Novel Approach to the Detection and Quantification of Phenolic Compounds in Olive Oil Based on ^{31}P Nuclear Magnetic Resonance Spectroscopy

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^{31}P NMR spectroscopy has been employed to detect and quantify phenolic compounds in the polar fraction of virgin olive oil. This novel analytical method is based on the derivatization of the hydroxyl and carboxyl groups of phenolic compounds with 2-chloro-4,4,5,5-tetramethyldioxaphospholane and the identification of the phosphitylated compounds on the basis of the ^{31}P chemical shifts. Quantification of a large number of phenolic compounds in virgin olive oil can be accomplished by integration of the appropriate signals in the ^{31}P NMR spectrum and the use of the phosphitylated cyclohexanol as internal standard. Finally, the validity of this technique for quantitative measurements was thoroughly examined.

KEYWORDS: Olive oil; phenolic compounds; ^{31}P NMR spectroscopy

INTRODUCTION

Phenolic compounds, formerly referred to as polyphenols, are an important class of minor components of olive oil (*1–3*). These compounds, acting as natural antioxidants against oxidation caused by the atmospheric oxygen, contribute significantly to olive oil's superior oxidative stability among the edible oils (*1–5*). Furthermore, phenolic compounds are directly linked to both nutritional and sensorial characteristics of olive oil (*1, 2, 6*). In addition, several epidemiological studies have demonstrated that phenolic compounds afford considerable protection against cancer (breast, skin, colon), coronary heart disease, and aging by inhibiting oxidative stress (*2, 7, 8*). The major phenolic compounds identified and quantified in olive oil can be classified into four categories: phenols, such as hydroxytyrosol, tyrosol, caffeic acid, and ferulic acid; the secoiridoids oleuropein glucoside, ligstroside, and their hydrolysis products; the lignans (+)-pinoresinol, (+)-1-acetoxypinoresinol and the newly found syringaresinol (*9*); and the flavonoids apigenin, luteolin, quercetin, and their glucoside derivatives. The composition of the phenolic fraction of olive oil is very complex depending on several factors, such as the variety and degree of ripeness of the olive fruit, geographical origin, climatic conditions, harvesting period, agricultural practices, and extraction procedure (*10–16*).

Various nonspecific and specific analytical methods have been employed in the past to quantify phenolic compounds. The well-established colorimetric method based on the Folin–Ciocalteu reagent (*17*) offers quantitative information for total phenolic compounds, although responses of single phenols to this reagent are significantly different from each other, thus making this
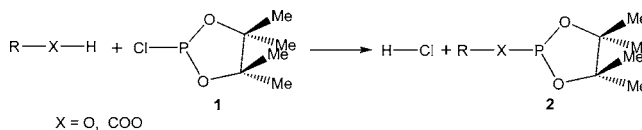

Figure 1. Reaction of hydroxyl and carboxyl groups of phenolic compounds with 2-chloro-4,4,5,5-tetramethyldioxaphospholane (**1**).

method less accurate than expected (*18*). Liquid/liquid solvent extraction followed by gas chromatography (GC) and especially liquid/liquid solvent extraction followed by high-performance liquid chromatography (HPLC) are specific for most phenolic compounds and give better results with very low detection limits (*1–3, 7, 8, 18–21*). Recent advancements in this field comprise solid-phase extraction (*22*) and hyphenated techniques, such as GC-MS, HPLC-MS, and HPLC-NMR (*9, 23–25*).

In this study, we have investigated an alternative methodology to detect and quantify phenolic compounds in olive oil by employing phosphorus-31 NMR spectroscopy. This method, introduced in an earlier publication (*26*), is based on the derivatization of the labile hydrogens of the hydroxyl and carboxyl groups of phenolic compounds by the phosphitylating reagent 2-chloro-4,4,5,5-tetramethyldioxaphospholane (**1**) according to the reaction scheme shown in **Figure 1** and the use of the ^{31}P chemical shifts to identify the labile centers (compound **2**). Compound **1** reacts rapidly (~15 min) and quantitatively under mild conditions within the NMR tube with the hydroxyl and carboxyl groups. This quantitative method has been already applied to determine diacylglycerols, total free sterols content, and free acidity of olive oil (*27, 28*). ^{31}P NMR spectra of phosphitylated phenolic compounds in olive oil were assigned on the basis of the ^{31}P chemical shifts of a large number of polyphenol-containing olive oil model compounds, the latter being determined by employing one- and two-dimensional (1D

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and 2D, respectively) NMR techniques (*29*). Assignment of additional phosphitylated polyphenol model compounds is presented in this study. Further validation tests of this technique have been performed with respect to known weights of model compounds.

MATERIALS AND METHODS

Samples, Reagents, and Reference Compounds. Four virgin olive oil samples (years of harvesting 2003−2004) from Messinia (samples 1 and 2) and Lakonia (samples 3 and 4) were used in the present study. Samples from Messinia were extracted from the olive variety Koroneiki, whereas those from Lakonia originated from the variety Athinolia. All samples, which were collected ∼48 h after extraction and stored in colored bottles at −20 °C, were virgin olive oils according to the official analytical methods and limits (*30, 31*).

Reference materials used in this study were as follows: Oleuropein, apigenin, apigenin-7-*O*-glucoside, apigenin-7-*O*-rutinosode (isorhoifolin), luteolin, and luteolin-7-*O*-glucoside were purchased from Extrasynthese (Genay France). Tyrosol, vanillin, homovanillyl alcohol, glycerol, and D-glucose were obtained from Sigma-Aldrich (Athens, Greece), whereas (+)-pinoresinol was provided by Separation Research (Turku, Finland). These materials were used without further purification. (+)-1-Acetoxypinoresinol, syringaresinol, and maslinic acid were isolated from olive oil extraction procedures. Protonated solvents for synthesis (reagent or analytical grade) and deuterated chloroform and pyridine solvents were purchased from Sigma-Aldrich (Athens, Greece). The derivatizing phosphorus reagent was synthesized from pinacol and phosphorus trichloride in the presence of triethylamine following the method described in the literature (*32*). However, to increase the yield of the reaction, we utilized hexane instead of benzene and pyridine instead of triethylamine, as suggested in the original method. This modification resulted in ∼45% yield of the product against 19% obtained with the original method. Hydroxytyrosol was synthesized upon reduction of 3,4-dihydroxybenzylacetic acid with LiAlH$_4$ in THF solution following the method described in the literature (*33*): ^{1}H NMR [acetone-d_6] δ 3.67 (t, $^3J_{1', 2'}$ = 7.1 Hz, 2H, H-1′), 2.65 (t, $^3J_{1', 2'}$ = 7.1 Hz, 2H, H-2′), 6.71 (d, $^4J_{2, 6}$ = 2.0 Hz, 1H, H-2), 6.71 (d, $^3J_{5, 6}$ = 8.0 Hz, 1H, H-5), 6.54 (dd, $^3J_{5, 6}$ = 8.00 Hz, $^4J_{2, 6}$ = 2.0 Hz, 1H, H-6); ^{13}C NMR [acetone-d_6] δ 63.61 (C-1′), 39.10 (C-2′), 131.37 (C-1), 115.31 (C-2), 145.05 (C-3), 143.49 (C-4), 116.25 (C-5), 120.43 (C-6).

Extraction of Minor Polar Components from Olive Oil. Phenolic compounds were extracted following the method developed by Montendoro et al. (*6*) using 35 g of olive oil and a mixture of ethanol/water (80:20, v/v). The polyphenol extracts were used immediately for sample preparation and ^{31}P NMR measurements.

Isolation of Lignans. A sample of olive oil containing the smallest amount of hydroxytyrosol was selected for the isolation of the lignans (+)-pinoresinol, (+)-1-acetoxypinoresinol, and syringaresinol. The olive oil was extracted initially with a mixture of methanol/water (50:50, v/v), and the lignans in the extract were separated by preparative thin-layer chromatography (PLC). Separation was conducted on glass plates (20 × 20 cm) precoated with silica gel 50 F$_{254}$ (Machery-Nagel). The extract (1 mL) was applied to the origin of the plate and developed in the mobile phase hexane/ethyl acetate (40:60, v/v) for 2 h, up to a height of 11.5 cm on the plate. Bands on the PLC plates were detected under UV light (254 nm). (+)-Pinoresinol eluted with R_f = 0.48, (+)-1-acetoxypinoresinol with R_f = 0.43, and syringaresinol with R_f = 0.30 cm. Finally, bands were scraped from the plates, pooled, and extracted three times with methanol, and the solvent was removed under reduced pressure.

Isolation of Maslinic Acid. A sample of olive oil containing the smallest amount of monoacylglycerols was selected for the isolation of maslinic acid. Olive oil was extracted initially with a mixture of methanol/water (50:50, v/v) for the removal of a large portion of phenols, followed by a second extraction with methanol/water (70:30, v/v) to minimize the coextraction of monoacylglycerols. Maslinic acid was obtained from the aqueous phase by distillation under reduced pressure. No attempt was made for further purification of the solid material.

Sample Preparation for Spectroscopic Analysis. A stock solution was prepared by dissolving 0.6 mg of chromium acetylacetonate, Cr-(acac)$_3$ (0.165 μM), and 13.5 mg of cyclohexanol (13.47 mM) in 10 mL of a mixture of pyridine and CDCl$_3$ solvents (1.6:1.0, v/v) and protected from moisture with 5A molecular sieves. An amount of 0.1−3.0 mg of each model compound, depending on its molecular weight and the number of hydroxyl groups, and/or 35−40 mg of the polar extract obtained from 35 g of olive oil was dissolved in 0.4 mL of stock solution, in which 50 μL of reagent 1 was added. The mixture was added directly into the 5 mm NMR tube and was left to react for ∼15 min at room temperature. Upon completion of the reaction, the solution was used to obtain the ^{31}P NMR spectra.

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. Some details of the two-dimensional NMR experiments in the present study are given below. Additional information for gradient COSY and HMQC experiments are given elsewhere (*34*).

^{13}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, 10 kHz; relaxation delay, 30 s; memory size, 16K (zero-filled to 32K). To ensure quantitative spectra, the magnitude of the relaxation delay adopted was >5 times the relaxation time (4.9 s) of the phosphitylated cyclohexanol. Line broadening of 1 Hz was applied, and drift correction was performed prior to Fourier transform. A polynomial fourth-order baseline correction was performed before integration. For each spectrum, 32 transients were accumulated. All ^{31}P chemical shifts are relative to the product of the reaction of **1** with water (moisture contained in all samples), which gives a sharp signal in pyridine/CDCl$_3$ at δ 132.20. It should be noted that the presence of the paramagnetic metal center of Cr(acac)$_3$ in the samples lowers the relaxation times of the phosphorus nuclei, thus shortening the duration of the measurements significantly.

Gradient ^{1}H−^{1}H Homonuclear Spectroscopy (g-H−H-COSY). Gradient selected ^{1}H−^{1}H COSY spectra were obtained by using 128 increments of 1K data points. Sixteen scans and eight dummy scans were acquired for each FID with a recycle delay of 1.0 s. The data set was 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.

Gradient ^{31}P−^{31}P Homonuclear Spectroscopy (g-P−P-COSY). Gradient selected ^{31}P−^{31}P COSY spectra were obtained with proton decoupling using 128 increments of 1K data points. Eight scans and four dummy scans were accumulated for each FID with a recycle delay of 1.0 s. The data set was 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.

Gradient ^{1}H−^{13}C Heteronuclear Multiple Quantum Coherence (g-H−C-HMQC). The gradient selected hydrogen−carbon HMQC spectra were acquired with 64 increments and 1K data points. The heteronuclear (^{13}C/^{1}H) GARP composite pulse decoupling was used during acquisition. Thirty-two scans and four dummy scans were accumulated for each FID with a recycle delay of 1.0 s. Before Fourier transformation, the data set was zero-filled to a 1K × 1K matrix. A squared sinusoidal window function was used in both dimensions. No phase correction was applied, and the 2D spectra were displayed in magnitude mode.

Gradient ^{1}H−^{31}P Heteronuclear Multiple Quantum Coherence (g-H−P-HMQC). The gradient selected hydrogen−phosphorus HMQC spectra were acquired with 64 increments and 1K data points. The experiment was optimized for long-range proton−phosphorus couplings by setting the evolution delay to 50 ms, corresponding to a coupling constant of $J_{H,P}$ ∼ 10 Hz. Sixteen scans and eight dummy scans were accumulated for each FID with a recycle delay of 1.0 s. Before Fourier transformation, the data set was zero-filled to a 1K × 1K matrix. A squared sinusoidal window function was used in both dimensions. No phase correction was applied, and the 2D spectra were displayed in magnitude mode.

Table 1. ^{31}P NMR Chemical Shifts and Multiplicities (in Parentheses) of Phosphitylated Polyphenol Model Compounds

compound	hydroxyl groups[b]	compound	hydroxyl group[b]
homovanillic acid	139.94 134.80 (COOH)	glycerol	1, 3, 147.37; 2, 146.32
homovanillyl alcohol	1, 139.84; 2, 147.00	1-monoacylglycerol	1, 147.64; 2, 146.45
vanillin	138.98	2-monoacylglycerol	148.00
apigenin	5, 136.46; 7, 137.43; 4′, 137.88	maslinic acid	1, 145.97; 2, 147.67; 134.95 (COOH)
apigenin-7-*O*-glucoside	5, 136.50; 4′, 137.85	α-D-glucopyranose	1, 146.70; 2, 146.79; 3, 147.28; 4, 148.08; 6, 147.91
isorhoifolin	4′, 137.84; 5, 136.41	β-D-gucopyranose	1, 145.71; 2″, 147.67; 3, 147.28; 4, 148.32; 6, 147.16
luteolin	5, 136.47; 7, 137.52; 4′, 138.60 (d); 3′, 139.80 (d)	oleuropein glucoside	1, 139.18 (d); 2, 139.11 (d); 2″, 147.30; 3″, 147.56; 4″, 148.49; 6″, 148.32
luteolin-7-*O*-glucoside	5, 136.52; 4′, 138.61 (d); 3′, 138.81 (d)		
(+)-pinoresinol	139.84		
(+)-1-acetoxypinoresinol	4′, 140.03; 4″, 139.86		
syringaresinol	142.89		

[a] Referenced from the phosphitylated water peak at 132.20 ppm. [b] Singlets unless otherwise stated.

The gradient selected hydrogen−phosphorus heteronuclear multiple bond correlation experiment (*g*-H−P-HMBC) to obtain long-range H−P couplings was not used. In fact, this experiment was not necessary, because the present phosphitylated compounds have no direct H−P couplings to be suppressed. Also, it is worth mentioning that 2D NMR experiments performed by using a larger number of increments (e.g., 256 for *g*-H−H-COSY and/or *g*-P−P-COSY and 128 for *g*-H−C-HMQC and *g*-H−P-HMQC) did not offer additional information. Therefore, we decided to use 128 and 64 increments to speed the analysis. The presentation of the gradient 2D spectra in the amplitude mode may be a disadvantage for acquiring complex spectra. Nevertheless, this is not the case for the present simple molecules.

RESULTS AND DISCUSSION

[31]P Chemical Shifts of Phosphitylated Model Compounds.
[31]P chemical shifts of most phosphitylated polyphenol model compounds have been assigned in a previous publication (*29*) and are summarized in Table 1 of this reference. It should be noted that the ^{31}P chemical shifts of the two phosphitylated hydroxyl groups of caffeic acid published previously (*29*) are incorrect and should be replaced by δ 139.58 and 138.63 for the phosphitylated hydroxyl groups OH-1 and OH-2, respectively. [31]P chemical shifts for the phosphitylated hydroxyl and carboxyl groups of additional model compounds used in the present study are summarized in **Table 1**, whereas their structural formulas and numbering system are depicted in **Figure 2**.

Assignment of ^{31}P signals of the phosphitylated (+)-pinoresinol and syringaresinol, each bearing equivalent hydroxyl groups (**Figure 2**), was not difficult. The lignan (+)-1-acetoxypinoresinol with two nonequivalent hydroxyl groups (**Figure 2**) shows two separate signals in its ^{31}P NMR spectrum, which were assigned unambiguously following the cross-peaks in the *g*-H−P-HMQC spectrum (not shown) connecting the magnetically nonequivalent aromatic protons H-5′ (δ 7.09) and H-5″ (δ 7.17) with the phosphorus-31 signals at δ 140.03 and 139.86 for the phosphitylated hydroxyl groups OH-4′ and OH-4″, respectively ($J_{H-5',P-4'} = J_{H-5'',P-4''} = 0.65$ Hz). Full assignment of the ^{1}H NMR spectrum of (+)-1-acetoxypinoresinol has been reported in previous publications (*7, 35*). The assignment of the ^{31}P signals of homovanillic acid, homovanillyl alcohol, and vanillin (**Table 1**) presented no serious problems, because the phosphitylated aromatic and aliphatic hydroxyl groups, as well as the phosphitylated carboxyl group, appear in different regions (*29*).

The ^{31}P NMR spectrum of phosphitylated glycerol shows two singlets at δ 147.37 and 146.32 with an intensity ratio of 2:1. These signals were assigned to the derivatized primary and secondary hydroxyl groups, respectively.

Oleuropein glucoside is a heterosidic ester of elenolic acid with hydroxytyrosol. Therefore, the assignment of the aromatic phosphitylated hydroxyl signals was obtained by a direct comparison of its ^{31}P NMR spectrum with that of free hydroxytyrosol (*29*). Four additional signals appear at low magnetic field strength due to the phosphitylated hydroxyl groups of the attached glucose moiety. A coupled ^{31}P NMR spectrum (not shown) differentiated immediately the primary hydroxyl group at the C-6 carbon. A doublet of doublets appeared in the spectrum at δ 148.32 due to the phosphorus coupling with the two diastereotopic protons H-6a″ and H-6b″ ($J_{H-6a'',P} = 13.84$, $J_{H-6b'',P} = 11.29$ Hz). The remaining signals appear as doublets owing to the vicinal coupling of each phosphorus nucleus with a single ring proton ($J_{H-2,P} = J_{H-4,P} = 9.27$ Hz and $J_{H-3,P} = 8.34$ Hz). **Figure 3A** shows an expansion of the *g*-H−H-COSY spectrum of the phosphitylated oleuropein glucoside in pyridine/chloroform solvents in the region where proton signals of the glucose segment appear. Following the cross-peaks pattern starting from the anomeric proton H-1″, which resonates at the lowest magnetic field strength, all glucose protons signals can be assigned unambiguously. Next, the phosphitylated hydroxyl groups of the sugar moiety can be assigned by performing a simple *g*-H−P-HMQC experiment. The cross-peaks between the phosphitylated OH-6 group at δ 148.32 and protons H-6a″ and H-6b″ in the spectrum shown in **Figure 3B** confirm the correct assignment of this signal. The other three signals at δ 147.30, 147.56, and 148.49 were attributed to the phosphitylated hydroxyl groups OH-2, OH-3, and OH-4 connected with ring protons H-2″, H-3″, and H-4″, respectively (**Figure 3B**).

The assignment of the phosphitylated luteolin molecule, which contains four hydroxyl groups at positions 5, 7, 3′, and 4′ (**Figure 2**), can be easily performed on the basis of ^{31}P chemical shifts of quercetin bearing four hydroxyl groups at the same positions as luteolin and one additional hydroxyl group at position 3 (*29*). It should be noted that the assignment of the phosphitylated hydroxyl groups OH-5 and OH-7 cited (*29*) should be reversed. This stems from the fact that the phosphitylated hydroxyl group OH-7 disappears in the ^{31}P NMR spectra

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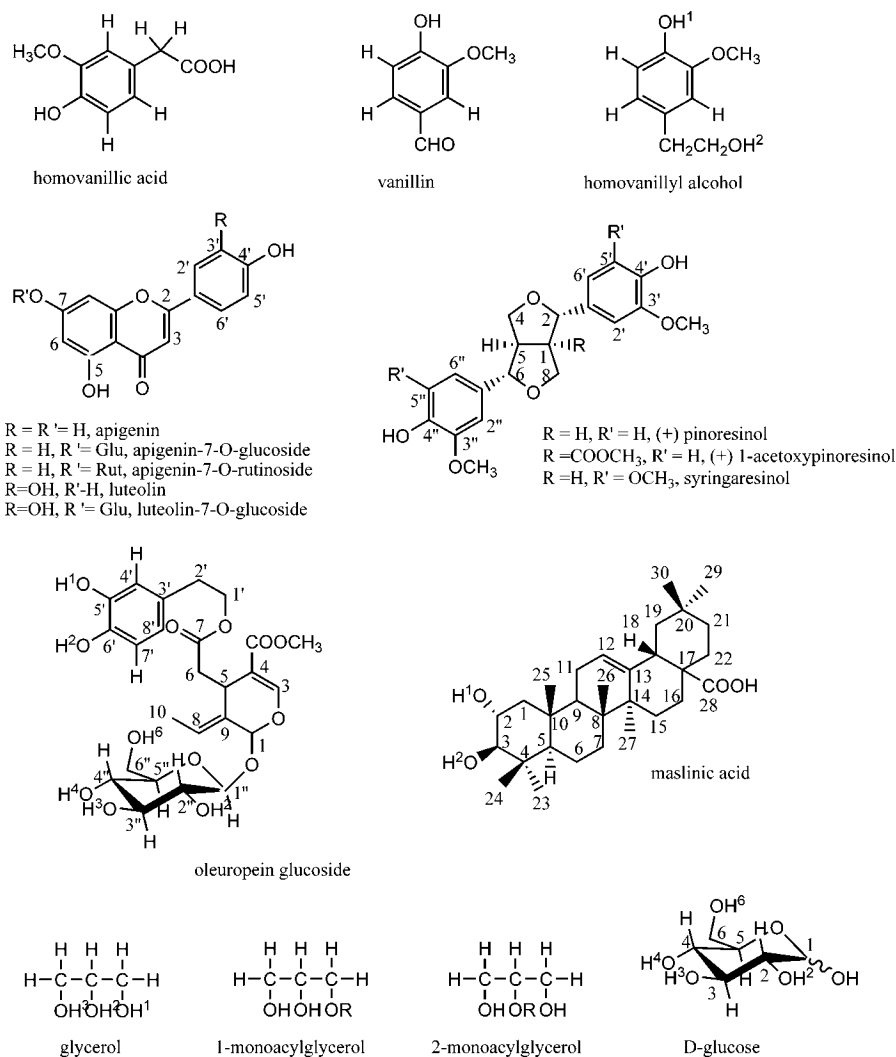*J. Agric. Food Chem.,* Vol. 54, No. 3, 2006   **659**

Figure 2. Chemical structures and numbering system of polyphenol model compounds used in this study.

of luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, and isorhoifolin (apigenin-7-*O*-rutinoside) (not shown). Four ^{31}P signals for each of the luteolin and apigenin glucosides and six signals for isorhoifolin at low magnetic field strength (below δ 145.30) belong to phosphitylated hydroxyl groups of the attached glucose and rutinose (6-*O*-α-L-ramnopyranosyl-D-glucose) moieties, respectively (**Table 1**), No further attempt was made to assign these signals.

It is well-known (*36*) that aqueous solutions of D-glucose contain in equilibrium the stable tautomers α-D- and β-D-glucopyranose at concentrations of 38 and 62%, respectively, whereas in pyridine solutions the tautomeric composition changes dramatically to 47% for α-D-glucopyranose and to 50% for β-D-glucopyranose (*36*). Both tautomers were observed in the mixture of solvents chloroform/pyridine after equilibration, α-D-glucose being the preponderant tautomer (58% for α-D-glucopyranose and 42% for β-D-glucopyranose). After addition of phosphorus reagent and completion of the phosphitylation reaction, the mixture composition remained unaltered for >1 week. The ^{31}P NMR spectrum of the mixture showed nine signals instead of ten, because the signals of the phosphitylated OH-3 groups of both tautomers coincided (**Figure 4B**). Signals ascribed to α-D-glucopyranose were easily identified by comparing the ^{31}P NMR spectrum of the mixture (**Figure 4B**) with that of a fresh solution of phosphitylated α-D-glucopyranose (**Figure 4A**). Our strategy and accompanying 1D and 2D experiments for the assignment of the various signals of the

mixture of α-D- and β-D-glucopyranose in the ^{31}P NMR spectrum are available as Supporting Information.

Maslinic acid is not a polyphenol. Its presence in virgin olive oil has been detected by employing the hyphenated technique HPLC-SPE-NMR (*9*). This molecule contains two hydroxyl groups and one carboxyl group (**Figure 2**), and therefore phosphitylation of the polar fraction of olive oil is expected to show three additional signals. Assignment of the phosphitylated hydroxyl groups of maslinic acid was afforded by recording a *g*-H−P-HMQC spectrum (**Figure 5**) in which the phosphitylated hydroxyl groups were connected with proton signals at δ 3.63 (dd) and 4.26 (m) assigned to H-3α and H-2β protons, respectively (*9*, *26*). Signals due to the phosphitylated hydroxyl group of the internal standard cyclohexanol, as well as those of 1- and 2-monoacylglycerol impurities were observed in the 2D spectrum (**Figure 5**).

Concentration and Temperature Effects on the ^{31}P Chemical Shifts. Because several phenolic compounds in olive oil occur in smaller amounts than those used for the spectroscopic analysis of model compounds, it was found to be appropriate to examine possible concentration effects on the ^{31}P chemical shifts. Accordingly, several ^{31}P NMR spectra were recorded by changing the concentration of model compounds. No significant differences in the chemical shifts were observed with changing concentration. For instance, no measurable chemical shift changes (0.01−0.03 ppm) were observed when the tyrosol and hydroxytyrosol concentrations were increased by a factor of 400
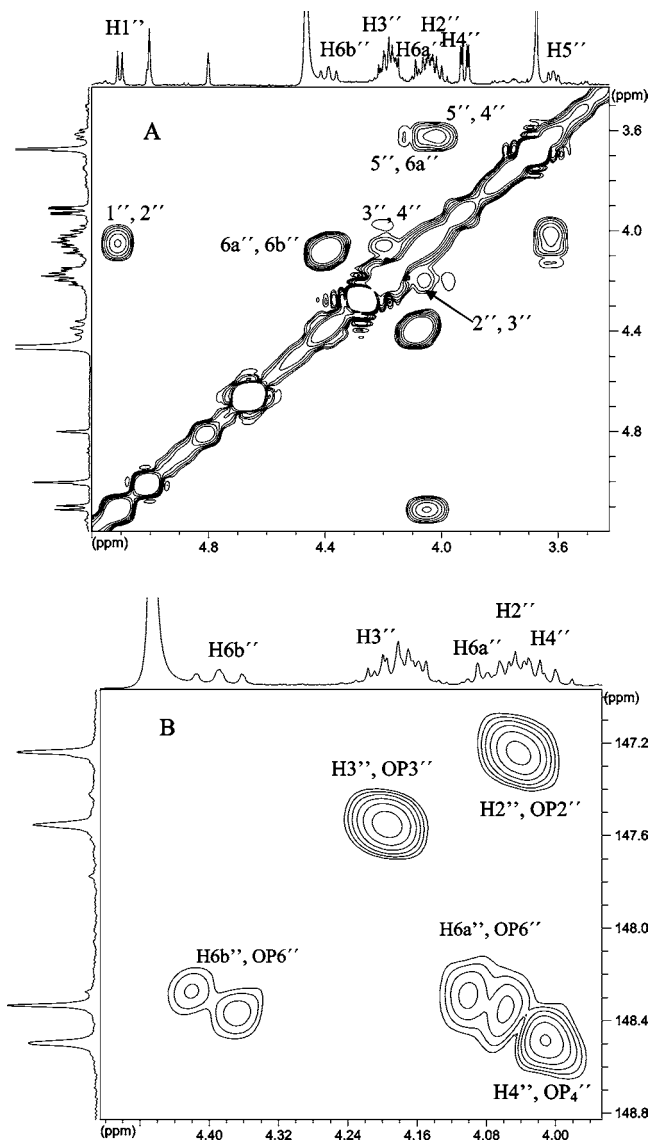
Figure 3. (**A**) *g*-H–H-COSY spectrum and (**B**) *g*-H–P-HMQC spectrum of the phosphitylated oleuropein glucoside in chloroform/pyridine solution. The regions of ¹H and ³¹P NMR signals of *β*-D-glucose are shown.
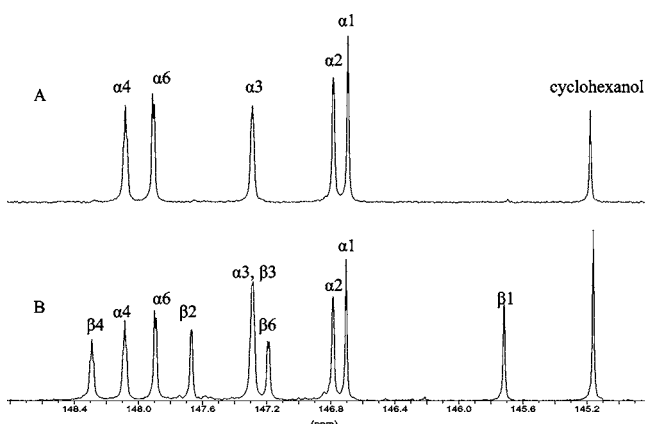

Figure 4. 202.2 MHz ³¹P NMR spectra of (**A**) α-D-glucopyranose and (**B**) an equilibrium mixture of α-D-glucopyranose and *β*-D-glucopyranose in chloroform/pyridine solution. α and *β* denote signals of α- and *β*-D-glucopyranose, respectively.

(from 0.05 to 20 *μ*mol/0.4 mL). Nevertheless, somewhat larger changes in the chemical shifts are expected owing to different
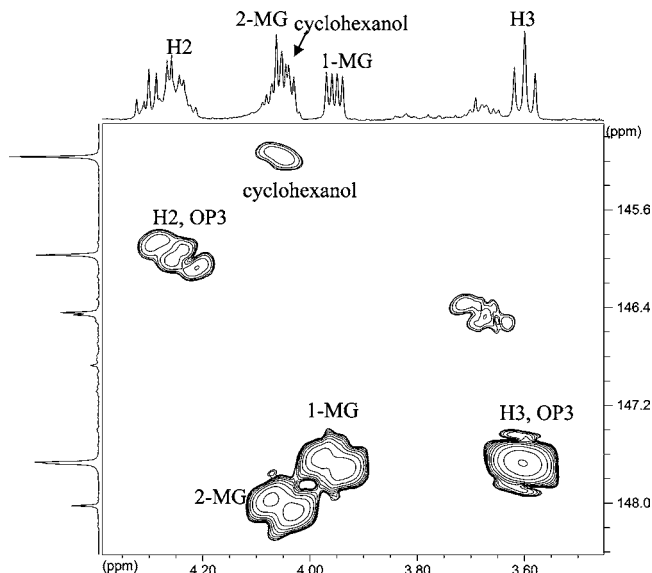

Figure 5. *g*-H–P-HMQC spectrum of the phosphitylated maslinic acid in chloroform/pyridine solution. The region of the ¹H and ³¹P NMR signals of the phosphitylated hydroxyl signals is shown. Signals of the phosphitylated hydroxyl groups of the internal standard cyclohexanol and 1- and 2-monoacylglycerols are observed in the spectrum.

polarity of the polar fraction of olive oil as compared to that of solutions of isolated phenols. Measurements of the chemical shifts of hydroxytyrosol and tyrosol at 20 and 30 °C showed a small upfield shift with temperature, ranging from 0.02 to 0.06 ppm. However, as the temperature in the probe was controlled within ±1 °C, stray fluctuations at ambient temperature would not affect the chemical shift values.

Quantitation, Sensitivity, Repeatability, and Reproducibility of the ³¹P NMR Methodology. The applicability of the ³¹P NMR method to quantitative analysis has been tested against ¹H NMR spectroscopy and known weights of standards (*26, 29*). To further check the ability of this method to quantify phenolic compounds in olive oil, we repeatedly recorded ³¹P NMR spectra for a series of solutions prepared according to the protocol described previously upon adding small amounts of standard hydroxytyrosol and tyrosol at a concentration range from 0.05 to ~20 *μ*mol/0.4 mL of solvent mixture and keeping fixed the concentration of the internal standard of cyclohexanol. **Figure 6** shows typical ³¹P NMR spectra of hydroxytyrosol and tyrosol. The aromatic phosphitylated hydroxyl groups of hydroxytyrosol form an AB spectrum due to the five strong bonds (homoallylic coupling) between the neighboring phosphorus nuclei (*29*). When the ratios of the signal integrals of the phosphitylated hydroxyl groups of hydroxytyrol and tyrosol over the phosphitylated cyclohexanol signal integral at various concentrations were plotted against the corresponding concentrations, linear relationships were observed (not shown) with very good correlation coefficients ($R = 0.998-0.999$) and other statistical parameters. From these results, and assuming a minimum workable sample volume of 0.4 mL and a minimum signal-to-noise ratio of 3, the minimum detectable amounts of tyrosol and hydroxytyrosol is on average 0.06 *μ*mol/0.4 mL of mixture of solvents. Taking into account that 35 g of olive oil was extracted and dissolved in 0.4 mL of solvent, the detection limit of ³¹P NMR spectroscopy was calculated to be 0.17 *μ*mol/100 g of olive oil. Because the expected molecular weights of phenolic compounds in olive oil lie between 150 and 500 Da, the detection limit expressed in ranges from 0.26 to 0.85 mg/kg for the present instrumentation. The repeatability of eight
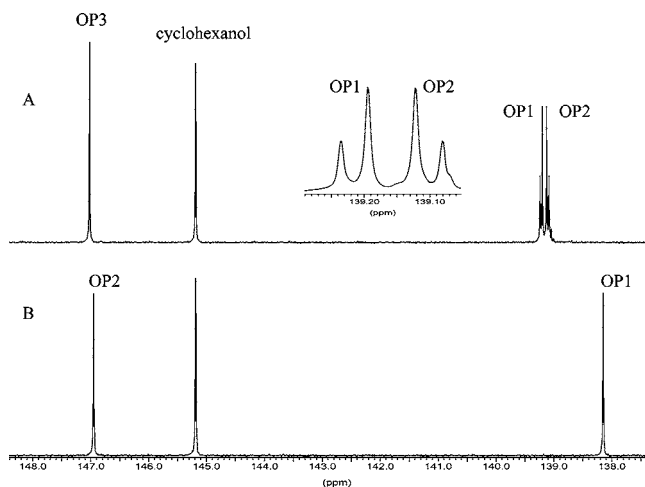
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Figure 6. 202.2 MHz ^{31}P NMR spectra of (**A**) hydroxytyrosol and (**B**) tyrosol in chloroform/pyridine solutions. (Inset) AB spectrum formed for the two phosphitylated neighboring aromatic hydroxyl groups of hydroxytyrosol.

Figure 7. 202.2 MHz ^{31}P NMR spectra of (**A**) a mixture of phosphitylated monohydroxyphenols and (**B**) a mixture of phosphitylated polyhydroxyphenols in chloroform/pyridine solutions: (1) *o*-coumaric acid; (2) *p*-coumaric acid; (3) ferulic acid; (4) caffeic acid; (5) 3-hydroxy-4-methoxycinnamic acid; (6) sinapic acid; (7) *p*-hydroxybenzoic acid; (8) vanillic acid; (9) protocatechuic acid; (10) gallic acid; (11) syringic acid; (12) gentisic acid; (13) *p*-hydroxyphenylacetic acid; (14) homoprotocatechuic acid; (15) tyrosol; (16) hydroxytyrosol; (17) shikimic acid; (18) homovanillic acid; (19) apigenin; (20) oleuropein.

consecutive spectra using the same sample of phosphitylated hydroxytyrosol was found to be 1.04%, whereas the reproducibility (2.44%) was tested by performing measurements on six different hydroxytyrosol samples and using the same experimental protocol for each measurement. These values are not different from those obtained previously (*26*−*29*).

It is interesting at this stage to assess the repeatability of the whole methodology (extraction−phosphitylation−spectrum). In this respect, we repeated three times the whole procedure using the same olive oil sample and the same protocol for liquid−liquid extraction of the polar fraction of olive oil, sample preparation, and recording of ^{31}P NMR spectra as described. Next, we calculated the average concentration (in micromoles per 100 g of olive oil) upon integration of each unambiguously assigned signal in the ^{31}P NMR spectra, standard deviation, and relative standard deviation of measurements. Only 5 of 30 signals showed relative errors lying between 10 and 16% corresponding to concentrations of <0.4 μmol/100 g of olive oil. Twenty-two signals showed errors of <4% and three between 4 and 10%. The smallest integration error (<4%) was observed for the largest signals as expected. The overall relative standard deviation was estimated to be 3.9%. This analysis demonstrates that the present method is accurate and precise.

^{31}P Chemical Shifts and Quantitation of Mixtures of Polyphenol-Containing Olive Oil Model Compounds. Figure 7 shows the ^{31}P NMR spectrum of a mixture of several monohydroxy- and polyhydroxyphenol model compounds. No significant chemical shift change was observed for each polyphenol in the mixtures relative to that of the individual polyphenol model compound in its own solution. Integration of the ^{31}P signals and the use of the internal standard cyclohexanol allowed the quantification of each polyphenol in the mixtures. However, a few phosphitylated hydroxyl groups of different phenols gave overlapping signals in the spectrum. For instance, several phosphitylated aromatic hydroxyl groups contribute to the strong signal at δ 139.2, namely, two from each hydroxytyrosol (δ 139.25, d; and δ 139.13, d), oleuropein glucoside (δ 139.18, d; and δ 139.11, d), and homoprotocatechuic acid (δ 139.14, s) and one from protocatechuic acid (δ 139.14, d) (*29*). Quantification of these phenolic compounds was achieved by considering other signals in the spectrum, which are unique for each model compound. The concentration of hydroxytyrosol was calculated from the signal of its phos-
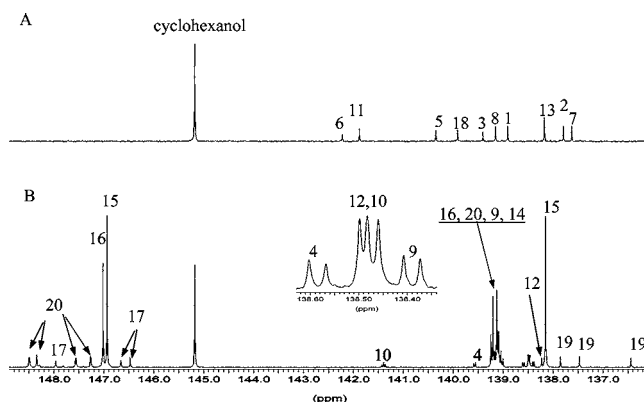
phitylated aliphatic hydroxyl group at δ 146.99 (*29*), whereas that of protocatechuic acid was calculated from the doublet at δ 138.51 assigned to the second phosphitylated aromatic hydroxyl group of this molecule (*29*). The concentration of oleuropein glucoside was estimated from the signal integrals of the phosphitylated hydroxyl groups of the β-D-glucopyranosyl ring. The difference of these integrals from that of the signal at δ 139.2 divided by 2 gave the amount of homoprotocatechuic acid. The amount of each polyphenol in the mixture calculated from ^{31}P NMR spectra was compared with that actually added to the mixture. A good linear relationship was obtained with a correlation coefficient of $r = 0.999$, slope = 1.04, and intercept = 0.02. These results comprise of a second piece of evidence that the present methodology is accurate and reproducible.

Detection and Quantitation of Phenolic Compounds in Olive Oil. Panels A and **B of Figure 8** show the ^{31}P NMR spectrum of the polar fraction of a virgin olive oil sample from Messinia in the regions where the aromatic and aliphatic phosphitylated hydroxyl groups of phenolic compounds appear, respectively. Similar spectra were obtained for the remaining three olive oil samples, although variation in concentration of phenolic compounds was observed for the reasons mentioned. The singlets at δ 136.42 (OH-5), 137.52 (OH-7), and 138.86 (OH-4′) in **Figure 8A** indicate the presence of apigenin. Also, the singlets at δ 136.46 (OH-5) and 137.56 (OH-7) and the doublet at δ 138.62 (OH-4′) reveal the presence of luteolin. The small signal at δ 139.82 appears to be half of the doublet of the phosphitylated hydroxyl group OH-3′ of luteolin, the other half being overlapped by the strong signal at δ 139.78. Spiking the sample with pure luteolin resulted in an increase of the signal intensity at 139.82, thus confirming the identity of this signal. *p*-Coumaric acid can be quantified from its phosphitylated hydroxyl group at δ 137.80 (*29*) (**Figure 8A**). The presence of this compound was verified by spiking the sample with pure substance. Tyrosol (δ 138.20), gentisic acid (δ 138.18), and *p*-hydroxyphenylacetic acid (δ 138.21) (*29*) contribute to the strong signal at δ 138.19. The concentration of the latter phenolic compound in Greek virgin olive oils is too low to be detected by the present method, whereas lack of a signal at δ
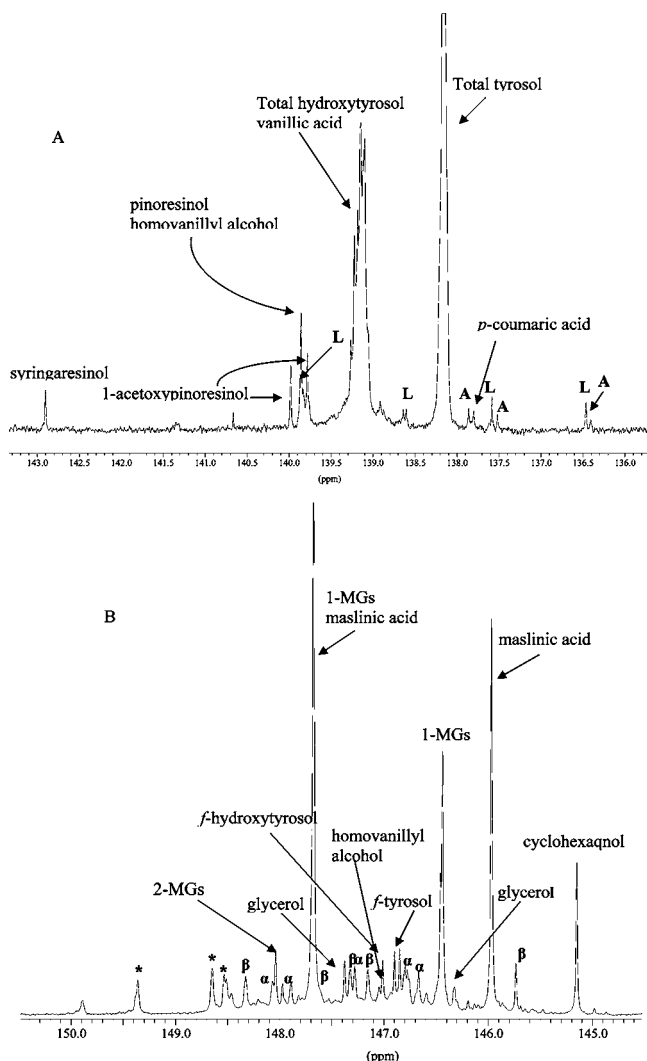
Figure 8. 202.2 MHz ^{31}P NMR spectrum of the phosphitylated polar fraction of a virgin olive oil sample from Messinia in chloroform/pyridine solution: (**A**) aromatic region; (**B**) aliphatic region. A, apigenin; L, luteolin; 1-MGs, 1-monoacylglycerols; 2-MGs, 2-monoacylglycerols; *f*-hydroxytyrosol, free hydroxytyrosol; *f*-tyrosol, free tyrosol; α, α-D-glucopyranose; β, β-D-glucopyranose. The unidentified signals of the hydrolysis products of oleuropein glucoside (and ligstroside) are denoted by asterisks.

Table 2. Quantification (in Micromoles per 100 g of Olive Oil) of Phenolic Compounds in the Polar Fraction of Virgin Olive Oil Samples by Employing the Methodology Based on ^{31}P NMR Spectroscopy

	olive oil sample			
polyphenol	1	2	3	4
free hydroxytyrosol	3.66	5.14	11.71	8.23
free tyrosol	5.60	4.97	6.89	5.06
total hydroxytyrosol	36.66	51.23	90.63	100.20
total tyrosol	64.03	48.91	76.06	63.74
esterified hydroxytyrosol	33.00	46.09	78.92	91.97
esterified tyrosol	58.43	43.94	69.17	58.68
(+)-pinoresinol	2.74	2.07	2.33	2.94
(+)-1-acetoxypinoresinol	3.49	3.14	2.29	11.10
syringaresinol	1.06	1.20	1.03	0.89
luteolin	0.37	0.57	0.60	4.90
apigenin	0.31	0.51	0.43	1.40
p-coumaric acid	0.43	0.37	0.57	0.00
vanillic acid	0.00	0.29	0.37	0.00
homovanillyl alcohol	1.43	6.14	0.00	0.00
D-glucose (α and β anomers)	0.00	3.31	7.46	0.83

hydroxyl group. Two signals at δ 139.97 and 139.78 belong to the phosphitylated hydroxyl groups of (+)-1-acetoxypinoresinol. Finally, the signal at δ 142.89 was attributed to syringaresinol. Other phenolic compounds, such as *o*-coumaric acid, vanillin, gallic acid, *p*-hydroxybenzoic acid, caffeic acid, and ferulic acid, were not detected in the spectrum, presumably because of their absence and/or their low concentration. Spiking of olive oil with these pure substances resulted in new peaks in the spectrum.

The aliphatic region of the ^{31}P NMR spectrum of the polar fraction of olive oil is shown in **Figure 8B**. On the basis of the known phosphorus-31 chemical shifts of model compounds (*26, 29*), the strong signals at δ 146.47 and 147.66 were attributed to 1-monoacylglycerols, and that at δ 148.05 was attributed to 2-monoacylglycerols (*26*). Maslinic acid is detected from signals at δ 145.95 and 147.66, the latter being overlapped by the strong signal of 1-monoacylglycerols. The presence of these signals in the olive oil indicate that monoacylglycerols and maslinic acid were coextracted with phenols by the liquid–liquid extraction procedures used in this study. Free hydroxytyrosol and tyrosol can be quantified from the phosphitylated aliphatic hydroxyl groups at δ 147.00 and 146.89, respectively. This observation is very important, because the combination of these two signal integrals with those of the phosphitylated aromatic hydroxyl groups of the same compounds discussed previously allows the quantification of the free and esterified forms of tyrosol and hydroxytyrosol in olive oil. A number of signals denoted by letters α and β in **Figure 8B** were attributed to the two tautomers α-D- and β-D-glucopyranose, respectively, produced by the hydrolysis of the various polyphenol glucosides. These signals were assigned upon comparison with the corresponding signals of a mixture of the tautomers of D-glucose model compound in pyridine/chloroform at room temperature (**Figure 4B**) and by spiking the sample with an equilibrium mixture of α-D- and β-D-glucopyranose in the same solvents. Glycerol was identified from signals at δ 147.38 and 146.33, in agreement with the chemical shifts of model compound (**Table 1**).

The spectrum in **Figure 8B** contains three signals at low magnetic fields (denoted with asterisks), which appear at the same chemical shifts as those obtained upon phosphitylation of the hydrolysis products of pure oleuropein glucoside with β-glucosidase in chloroform/pyridine solution. Nevertheless, identification of these hydrolysis products is not an easy task, because their structure and proportion in the mixture depend heavily on the nature of solvent and the pH of the hydrolysis

138.55 indicates the absence of gentisic acid (*29*). Therefore, the signal at δ 138.19 is indicative of the presence of tyrosol alone.

As mentioned earlier, several phosphitylated aromatic hydroxyl groups of different phenolic compounds contribute to a number of overlapping signals in the range of 139.22–139.14 ppm (*29*). Vanillic and homoprotocatechuic acids can be quantified from the signals of the phosphitylated carboxyl group at δ 135.15 and 134.63 (not shown), respectively, which are very close to those assigned from model compounds (*29*). Lack of signal at δ 138.51 indicates the absence of protocatechuic acid. It is important to note that the strong signals at δ 138.19 and 139.20 reflect the total content of tyrosol and hydroxytyrosol contained in olive oil, respectively. The phosphitylated aromatic hydroxyl groups of these compounds in their free and esterified forms are expected to show about the same chemical shifts.

Homovanillyl alcohol and (+)-pinoresinol show overlapping signals at δ 139.84. Nevertheless, both compounds can be quantified, because the concentration of homovanillyl alcohol can be calculated from the signal of its phosphitylated aliphatic

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medium (*37, 38*). A systematic study, using appropriate model compounds bearing carbonyl groups, is currently in progress in our laboratory to identify the products of the phosphitylation reaction with the hydrolysis products of oleuropein glucoside and finally to assign the three unknown signals in the ^{31}P NMR spectra of olive oil. Finally, **Table 2** contains the concentration of the phenolic compounds for four virgin olive oil samples determined by the present methodology.

In summary, this study demonstrated the potential of ^{31}P NMR spectroscopy to detect and quantify a large number of phenolic compounds in olive oil extracts in a single experiment. Future automation of this technique will make it very effective for fast screening of large numbers of samples and for setting up a comprehensive data bank of authentic oils. Moreover, application of stronger magnetic fields and the use of cryoprobes are expected to lower significantly the detection limit for these minor compounds.

Supporting Information Available: Strategy and accompanying 1D and 2D experiments for the assignment of the ^{31}P NMR spectrum of the phosphitylated mixture of α-D- and β-D-glucopyranose; ^{31}P NMR spectra of the phosphitylated luteolin, apigenin, their glycosides, and the β-D-glucopyranose moiety of oleuropein glucoside; detailed tables for validation tests and reproducibility of the present methodology. This material is available free of charge via the Internet at http://pubs.acs.org.

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