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Low-temperature clean-up method for the determination of organophosphorus insecticides in olive oil

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Abstract

A simple, extremely low-cost method using low-temperature lipid precipitation has been developed for the rapid analysis of virgin olive oil for organophosphorus insecticides and triazine herbicides commonly used in olive groves. The method gives good clean-up for GC analysis with nitrogen–phosphorus detection and recoveries between 77 and 104%, with RSD values of 7–16%. Matrix enhancement was observed for some pesticides and metabolites.

Keywords: Food analysis; Olive oil; Matrix effects; Pesticides; Organophosphorus compounds; Triazines

1. Introduction

Due to the importance of olive oil for the economy of Greece and for the diet of the Greek population (extreme daily consumption 92 g/person), a continuous effort is being made by the Ministry of Agriculture and the National Agricultural Research Foundation to preserve its high quality characteristics. One of the most important quality criteria is low concentration or not detectable pesticide residues. Therefore, olive oil, and especially virgin olive oil – oil obtained from the fruit of the olive tree solely by mechanical or other physical means without any treatment – is controlled regularly. Samples are collected by officials of the Ministry of Agriculture directly from olive mills during oil extraction and the identities of the growers are known. Although the harvest period of the olive fruit throughout Greece is relatively extended (from early December to the end of March), most samples are dispatched to the laboratory during a rather limited time period and the need for a fast and efficient method for the determination of pesticide residues in olive oil is evident. Organophosphorus insecticides, which are the pesticides used in the largest quantities in olive groves, are the main target compounds of the analysis. In addition, triazine herbicides are included within its scope, since they may be determined under the same chromatographic conditions, and their use (on the soil) may sometimes leave detectable residues on olive fruits by contamination.

Analytical problems associated with fatty substrates are well known [1,2]. Although some researchers have used direct injection of olive oil into the gas chromatography (GC) system [3], it seems that this technique can be used only with a specific

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injection system and the number of samples that may be analyzed is limited due to rapidly decreasing column resolution efficiency. With fatty substances, rigorous clean-up is necessary for satisfactory peak separation, sensitivity and overall performance of the chromatographic system. The clean-up methods applied to olive oil have been reviewed recently [4]. Most of them are based on liquid–liquid partitioning with solvents of different polarity, size-exclusion chromatography, sweep co-distillation or adsorption column chromatography. These procedures involve time-consuming operations, a number of pieces of glassware, large amounts of solvents and reagents, and skilled operators. Cabras et al. [5] used a simplified hexane–acetonitrile partitioning method and achieved acceptable recoveries for 13 organophosphorus insecticides. With 2 g of olive oil as the analytical sample, 1.20% of oil was co-extracted and the amount of lipid present in the final solution was of the order of 16 ± 1 mg/ml.

Low-temperature fat precipitation has been used in the past to a limited extend to isolate pesticides from plant or animal matrices. Juhler [6] reviewed the reported applications of this technique and concluded that the temperature used so far for fat precipitation (-78°C) precluded its extended application. Recent work done with meat and fatty matrices have shown that gravimetric fat removal through ice cooling gives sufficient clean-up for organophosphorus pesticide determination when combined with solid-phase extraction [6].

The aim of this work was to develop a simple, cheap, rapid and efficient clean-up method suitable for routine analysis for the determination of pesticide residues in olive oil by precipitation using easily achievable temperatures.

2. Experimental

2.1. Matrices

Virgin olive oil samples from several producers and different tree varieties were used as matrices for the fortified samples. These had already been analyzed by the liquid–liquid partitioning method routinely used in our laboratory and found not to contain detectable residues of the pesticides under consideration. In addition, samples that had been found to contain residues of pesticides were reanalyzed with the new method in order to assess its efficiency using samples with incurred residues.

2.2. Pesticides

The target pesticides were selected according to their importance in oleiculture. Fenthion and dimethoate are the most extensively used, followed by methidathion, diazinon, parathion-ethyl, parathion-methyl, and phosalone. Azinphos-ethyl and chlorpyriphos were also included in the list of target insecticides because their use on olives, which used to be important, has recently been withdrawn in compliance with European Union (EU) directives. Atrazine, simazine and prometryne were selected for reasons already explained. The oxidative metabolites of fenthion, which are known to contribute significantly to the total residue [7,8], were also included within the scope of the method.

2.3. Chemicals and materials

All solvents used were pesticide residue analysis grade. Analytical standards were kindly supplied by manufacturers or were purchased. Details are provided in Table 1, which also gives their log $K_{o/w}$ values.

Table 1
Target pesticides

Compound	Log $K_{o/w}$	Purity (%)	Supplier
Atrazine	2.5	98	Chem Service
Azinphos-ethyl	3.2	99.2	Bayer
Chlorpyriphos	4.7	98.7	Dr. Ehrenstorfer
Diazinon	3.3	99.3	Chem Service
Dimethoate	0.7	99.8	BASF
Fenoxon	2.3	98.5	Bayer
Fenoxon sulfoxide	0.2	98	Bayer
Fenoxon sulfone	0.3	99.5	Bayer
Fenthion	4.8	99.7	Bayer
Fenthion sulfoxide	1.9	98.5	Bayer
Fenthion sulfone	2.0	98	Bayer
Methidathion	2.2	99	Chem Service
Parathion-ethyl	3.8	99.3	Chem Service
Parathion-methyl	3.0	99	Chem Service
Phosalone	4.0	99.5	Rhone Poulenc
Prometryn	3.1	99.8	Novartis
Simazine	2.1	99	Chem Service

2.4. Extraction and clean-up

In nearly all the tests, the analytical sample size was 5 g. This sample size allowed good sensitivity to be achieved while keeping the volume of solvent used and the amount of lipid to be eliminated to a minimum. Various extraction solvents, containers and techniques were assessed with spiked samples in order to optimize the method for maximum pesticide recoveries and minimum oil co-extraction. The extraction solvents tested were acetonitrile alone (20, 25 or 50 ml) and mixtures of acetonitrile (15 ml) with acetone or hexane (10 ml). These volumes were chosen on the basis of previous experience with the liquid–liquid partitioning method. Different containers were used, with appropriate methods of mixing for each. The containers were a 100-ml beaker, a 25-ml test tube (with only 2 g of oil and 20 ml of acetonitrile) and a 100-ml separatory funnel. For the beakers, homogenization and extraction were carried out in an ultrasonic bath for 15 or 40 min; the test tubes were shaken either vigorously by hand for 1 min or by a mechanical test tube shaker (Heidolph REAX 2000, Kelheim, Germany) for 10 min; the funnels were again either shaken vigorously by hand for 1 min or on an horizontal shaker (HS-501 digital IKA Labortechnik, Staufen, Germany) for 20 or 40 min. After agitation, the samples were stored, horizontally for the separatory funnels, on the shelves of a freezer at −20°C and allowed to stand overnight for lipid precipitation and separation. Since freezing did not collect the oil into a single frozen mass under all the conditions tested, the effect of filtration through a filter paper or glass wool was also assessed. However, after initial trials it was found that small pieces of frozen oil were best removed, where necessary, by transfer of the extract to a small beaker, where they tended to adhere to the glass walls. Because of the temperature dependence of volume and the requirement to maintain the low temperature of the samples while separating the solvent from the lipid, a process which must be carried out as rapidly as possible on removing the sample from the freezer, an aliquot corresponding to 10 ml at 20°C was taken by weighing out 7.86 g of cold solvent into a pre-weighed round-bottomed 50-ml flask. The solvent was evaporated almost to dryness using a rotary evaporator. Traces of acetonitrile were removed by rinsing twice with a small volume of acetone and the sample was evaporated to dryness. At this stage, the flask was re-weighed in order to find the amount of oil co-extracted. The residue was collected in 2 ml of acetone for GC injection.

2.5. Gas chromatographic analysis

Three different gas chromatographs were used for the analyses, depending on availability. These were a Hewlett-Packard (HP) 6890 Series gas chromatograph, a Varian 3600 and a Fisons HRGC MEGA 2 series. All were equipped with a nitrogen–phosphorus detection (NPD) system (280°C) and were operated in the splitless mode (1 min for HP and Fisons, 1.5 min for Varian; 1 μl injection). Details of capillary columns and operating conditions are as follows. HP6890: Rtx-50 column (50% phenyl, 30 m×0.25 mm I.D., 0.25 μm film thickness); injector temperature 250°C; temperature programme 80°C, 1 min; 170°C, 15°C/min, 1 min; 200°C, 3°C/min; 260°C, 15°C/min, 20 min. Varian 3600: Rtx-1701 (14% cyanopropylphenyl, 30 m×0.25 mm I.D., 0.25 μm film thickness), injector temperature programme 140°C; 240°C, 100°C/min, 20 min; temperature programme 75°C, 2 min; 170°C, 12°C/min, 2 min; 200°C, 1.5°C/min; 260°C, 15°C/min, 10 min. Fisons: Rtx-1701 (14% cyanopropylphenyl, 30 m× 0.53 mm I.D., 1 μm film thickness); injector temperature 250°C; temperature programme 80°C, 1 min; 170°C, 15°C/min, 1 min; 200°C, 3°C/min; 260°C, 15°C/min, 20 min temperature programme 80°C, 1 min; 170°C, 15°C/min, 1 min; 200°C, 3°C/min; 240°C, 15°C/min, 30 min.

Quantification was carried out both using standards in acetone and standards in matrix extract, since the gas chromatographic response for many pesticides is known to be matrix dependent [9–11].

3. Results and discussion

3.1. Extraction technique

Acetonitrile alone was shown to be the most appropriate extraction solvent. When a mixture of acetonitrile and hexane was used, the oil did not

freeze at −20°C and separation of the phases was difficult. The amount of oil co-extracted was higher than for acetonitrile alone and there was a very marked enhancement effect for the chromatographic analysis, leading to recoveries above 100% for nearly all compounds, even when matrix-matched standards were used. For acetonitrile mixed with acetone, recoveries were satisfactory, but the amount of oil co-extracted was approximately double that for acetonitrile alone. The minimum volume of acetonitrile that gave satisfactory recoveries was 25 ml. The larger volume increased the amount of co-extracted oil by 20% without increasing these recoveries. The combination of funnel and horizontal shaker showed several advantages over the other techniques tested such as: excellent homogenization, automation, standardization and easy separation of the desired aliquot of extract from the single mass of frozen oil, by first emptying it into a small beaker or by taking the aliquot through the neck of the flask. The most effective extraction technique of those tested was, therefore, as follows: a 5-g analytical sample was weighed out into a 100-ml separatory funnel, 25 ml of acetonitrile was added, the flask was closed tightly with a glass stopper, and the mixture was shaken on an horizontal shaker at 250 rpm for 20 min. The funnels were stored horizontally in the freezer overnight (−20°C) for oil precipitation. The following day each funnel was removed in turn by grasping around the neck (in order to warm only this part of the flask for the stopper to be removed), and a 10-ml aliquot (7.86 g) was weighed out into a 100-ml round-bottomed flask. The solvent was removed by rotary evaporation and the residue was collected in 2 ml of acetone after removing traces of acetonitrile by rinsing twice with a small volume of acetone.

3.2. Clean-up efficiency

In order to assess the clean-up efficiency of the new method, the amount of oil co-extracted was measured for each sample. The mean value for 204 samples analyzed was compared to the mean mass of oil co-extracted with the old method (349 samples from the previous year's analyses), in which lipid was eliminated by successive partitioning steps between hexane and acetonitrile. The amount of oil residue, adjusted in the case of the new method to refer to the whole 5 g sample (the aliquot taken was 2/5 of this), was almost exactly the same for both methods with values of 0.055±0.035 and 0.054±0.038 g, respectively. This was 1.1% of the original sample mass and corresponded to a concentration of co-extractives in the final extract of 11 mg/ml. Our experience with virgin olive oil analyses had shown that the residue of oil present in the final extract was related to the chemical characteristics of the oil, which might depend to a certain extent on the tree variety. Approximately 70% of the oil samples

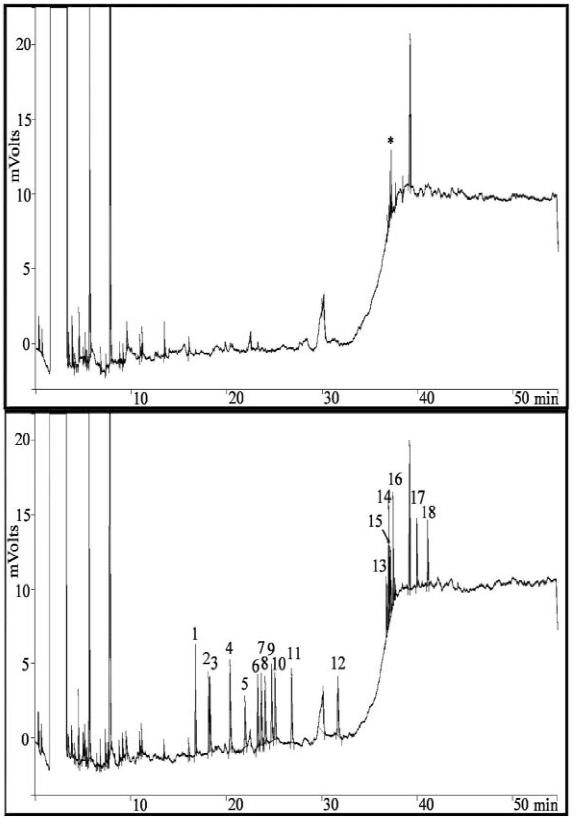

Fig. 1. Chromatograms on the Varian 3600 GC system of a blank oil extract and a mixture of pesticides at a concentration of 0.01 μg/ml, matrix-matched with the same oil extract. 1=Diazinon, 2=atrazine, 3=simazine, 4=dimethoate, 5=prometryn, 6=chlorpyriphos, 7=parathion-methyl, 8=fenoxon, 9=fenthion, 10=malathion, 11=parathion, 12=methidathion, 13=fenoxon sulfoxide, 14=fenthion sulfoxide, 15=fenoxon sulfone, 16=fenthion sulfone, 17=phosalone, 18=azinphos-ethyl, *=interference peak for fenoxon sulfone.

analyses with both methods gave an oil residue less than or equal to the mean value.

Repeat extractions of the same oil sample, which were carried out during recovery studies, showed that the oil residue had a high reproducibility for any given sample with a relative standard deviation (RSD) of the order of 10% for 20–50 repeat analyses of the same sample.

The sample clean-up achieved with this simple method was sufficient for the chromatographic system to maintain its separation efficiency for at least 100 sample injections. Fig. 1 shows chromatograms of a typical blank oil sample and of a mixture of the pesticides at 0.01 μg/ml, matrix-matched with the same oil extract. Gas chromatograms were generally free of interfering oil peaks, although a small interference peak typically occurred close to the retention time of fenoxon sulfone on both the columns used, restricting the accuracy of its quantification at low residue levels. However, previous analyses on olive oil have shown that the oxon metabolites of fenthion are rarely detected and, when they are, make only a small contribution to the total fenthion residue [8]. This restriction is not, therefore, considered to be of practical importance.

3.3. Recoveries of pesticides

Recoveries were measured for five replicate samples at each of four spiking levels: 1.0, 0.2, 0.05 and 0.01 mg/kg. The results obtained using matrix-matched standards for the GC analysis are given in Table 2. Overall, recoveries were between 77 and 104% and RSD values between 7 and 16%. The greatest variability was found for the oxon metabolites of fenthion which are known to be difficult to analyze using GC.

3.4. Samples with incurred residues

The efficiency of the new method was compared to that of the liquid–liquid partitioning method through the repeated analysis of 14 samples with incurred residues containing a variety of pesticides. The results, given in Table 3, showed that there was

Table 2
Percentage recoveries[a] using matrix-matched standards at different fortification levels

Pesticide	Recovery (%)±RSD (%)				
	1.0 mg/kg	0.2 mg/kg	0.05 mg/kg	0.01 mg/kg	Overall
Atrazine	80±4	83±9	85±6	–[c]	83±7
Azinphos-ethyl	87±6	91±9	88±6	104±4	93±9
Chlorpyriphos	78±6	85±5	91±9	83±3	85±9
Diazinon	82±6	86±9	91±4	86±5	86±7
Dimethoate	92±4	95±11	102±6	97±8	96±8
Fenoxon	87±12	71±14	91±18	85±11	84±16
Fenoxon sulfone	94±9	100±10	–[b]	118±4	104±15
Fenoxon sulfoxide	93±6	97±8	110±7	102±10	101±10
Fenthion	81±9	70±5	77±13	84±5	78±10
Fenthion sulfone	94±9	105±7	93±5	96±6	97±8
Fenthion sulfoxide	94±9	98±5	97±6	106±7	99±8
Malathion	85±4	95±7	102±10	96±8	94±10
Methidathion	87±4	86±8	95±8	97±6	91±8
Parathion-ethyl	88±10	81±7	79±6	93±7	86±9
Parathion-methyl	88±6	89±12	103±9	94±4	93±10
Phosalone	86±7	91±12	93±5	93±10	91±9
Prometryn	71±8	76±5	82±10	–[c]	77±11
Simazine	76±15	–[b]	89±9	–[c]	83±14

[a] Five replicate samples.
[b] Not analyzed.
[c] Fortification level below LOQ.

Table 3
Comparison of recoveries with the old and new methods for samples with incurred residues

Sample	Method[a]	Residue (mg/kg)								
		Fenthion	Fenthion sulfoxide	Fenthion sulfone	Fenoxon	Fenoxon sulfoxide	Dimethoate	Methidathion	Chlorpyriphos	Atrazine
1	A	0.006	0.068	0.008			0.037	0.038		
	B	0.005	0.053	0.005			0.033	0.036		
2	A	0.014	0.34	0.021			0.006			
	B	0.013	0.29	0.017			0.004			
3	A	0.012	0.28	0.018						
	B	0.012	0.32	0.019						
4	A	0.041	0.55	0.032						
	B	0.035	0.49	0.026						
5	A	0.20	0.72	0.047	0.011	0.014	0.011		0.004	
	B	0.21	0.72	0.045	0.010	0.013	0.011		0.005	
6	A	–	0.038	0.007						
	B	–	0.038	0.006						
7	A	0.027	0.070	0.017			0.015	0.007		
	B	0.024	0.058	0.014			0.014	0.007		
8	A	0.014	0.069	0.008			0.009			
	B	0.015	0.075	0.008			0.008			
9	A	0.004	0.025	0.016			0.007	0.040		
	B	0.005	0.026	0.013			0.009	0.047		
10	A	0.013	0.037	0.009			0.008			
	B	0.018	0.047	0.005			0.010			
11	A	0.019	0.20	0.012						0.062
	B	0.024	0.28	0.014						0.062
12	A								1.72	
	B								1.84	
13	A								0.14	
	B								0.15	
14	A							0.043		
	B							0.048		

[a] Method A: liquid–liquid partitioning; method B: low-temperature precipitation.

good agreement between the two methods for all the compounds present in the samples.

3.5. Matrix effects

In order to assess the influence of the oil matrix on recoveries, the final extracts of a few fortified samples and samples with incurred residues were quantified both against standards in acetone and against standards in matrix extract. The results are shown in Tables 4 and 5. An enhancement effect was observed for some pesticides, which led to significantly higher recoveries than when non-matrix-matched standards were used. This was particularly pronounced for fenoxon sulfoxide and fenoxon sulfone, which appeared to be easily lost in the chromatographic system when in solvent alone. In addition, the peaks for the non-matrix-matched standards were broader, leading to a slight increase in the retention times of all compounds. These results are

Table 4
Residue quantifications in samples with incurred residues using matrix-matched and non-matrix-matched standards

Sample	GC standard	Residue (mg/kg)								
		Fenthion	Fenthion sulfoxide	Fenthion sulfone	Fenoxon	Fenoxon sulfoxide	Dimethoate	Methidathion	Chlorpyriphos	Atrazine
1	In matrix	0.005	0.053	0.005			0.033	0.036		
	In solvent	0.003	0.088	0.009			0.059			
2	In matrix	0.013	0.29	0.017			0.004			
	In solvent	0.011	0.44	0.023			0.004			
3	In matrix	0.012	0.32	0.019						
	In solvent	0.009	0.49	0.027						
4	In matrix	0.035	0.49	0.026						
	In solvent	0.034	0.75	0.037						
5	In matrix	0.21	0.72	0.045	0.010	0.013	0.011		0.005	
	In solvent	0.22	0.99	0.063	0.018	0.12	0.016		0.003	
6	In matrix		0.038	0.006						
	In solvent		0.072	0.010						
7	In matrix	0.024	0.058	0.014			0.014	0.007		
	In solvent	0.022	0.095	0.020			0.022	0.008		
8	In matrix	0.015	0.075	0.008			0.008			
	In solvent	0.014	0.12	0.012			0.012			
9	In matrix	0.005	0.026	0.013			0.009	0.047		
	In solvent	0.003	0.047	0.019			0.013	0.061		
10	In matrix	0.018	0.047	0.005			0.010			
	In solvent	0.015	0.09	0.009			0.013			
11	In matrix	0.024	0.28	0.014						0.062
	In solvent	0.023	0.42	0.021						0.059
12	In matrix								1.84	
	In solvent								1.88	

similar to those obtained by Molinari et al. [9] for analyses of fenthion and its oxidative metabolites in olives and olive oil and are in agreement with those of Erney et al. [10], who have noted a marked matrix effect, particularly for compounds with P=O bonds such as the fenoxon metabolites of fenthion, and have attributed it to the enhanced transmission of the analytes from the injector to the column when protected by the matrix from adsorption or decomposition.

3.6. Limits of quantification

The limits of detection (LODs) and quantification (LOQs) measured depended on the gas chromatograph used. With the Varian 3600 GC system, for which the greatest sensitivity was consistently achieved, LOQ values for the organophosphorus compounds were mostly ≈0.005 mg/kg, calculated on the basis of the standard deviation for 10 repeated injections of a sample fortified at the expected LOQ. The value for fenoxon sulfone was higher (≤0.02 mg/kg), because of the small interference peak previously referred to, and the LOQs for the triazine herbicides were ≤0.04 mg/kg. On the HP6890, the same sensitivity was sometimes, but not always, achievable, depending on the characteristics and age of the NPD bead. For the Fisons, in which a wide bore column was installed, the LOQ values were a factor of 2–5 larger.

Table 5
Quantification of residues in samples fortified at 1 mg/kg using matrix-matched and non-matrix-matched standards

Pesticide	Recovery (%)					
	Sample 1		Sample 2		Sample 3	
	In matrix	In solvent	In matrix	In solvent	In matrix	In solvent
Diazinon	88	89	87	86	89	87
Atrazine	93	115	87	96	90	98
Simazine	96	100	88	100	92	105
Dimethoate	89	128	90	131	92	135
Prometryn	79	91	84	79	86	81
Chlorpyriphos	81	86	82	83	83	85
Parathion-methyl	90	106	89	97	92	100
Fenoxon	101	104	91	104	94	107
Fenthion	94	91	86	84	89	87
Malathion	92	100	93	79	95	81
Parathion-ethyl	94	90	86	85	90	86
Methidathion	91	115	91	110	93	113
Fenoxon sulfoxide	87	195	96	378	93	365
Fenthion sulfoxide	84	135	94	130	94	130
Fenoxon sulfone	91	155	91	233	87	227
Fenthion sulfone	92	123	94	114	94	115
Phosalone	89	101	89	103	87	101
Azinphos-ethyl	93	100	90	110	88	107

4. Conclusions

The method described gave satisfactory recoveries for all the pesticides most commonly used on olive trees in Greece. A low, reproducible mass of oil residue remained in the final extract and this allowed the chromatographic system to maintain its separation efficiency for a large number of injections. The pesticides tested have a wide range of polarity (log $K_{o/w}$ 0.2–4.8), indicating that it should be possible to include other compounds in the method. The low solvent volume and analyst time required per sample make it very economical, while its simplicity means that it requires only standard laboratory equipment and is sufficiently robust to be applied successfully by inexperienced technicians.

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