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Analysis of Pesticides in Nuts by Online Reversed-Phase Liquid Chromatography−Gas Chromatography Using the Through-Oven Transfer Adsorption/Desorption Interface

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A new method to determine pesticide residues in nuts is presented, in which the pesticides are extracted from samples with a small amount of ethyl acetate and anhydrous sodium sulfate. No additional cleanup or concentration steps are necessary. The extract is directly injected into the high-pressure liquid chromatograph, where preseparation of the pesticide residues from other components coextracted from the nuts is carried out using methanol/water as the eluent. The selected liquid chromatography fraction containing the pesticides is automatically transferred to the gas chromatograph using the through-oven transfer adsorption/desorption interface. The calculated limits of detection for each pesticide varied from 0.1 to 61.3 μg/kg. The repeatabilities of the analysis and the overall procedure (extraction and analysis) were satisfactory. No variations in the retention time were observed. The method was applied to the analysis of pistachio nut, peanut, walnut, hazelnut, and sunflower seed.

KEYWORDS: Reversed-phase liquid chromatography−gas chromatography (RPLC−GC); TOTAD interface; pesticide residue analysis; nuts

INTRODUCTION

Pistachio nut has recently been ranked among the 50 food products highest in antioxidant potential (*1*). Pistachio nuts are a rich source of bioactive antioxidant compounds, especially phytoestrogens, and their dietary consumption is associated with a decreased incidence of cardiovascular diseases and some tumors (*2*). Positive effects of pistachio nuts on the oxidative status of healthy individuals have recently been reported (*3*).

Pesticides are widely used in the cultivation of pistachio nuts, and strict control is necessary to protect the consumer from the harmful impact of pesticide residues. Most pesticides are hydrophobic and absorbed in the lipidic matrix of the nuts (fat content 50−70%) passing to the consumer.

Pesticide residue control is usually performed by analytical methods that involve solvent extraction, cleanup, and concentration steps followed by gas chromatography (GC) using various detectors. Isolation of the analytes from pistachio and other nuts is a complicated and laborious task due to the fatty nature of the matrix. The crucial step in the analytical procedure is the separation of the pesticide residues from the bulk of the lipidic material, since even a small amount of lipids can harm the gas chromatographic injectors, capillary columns, and detectors. Moreover, interference from the lipid matrix may cause problems in the analytical signal of the target pesticides. Therefore, after extraction, a subsequent cleanup step is

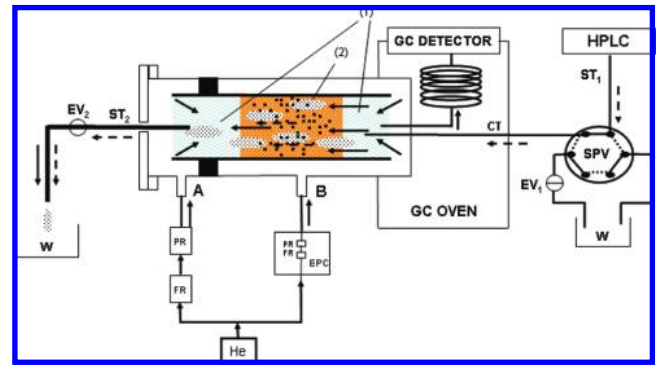

Figure 1. Automated TOTAD interface during the transfer step. Key: (1) glass wool; (2) sorbent (Tenax TA); (SPV) six-port valve; (EV_1 and EV_2) electrovalves 1 and 2; (EPC) electronic pressure control; (PR) pressure regulator; (FR) flow regulator; (solid arrows) gas flow; (dotted arrows) liquid flow; (ST_1) stainless steel tubing, 0.25 mm i.d., to transfer eluent from the LC instrument to the six-port valve; (ST_2) stainless steel tubing, 1 mm i.d., to allow the exit of liquids and gases; (CT) silica capillary tubing, 0.32 mm i.d.; (W) waste; (blue shading) solvent; (dots) analytes.

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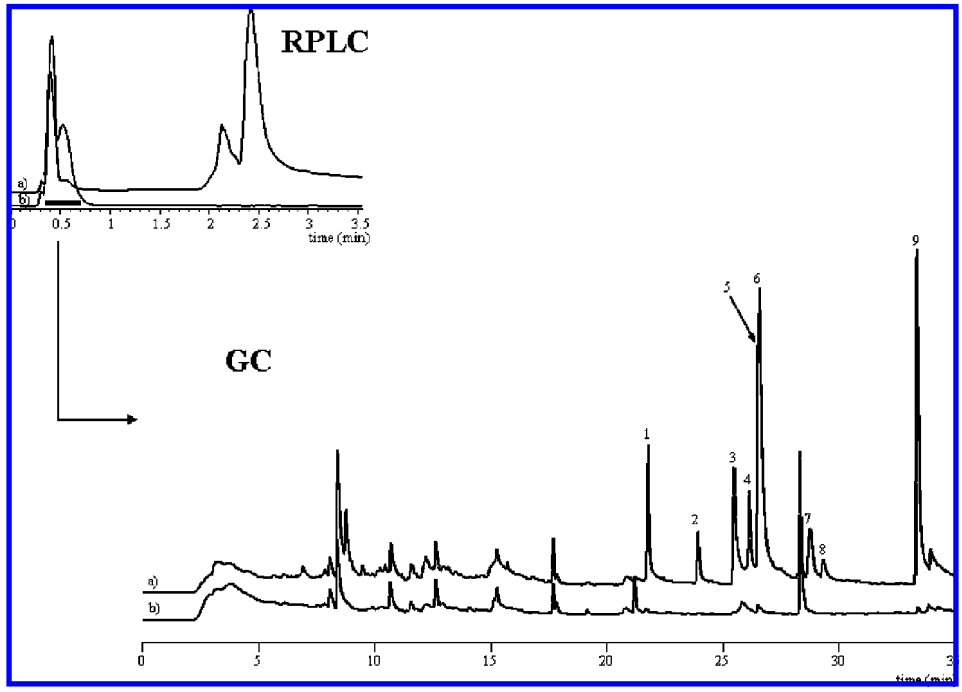

Figure 2. GC chromatograms obtained from the RPLC−GC−NPD analysis of an extract obtained from (**a**) a pistachio nut sample fortified at a 0.05 mg/kg concentration of each pesticide and (**b**) an unfortified pistachio nut sample. In the left corner are shown LC chromatograms from (**a**) a pistachio nut extract and (**b**) a solution of each pesticide in methanol at 50 mg/L. The flow rate was 2 mL/min. The thick line situated between the time axis and the chromatogram indicates the LC fraction transferred from the LC instrument to the GC instrument. The conditions are as indicated in the Experimental Procedures. Identification peaks: (1) diazinon; (2) methylchlorpyrifos; (3) fenitrothion; (4) malathion; (5) chlorpyrifos; (6) parathion; (7) phenthoate and chlorfenvinphos; (8) methidathion; (9) ethion.

needed. These steps make analysis very expensive and time-consuming, and a large amount of organic solvent is required. Furthermore, analytes may be contaminated during these procedures and lost as the samples are manipulated. Immunoaffinity (IA) chromatography has been proposed as a cleanup procedure for the determination of thifluzamide from peanut samples (*4*).

Alternative methods, such as supercritical fluid extraction (SFE), pressurized liquid extraction, or microwave-assisted extraction (MAE), are little used. Solid-phase extraction (SPE) cannot be used with nut samples because any sample should be in a homogeneous liquid state before addition to an SPE column or disk device. Matrix solid-phase dispersion (MSPD) was introduced in 1989 by Barker et al. (*5*) for the simultaneous disruption and extraction of semisolid and solid samples. The obtained extracts are ready for analysis (*6*) or subjected to a cleanup step. Garcinuño et al. have used MSPD to extract carbamate residues from almond samples with a subsequent cleanup step by SPE using alumina and C-18 as the sorbent, but the results were unsatisfactory because large amounts of fat were present. To overcome this problem, the sample was first defatted by alkaline hydrolysis with NaOH (*7*). Husain and co-workers analyzed diazinon and ethion in pistachio nuts by using MSPD and thin-layer chromatography (TLC) to separate pesticides from the pistachio extract followed by GC with nitrogen−phosphorus detection (NPD) (*8*).

A simple, rapid, and sensitive multiresidue method was developed by our research group (*9*) for the determination of organophosphorus pesticides in vegetable samples. Pesticide residues are extracted from samples with a small amount of ethyl acetate and anhydrous sodium sulfate, while no additional concentration or cleanup steps are necessary. Analyses are performed by large-volume GC injection using the through-oven transfer adsorption/desorption (TOTAD) interface. However, this method could not be applied to the analysis of pesticides in nut samples due to the fatty nature of the matrix. Therefore, after extraction, liquid chromatography (LC) could be used as the cleanup step. In online liquid chromatography−gas chromatography (LC−GC), the LC step separates pesticides from the fatty material, while the LC fraction containing the pesticides is automatically transferred to the gas chromatograph. The TOTAD interface is a modified PTV injector that allows large-volume injection of polar solvents into the capillary GC instrument and the online-coupled reversed-phase liquid chromatography−gas chromatography (RPLC−GC) system. A multiresidue method to analyze pesticide residues in olive oil by RPLC−GC using the TOTAD interface was previously developed. In this fully automated system, olive oil can be injected directly with no sample pretreatment step other than filtration (*10–12*).

Modeled on this, the aim of this work is to develop a new method to analyze pesticide residues in nut samples using a low-solvent-consuming extraction step and online RPLC−GC using the TOTAD interface for the rapid, easy, and sensitive analysis of nuts, with very little manipulation of the sample.

EXPERIMENTAL PROCEDURES

Materials. Nuts (pistachio, peanut, walnut, hazelnut) and sunflower seeds were purchased from a local market. Pesticide standards were obtained from Chem Service Inc. (West Chester, PA). Organophosphorus pesticides (OPs) used were diazinon, methylchlorpyrifos, malathion, fenitrothion, chlorpyrifos, parathion, phenthoate, chlorfenvinphos, methidathion, and ethion. The following chlorinated pesticides (CPs) were used: captan, oxyfluorfen, α-endosulfan, β-endosulfan, and lindane. Solutions of each pesticide were individually prepared in methanol at 50 mg/L to fix the LC fraction to transfer to the GC instrument. Two different stock solutions (1000 mg/L) of each pesticide, one of OPs and another of CPs, were prepared in methanol and stored

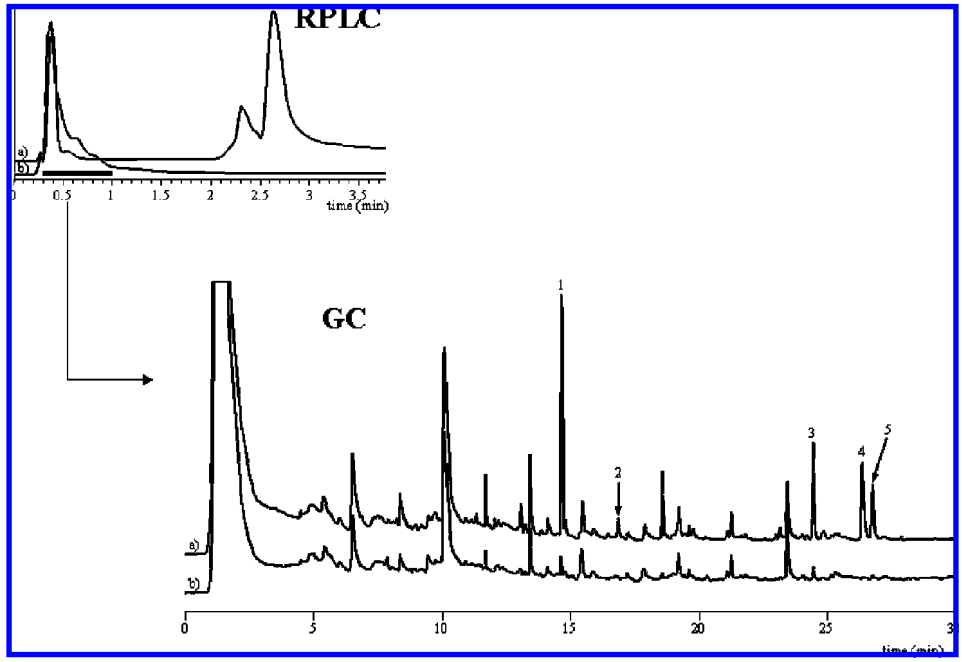

Figure 3. GC chromatograms obtained from the RPLC−GC−ECD analysis of an extract obtained from (**a**) a pistachio nut sample fortified at a 0.2 mg/kg concentration of each pesticide and (**b**) an unfortified pistachio nut sample. In the left corner are shown LC chromatograms from (**a**) a pistachio nut extract and (**b**) a solution of each pesticide in methanol at 50 mg/L. The thick line situated between the time axis and the chromatogram indicates the LC fraction transferred from the LC system to the GC system. The conditions are as indicated in the Experimental Procedures. Identification peaks: (1) captan; (2) lindane; (3) α-endosulfan; (4) oxyfluorfen; (5) β-endosulfan.

Table 1. Correlation Coefficients for the Linear Calibration (R^2) (Range 0.01−0.15 mg/kg), Detection Limits (LODs) Calculated as the Amount of Product Giving a Signal Equal to 5 Times the Background Noise, Maximum Residue Limits (MRLs) Established by European Legislation, and Coefficients of Variation (CVs) from the Retention Time and from the Absolute Peak Area, $n = 5$, for the Same Pistachio Nut Extract (CV) and for the Whole Analytical Procedure (CV*)[a]

pesticide	R^2	LOD (μg/kg)	MRL (mg/kg)	CV (t_R)	CV (area)	CV* (area)
diazinon	0.988	0.4	0.05	0.03	12	13.7
methylchlorpyrifos	0.978	0.7	0.05	0.02	14.8	14.6
fenitrothion	0.981	0.3	0.01	0.02	3.2	10.1
malathion	0.983	0.6	0.05	0.03	7	13.1
chlorpyrifos	0.999	0.2	0.1	0.03	15.2	19.3
parathion	0.977	0.2	0.05	0.06	6.6	18.9
phenthoate + chlorfenvinphos	0.991	0.9	0.05/0.02	0.12	10.1	9.9
methidathion	0.981	1.5	0.05	0.05	13.4	10.6
ethion	0.957	0.1	0.01	0.06	9.8	5.8

[a] The pistachio nuts were fortified at 0.05 mg/kg for each pesticide. NPD was used.

Table 2. Correlation Coefficients for the Linear Calibration (R^2) (Range 0.01−0.25 mg/kg), Detection Limits (LODs) Calculated as the Amount of Product Giving a Signal Equal to 5 Times the Background Noise, Maximum Residue Limits (MRLs) Established by European Legislation, and Coefficients of Variation (CVs) from the Retention Time and from the Absolute Peak Area, $n = 5$, for the Same Pistachio Nut Extract[a]

pesticide	R^2	LOD (μg/kg)	MRL (mg/kg)	CV(t_R)	CV(area)
captan	0.996	0.8	0.02	0.09	0.8
lindane	0.997	7.0	0.01	0.1	5.56
α-endosulfan	0.996	2.1	0.1[b]	0.09	7.33
oxyfluorfen	0.986	2.4	0.05	0.09	2.89
β-endosulfan	0.996	4.0	0.1[b]	0.11	8.83

[a] The pistachio nuts were fortified at 0.05 mg/kg for each pesticide. ECD was used. [b] The MRL for endosulfan is given as the sum of those of both isomers and endosulfan sulfate.

at 4 °C. Working pesticide solutions (1 mg/L) used for sample fortification were prepared by diluting the stock solution in methanol. Pesticide-grade ethyl acetate and anhydrous sodium sulfate were obtained from Merck (Darmstadt, Germany). Ethyl acetate was used to extract the samples. The methanol, water, and 1-propanol used as the LC mobile phase were HPLC grade from pestican (LabScan, Dublin, Ireland). Tenax TA, 80−100 mesh (Chrompack, Middelburg, The Netherlands), was used as the packing material in the liner of the modified PTV injector (TOTAD interface). The glass liner was packed with a 1 cm length of Tenax TA between two plugs of glass wool to keep it in place and was then conditioned under a helium stream by heating from 50 to 350 °C, increasing 50 °C every 10 min, and maintaining the final temperature for 60 min.

Sample Preparation. A 200 g mass of nuts (in this term, we include sunflower seeds) were shelled, and the kernels were powdered with a pestle until a homogeneous mash was obtained. In the case of pistachio nuts, two types of mash were obtained, the first one from kernels and the second one from kernels with shells. A 2.5 g sample of the mash was weighed and fortified with aliquots of the working solutions to provide a pesticide concentration in the sample ranging from 0.01 to 0.25 mg/kg. The fortified samples were mixed with 5 mL of ethyl acetate and 2 g of anhydrous sodium sulfate. Extraction takes places at room temperature. After extraction for 5 min with a high-speed blender, the extract was filtered through a 0.20 μm (Millex-GN SLGN 013 NL) filter.

Online-Coupled LC−GC System. The analyses were performed using online LC−GC equipment. The HPLC system (Konik model 550) was provided with a manual injection valve (model 7125, Rheodyne, California) with a 50 μL loop, an oven column, and an ultraviolet (UV) detector operated at 205 nm. The gas chromatograph (Konik 4000B) was equipped with an ECD and NPD system. The LC and GC instruments were fitted with a TOTAD interface (U.S. Patent 6,402,947 B1, exclusive rights assigned to KONIK-Tech, Sant Cugat del Vallés, Barcelona, Spain).

Data acquisition and processing were performed with KoniKrom 32 (Konik, Sant Cugat del Vallés, Barcelona) software.

LC Preseparation. The LC column temperature was set at 45 °C. The LC column used was a 50 × 4.6 mm i.d. column packed with modified silica (C4, Kromasil 100-10, Hichrom, Theale, U.K.).

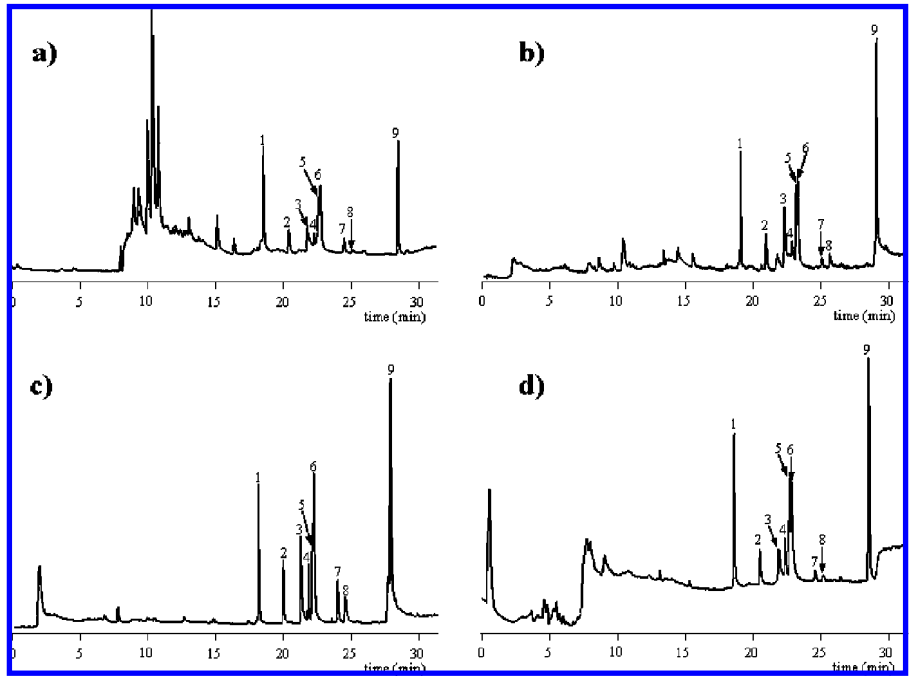

Figure 4. GC chromatograms obtained from the direct RPLC−GC−NPD analysis of extracts from different nuts: (**a**) peanut; (**b**) sunflower seed; (**c**) walnut; (**d**) hazelnut. The conditions and identification of peaks are as in **Figure 2**.

Table 3. Maximum Residue Limits (MRLs) Established by European Legislation and Detection Limits (LODs) Calculated as the Amount of Product Giving a Signal Equal to 5 Times the Background Noise for Different Nuts

	walnut		hazelnut		peanut		sunflower seed	
pesticide	MRL (mg/kg)	LOD (μg/kg)	MRL (mg/kg)	LOD (μg/kg)	MRL (mg/kg)	LOD (μg/kg)	MRL (mg/kg)	LOD (μg/kg)
diazinon	0.05	2.5	0.05	2.1	0.05	1.9	0.05	1
methylchlorpyrifos	0.05	6.3	0.05	10.8	0.05	7.5	0.05	3.2
fenitrothion	0.01	4.6	0.01	12.9	0.01	6.6	0.01	2.5
malathion	0.5	6.3	0.5	9.1	0.5	13.5	0.5	6.5
chlorpyrifos	0.05	4.8	0.05	3.8	0.05	3.7	0.05	1.4
parathion	0.05	2.5	0.05	3.9	0.05	2.7	0.05	1.4
phenthoate + chlorfenvinphos	0.05/ 0.02	11.3	0.05/ 0.02	35.7	0.05/ 0.02	12.2	0.05/ 0.02	13.9
methidathion	0.05	16.3	0.05	61.3	0.02	50.2	0.02	8.7
ethion	0.01	1.8	0.01	1.5	0.02	1.6	0.02	0.5

Table 4. Coefficients of Variation (CVs) from the Retention Time and from the Absolute Peak Area, $n = 5$, for the Same Nut Extract[a]

	walnut		hazelnut		peanut		sunflower seed	
pesticide	CV (t_R)	CV (area)	CV (t_R)	CV (area)	CV (t_R)	CV (area)	CV (t_R)	CV (area)
diazinon	0.08	4.4	0.01	10.7	0.05	4.8	0.11	19.7
methylchlorpyrifos	0.06	10.1	0.02	14.9	0.05	14.5	0.11	14.7
fenitrothion	0.04	9.3	0.04	12.6	0.03	9.8	0.12	11.7
malathion	0.06	11.7	0.03	15.8	0.08	19.4	0.09	16.7
chlorpyrifos	0.06	4.8	0.01	18.2	0.07	2.1	0.08	16.5
parathion	0.04	11.7	0.02	11.6	0.06	6.7	0.1	8.4
phenthoate + chlorfenvinphos	0.04	14.9	0.01	13.6	0.05	8.8	0.07	16.4
methidathion	0.06	14.2	0.08	12.6	0.2	15.1	0.1	14.3
ethion	0.05	6.3	0.01	13.2	0.7	7.7	0.05	10.9

[a] The nut samples were fortified at 0.05 mg/kg for each pesticide. NPD was used.

Methanol/water (80:20, v/v) was used as the mobile phase. To fix the LC fraction to be transferred to the GC instrument, the pesticide solutions at 50 mg/L were injected into the LC instrument. The flow rate was 2 mL/min. In the RPLC−GC analysis the filtered extracts were injected into the LC system. The flow rate was 2 mL/min until the fraction containing the pesticides was eluted and changed to 0.1 mL/min during the transfer time. After transfer, the eluent was changed in 1 min to 100% water at 1 mL/min for 8 min and then to 100% 1-propanol at 1 mL/min for 10 min to ensure complete elimination of the retained compounds.

LC−GC System Transfer. Initially, the interface temperature was stabilized at 125 °C. The carrier gas (helium) stream entered the packed liner through the oven side (B) and through the opposite side (A), both at 500 mL/min. EV_1 was closed and EV_2 opened (**Figure 1**). At the beginning, the eluent from the HPLC system was sent to waste. When the front of the pesticide fraction reached the six-port valve (0.35 min for OPs and 0.3 for CPs), this was automatically switched, transferring the fraction to the GC system. During LC−GC system transfer (7 min for OPs and 14 min for CPs), the analytes were retained on the packed material in the liner and the solvent was vented to waste through the ST_2 tubing. When the transfer step was completed, the six-port valve was automatically switched, so that the LC eluent was sent to waste. EV_1 was opened. The temperature and helium flow were maintained constant for 1 min to ensure elimination of the remaining solvent in the glass liner and the CT tubing. After this time, EV_1 and EV_2 were closed and the flow through B was interrupted while the flow through A was changed to 1.8 mL/min. Then the TOTAD interface was quickly heated to 275 °C for 5 min, leading to the thermal desorption of the analytes, which were transferred to the GC column, pushed by the helium. GC analysis was then carried out, after which EV_2 was opened

and the interface was cleaned by maintaining the helium stream for 5 min at 300 °C. Finally, it was cooled to 125 °C so that another analysis could be carried out.

GC Conditions. During the transfer and the solvent elimination steps, the oven temperature was kept at 50 °C. The transferred LC fraction was analyzed using a 5% phenylmethylsilicone fused-silica column (30 m × 0.32 mm i.d., 0.25 μm film thickness) (Quadrex, Weybridge, U.K.) with helium as the carrier gas at a flow rate of 1.8 mL/min. During GC analysis, the oven temperature was programmed as follows: initially 50 °C; 10 °C/min to 160 °C; 2 °C/min to 170 °C; 5 °C/min to 230 °C; 10 °C/min to 300 °C; hold for 5 min. Analyses were carried out without split. The NPD and ECD temperatures were kept at 270 and 300 °C, respectively.

RESULTS AND DISCUSSION

Initially, experimental conditions were established on the basis of our previous experience in the determination of pesticides in vegetables (*9*); however, because of the fatty nature of the nuts, the extract obtained contained a large amount of fat, so that large-volume injection of the extract into the GC system was not possible. To solve this problem, an additional cleanup of the extract could be used before chromatographic determination, but to avoid such a step, RPLC−GC analyses were carried out. The LC step replaces the cleanup and concentration steps. In the LC step, analytes are separated from the fatty material extracted and the LC fraction containing the pesticides is automatically transferred to the GC system to carry out the analysis.

First, the start and end times of the fraction to be transferred from the LC system to the GC syste, must be selected in the LC chromatogram. Solutions of each pesticide in methanol at 50 mg/L were used with this aim, so that their peaks could be monitored with the LC detector. The fractions were settled by taking into consideration the elution time of the first and the last pesticides. As can be observed in the LC chromatograms (**b**) in **Figures 2** and **3**, pesticides eluted rapidly (from 0.35 to 0.7 min for OPs and from 0.30 to 1.0 min for CPs). The LC chromatograms of the pistachio nut extract (**a**) showed a peak of elution from 0.3 to 0.6 min, but the bulk of the fatty material eluted later (after 2 min) because it was retained more strongly than pesticides in the LC system since reversed-phase liquid chromatography was used in the preseparation step. In the LC chromatograms of **Figures 2** and **3** the initial and final times of the transferred fraction for OPs and CPs, respectively, are indicated. Bearing in mind the flow in the LC system during the preseparation and transfer steps, the resulting volume of the fraction containing the pesticides (0.7 mL for OPs and 1.4 mL for CPs) and the time of the transfer step (7 min for OPs and 14 min for CPs) can easily be calculated. The overall procedure, including extraction, LC preseparation, LC−GC system transfer, and GC analysis, required approximately 1 h, a short time compared with the time taken by traditional techniques, which tend to be time-consuming and tedious.

Figures 2 and **3** show the GC chromatograms of a pistachio nut extract with NPD and ECD, respectively. Chromatographic conditions were chosen to maximize the resolution between peaks while minimizing the total run time (about 30 min). The pesticide peaks are indicated by numbers. Unidentified peaks correspond to matrix components which were coeluted with pesticides in the LC preseparation step. The retention times of these compounds in GC do not match those of the target pesticide in the ECD or in the NPD chromatograms except in a few cases, when α-endosulfan overlaps a compound from the matrix, as can be observed in **Figure 3**. Technical endosulfan is a mixture of isomers α and β. When endosulfan is used as an insecticide in crop protection, both isomers appear in the GC chromatogram. α-Endosulfan overlaps, in some cases, a compound from the matrix so that we cannot guarantee the presence of endosulfan although a peak appears at that retention time, but the presence of a peak at the retention time of β-endosulfan confirms the presence of endosulfan in the sample, because no interference is observed with β-endosulfan. As can be observed in **Figure 2** phenthoate and chlorfenvinphos are not resolved because of partial overlapping, so both pesticides were quantified together.

Method Validation. To confirm that the method is suitable for the quantitative determination of OPs and CPs in nuts, the precision, linearity, and detection limits were evaluated in pistachio nuts. Validation parameters were calculated for pistachio kernels and for kernels with shells. The differences were not significant. Data given later correspond to kernels without shells. The precision and detection limits were also evaluated for kernels from other nuts (peanut, walnut, hazelnut, and sunflower seed).

Linearity. The linearity of the proposed method (sample preparation plus RPLC−GC analysis) was studied. Calibration curves were obtained for pistachio nut samples fortified in the range from 0.01 to 0.15 mg/kg for OPs and from 0.01 to 0.25 mg/kg for CPs and considering the absolute peak areas. The correlation coefficients are shown in **Table 1** for OPs and in **Table 2** for CPs. Better linearity was obtained for CPs than for OPs.

Precision. The precision of the chromatographic method was evaluated by analyzing an extract obtained from a pistachio nut sample fortified at 0.05 mg/kg. The same extract was injected five times. The repeatability for the overall analytical procedure was also determined carrying out the whole procedure (extraction and RPLC−GC analysis) with the same fortified sample five times. The CVs for the retention time and for the absolute peak area are indicated in **Tables 1** and **2** for OPs and CPs, respectively.

Detection Limits. The LODs were calculated as the amount of product giving a signal equal to 5 times the background noise. MRLs for the target pesticides in pistachio nuts established by European legislation are much higher than the detection limits obtained in the present work. Lower LODs were obtained for OPs than for CPs (**Tables 1** and **2**).

Analysis of Different Nuts. The proposed method was applied to the analyses of different nuts (walnut, hazelnut, peanut, and sunflower seed). **Figure 4** shows the GC chromatograms obtained in the analysis of the extracts of different nuts. Components of the matrixes coeluted with pesticides in the LC preseparation step were different for each type of nuts. Nevertheless, no interference with pesticide peaks was observed in any of these cases so that pesticide quantification was feasible. **Tables 3** and **4** show the validation parameters for different nuts. The data obtained were similar to those obtained for pistachio nuts.

The technique described, based on rapid extraction with ethyl acetate and RPLC−GC analysis of an extract, allows the determination of OPs and CPs in different nuts. The method can be indistinctly used for the kernel with or without the shell. It reduces the amount of solvent used, minimizes the number of analytical steps necessary, and avoids laborious and time-consuming cleanup steps. The method shows good linearity and repeatability. For the majority of the pesticides in the different nuts, the sensitivity was good enough to ensure reliable determination at levels much lower than the respective MRLs established by European legislation.

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