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Determination of Phospholipids in Olive Oil by ^{31}P NMR Spectroscopy

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A nondestructive analytical method based on NMR spectroscopy was developed for the determination of phospholipids in olive oil. The phospholipids extracted from virgin olive oil with a mixture of ethanol/water (2:1 v/v) were identified and quantified by high resolution ^{31}P NMR spectroscopy. The main phospholipids found in olive oil were phosphatidic acid, lyso-phosphatidic acid, and phosphatidylinositol. Validation of the ^{31}P NMR methodology for quantitative analysis of phospholipids in olive oil was performed. Sensitivity was satisfactory with detection limits of 0.25−1.24 μmol /mL. In addition, the composition of fatty acids in phospholipids model compounds and those in olive oil samples was estimated by employing one- and two-dimensional ^{1}H NMR. The results indicated that the fatty acid composition in phospholipids and triacylglycerols of olive oil was similar.

KEYWORDS: Olive oil; phospholipids; ^{1}H NMR spectroscopy; ^{31}P NMR spectroscopy

INTRODUCTION

Glycerophospholipids or simply phospholipids constitute a particular class of membrane lipids comprising a glycerol backbone in which the *sn*-1 and *sn*-2 hydroxyl groups have been esterified by fatty acids, whereas the third hydroxyl has been phosphorylated. The phosphate group could be esterified by the hydroxyl groups of various amino-alcohols and alcohols, such as ethanolamine, choline, inositol, glycerol, and others. Olive oil phospholipids have been poorly studied. Still, their presence in these oils may affect their oxidative stability (*1*) or the physicochemical state of cloudy (veiled) olive oil. The antioxidant activity of phospholipids in olive oil has been attributed to their catalytic activity in hydroperoxide decomposition or to form complexes with prooxidant metals (*2*).

Levels of total phospholipids in veiled and filtered virgin olive oil have been reported to range from 21 to 124 mg/kg (*3*). However, data published in the literature pointed out distinct amounts of individual phospholipids in olive oils (*4, 5*) that can probably result from the different samples and/or the different analytical techniques used. In this sense, phosphatidylcholine (**1**), phospatidylethanolamine (**2**), phosphatidylinositol (**3**), and phosphatidylserine (**4**) were the main phospholipids detected by thin layer chromatography (TLC) in olive, avocado, cottonseed, corn, and rapeseed oils (*4*), whereas phosphatidylglycerol (**5**) was the major phospholipids detected in commercial olive oil samples, when using liquid chromatography−mass spectrometry (*5*). Other phospholipids identified in these last samples were phosphatidic acid (**6**), **1**, **2**, and **3**, but **4** was not detected. **Figure 1** depicts the chemical structures of phospholipids detected in olive oil. Apart from the aforementioned phospholipids, the so-called lyso-phospholipids (e.g., lyso-phosphatidic acid) were detected in olive oils; they resulted from the partial hydrolysis of phospholipids losing one acyl chain preferably at the *sn*-2 position of the glycerol moiety. The chemical structures of two lyso-phospholipids are shown in **Figure 1**.

In the past decade, high resolution ^{1}H and ^{13}C NMR spectroscopy has emerged as a potential analytical tool for the analysis of vegetable oils and in particular olive oil (*6–9*). The amount of information contained in an NMR spectrum obtained in a fairly rapid manner, combined with the easy sample preparation, renders this spectroscopic technique very attractive for the composition determination of olive oil. ^{1}H NMR spectroscopy has provided valuable information (*6, 8, 9*) about lipid classes, fatty acid composition, unsaturation levels, and several minor compounds (sterols, squalene, terpenes, volatile compounds, etc.), whereas ^{13}C NMR among others gave unique information (*7*) about the positional distribution of fatty acids on glycerol moiety and the stereochemistry of unsaturation.

Although ^{1}H and ^{13}C NMR may provide useful data for individual phospholipids (*10, 11*), they are not practical to analyze mixtures of phospholipids (*12*). Strong signal overlap and dynamic range problems in ^{1}H NMR spectra and/or long relaxation times of the insensitive ^{13}C nuclei, render the analysis of multicomponent mixtures a difficult task. An alternative, quantitative NMR method based on the ^{31}P nucleus has been proposed (*13*). This technique exploits the 100% abundance and

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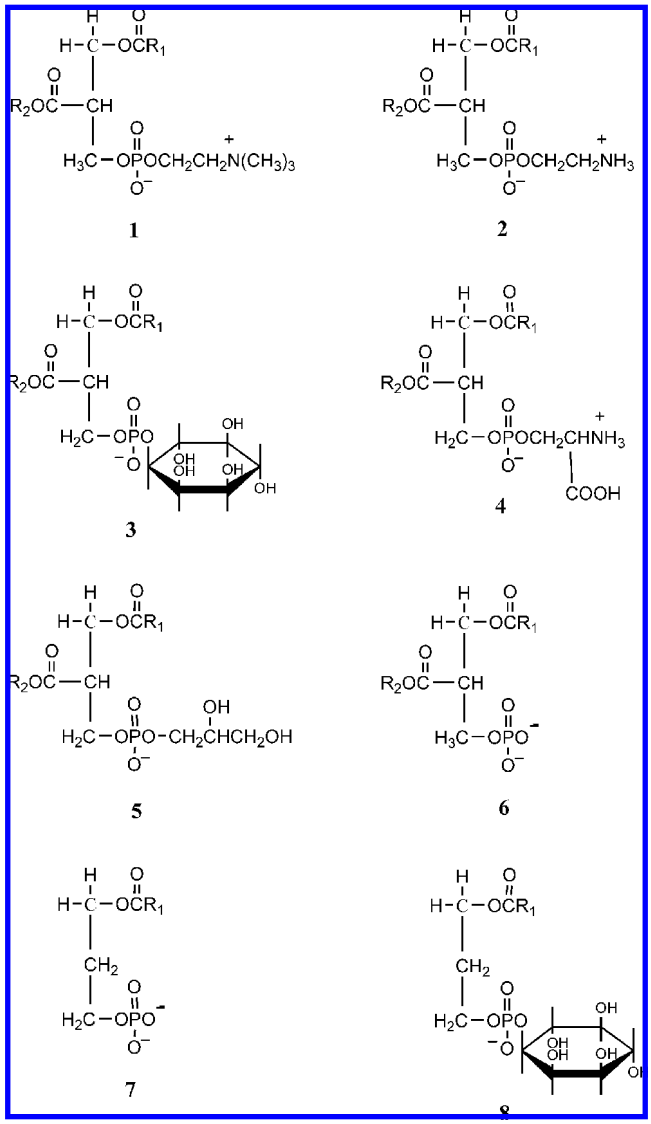

Figure 1. Chemical structures of phospholipids detected in virgin olive oil. Phosphatidylcholine, **1**; phosphatidylethanolamine, **2**; phosphatidylinositol, **3**; phosphatidylserine, **4**; phosphatidylglycerol, **5**; phosphatidic acid, **6**; lyso-phosphatidic acid, **7**; lyso-phosphatidylinositol, **8**.

the high sensitivity of the phosphorus-31 nucleus (only 15 times less sensitive than proton), and the simplicity of the decoupled ^{31}P NMR spectra. As phospholipids give a single signal in the spectrum, and different phospholipids are characterized by specific resonance frequencies (due to different chemical structures of the phospholipids), separation of the mixture components is not necessary to analyze it. ^{31}P NMR spectroscopy has been used to quantitate phospholipids from different sources, including lecithins (*14–17*), lipids (*18, 19*), and biological fluids and tissues (*17–24*). A comparison study (*17*) for the determination of phospholipids in lecithins has shown that TLC, HPLC, and ^{31}P NMR methods gave comparable results concerning the total amounts of phospholipids, as well as the amounts of phospholipids classes in the samples used. These analytical methods differ somewhat in terms of selectivity, reproducibility, detection limit, and duration of the analysis. Overall, ^{31}P NMR methodology was recommended as the method with the highest selectivity, ease of performance, and the lowest duration of analysis (*17*).

In the present study, we describe for the first time the application of ^{31}P NMR spectroscopy for the detection and quantification of phospholipids extracted from Greek olive oils. To the best of our knowledge, there is no other NMR study in the literature dealing with the detection and quantification of phospholipids in olive oil. Also, high resolution ^{1}H NMR spectroscopy was employed to determine the fatty acid composition in phospholipids.

MATERIALS AND METHODS

Materials. Twelve Greek virgin olive oil samples were provided by local cooperatives. Samples originated from Crete (6), Messinia (1), Lakonia (2), Lesvos (1), and Halkidiki (2); they were extracted from the olive cultivars KORONEIKI, HONDROELIA, TSUNATI, KOLOVI, and a local cultivar cultivated in northern Greece (HALKIDIKI). Also, three samples of refined olive oils and two samples of olive-pomace oil were used in this study. The olive-pomace oils were produced in Lakonia and Inofita.

The phospholipids **1**, **2**, **3**, **5**, **6**, the lyso-phospholipids, ethylenediaminetetraacetic acid (EDTA), and cesium hydroxide were purchased from Fluka (Buchs, Switzerland). The fatty acid composition of commercial phospholipids was unknown, except that of **6** bearing the saturated palmitoyl acid chains at the *sn*-1 and *sn*-2 positions of the glycerol moiety. Triphenylphosphate, citric acid, hexane, ethanol, methanol, chloroform solvents, deuterated chloroform, and methanol were obtained from Sigma-Aldrich (Athens-Greece).

Preparation of the EDTA Cesium Salt. The cesium salt of EDTA (EDTA-Cs^+) was generated by titrating a solution of 0.58 g of the free acid in 2 mL of water with cesium hydroxide to a pH value of 7.0.

Preparation of Citric Cesium Salt. The cesium salt of citric acid was prepared by titrating a suspension of 9.21 g of the free acid in 50 mL of water with cesium hydroxide to a pH value of 7.

Preparation of Triphenylphosphate Solution. Two stock solutions of the internal standard were prepared by dissolving 0.033 g of triphenylphosphate (TPP) in 50 mL of a mixture of $CDCl_3$/MeOH (2:1 v/v) or in 50 mL of a mixture of $CDCl_3$/MeOH/D_2O-EDTA-Cs^+ (400:80:5 v/v/v), depending on the experiment.

Extraction of Phospholipids from Olive Oils. Phospholipids were extracted from olive oil samples following the method developed by Galanos et al. (*25*). Briefly, 100 g of olive oil was dissolved in 400 mL of hexane, and the solution was extracted by 3 × 100 mL of ethanol/water (87:13 v/v). The ethanolic extracts were washed with 100 mL of hexane, and the phospholipids were obtained upon removal of the solvent under vacuum.

Sample Preparation. After solvent evaporation, the residue was dissolved in 10 mL of chloroform/methanol 2:1 (v/v), and the mixture was washed with 2 mL of aqueous solution of citric acid-Cs^+ or an aqueous solution of EDTA-Cs^+ prepared as described above. The two phases were allowed to separate for 2 h. The bottom chloroform phase was collected, and the solvent was evaporated on a rotary evaporator at 30 °C.

NMR Experiments. All NMR experiments were conducted on a Bruker AMX500 spectrometer operating at 500.1 and 202.2 MHz for proton and phosphorus-31 nuclei, respectively, at 30 ± 1 °C using temperature stabilization. The phospholipids were dissolved in $CDCl_3$/MeOH/D_2O-EDTA-Cs^+ for the ^{31}P NMR experiments; an amount of 1−60 mg of the extracted phospholipids obtained from 100 g of olive oil was dissolved in 0.6 mL of the stock solutions of TPP prepared as described above. The mixture was added directly into the 5 mm NMR tube and used to obtain the NMR spectra. For the ^{1}H NMR experiments, phospholipids were dissolved in the mixture of deuterated solvent $CDCl_3$/CD_3OD/D_2O-EDTA-Cs^+.

One-Dimensional ^{31}P NMR Spectra. These spectra were recorded by employing the inverse gated decoupling technique to suppress NOE. Typical parameters for quantitative studies were as follows: 90° pulse width, 12.5 μs; sweep width, 5 kHz (25 ppm); acquisition time, 6.3 s; repetition time (relaxation delay + acquisition time), 10.5 s; memory size, 32K (zero-filled to 64K). The large sweep width was dictated by the ^{31}P chemical shift (δ −17.30) of the internal standard triphenylphosphate. To ensure quantitative spectra, the magnitude of the repetition time adopted was greater than 5 times the longest spin−lattice relaxation time (T_1 = 2.12 s) presented by the internal standard

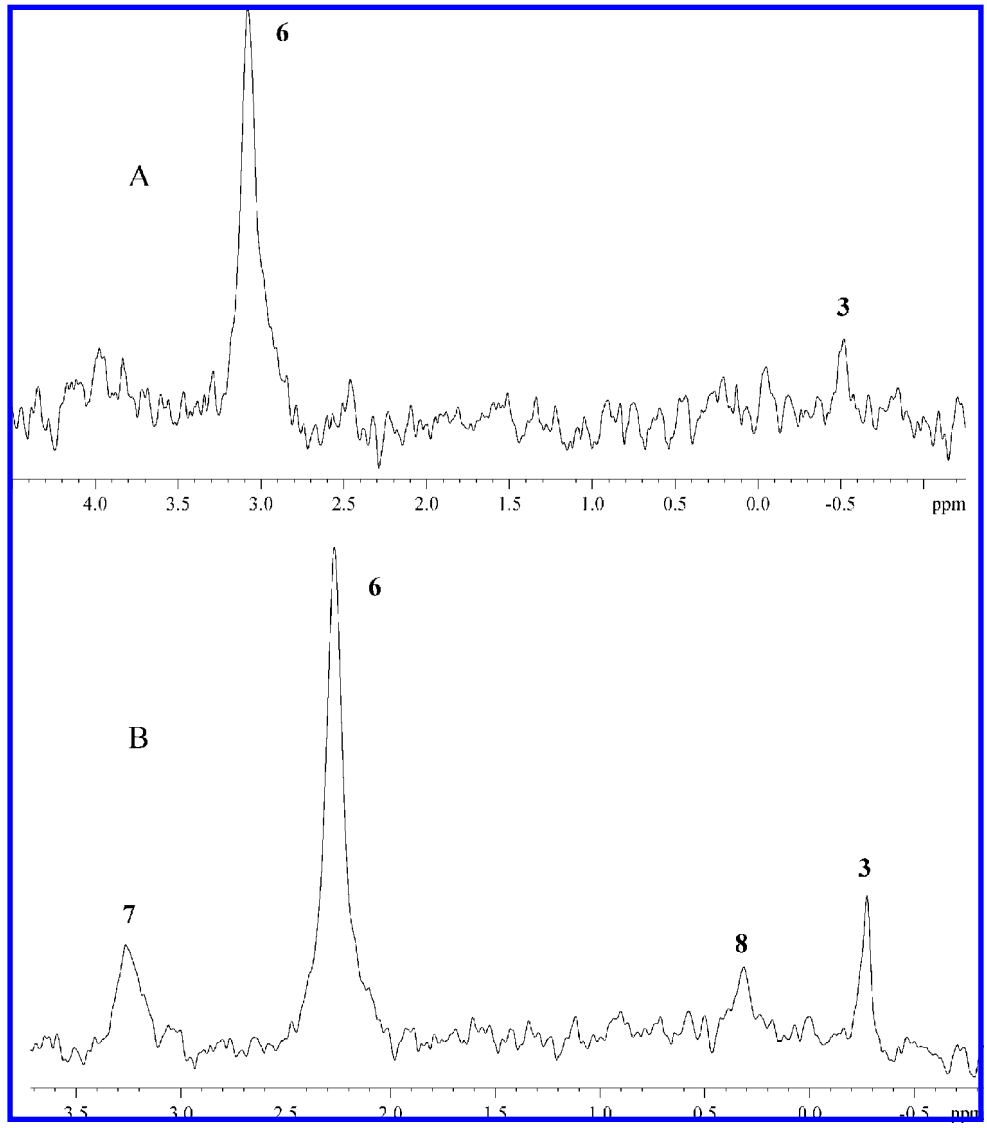

Figure 2. ^{31}P NMR spectra (202.2 MHz) of phospholipids extracted from a virgin olive oil sample of KORONEIKI cultivar originating from Heraklion in (**A**) chloroform-*d* solvent and (**B**) a mixture of $CDCl_3$/MeOH/D_2O-EDTA-Cs^+ (400:80:5 v/v/v).

triphenylphosphate. Line broadening (lb) of 3 Hz was applied, and drift correction was performed prior to Fourier transform. A polynomial fourth-order baseline correction was made before integration. The lb = 3 Hz was adopted to increase the sensitivity (S/N) of the experiment. It did not harm resolution inasmuch the line-widths (8−10 Hz) of the various ^{31}P signals of phospholipids were much broader than this window function. For each spectrum, 400−800 transients were accumulated depending on the concentration of phospholipids in olive oil samples.

One-Dimensional ^{1}H NMR Spectra. They were obtained with the following parameters: time domain, 32K; 90° pulse width, 9.3 μs; spectral width, 12 ppm; relaxation delay, 2 s. Thirty-two scans and 4 dummy scans were accumulated for each free induction decay. Baseline correction was performed carefully by applying a polynomial fourth-order function in order to achieve a quantitative evaluation of all signals of interest. The spectra were acquired without spinning the NMR tube in order to avoid artificial signals, such as spinning sidebands of the first or higher order.

Gradient ^{1}H−^{1}H Homonuclear Spectroscopy (g-H−H-COSY). Gradient selected H−H-COSY spectra were collected by using 128 increments of 1K data points. Thirty-two scans and 8 dummy scans were acquired for each FID with a recycle delay of 1.0 s. The data set were zero-filled to a 1K × 1K matrix prior to Fourier transformation. A squared sinusoidal window function was used in both dimensions. No phase correction was applied, and the 2D spectra were displayed in magnitude mode.

^{1}H−^{1}H Total Correlation Homonuclear Spectroscopy (H−H-TOCSY). These spectra were acquired in the phase sensitive mode with TPPI, using the MLEV17 pulse sequence for the spin lock. Typically, 16 dummy scans and 32 scans were collected for each of 512 increments with a spectral width of 7183.91 Hz in both dimensions, 2048 data points, mixing time of 60 ms, and a relaxation time of 1 s. The data points in the second dimension were increased to 1K real data points by linear prediction, and the spectra were zero-filled to a final size of 2K × 2K prior to Fourier transformation. A sine-bell squared window function was used in both dimensions.

^{31}P Spin−lattice Relaxation Measurements. ^{31}P spin−lattice relaxation times (T_1) for the various phospholipids and internal references were measured by the standard inversion recovery Fourier transform (IRFT) method with a repetition time longer than 5 × T_1 (12 s). A total of 256−720 transients were accumulated, for a set of 8−10 arrayed t values. The 180° pulse width was set to 25 μs. Values of T_1 were determined by a three-parameter nonlinear procedure with a rms error of ± 8% or better.

RESULTS AND DISCUSSION

High-Resolution ^{31}P NMR Spectra. As previously described (*21*), high-resolution ^{31}P NMR spectra of extracted phospholipids from various sources are hampered mainly by two factors: the formation of aggregates and the formation of electrostatic

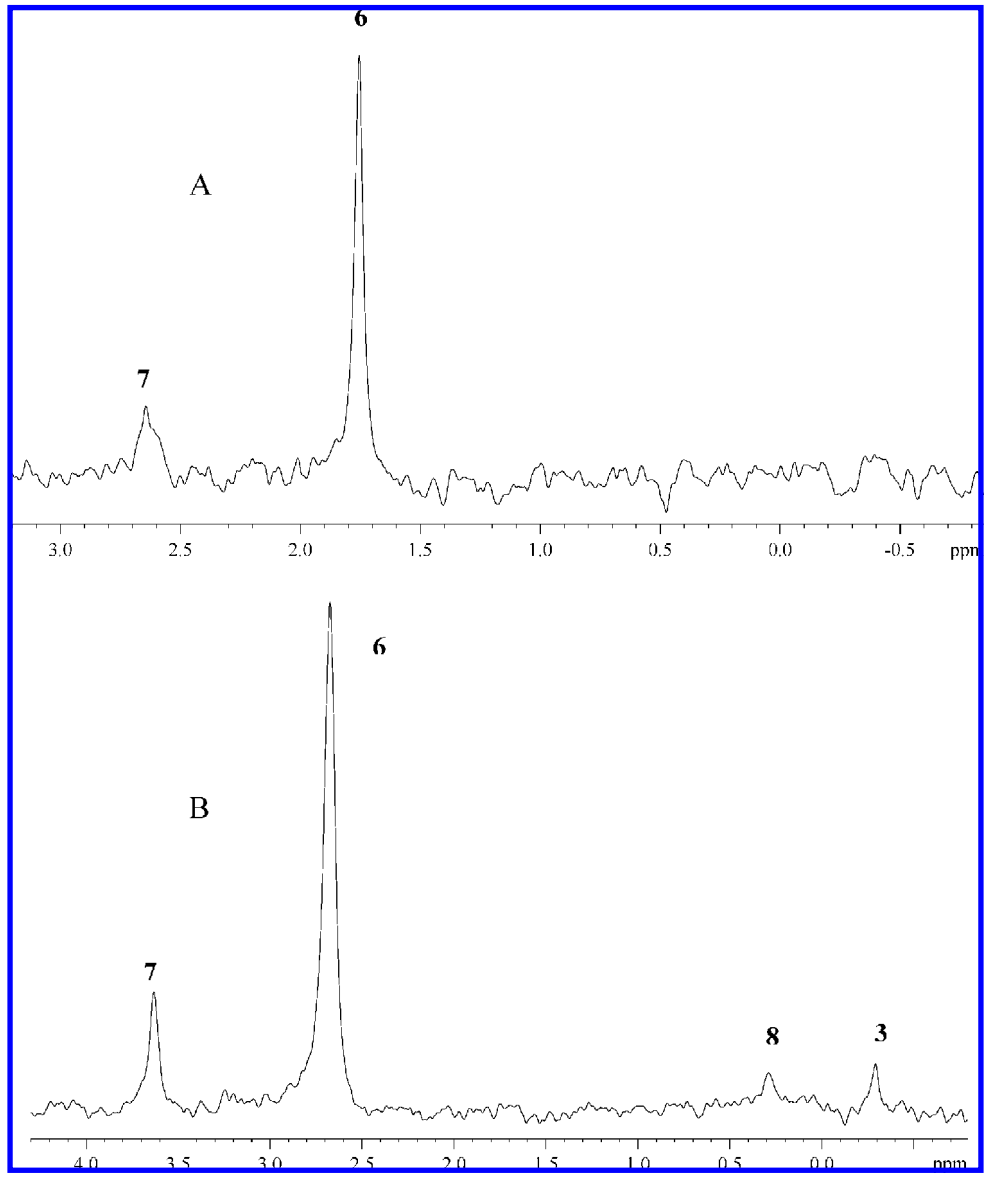

Figure 3. ^{31}P NMR spectra (202.2 MHz) of phospholipids extracted from a virgin olive oil sample of KORONEIKI cultivar originating from Heraklion (**A**) washed with EDTA-Cs^+ salt solution at pH 7 and (**B**) washed with citric acid-Cs^+. Both spectra were recorded in a mixture of $CDCl_3$/MeOH/D_2O-EDTA-Cs^+ (400:80:5 v/v/v).

Table 1. Comparison of the Measured Amounts (μmol/0.5 mL) of the Phospholipids Phosphatidylcholine (**1**), Phosphatidylethanolamine (**2**), and Phosphatidylglycerol (**5**) Model Compounds Obtained From Integration of the Corresponding ^{31}P NMR Signals to the Weighed Amounts[a]

weighed	1	2	5
0.10	0.12	0.14	0.11
0.30	0.32	0.28	0.26
0.50	0.51	0.61	0.55
0.70	0.64	0.71	0.65
1.00	0.98	1.12	1.21
2.00	2.01	1.94	2.02
3.00	3.16	3.21	3.01
5.00	5.21	4.78	5.43
7.00	6.47	6.94	6.86
10.00	10.29	10.36	9.94
15.00	15.11	14.88	15.42
R	0.99 ± 0.01	0.99 ± 0.01	0.99 ± 0.01
Intercept	−0.01 ± 0.09	0.04 ± 0.07	0.04 ± 0.07
Slope	1.01 ± 0.01	1.00 ± 0.01	1.01 ± 0.01

[a] ^{31}P NMR spectra were recorded in $CDCl_3$/MeOH/D_2O-EDTA-Cs^+ (400:80:5 v/v/v).

complexes with ions in solution. Both factors result in significantly broad ^{31}P NMR resonances that prevent resolution and hence quantitation of the constituents phospholipids. In previous works, the formation of aggregates has been confronted by using detergents (*16, 19, 26, 27*) or alternative mixtures of appropriate solvents (*16, 28*). In the present study, the solvent effect on the ^{31}P NMR spectra profile was tested by dissolving the olive oil extracts in chloroform and in a mixture of $CDCl_3$/MeOH/D_2O-EDTA-Cs^+ (1.0 M) (400:80:5 v/v). The mixture of $CDCl_3$/MeOH/ D_2O-EDTA-Cs^+ was found most useful among several other solvents that were investigated (*14, 16*). **Figure 2** compares the spectra of phospholipids extracted from a virgin olive oil sample of KORONEIKI cultivar originating from Heraklion in chloroform and $CDCl_3$/MeOH/D_2O-EDTA-Cs solvents. It appears that the presence of methanol in the mixtures of solvents destroys the phospholipid aggregates resulting in sharper resonances, thus revealing additional phospholipid signals. The mixture of solvents has the additional advantage that ^{1}H and ^{13}C NMR spectra of the same sample could be recorded, provided that deuterated solvents are used.

Table 2. Phospholipid Content (μmol/100 g) of Olive oil Samples Determined by ^{31}P NMR Spectroscopy[a]

no	origin	variety	quality	1[b]	2[b]	3[b]	5[b]	6[b]	7[b]	8[b]
1	Heraklion	KORONEIKI	virgin					0.59	0.09	
2	Heraklion	KORONEIKI	virgin					0.26		
3	Heraklion	KORONEIKI	virgin			0.22		0.81	0.13	0.30
4	Heraklion	KORONEIKI	virgin			0.09		1.63	0.21	0.13
5	Heraklion	KORONEIKI	virgin			0.43		9.17	1.77	0.87
6	Heraklion	KORONEIKI	virgin			1.55		10.88	2.09	1.48
7	Messinia	KORONEIKI	virgin			1.09		13.44	1.02	1.26
8	Lakonia	TSOUNATI	virgin			8.66		14.41	7.01	5.98
9	Lakonia	TSUNATI	virgin			1.94		13.76		
10	Halkidiki	LOCAL	virgin	0.18	0.20			0.38		
11	Halkidiki	LOCAL	virgin					1.25		
12	Lesvos	KOLOVI	virgin					0.97	1.97	
13	Inofyta		OPO[c]			1.51		22.76	4.88	2.13
14	Lakonia		OPO[c]			12.19	5.81	33.38	18.66	11.86

[a] ^{31}P NMR spectra were recorded in $CDCl_3$/MeOH/D_2O-EDTA-Cs^+ (400:80:5 v/v/v). [b] Phosphatidylcholine, **1**; phosphatidylethanolamine, **2**; phosphatidylinositol, **3**; phosphatidylglycerol, **5**; phosphatidic acid, **6**; lyso-phosphatidic acid, **7**; lyso-phosphatidylinositol, **8**. [c] OPO = olive-pomace oil.

Table 3. Chemical Shifts and Assignments of the Signals in the ^{1}H NMR Spectrum of Phosphatidylcholine in Chloroform-*d* Solvent (**Figure 4**)

signal	proton	δ	attribution
1	C*H*═C*H*	5.33	all unsaturated acyl chains
2 (a)	CH═CH-C*H*$_2$-CH═CH	2.81	linolenyl and linoleyl chains
3 (b)	C*H*$_2$-COOH	2.28	all acyl chains
4 (c)	C*H*$_2$-CH═CH	2.03	all unsaturated acyl chains
5 (d)	C*H*$_2$-CH$_2$COOH	1.58	all acyl chains
6	(C*H*$_2$)$_n$	1.28	all acyl chains
7 (f)	CH$_2$CH$_2$CH$_2$−C*H*$_3$	0.88	all acyl chains except linolenyl
1′a	C*H*$_2$-OCOR	4.12 $J_{1'a,1'b}$ = 11.4 Hz, $J_{1'a,2'}$ = 6.9 Hz	glycerol
1′b	C*H*$_2$-OCOR	4.41 $J_{1'b,2'}$ = 3.4 Hz	glycerol
2′	C*H*-OCOR	5.20 (m)	glycerol
3′	CH$_2$−OP(O)$_2$O	3.94 (m)	glycerol
4″	P−O-C*H*$_2$	4.31 (s)	choline
5″	C*H*$_2$-N(CH$_3$)$_3$	3.81 (s)	choline
6″	-N(C*H*$_3$)$_3$	3.36 (s)	choline

Also, washing of phospholipids with EDTA-cation salts could narrow further the phospholipid signals in the ^{31}P NMR spectra (*16, 19, 28*). Nevertheless, EDTA is unable to form complexes with Fe^{3+} at pH values close to 7 since at this pH range, hydrolysis of phospholipids is likely to occur (*27*); ferric cations form complexes with EDTA salts at pH ≥ 10. Therefore, all paramagnetic ions cannot be removed from phospholipids solutions using EDTA salts. Accordingly, we tried another complexation agent, the salt citric acid-Cs^+. The effectiveness of the two complexation agents in obtaining high-resolution ^{31}P NMR spectra is compared in **Figure 3**. **Figure 3A** shows the ^{31}P NMR spectrum of phospholipids extracted from the same virgin olive oil sample from Heraklion and washed with EDTA-Cs^+ salt solution at pH 7. Two signals are observed in the spectrum owing to phosphatidic acid (**6**) and lyso-phosphatidic acid (**7**). Washing the same phospholipids with citric acid-Cs^+ resulted in an ^{31}P NMR spectrum (**Figure 3B**) with two additional signals, indicating that the mixture of phospholipids has two more constituents, namely, phosphatidylinositol (**3**) and lyso-phosphatidylinositol (**8**), the signals of which were hidden under the noise level of the spectrum recorded after pretreatment with EDTA-Cs^+ (**Figure 3A**). The citric acid-Cs^+ salt should be used carefully, preferably right after its preparation, because it crystallizes after a few hours.

^{31}P NMR Chemical Shifts. The various signals of the phospholipid components in olive oils were assigned by spiking the samples with predetermined quantities of commercial phospholipids. The spiking procedure was necessary since ^{31}P chemical shifts, especially those of **6** and **7**, were highly pH-dependent (*22, 27, 29*). This observation is crucial because the spectroscopic region in which most of the phospholipids signals appear is relatively narrow (5−6 ppm), and therefore, spectroscopic overlap is likely to occur. In particular, significant chemical shift differences were observed for phospholipids extracted from different sources, such as olive oils and olive oil dregs. The ^{31}P chemical shifts of **3**, **6**, **7**, and **8** in olive oil at δ − 0.26, +2.26, δ + 3.30, and δ + 0.31 (**Figure 2**B), respectively, were shifted to δ +0.46, δ +0.90, δ +1.50, and δ −0.37, respectively, in dregs. These chemical shifts differences reflected the different environments of phospholipids in olive oil and dregs. Also, chemical shifts are affected to a smaller or greater degree by solvent modifications, temperature changes, addition of acids and bases, water content, and by large differences in the proportion of the various phospholipid components (*13, 14, 22, 27*). For instance, compare the ^{31}P chemical shifts of the same phospholipids extracted from the same olive oil sample in **Figures 2B** and **3B**. Both spectra were recorded in the same solvent mixtures following the same experimental protocol, but the extracts were washed by different complexation agents. The largest chemical shift differences were observed for phosphatidic acid, $\Delta\delta$ 0.36, and lyso-phosphatidic acid, $\Delta\delta$ 0.34. In the present study, all ^{31}P chemical shifts of the various phospholipids detected were referenced to trimethylphosphate, which gives a single signal at δ 2.43.

Quantitative Analysis of Phospholipids in Olive Oil. Quantitative analysis by using NMR spectroscopy requires the addition of an internal (and some times external standard) of known quantity and well controlled experimental conditions. The latter should guaranty that integration of the respective signals in the spectrum is quantitative. These are the relaxation delay between consecutive pulse sequences, which has to be at least five times the longest spin−lattice relaxation times (T_1), and the technique of the inverse gated decoupling to suppress NOE effects. Both dynamic parameters may considerably affect the intensity (integrals) of the various signals in the spectrum. The ^{31}P T_1 values of phospholipids determined by inversion recovery at 30 °C in the $CDCl_3$/MeOH/D_2O-EDTA-Cs^+ mixture of solvents were rather short ranging from 0.72 for the heavier

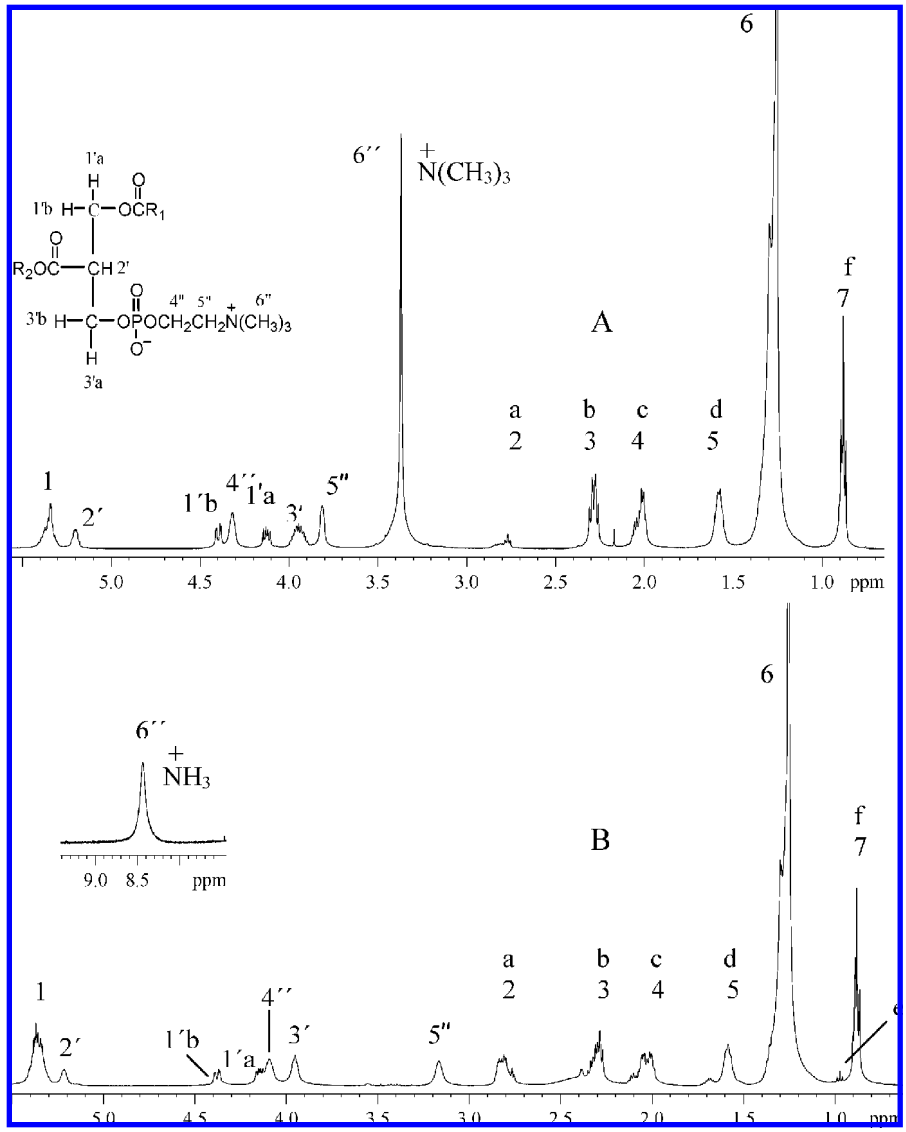

Figure 4. ^{1}H NMR spectra (500 MHz) of the phospholipids (**A**) phosphtidylcholine **1** and (**B**) phosphtidylethanolamine **2** in chloroform-*d*. Signals denoted by letters a−f belong to protons of the fatty acyl chains (see text).

phosphatidylglycerol to 1.64 s for the lighter phosphatidic acid. The T_1 values of phaphatidylcholine and phosphatidylethanolamine were 1.13 and 0.91 s, respectively. However, the internal standards suggested in the literatures were characterized by rather large T_1 values, namely, 9.55 s for the trimethylphosphate and 6.52 for the triethylphospate. To avoid such long relaxation times, which may increase considerably the duration of the experiment, we selected triphenyphosphate (^{31}P chemical shift δ −17.30) as an internal standard; this molecule is characterized by a much shorter T_1 value (2.12 s). Addition of paramagnetic metal centers such as Cr(acac)$_3$ in solution could be another option to reduce the relaxation times. Nevertheless, Cr(acac)$_3$ was not used in the present study since its presence generates further increase in the line-widths of the phosphorus signals. Quantification of phospholipids in μmol/100 g of olive oil was performed on the basis of the following equation:

$$PL(\mu\text{mol}/100g) = \frac{I_{PL} \times A \times m}{I_{\text{TPP}} \times 100} \quad (1)$$

where I_{PL} and I_{TPP} are the signal integrals of phospholipids and triphenylphosphate, respectively, A is the μmol of the internal standard, and m the amount in mg of olive oil. The results may be given in mg/kg whenever the molecular weight of each phospholipid is known. The latter depends on the fatty acids that esterify glycerol at *sn*-1 and *sn*-2 positions. The identity and percentage of each fatty acid were obtained from ^{1}H NMR.

Validation of the ^{31}P NMR Methodology. The ability of ^{31}P NMR for quantitative analysis has been tested rigorously in earlier reports (*13, 14, 30*). However, we found it necessary to validate once more this spectroscopic technique for the determination of phospholipids in olive oil by comparing the amounts of a pure phospholipid samples determined by ^{31}P NMR with the weighed ones. Comparison of the NMR data with known amounts of phospholipids **1**, **2** and **5** at the specified range (0.1−15.0 μmol/0.5 mL) is shown in **Table 1**. Linear regression of the data in **Table 1** resulted in very good correlation coefficients (R), intercepts close to zero, and unity regression coefficients (slopes). The method showed good linearity over the concentration range of phospholipids found in the studied oils.

The repeatability and reproducibility of our ^{31}P NMR method for the determination of phospholipids was also examined. The repeatability ($\%CV$ = 2.74) was calculated by recording five consecutive spectra on the same day (intraday runs) and using the same solution of solvents CDCl$_3$/MeOH/D$_2$O-EDTA-Cs$^+$

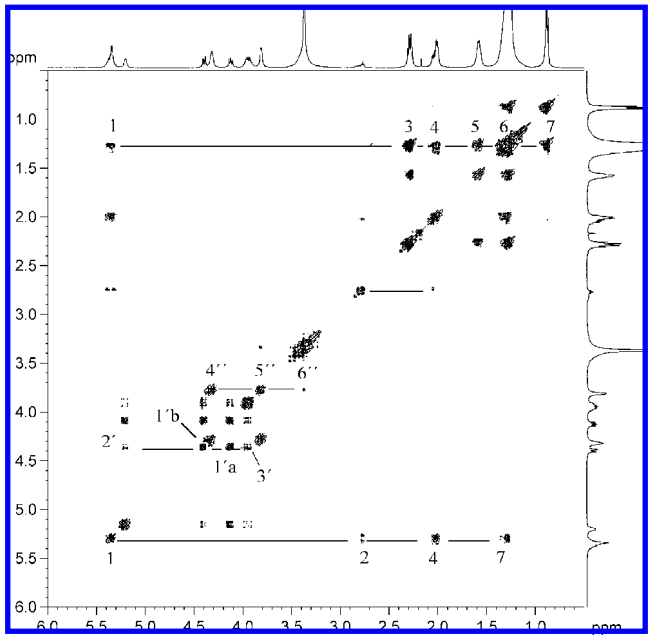

Figure 5. TOCSY spectrum (500 MHz) of phosphatidylcholine **2** in chloroform-*d*.

containing 6 μmol (4.5 mg) of **1**, whereas the repeatability (*%CV* = 2.84) was estimated by performing measurements on different days (interday runs) on five different samples of **1**, using the same experimental protocol for each measurement.

The limit of detection (LOD) and limit of quantification (LOQ) were determined by analyzing decreasing concentrations of phospholipids until the NMR signal-to-noise ratio reaches the minimum value 3. The LOD with the present instrumentation ranged from 0.25 μmol/mL to 1.24 μmol/mL and it was dependent on the phospholipids. The sensitivity of the ^{31}P NMR method is inferior to that estimated by TLC and HPLC measurements by a factor of 10−70 (*17, 31*). For instance, the LOD for phosphatidylcholine was estimated to be 0.1 mg/mL, 0.02 mg/mL, and 1.4 mg/mL by TLC, HPLC, and ^{31}P NMR, respectively (*17*). The estimated LOD for the same phospholipids in this study was 1.2 mg/mL.

Phospholipids Concentration in Olive Oils. Table 2 contains the phospholipids and their concentration determined in olive oils by the present ^{31}P NMR method. Inspection of **Table 2** reveals the following trends: the dominant phospholipid in olive oils is the phosphatidic acid (**6**), whereas phospholipids **1** and **2** are found in one olive oil sample, and in small proportions. This finding is in contrast to a recent report (*31*) asserting that the most abundant phospholipids in olive oil are phosphatidylcholine and phosphatidylethanolamine. No phosphatidylserine is detected contrary to what was observed in an earlier study (*4*). Moreover, the presence of the lyso-phosphatidic acid and lyso-phosphatidylinositol in olive oils is reported for the first time. No phospholipids were detected in the three refined olive oil samples analyzed in this study. It is likely that phospholipids were removed upon refining. A large diversity of phospholipids with high concentrations was found in olive-pomace oil. This observation is in accord with an earlier report (*32*) that phospholipids can be found mainly in the olive kernel. Although more data should be collected to draw definite conclusions, the present measurements show that it is unlikely that correlations can be observed between phospholipids and the geographical origin and/or varietal characteristics of olive oils.

The concentration range of the phospholipids in the present virgin olive oil samples was estimated to be 11−157 mg/kg, by taking into account the data in **Table 2**, the fatty acid profile of phospholipids in **Table 4**, and assuming that 50% of the unsaturated fatty acids were removed in the lyso-phospholipids. These results are not very far from those calculated indirectly by means of the total phosphorus content; 21−124 mg/kg in ref *3*, and 40−135 mg/kg in ref *33*.

Fatty Acid Composition in Phospholipids. It is interesting at this stage to estimate the fatty acid composition in phospholipids extracted from olive oils and to compare this parameter with the fatty acid profile in triacylglycerols of the same olive oil samples. The best way to do this without the need for hydrolyzing the acyl chains in glycerol is high-resolution ^{1}H NMR spectroscopy (*6, 34*). As an example, **Figure 4** illustrates the 500 MHz ^{1}H NMR spectra of commercial samples of **1** and **2** of unknown fatty acid composition. The spectrum of phospholipid **2** appears a bit noisy because of its lower solubility in chloroform than compound **1**. The assignment of the various signals, shown in the spectra, was made by employing the gradient-selected COSY and TOCSY experiments. As an example, **Figure 5** shows the TOCSY spectrum of **1** connecting proton nuclei that are part of the same spin network via magnetization transfer. Apart from signals corresponding to protons of the glycerol backbone, the methylene and methyl protons of the choline moiety of **1**, the methylene and amino protons of the ethanolamine group of **2**, the remaining signals denoted by letters a−f belong to the fatty acyl chains of the phospholipids (*6, 34*). The two methylene protons H1′ and H3′ are diastereotopic because of the presence of the *sn*-2 asymmetric carbon; each should appear as a doublet of doublets because of coupling with each other

Table 4. Fatty Acid Composition (% w/w) of the Triacylglycerols and the Extracted Phospholipids of Virgin Olive Oil (VOO) Samples Determined by ^{1}H NMR Spectroscopy[a]

	linolenic acid		linoleic acid		oleic acid		saturated fatty acids	
no[b]	phospholipids	VOO	phospholipids	VOO	phospholipids	VOO	phospholipids	VOO
1	0.19	0.23	5.13	5.11	80.54	79.79	14.14	14.87
2	0.25	0.32	3.92	5.20	78.45	76.23	17.38	18.57
3	0.14	0.12	5.84	6.02	82.87	81.56	11.15	12.30
4	0.18	0.20	4.74	5.44	77.96	80.56	17.12	13.80
5	0.47	0.53	2.73	4.68	80.46	78.86	16.34	15.93
6	0.19	0.26	5.23	6.59	78.11	79.51	16.47	13.64
7	0.15	0.22	3.98	5.80	80.43	78.10	15.44	15.88
8	0.11	0.22	5.68	5.13	74.83	75.00	19.38	19.64
9	0.35	0.30	3.26	4.57	80.54	79.16	15.95	15.97
11	0.13	0.17	5.69	4.57	75.53	75.37	17.65	19.70
12	0.33	0.21	7.68	7.87	76.46	77.54	15.53	14.38
13[c]	0.12	0.08	12.02	11.02	72.48	72.00	15.50	16.42

[a] ^{1}H NMR spectra were recorded in $CDCl_3/CD_3OD/D_2O$-EDTA-Cs^+ (400:80:5 v/v/v). [b] For numbering of olive oil samples, see **Table 2**. [c] Olive-pomace oil.

and with the H2′ proton. The proton H1′a at δ 4.12 shows a stronger coupling to H2′ proton than that at δ 4.41 (H1′b). The methylene protons H3′ at δ 3.94 appear as a broad signal, arising from two overlapping pairs of quartets with a very small chemical shift difference. The proton H2′ should appear as a quartet of doublets because of coupling to the four nonequivalent H1′ and H3′ glycerol backbone protons. However, splitting is not clear in the spectrum showing a broad signal (**Figure 4**). The assignment of the various protons of compound **1** is summarized in **Table 3**. The ^{1}H NMR spectrum of phospholipid **2** (**Figure 4**) is similar to that of **1**, except for the appearance of the amino protons at δ 8.46, the methane proton H5″ at δ 3.16, and the methyl protons (e) of linolenic acid at δ 0.98.

Since the fatty acid proton signals are common to the various fatty acyl chains attached at *sn*-1 and *sn*-2 positions of glycerol, the composition of the fatty acids can only be estimated by using pertinent equations that combine the various peak integrals in the spectrum (*6, 34*). The fatty acid composition in commercial phosphatidylcholine was found to be 49% oleic acid, 1% linoleic acid, and 50% saturated fatty acids (SFA), whereas the fatty acid composition in commercial phosphatidylethanolamine was estimated to be 4% linolenic acid, 66% limoleic acid, and 30% SFA. The fatty acid compositions for the other commercial phospholipids were phosphatidic acid, 100% SFA and phosphatidylinositol, 4% linolenic acid, 2% linoleic acid, 14% oleic acid, and 80% SFA. **Table 4** summarizes the fatty acid composition of phospholipids extracted from the olive oil samples and calculated in a similar way. Also, **Table 4** contains the fatty acids composition of triacylglycerols of olive oil samples, from which phospholipids were extracted. The amount of each fatty acid was calculated as before by using ^{1}H NMR spectroscopy. As can be seen, the fatty acids concentration in phospholipids is similar to that found in triacylglycerols. This pattern is in agreement with an earlier study (*31*). The similarity of the fatty acid profiles between the phospholipids and normal lipids in virgin olive oils is expected since these lipids have a common biosynthetic pathway (*35*). According to Kennedy's biosynthetic scheme, positions *sn*-1 and *sn*-3 of glycerol are occupied not only by saturated acyl chains but also by unsaturated ones since the unsaturated chains (especially the oleoyl chains) are in high proportions in olive oil. However, the formation of phospholipids precedes that of triacylglycerols. Therefore, the void *sn*-3 position of glycerol after the removal of the phosphate group (by the dephosphorylating enzyme PAP) can be occupied by either saturated or unsaturated acyl chains, thus retaining in triacylglycerols the same proportion of acyl chains made up initially in phospholipids.

In summary, this work showed that quantitative ^{31}P NMR spectroscopy was suitable and valid for the detection and quantification of phospholipids in olive oils. It requires minimal sample preparation and a readily available internal standard. Nevertheless, the low concentration of phospholipids in olive oil results in lengthy acquisitions (average 1 h) to achieve a reasonable S/N ratio. Moreover, this study revealed that, in contrast to what was believed, the main phospholipids in olive oil were **3**, **6**, and **7** and that the fatty acid composition of phospholipids and triacylglycerols of an olive oil sample is the same.

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