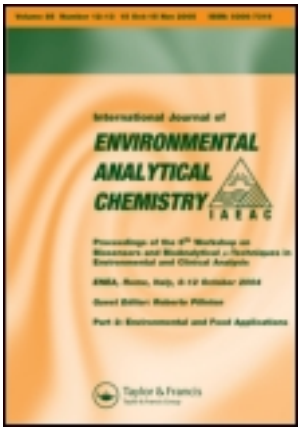

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Determination of dimethoate and omethoate in human serum samples. Risk assessment for the operator

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A simple and effective analytical procedure has been developed for the determination of dimethoate (DIM) residues and its metabolite, omethoate, in serum samples of pesticide operators. For the selection of the most appropriate method for sample treatment, techniques such as headspace solid phase micro extraction and solid phase extraction and liquid–liquid extraction were applied. The applied method was based on toluene (2 mL) extraction of a 0.5 mL serum sample. In this report, it was observed that DIM concentration level affected the ratio of the area response of DIM and one of its oxygenated metabolite, omethoate. In this context, higher concentrations favoured the predominance of DIM while lower concentrations lead to the formation of omethoate. The method was validated using human serum samples spiked with DIM. Good linearity was obtained in the range of 1–10 ng/mL co-calculating DIM and omethoate. Various concentrations of DIM were mixed with serum and stored up to five days at −20°C. Recoveries ranged from 72% to 88% at two spiking levels for six replicates. The detection and quantification limit were calculated at 0.12 and 0.36 ng/mL of serum, respectively. Finally the comparison with the Acceptable Operator Exposure Level (AOEL) of DIM revealed that the maximum exposure of the operators reached the 30% of the AOEL for only two cases.

Keywords: organophosphorous; insecticides; dimethoate; metabolite; human serum; GC/MS; AOEL

1. Introduction

Olive oil is a traditional product of the countries of the Mediterranean basin and a significantly important component of their diet [1]. Its essentiality is reported in various scientific reports which highlight the antioxidant properties of its phenolic components [2,3]. DIM [dimethyl *S*-(*N*-methylcarbamoylmethyl)phosphorothiolothionate] is an organophosphorous insecticide with various uses on agricultural crops and ornamentals. In particular, the significance of DIM is exemplified by its application in olive groves to control the *Bactrocera oleae* [4] and *Dacus oleae*. DIM acts via inhibition of acetylcholinesterase in the nervous tissue. The demands for increased use of insecticides as DIM can potentially affect applicators and humans associated with agricultural work, especially if they do not use the appropriate personal protection equipment measures.

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In this context, the determination of DIM or its metabolites' residues in biological fluids has attracted scientific attention since it is a reliable biomonitoring factor. Such determination has been achieved using solid-phase extraction (SPE) [5,6], solid-phase micro extraction (SPME) [7] and liquid–liquid extraction (LLE) [8] in combination with gas chromatography (GC) with various detectors including mass spectrometry detector (MS).

DIM is metabolised to various metabolites, such as omethoate, an oxygenated analogue of DIM. The toxicity of these metabolites can possibly differentiate from that of the parent compound. Thus, their detection [9] is of equal importance (as for DIM) for risk assessment purposes. Therefore, when we refer to humans it is critical to compare their systemic exposure with the AOEL which corresponds to a specific amount of the active substance dependent on the body weight of each applicator. On the whole, conclusions can be derived from the direct comparison of the operator exposure with AOEL expressed as %AOEL [10].

2. Experimental

2.1 *Operators*

A total of 11 operators with adequate experience in spraying pesticides were selected. They were given the study details, procedures, safety precautions and their obligations throughout the monitoring phase. All the operators signed a consent form to express their willingness to participate in the study. None of the pesticide applicators was involved in spraying activities, for at least two months, before DIM applications and the respective monitoring.

The applications were carried out in olive groves in Agrinio, Etoloakarnania, Greece and the application practice was the knapsack sprayer. This sprayer is carried by the operator (backpack) and it includes a lance and spray nozzle. It is suitable for a wide range of spraying jobs and is ideal for tree (like the olive grove tree), shrub and plant protection.

2.2 *Blood samples handling*

Before starting the study, blood was taken from volunteers for normal blood tests, for the assessment of their physical condition and for the estimation of their previous exposure to pesticides, especially DIM. At the end of the application day, 10 mL of blood samples were taken in Wasserman tubes. The serum was separated by centrifiguration of the samples for 5 min at 3500 rpm in a Hettich Rotofix 32 centrifuge and transferred to a deep freezer, located in storage near the application area. At the end of the sampling period, the samples were transported in dry ice to the Laboratory of Pesticides Toxicology at the Benaki Phytopathological Institute and stored in deep freezers for one week until analysis. An equal number of blood samples was collected from non-exposed volunteers, transported and stored as previously mentioned.

2.3 *Reagents and materials*

DIM (99.4%) was purchased from Fluka. Methanol and toluene were purchased from Merck (Suprasolv, Merck, Darmstadt, Germany).

2.3.1 *SPME holder and fibres*

The SPME holder and coated fibres (85 μm PolyAcrylate (PA), 100 μm Polydimethylsiloxane (PDMS) and 65 μm CarboWax/divinylbenzene (CW/DVB)) were supplied by Supelco (Bellefonte, PA, USA).

2.3.2 *Stock solutions*

Stock methanolic solution of DIM (100 μg/mL) was prepared in the lab and kept stored at −20°C. Stock solutions were diluted with methanol to get appropriate pesticide standard solutions for preparation of spiked samples which will be used for the calibration curve and the recovery study. The preparation of the spiked samples consisted of evaporation under a gentle stream of nitrogen of the various methanolic solutions and then reconstitution with 0.5 mL of serum.

2.3.3 *Experimental procedure*

An aliquot of 0.5 mL of serum sample was mixed with 2 mL of toluene (vortex, a MS1 Minishaker, IKA) for 2 min. Then the mixture was placed in an appropriate falcon tube (15 mL) and centrifuged at 4000 rpm for 5 min, at 4°C. The organic layer was collected and filtered through Acrodisk filter (Whatman) and then an amount of 1μL was eluted directly for analysis to the gas chromatographer.

2.3.4 *Gas chromatographic conditions*

Analysis was carried out on an Agilent 6890N chromatograph equipped with a split–splitless injector and a 5975B inert XL EI/CI MSD (Agilent Technologies) connected to MSDChemStation G1701 DA MSD software, version D.03.00.611. The capillary column was a DB-5MS (30m × 0.25 mm × 1.0 μm) with 5% diphenyl–95% dimethylsiloxane. The injector and detector were operated at 240°C and 280°C, respectively. The sample (1 μL) was injected into the pulsed splitless mode and the oven temperature was programmed as follows: 80°C for 2 min, raised to 120°C (40°C/min), raised to 280°C (15°C/min) for 3 min and to 300°C (10°C/min) for 3 min. Helium was the carrier gas (1.0 mL/min) and nitrogen (30 mL/min) the make-up gas.

3. Results and discussion

3.1 *Extraction techniques*

All endeavours were focussed on the efficient extraction of thermally labile DIM that decomposes at low temperature [11]. Various extraction techniques for the determination of DIM were applied. Headspace (HS) SPME or SPME were practiced with and without heating at 35°C. Three types of fibre coating were assayed: 85 μm PA, 100 μm PDMS and 65 μm CW/DVB. The recoveries of DIM for the aforementioned cases ranged from 39% to 62% and were not reproducible. Thus, HS-SPME or SPME were not sufficient in our case for the quantitative determination of DIM, although it was reported that in other matrices, such as blood and urine, SPME was quite efficacious [7]. The most effective technique was the LLE. Its conjunction with gas chromatography mass spectrometry (GC/MS) was sufficient for the detection of DIM. In particular, and as pointed out by Tarbah *et al*. [8] the use of toluene – as the solvent of choice, for the extraction of DIM

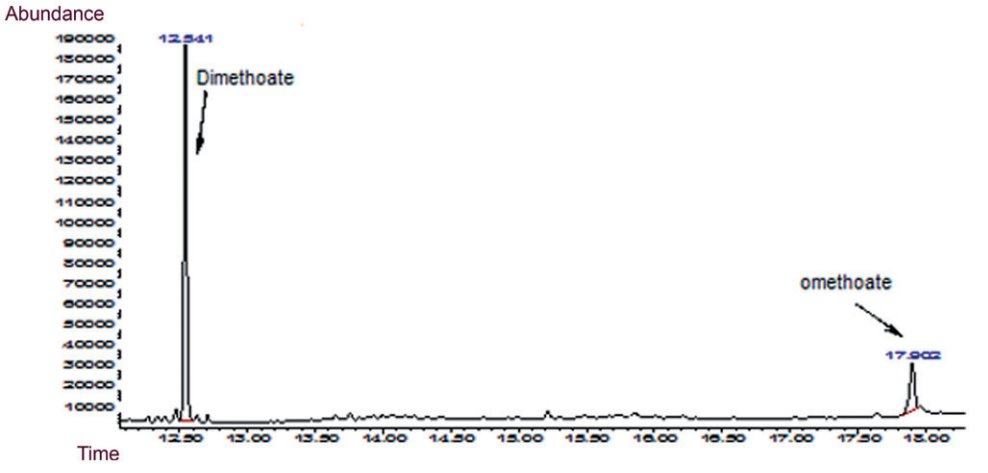

Figure 1. SIM chromatogram of spiked human serum sample with DIM at concentration of 3 ng/mL.

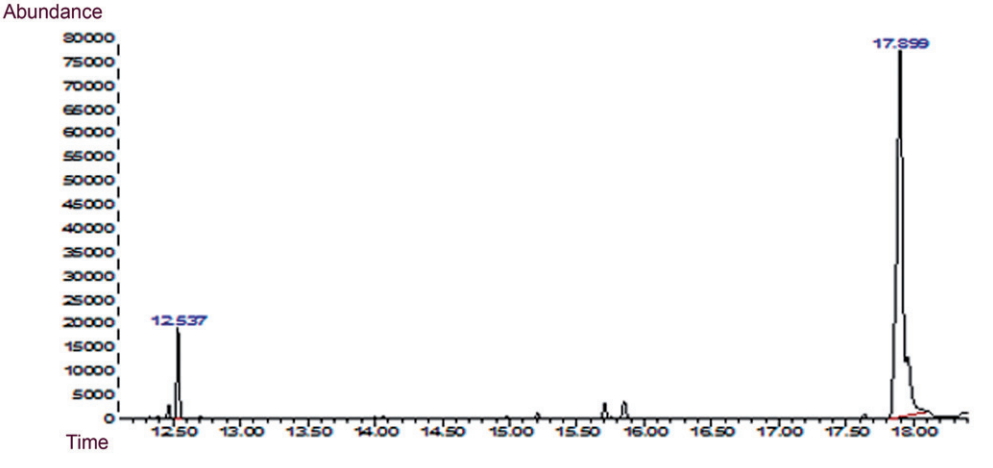

Figure 2. SIM chromatogram of spiked human serum sample with DIM at concentration of 0.9 ng/mL.

from human serum samples – proved to function satisfactorily. In this report, it was also found that the DIM concentration level affected the ratio of the area response of DIM and omethoate. In this regard, higher concentrations favoured the predominance of DIM, while lower concentrations lead to the predominant formation of omethoate (Figures 1 and 2).

3.2 *Linearity*

DIM and omethoate were determined and quantitated using the LLE-GC/MS technique from the sum areas of the corresponding peaks (12.54 min for DIM and 17.90 min for

Table 1. Retention times and detected masses of DIM and omethoate.

Organophosphates	Retention time (min)	Detected masses (m/z)
Dimethoate	12.54	87, 93, 125
Omethoate	17.90	109, 182

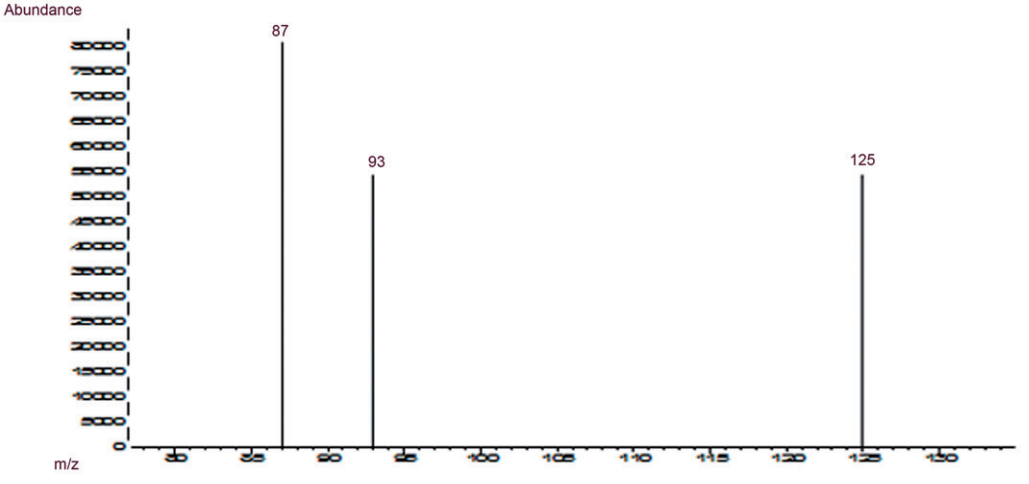

Figure 3. MS characteristic ions of DIM.

omethoate, Table 1, Figures 1 and 2), based on the characteristic ions at m/z 87, 93 and 125 (Figure 3). Identification of omethoate was based on the characteristic peaks at m/z 109 and 182 with decreasing order of magnitude (Figure 4). Therefore, Selected Ion Monitoring (SIM) method was applied producing good response of linearity for concentrations ranging from 1 to 10 ng/mL, with a correlation coefficient of $r^2 > 0.994$.

3.3 *Limit of detection (LOD) – Limit of quantification (LOQ)*

The limits of detection (LOD) and quantification (LOQ) were determined via statistical calculations using a calibration plot ($y = 92255x + 338772$) of DIM and its metabolite which was established at concentration levels ranging from 1 to 10 ng/mL. The LOD was defined as 3.3 $(S_{Y/x})/\alpha$ and the LOQ as $10(S_{Y/x})/\alpha$, where $S_{Y/x}$ represents the residual standard deviation and α is the slope of the calibration plot. Thus, LOD and LOQ were calculated at 0.12 and 0.36 ng/mL of serum respectively.

3.4 *Recovery study*

Various concentrations of DIM were mixed with serum and stored up to five days at −20°C. Recoveries were satisfactory and ranged from 72% to 88% at two spiking levels (3 and 8 ng/mL, respectively) for six replicates. When the recovery study was applied at 1 and 9 ng/mL, the recoveries were not acceptable (54 and 62% respectively). RSDs for low

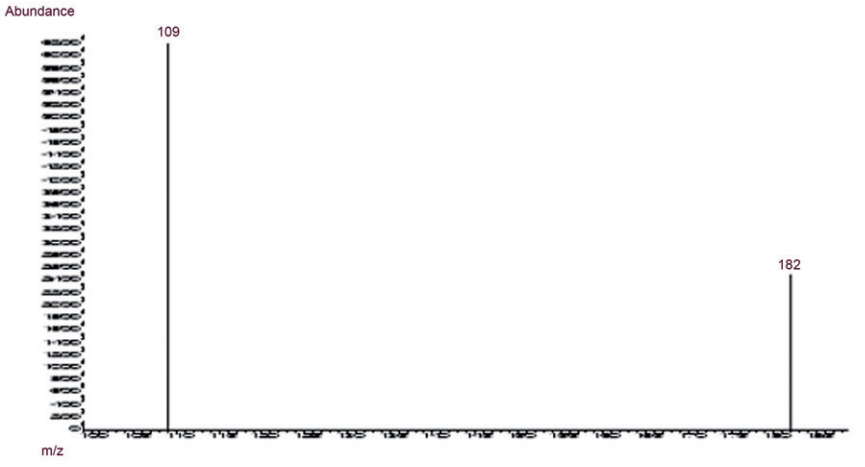

Figure 4. MS characteristic ions of omethoate.

concentrations were between 3.5% and 9.3% and for high concentration were found between 2.2% and 7.9%.

3.5 *Assessment of applicators' exposure*

Various diseases have been reported to occur at high rates among agricultural populations indicating that pesticides might be the causal agents [12–15]. Thus the need for estimation of pesticides levels especially among rural populations is a crucial matter. One route which is applied is the calculation of the Estimated Dietary Intakes (EDIs) in comparison with Acceptable Daily Intakes (ADIs). The latter is applied when consumers of agricultural products, such as olive oil [16], are involved. In addition, concentrations of urinary pesticide metabolites like dimethyl phosphate (DMP), diethyl phosphate (DEP) and other phosphates which correspond to dimethyl or diethyl substituted organophosphorous insecticides can serve as a conventional tool for the assessment of the latter [17]. In our case – pesticide applicators – we propose a straightforward method which targets the direct calculation of pesticide levels in the serum of the operators. The results of dimethoate-omethoate concentration assessment in human serum samples are expressed as μg of DIM-omethoate per mL of human serum and have been summarised in Table 2. Furthermore, the total amount per applicant was estimated as the insecticide accumulation in 5 L of human blood.

It must also be noted that no DIM-omethoate contamination was detected in non-exposed human volunteers' samples and that no residues of dimethoate or omethoate were observed at time $t = 0$ min for the applicators signifying that the concentrations observed are indicative of their exposure. We have to mention that human volunteers excreted the 76–100% of administered dimethoate within 24 hours [18]. Thus, immediate blood sampling is important for the liability of the results since time is important in the excretion of dimethoate from the human body.

Table 2. Estimated exposure levels of applicators (1–11) to dimethoate (-omethoate).

Serum	0.5 mL										
Operator	1	2	3	4	5	6	7	8	9	10	11
Body weight (kg)	72	68	81	83	76	84	87	79	71	80	81
Conc.[1] μg/mL	2.49 E−03	1.42 E−03	1.34 E−03	4.77 E−03	1.41 E−03	4.89 E−03	3.35 E−03	1.73 E−03	1.80 E−03	2.66 E−03	1.43 E−03
Total amount in serum (μg).[2]	12.43	7.09	6.72	23.83	7.03	24.43	16.76	8.66	8.99	13.29	7.17
%AOEL[3]	17.26	10.42	8.29	28.71	9.25	29.08	19.26	10.96	12.66	16.61	8.85

Notes: [1]Concentration as the sum of dimethoate detected and dimethoate equivalent of omethoate.
[2]Extrapolation to the volume of 5 L (5000 mL blood).
[3]AOEL for dimethoate is 1 μg/kg body weight per day.

Operators **4** and **6** were the most exposed compared to other applicators. However, the values of exposure approached only the 30% of the AOEL in the latter cases (operators **4** and **6**). In general, the calculated levels of DIM-omethoate residues indicate that the field application was effective as it can be derived from the systemic exposure of all applicators to DIM which was far away from the AOEL of DIM. Finally, supportive information from the field application observations confirmed the correct application of DIM formulation and the safety precautions that were adopted by the operators.

4. Conclusion

A simple analytical procedure was developed for the determination of DIM and omethoate residues in human serum samples of pesticide operators. The method was based on the liquid extraction of DIM and omethoate and their subsequent GC-MS-SIM determination. The matrix (serum) does not inhibit absorption efficacy, which is evident by the observed recoveries. The operators' systemic exposure with respect to the AOEL of DIM indicated that the operators are on the safe side and no serious contamination was observed. The latter is based on the strong possibility that during the time of blood sampling (immediately after the operation) no excretion of dimethoate occurs or its excretion is negligible.

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