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^{31}P NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY OF POLYPHENOL-CONTAINING OLIVE OIL MODEL COMPOUNDS

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Phosphitylation of the free hydroxyl and carboxyl groups of a series of polyphenol-containing olive oil model compounds with 2-chloro-4,4,5,5-tetramethyl-dioxaphospholane allows their classification on the basis of ^{31}P NMR chemical shifts. Unambiguous assignment of the ^{31}P NMR chemical shifts of the dihydroxy- and polyhydroxy-phenols has been achieved by employing one and two-dimensional NMR techniques. Furthermore, integration of the appropriate signals of the hydroxyl derivatives in the corresponding ^{31}P NMR spectra allows the quantification of these compounds. This study forms the basis for the development of a novel and sensitive method that can be used to obtain valuable information about polyphenols that are present in olive oils.

Keywords: ^{31}P NMR spectroscopy; chemical shifts; 2D NMR; polyphenols

INTRODUCTION

Polyphenols are part of the polar fraction of virgin olive oil, which is usually obtained by extraction with methanol/water mixtures. It has been suggested [1] that the stability of virgin olive oil against oxidation from atmospheric oxygen is partly due to the total polyphenol content. Also, polyphenols have been related to the sensory (taste and aroma) and nutritional characters of olive oil [2]. Recent clinical research has demonstrated that polyphenols provide a defense mechanism that delays ageing, and pre-

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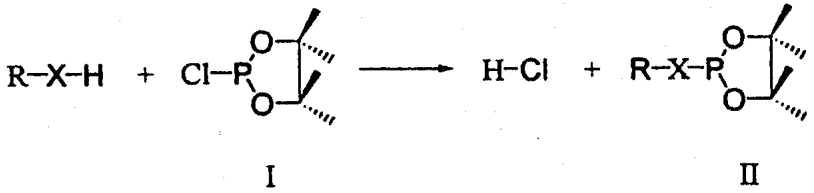
vents carcinogenesis, cardiovascular diseases, liver disorders and inflammations [3,4]. In this respect, the detection and quantification of polyphenols in virgin olive oil is an essential step for understanding their antioxidant, nutritional and biological properties.

In our laboratory, we are developing a facile magnetic resonance method, which is based on the derivatization of the labile hydrogens of the hydroxyl and carboxyl groups with 2-chloro-4, 4, 5, 5-tetramethyldioxaphospholane (I) according to the reaction scheme 1. This procedure is followed by the use of the ^{31}P chemical shifts to identify the labile centers. In fact, compound I reacts rapidly and quantitatively under mild conditions with functional groups bearing labile protons [5]. This analytical method has been used successfully by us to detect and quantify mono- and diglycerides in virgin olive oils [6]. The same method has been used earlier for the analysis of lignocellulosic substances [7,8], and for the examination of the functional group distributions in coal-derived materials [9].



I II

X = O, COO, CO

SCHEME 1

In this work, a series of polyphenol model compounds, including major phenolic compounds detected in olive oil, were subjected to phosphitylation by reagent I. The resulting phosphorous-containing derivatives were examined by ^{13}P NMR spectroscopy. Unambiguous assignment of the ^{13}P NMR chemical shifts of the phosphitylated phenols was achieved by using one- and two-dimensional NMR techniques. Moreover, by introducing an internal standard (cyclohexanol) in the reaction mixture, the concentration of the product II (scheme 1) is obtained avoiding normalization conditions.

This work is a preliminary study for the development of a novel sensitive method that can be used to detect and quantify polyphenols that are present in virgin olive oil.

EXPERIMENTAL

All solvents and model compounds were of reagent or analytical grade and used without further purification. Most of the model compounds were purchased from Sigma. The derivatizing reagent I was commercially available from Fluka Chem. Co. Hydroxytyrosol was synthesized according to the literature [10]. Finally, the deuterated pyridine and chloroform solvents were purchased from Aldrich.

Sample preparation

The phosphitylation and acquisition protocol was essentially that described by Argyropoulos and Jiang [5]. A stock solution (10 mL) composed of deuterated pyridine and $CDCl_3$ in 1.6:1.0 volume ratio containing 0.6 mg of chromium acetylacetonate, Cr (acac)$_3$, (0.165 μM) and 13.5 mg cyclohexanol (13.47 μM) was prepared and protected from moisture with 5A molecular sieves. Predetermined quantities of model compounds (0.1–3 mg) were placed in 5 mm NMR tube. The required volume of the stock solution (0.4 mL) and the reagent I (5–30 pL, depending on the number of the functional groups) were added. The reaction mixture was left to react for 0.5 h at room temperature.

Gentistic acid was decomposed upon derivatization with I at room temperature. This acid has vicinal hydroxy and carboxyl groups and phosphitylation may lead to a transesterification reaction of the phosphite esters similar to those observed for vicinal and/or *alpha*-hydroxy acids [11]. Similar decomposition was observed for p-hydroxyphenylacetic acid, and homoprotocatehuic acid bearing saturated aliphatic side chains which has been attributed to the formation of phosphate esters [11]. In order to avoid decomposition, these three phenolic compounds were carefully derivatized under stirring and cooling over an ice bath.

One-dimensional ^{31}P NMR experiments

^{31}P NMR spectra were obtained on a Bruker AMX500 spectrometer operating at 202.2 MHz for the phosphorus-31 nucleus at 30 °C. In order to

suppress the NOE, the inverse gated decoupling technique was used. Typical spectral parameters for quantitative studies were: 90° pulse width 12.5 μs, spectral width 10 kHz, relaxation delay 30 s, memory size 16 k (zero-filled to 32 k). Line broadening of 1 Hz was applied and drift correction was performed prior to Fourier transform. Polynomial fourth-order baseline correction was performed before integration. For each spectrum 32 transients were acquired. All ^{31}P chemical shifts reported in this paper are relative to the signal at δ 132.2 due to the product of the reaction of I with water (moisture content in commercial chemicals), which has been observed to give a sharp signal in pyridine-$CDCl_3$ mixture [5]. 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 phosphorous nuclei, shortening thus the duration of the measurements significantly [8].

Two-dimensional NMR experiments

2D NMR experiments were performed on a Bruker AMX500 spectrometer operating at 500.1, 202.2 and 125.7 MHz for proton, phosphorous-31, and carbon-13 nuclei, respectively. Some details of the two -dimensional NMR experiments used in the present study are given below. Additional information for gradient COSY, HMQC, and phase cycling HOESY experiments are given elsewhere [12].

Gradient ^1H-^1H homonuclear spectroscopy (g-H-H-COSY)

Gradient-selected ^1H-^1H COSY spectra were obtained by using 128 increments of 1K data points. 16 scans and 8 dummy scans were accumulated for each free induction decay 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 ^{31}P-^{31}P homonuclear spectroscopy (g-P-P-COSY)

Gradient-selected ^{31}P-^{31}P COSY spectra were obtained by using 128 increments of 1K data points. 8 scans and 4 dummy scans were accumulated for each free induction decay 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 ^1H-^{13}C heteronuclear multiple quantum coherence (g-H-C-HMQC)

The gradient selected hydrogen-carbon Heteronuclear Multiple Quantum Coherence spectra were acquired with 64 increments and 1K data points. The heteronuclear ($^{13}C/^1H$) GARP composite pulse decoupling was used during acquisition. 32 scans and 4 dummy scans were accumulated for each free induction decay 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 ^1H-^{31}P heteronuclear multiple quantum coherence (g-H-P-HMQC)

The gradient selected hydrogen-phosphorous Heteronuclear Multiple Quantum Coherence spectra were acquired with 64 increments and 1K data points. The experiment was optimized for long-range proton-phosphorous couplings by setting the evolution delay to 50 ms, corresponding to a coupling constant $J_{H,P} \sim 10$ Hz. Optimized experiments for couplings of $J_{H,P} \sim 3$ resulted in identical spectra. 16 scans and 8 dummy scans were accumulated for each free induction decay 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.

It should be noted that the gradient selected hydrogen-phosphorous Heteronuclear Multiple Bond Correlation experiment (g-H-P-HMBC) to obtain long-range H-P couplings was not used. In fact this experiment is not necessary since the present phosphitylated compounds have no direct H-P couplings to be suppressed.

^1H-^{31}P heteronuclear NOE (H-P-HOESY)

^1H-^{31}P NOE spectra were obtained by using 64 increments of 1K real data points. The WALTZ-16 composite pulse sequence for proton decoupling during acquisition was used. The mixing time was set to 3 s. 48 scans and 4 dummy scans were accumulated for each free induction decay 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.

It is worth mentioning that experiments performed by using a larger number of increments (e. g. 256 for g-H-H-COSY, g-P-P-COSY, or 128 for g-H-C-HMQC, H-P-HOESY) did not offer additional information. Therefore, we decided to use 128 and 64 increments to speed up the analysis. The presentation of the gradient 2D spectra in the amplitude mode may be a disadvantage for acquiring complex spectra. However, this is not the case for the present simple molecules (see following spectra).

RESULTS AND DISCUSSION

^{31}P chemical shifts of the phosphitylated polyphenols

The ^{31}P chemical shifts of the phosphitylated polyphenol model compounds are summarized in Table I. The structural formula of each compound and the numbering system, when necessary is depicted in Figure 1. Compounds **1–6** are derivatives of hydroxycinnamic acid, compounds **7–12** are derivatives of hydroxybenzoic acid, and compounds **13** and **14** are derivatives of phenylacetic acid.

TABLE I ^{31}P NMR Chemical Shifts[a] and multiplicity[b] (in parenthesis) of Phospitylated Polyphenols Model Compounds at 30 °C

Model Compound	*Hydroxyl Group*[c]	*Carboxyl Group*[c]
1	138.94 (s)	135.04 (s)
2	137.82 (s)	135.13 (s)
3	139.46 (s)	135.20 (s)
4[d]	*1*: 139.85 (d)	135.22 (s)
	2: 138.92 (d)	
5	140.37 (s)	135.20 (s)
6	142.23 (s)	135.25 (s)
7	137.68 (s)	135.21 (s)
8	139.20 (s)	135.17 (s)
9[d]	*1*: 139.14 (d)	134.94 (s)
	2: 138.51 (d)	
10[d]	*1, 3*: 138.51 (d)	134.58 (s)
	2: 141.43 (t)	
11	141.88 (s)	135.19 (s)
12[d]	*1*: 138.27 (d)	135.03 (s)
	2: 138.55 (d)	
13	138.21 (s)	134.75 (s)
14[d]	*1*: 139.14 (s)	134.65 (s)
	2: 139.14 (s)	
15[d]	*1*: 138.20 (d)	
	2: 146.91 (d)	
16[d]	*1*: 139.25 (d)	
	2: 139.13 (d)	
	3: 146.99 (s)	
17	138.64 (s)	
18	138.88 (s)	
19[d]	*3*: 147.98 (s)	134.81 (s)
	4: 146.69 (s)	
	5: 146.47 (s)	
20[d]	*5*: 137.44 (s)	
	7: 136.46 (s)	
	4': 137.88 (s)	
21[d]	*3*: 141.70 (s)	
	5: 137.44 (s)	
	7: 136.46 (s)	
	4': 137.95 (s)	
22[d]	*3*: 141.78 (s)	
	5: 137.44 (s)	
	7: 136.44 (s)	
	3': 140.47 (d)	
	4': 138.55 (d)	

a. Referenced from the phosphitylated water peak at 132.20 ppm.
b. The P-P homoallylic coupling constants, $^{5}J_{P,P}$, ranged between 6.8 and 7.0 Hz.
c. The OH and COOH groups were all phosphytilated with reagent I.
d. See numbering system of the hydroxyl groups in Fignre I

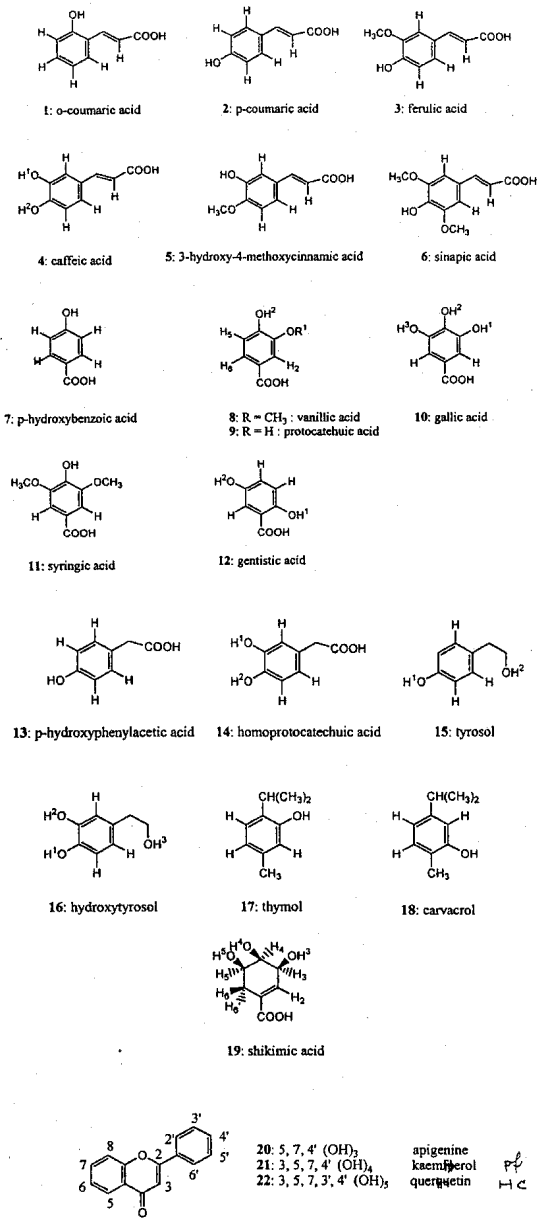


FIGURE 1 Chemical structures of polyphenol model compounds

The assignment of the ^{31}P signals of the phosphitylated carboxyl groups and those of the hydroxyl derivatives bearing one hydroxyl group is not difficult, since the ^{31}P chemical shifts of these two derivatizable groups appear in different spectral regions (see Table I, and reference 5). However, the assignment of the signals in the ^{31}P NMR spectra of model phenolic compounds bearing two phosphitylated hydroxyls is not an easy task. Attempts to assign these signals by adopting the additivity rules of substituent effects on ^{31}P chemical shifts of phenolic compounds derivatized with reagent I, as derived in the literature [5], failed to reproduce the present experimental ^{31}P chemical shifts in most cases. Moreover, the signal multiplicity in the one-dimensional ^{31}P NMR spectra does not allow a complete identification of the different P-P and H-P coupling pathways. Therefore, we decided to perform a series of two-dimensional NMR experiments in order to assign unambiguously the phosphorous signals of the dihydroxy derivatives.

Our strategy includes the following steps, the elucidation of the splitting patterns observed in the one-dimensional ^{31}P NMR spectra; the assignment of the aromatic proton and carbon chemical shifts of the phosphitylated polyphenols by using 2D NMR experiments; the assignment of the phosphorous signals by employing NMR experiments connecting signals via H-P and C-P long-range scalar interactions or via through space H-P dipole-dipole interactions.

Protocatehuic acid (**9**), derivatized with compound I, can be used as model analysis of the *ortho*-dihydroxy derivatives. The ^{31}P NMR spectrum of compound 9 shows two doublets at δ 138.43 and 139.14 reflecting the possibility of homoallylic coupling between two phosphorous nuclei. Indeed, the two-dimensional P-P homonuclear experiment in Figure 2 confirms the presence of such a coupling ($^5J_{PP}$ = 6.88 Hz). This coupling is observed in all the *ortho*-dihydroxy derivatives. The homoallylic coupling between the *ortho* phosphitylated hydroxyl groups allows the direct assignment of phosphorous signals in the spectrum of gallic acid (**10**) bearing three hydroxyl groups. The ^{31}P NMR spectrum of phosphitylated gallic acid shows a triplet at δ 141.43 and a doublet at δ 138.51 with intensity ratio 1:2. These signals have been assigned to the derivatized hydroxyls in *para* and *meta* position to the carboxyl group, respectively. The assignment of the aromatic protons of the phosphitylated protocatehuic acid is obtained from its g-H-H-COSY spectrum shown in Figure 3, where cross-peaks connect protons H-2 and H-6, as well as protons H-5 and H-6.

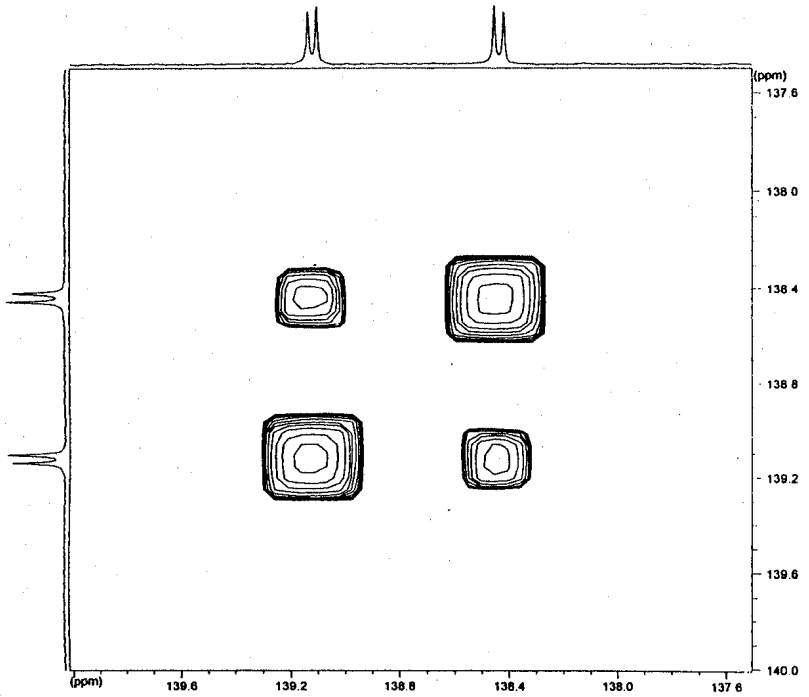


FIGURE 2 500 MHz $^{31}P - ^{31}P$ gCOSY spectrum of phosphitylated protocatehuic acid (**9**)

Having assigned the proton spectrum of the phosphitylated protocatehuic acid, the ^{31}P NMR spectrum can be assigned using a long-range g-H-P-HMQC experiment (Figure 4). Inspection of this spectrum reveals correlations between the phosphorous signal at δ 139.14 with protons H-2 and H-6, and between the phosphorous signal at δ 138.43 with proton H-5. Nevertheless, these correlations cannot establish unambiguously the assignment of the phosphorous signals unless the coupling pathways between proton and phosphorous nuclei are known. For instance, if the phosphorous signal at δ 139.14 belongs to the phosphorous nuclei of the derivatized hydroxyl at *para* position to the carboxyl group of the protocatehuic acid then the correlation with protons H-2 and H-6 indicate $^{5}J_{H\text{-}P}$couplings. However, if the same signal belongs to the phosphorous nuclei of the derivatized hydroxyl at *meta* position to the carboxyl group, the couplings are

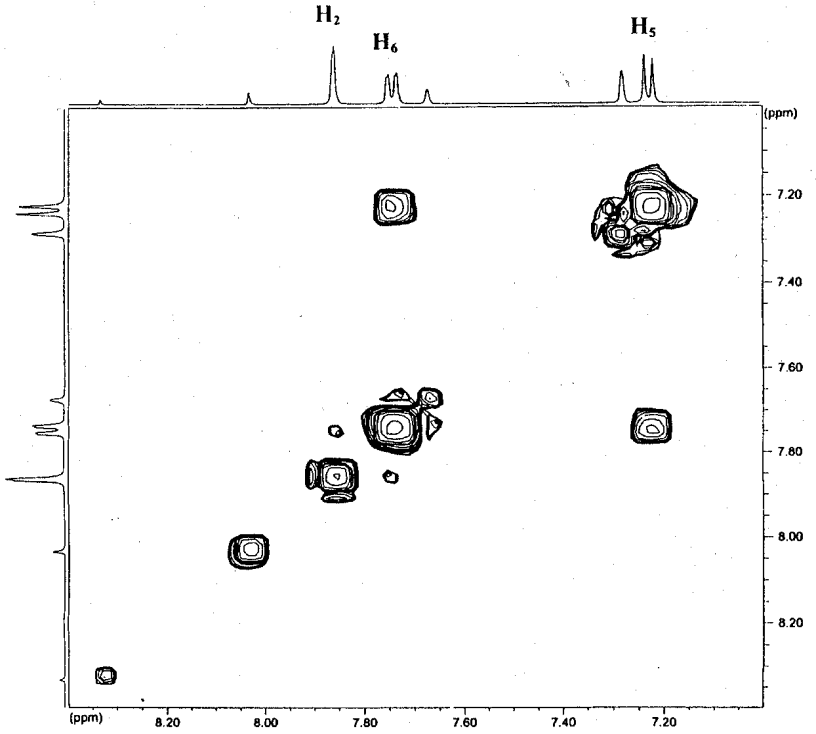


FIGURE 3 500 MHz $^{1}H - ^{1}H$ gCOSY spectrum of phosphitylated protocatehuic acid (**9**)

$^{4}J_{H\text{-}P}$ and $^{6}J_{H\text{-}P}$, respectively. To the same conclusions we are led by observing the carbon-phosphorous splitting pattern of the proton-decoupled one-dimensional carbon-13 spectrum of the same compound (not shown). Here, ambiguity arises between $^{2}J_{C\text{-}P}$, $^{3}J_{C\text{-}P}$, and $^{4}J_{C\text{-}P}$ couplings.

To resolve this ambiguity, we exploit the possibility of the existence of through space dipole-dipole interactions of the phosphorous nuclei with neighboring aromatic protons by performing a $^{1}H\text{-}^{31}P$ heteronuclear NOE experiment. The recorded H-P-HOESY spectrum of protocatehuic acid in Figure 5 shows clearly that the phosphorous signals at δ 139.14 and 138.43 correlate with protons H-2 and H-5, respectively. These signals are assigned to the derivatized hydroxyls at *meta* and *para* positions to the carboxyl group in the protocatehuic acid.

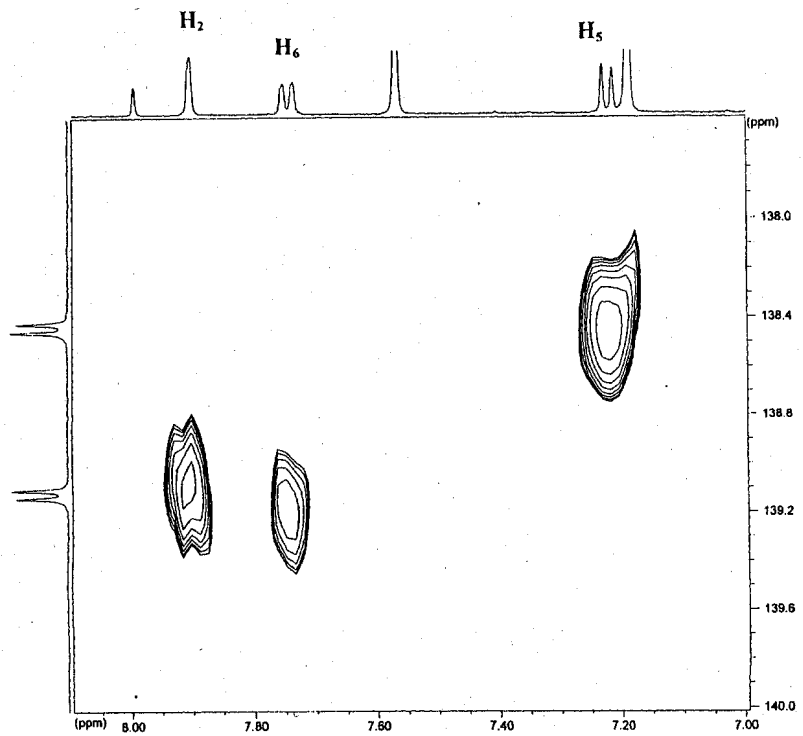


FIGURE 4 500 MHz $^{1}H - ^{31}P$ gHMQC spectrum of phosphitylated protocatehuic acid (**9**)

Turning back into the g-H-P-HMQC spectrum (Figure 4), we conclude that correlations between proton and phosphorous signals are observed only via the $^{4}J_{H\text{-}P}$ and $^{6}J_{H\text{-}P}$ long-range couplings. It appears that the absence of $^{5}J_{H\text{-}P}$ long-range coupling is a general phenomenon for all the mono- and dihydroxy model compounds. This conclusion has been verified by performing g-H-P-COSY and H-P-HOSY experiments for other phosphitylated mono- and dihydroxy phenolic model compounds, such as vanillic acid (**8**), hydroxytyrosol (**16**), caffeic acid (**4**) and gentistic acid (**12**). The knowledge of the H-P splitting pathway allows the assignment of the phosphorous signals through the much simpler g-H-P-COSY experiment. The ^{31}P NMR spectrum of shikimic acid (**19**), bearing three phosphotylated aliphatic hydroxyl groups, shows three singlets at δ 146.47,

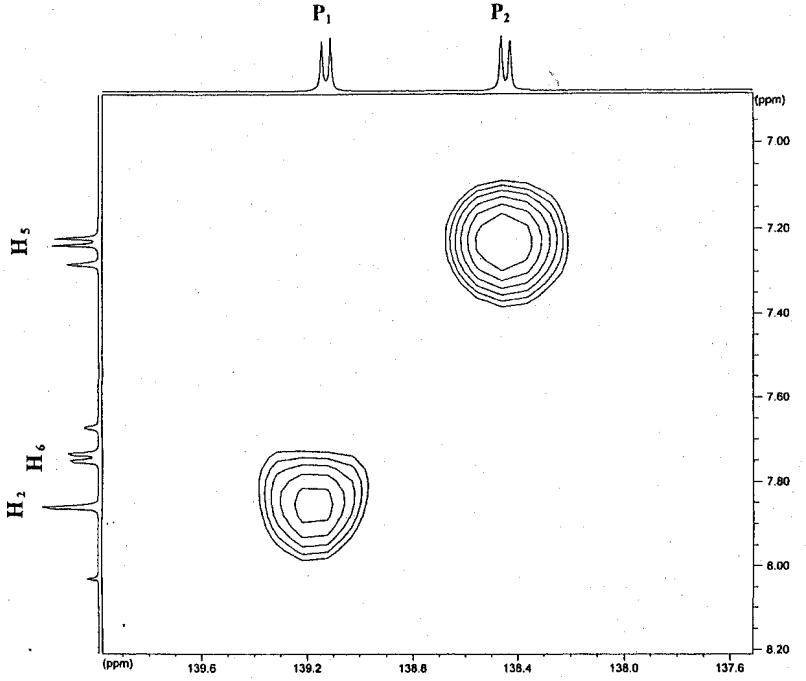


FIGURE 5 500 MHz $^{1}H - ^{31}P$ HOESY spectrum of phosphitylated protocatehuic acid (**9**)

146.69, and 147.98, at lower fields than the corresponding signals of the aromatic hydroxyl derivatives as expected [15]. The assignment of the three ^{31}P signals can be obtained by performing a g-H-P-HMQC experiment. Since this experiment connects proton and phosphorous chemical shifts, the proton spectrum of shikimic acid should be assigned first. The assignment is obtained through a g-H-H-COSY experiment. The recorded 2D spectrum is shown in Figure 6. Starting with the unequivocal more de shielded H-2 proton at δ 6.88, the spectrum shows connectivity of this proton with the H-3 proton at δ 5.14 and the two geminal protons H-6 and H-6′ (allylic coupling) at δ 2.78 and 2.54, respectively. In turn, the H-3 proton shows a cross peak with signal at δ 4.39, which should be attributed to the H-4 proton. Also, the spectrum shows a cross peak connecting proton H-3 with the geminal proton H-6 via long-range coupling. The signal

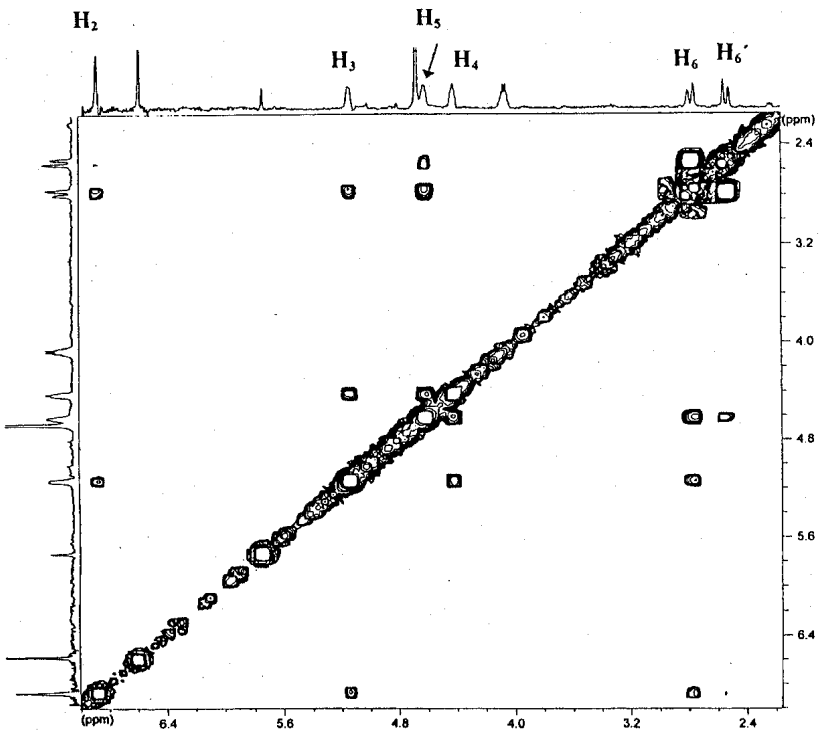


FIGURE 6 500 MHz $^{1}H - ^{1}H$ gCOSY spectrum of phosphitylated shikimic acid (**19**)

for the H-5 proton (δ 4.62) is assigned by following the cross peak with the H-4 proton. The g-H-P-HMQC spectrum shown in Figure 7 identifies clearly the phosphorous signals.

The assignment of the phosphorous signals for the phosphitylated polyhydroxy model compounds such as, apigenine (**20**) kaempferol (**21**), and quercetin (**22**) is obtained by a direct comparison of their ^{31}P NMR spectra depicted collectively in Figure 8. The phosphorous signals at δ 141.78 and 141.70 in the spectra of quercetin and kaempferol are assigned to the phosphitylated hydroxyl OH-3, since this signal is missing in the spectrum of apigenine. The signal at δ 140.47 in the spectrum of quercetin, which is missing in the spectra of apigenine and kaempferol, is attributed to the phosphitylated hydroxyl OH-3′. The signal at δ 138.55 in the same spectrum is attributed to the phosphitylated hydroxyl OH-4′. Both signals

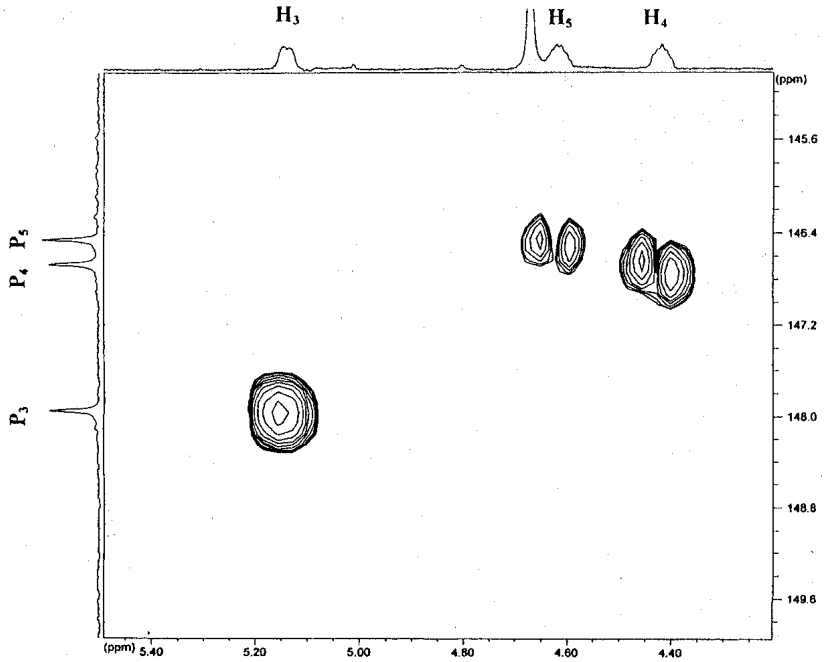


FIGURE 7 500 MHz $^{1}H - ^{31}P$ gHMQC spectrum of phosphitylated shikimic acid (**19**)

appear as doublets reflecting the usual homoallylic coupling ($^{5}J_{PP}$= 6.88 Hz), that is observed in the phosphitylated *ortho* dihydroxy model compounds. The signals at δ at 137.88 and 137.95 in the spectra of apigenin and kaempferol, respectively, are attributed to the phosphitylated hydroxyls OH-4′. The higher shift (~ 0.60 ppm) of these signals relative to the OH-4′ signal of quercetin is attributed to the shielding effect of the *ortho* hydroxyl group in the molecule. Finally, the remaining two signals of each model compound are assigned to the phosphitylated hydroxyls OH-5 and OH-7 (Figure 8). No further experiments have been performed to differentiate the chemical shifts of these phosphitylated hydroxyls. Nevertheless, the chemical shift of the phosphitylated hydroxyl OH-5 is expected to be larger than that of the phosphitylated hydroxyl OH-7, since the former is within the deshielding zone of the nearby carbonyl group (see Figure 1). The additional small signals observed in the NMR spectrum of quercetin (at ~ δ 137.2, 140.5 and 143.5) are due to impurities in the commercial compound.

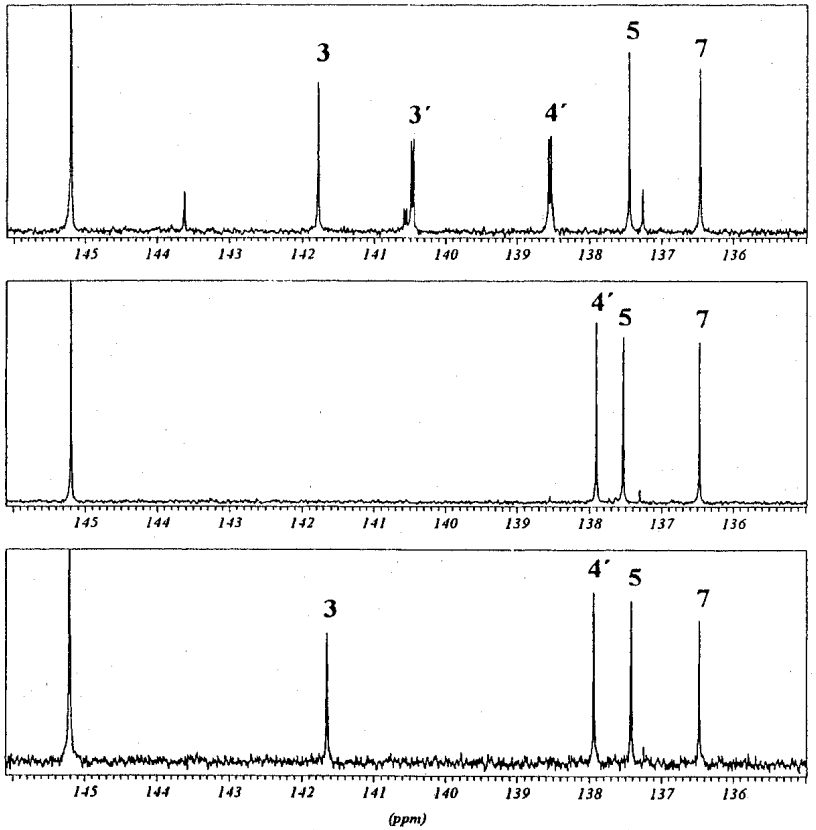


FIGURE 8 ^{31}P NMR spectra of the phosphitylated model compounds (top) quercetin (**22**); (middle) apigenine (**20**); (bottom) kaempferol (**21**)

It is important to examine the effect of concentration on the ^{13}P chemical shifts of the phosphitylated polyphenols model compounds; in fact polyphenols in olive oils are found in different quantities that those used in the present study. The concentration-dependence of the ^{13}P chemical shifts has been examined by performing experiments with samples of varying concentrations of model compounds. The results clearly indicate that concentration has a negligible effect on the ^{13}P chemical shifts of the phosphitylated hydroxyl groups. For instance, a 50-fold increase in the concentration (from 2.5 to 49 μmols) of carvacrol (**18**) showed no effect

on the chemical shift of the phosphitylated hydroxyl group. Also, a 30-fold increase in the concentration (from 1.5 to 31 μmols) of shikimic acid (**19**) resulted in an upfield shift of 0.01–0.02 ppm for the three phosphitylated hydroxyl groups.

Quantitative analysis of polyphenol model compounds

The one-dimensional ^{13}P NMR spectra of all polyphenol model compounds were run allowing a long relaxation delay for full magnetization recovery before the next pulse sequence (see experimental part). The long delay guarantees that the concentrations of the model compounds estimated by integrating the phosphorous spectra are closer to the actual concentrations. Moreover, the quantification of each model compound requires a suitable internal standard. Adding a known amount of the internal standard to an unknown amount of polyphenol, the concentration of polyphenol can be calculated from the integration ratio of the sample and internal standard of the corresponding ^{13}P signals. Cyclohexanol was found to be a useful internal standard in the present analysis [5–7]. The phosphitylated cyclohexanol affords a single ^{13}P signal at δ 145.20.

The applicability of this method to the quantitative determination is demonstrated in Figure 9, which depicts the correlation of the measured molar concentration of each model compound through integration of the phosphorous signal of the phosphitylated hydroxyl to that of the actual concentration. The measured concentration of each polyhydroxy phenol by ^{31}P NMR spectroscopy represents an average value of the concentrations that have been determined from each phosphitylated hydroxyl group in the molecule separately. As can be seen from Figure 9, the correlation is linear with correlation coefficient $r = 0.999$, slope $= 1.01 \pm 0.01$, and intercept $= -0.11 \pm 0.07$, indicating that this method is reliable for quantitative analysis.

Additional evidence for the reliability of this method for quantitative analysis is offered by performing a series of ^{13}P NMR experiments with tyrosol (**15**) model compound, followed by statistical analysis of the integration data in order to obtain the repeatability and reproducibility of the measurements. The repeatability of twelve consecutive spectra, using the same sample of phosphitylated tyrosol, was found to be 1.07% (or in terms of standard deviation, 12.19 ± 0.13, where 12.19 is the mean value of the measurements). The reproducibility was estimated by performing eight

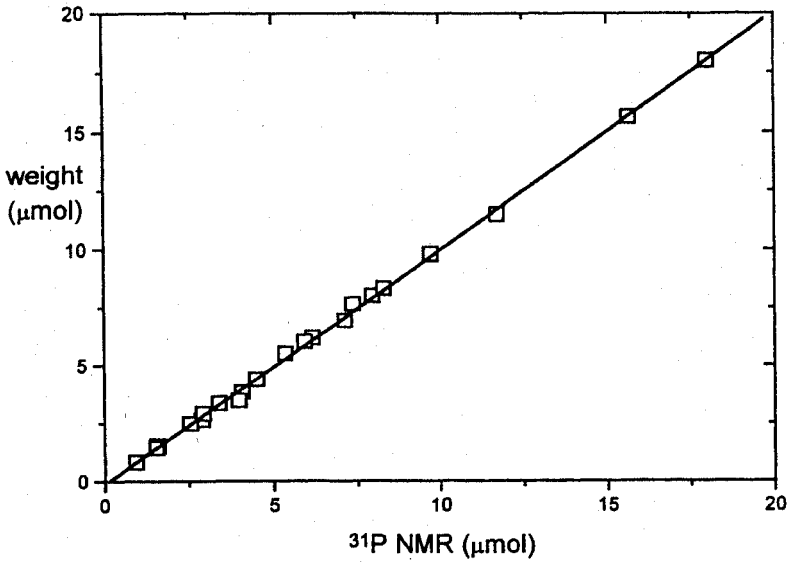


FIGURE 9 Correlation of the measured amounts (in μmols) for polyphenols model compounds from integration of the corresponding ^{31}P signals to those of the weighed amounts. The amounts for the dihydroxy and polyhydroxy phenols represents an average value of the amounts that have been determined from each phosphitylated hydroxyl group in the molecule separately

measurements on eight different samples of tyrosol using the same experimental protocol for each measurement. The reproducibility was calculated to be 2.44% (or 12.32 ± 0.30 in terms mean value and standard deviation). This statistical analysis demonstrates clearly that this method is precise and reproducible.

CONCLUSION

^{31}P NMR spectroscopy has been used for quantification of polyphenol-containing olive oil model compounds. This technique is based on the phosphitylation of the hydroxyl groups of these compounds with 2-chloro-4, 4, 5, 5-tetramethyldioxa-phospholane followed by a quantitative analysis of the corresponding ^{31}P NMR spectra. The ^{31}P NMR chemical shifts of the phosphitylated hydroxyls have been assigned unambiguously by employing 2D NMR experiments. The excellent resolution and high sensitivity to the environment of the phosphitylated hydroxyls can be exploited to detect and quantify polyphenols in olive oil.

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