to 0.07 mg/kg of olive oil. In samples collected during the period 2001–2002, higher concentrations of the parent compound and metabolites were encountered compared with previous periods, since 25% of the samples were in the range 0.1–0.2 mg/kg and 50% in the range 0.20–1.0 mg/kg. Three of the samples collected (18%) were found to contain total fenthion in the range 1.0–1.5 mg/kg. The concentration ranges of fenthion and its metabolites for each period are presented in

TABLE II Concentration ranges (mg/kg), for fenthion and metabolites, of olive oil samples collected during the three harvest periods, 1999–2000, 2000–2001, 2001–2002, from the island of Corfu

	1999–2000[a] (13)	*2000–2001*[a] (19)	*2001–2002*[a] (16)
Total fenthion[b]	0.007–0.34 (12)	0.02–0.32 (14)	0.11–1.57 (15)
Fenthion[b]	0.007–0.24 (12)	0.01–0.03 (14)	0.01–0.15 (10)
Fenthion-sulfoxide[b]	0.005–0.18 (12)	0.004–0.26 (6)	0.06–1.35 (15)
Fenthion-sulfone[b]	0.009–0.03 (5)	0.005–0.03 (4)	0.009–0.68 (12)

[a]Number in parentheses represent the total number of samples for each period; [b]Number in parentheses represent the number of samples with positive findings of each compound each year.

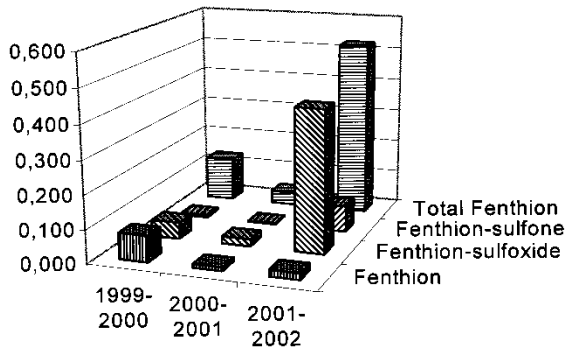

FIGURE 5 Mean values of fenthion and metabolites residues (mg/kg) in olive oil samples from Corfu during the three harvest periods.

Table II while the distribution of the mean values of the residues identified is shown in Fig. 5.

Additionally, the quality characteristics of oil samples have been thoroughly examined according to the EU olive oil Regulation (2568/91). The majority of the samples were of high acidity (> 2% w/w expressed as oleic acid) suggesting that samples could not be used for direct human consumption.

In the case of the island of Erikousa during the period 2001–2002, where the spraying was performed only from the ground, residues of fenthion and metabolites were determined in all the analyzed samples. 20% of them contained total fenthion in the concentration range 1.0–1.5 mg/kg. No residues were detected in olive oil samples originating from Paxoi, where no spraying was applied.

The olive oil samples tested gave negative results for the other organophosphorus pesticides listed in Table I. Dimethoate was detected at low levels (mean value 0.01 mg/kg) in 27% of the samples during the second harvest period 2000–2001.

Data relating to the samples collected during the two harvest periods (1999–2000, 2000–2001) showed that the parent compound fenthion was the most important residue. These findings are quite similar to the results of monitoring studies for olive oils previously reported [9–13]. During 2001–2002, the profile of residues (Fig. 5) was reversed indicating that the oxidative metabolite fenthion-sulfoxide was the major contributor to the total fenthion (Table II). Analysis of variance (one way) [18] used

to estimate the difference between the mean concentrations of each of the target compounds for the three harvest periods indicated that the mean values for fenthion do not differ significantly over the three periods since $F_{calc} = 2.40 < F_{theor,(2,45,95\%)} = 3.4$ while the means for fenthion-sulfoxide and fenthion-sulfone do differ significantly ($F_{calc} = 18$ and $F_{calc} = 19$ respectively). After subjecting the data concerning the concentrations of fenthion-sulfoxide over the three periods to successive comparisons with t-tests, [18], a statistically significant difference was present between the levels of 2000–2001 and 2001–2002 ($t_{calc} = 4.40$, $t_{theor} = 2.12$). A significant difference has also been derived between the levels of fenthion-sulfone during the first and third periods ($t_{calc} = 4.25$, $t_{theor} = 2.14$).

CONCLUSION

The overall findings of this investigation demonstrated the occurrence of fenthion and metabolites in olive oil samples produced in the years 1999–2002 in Corfu, indicating that the monitoring data may be used as a source of information on the pesticide levels taken through the food chain. A critical point is the determination of the total residue including the major metabolites. It is also important for assessing whether residues are increasing or decreasing. This study showed increased levels of residues during the last harvest season which are mainly attributed to the fenthion-sulfoxide content. This noticeable increase of the oxidative metabolite may be partially associated with the influence of various environmental conditions on the degradation mechanisms of the parent compound. No significant difference was observed among the results of olive oil samples collected from areas sprayed by different application methods.

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