Application of ^{31}P NMR Spectroscopy in Food Analysis. 1. Quantitative Determination of the Mono- and Diglyceride Composition of Olive Oils

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This paper introduces a facile method to determine the amount of mono- and diglycerides in virgin olive oils. This method is based on the phosphorylation of the free hydroxyls of the mono- and diglycerides with 2-chloro-4,4,5,5-tetramethyldioxaphospholane and the integration of the appropriate peaks in the ^{31}P NMR spectrum. Quantitative ^{31}P NMR spectroscopy can be extended to the quantification of other constituents of olive oils bearing functional groups with labile protons.

Keywords: *Olive oil; ^{31}P NMR; monoglycerides; diglycerides*

INTRODUCTION

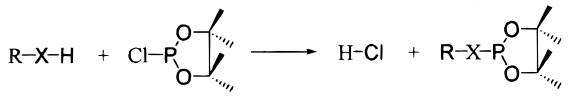
Over the past few years, NMR spectroscopy has been widely used for the analysis and quality control of foods (Belton et al., 1995). In particular, ^{1}H and ^{13}C NMR spectroscopies have been applied successfully to the analysis of virgin olive oil, showing a large number of structural and compositional features related to oil quality (Sacchi et al., 1996, 1997).

This paper introduces a facile magnetic resonance method that can supplement ^{1}H and ^{13}C NMR spectroscopies, especially in cases where overlapped peaks in the ^{1}H NMR spectra or long relaxation times of the insensitive ^{13}C nuclei render the analysis a difficult task. This method is based on the derivatization of labile hydrogens of functional groups, such as OH, COOH, and CHO, of olive oil constituents with 2-chloro-4,4,5,5-tetramethyldioxaphospholane (**I**) according to the reaction shown in Scheme 1 and the use of the ^{31}P chemical shifts to identify the labile centers. Compound **I** reacts rapidly and quantitatively under mild conditions with functional groups bearing labile protons (Jiang et al., 1995).

The large range of chemical shifts (∼700 ppm) reported for the ^{31}P nucleus, which ensures a good separation of signals in different environments, its 100% natural abundance, and its high sensitivity, which is only ∼15 times less than that of the proton nucleus, make ^{31}P NMR experiments a reliable analytical tool to determine very low concentrations of the order of micromoles. Moreover, by introducing an internal standard (cyclohexanol) in the reaction mixture, the concentration of the product, **II** (Scheme 1), and hence the concentration of the functional group bearing compound are obtained, avoiding normalization conditions.

The primary objective of this work was to test this simple technique with certain model compounds bearing labile protons and to determine quantitatively the ratio 1,2-diglycerides/1,3-diglycerides of virgin olive oils of different geographical origins and years of production.

Scheme 1



I II

X = O, COO, CO

MATERIALS AND METHODS

All solvents and model compounds used were of reagent or analytical grade and used without further purification. Model compounds, stearic acid, oleic acid, linoleic acid, linolenic acid, tyrosol, syringic acid, *n*-octadecanol-1, *n*-hexanol-1, hexanal, and *p*-hydroxybenzophenone, were purchased from Aldrich. 1-Monoolein, 2-monoolein, a mixture of ∼15% 1,2- and 85% 1,3-diolein, and β-sitosterol (containing campesterol) were purchased from Sigma. The derivatizing reagent **I** was commercially available from Fluka Chemical Co.

Sample Preparation. A stock solution (10 mL) composed of pyridine and $CDCl_3$ in 1.6:1 volume ratio containing 0.6 mg of $Cr(acac)_3$ (0.165 μM) and 13.5 mg of cyclohexanol (14.47 μM) was prepared and protected from moisture with molecular sieves. A predetermined quantity of the model compound (1−5 mg) or 150 mg of the olive oil samples was placed in a 5 mm NMR tube. The required volumes of the stock solution (0.4 mL) and the reagent I (5−30 μL, depending on the number of the functional groups) were added. The reaction mixture was left in the NMR tube to react for 0.5 h at room temperature. Upon completion of the reaction, the solution was used to obtain the ^{13}P NMR spectra.

NMR Experiments. ^{31}P NMR spectra were obtained on a Bruker AMX500 spectrometer operating at 202.2 MHz for the phosphorus-31 nucleus. The probe temperature was 25 °C. To eliminate NOE effects, the inverse gated decoupling technique was used. Typical spectral 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). Line broadening of 1 Hz was applied, and a drift correction was performed prior to Fourier transform. Polynomial fourth-order baseline correction was performed before integration. For each spectrum 32 and 64 transients were acquired for model compounds and olive oil samples, respectively. All chemical shifts reported in this paper are relative to the product of the reaction of **I** with water, which has been observed to give a sharp signal in pyridine/$CDCl_3$ at 132.2 ppm (Jiang et al., 1995).

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Table 1. ^{31}P NMR Chemical Shifts (Parts per Million) of Phosphorylated Derivatives of Model Compounds

compound	hydroxyl protons	carboxyl protons	aldehydic protons
1,3-diolein	146.7		
1,2-diolein	148.2		
1-monoolein	146.4 (secondary)		
	147.6 (primary)		
2-monoolein	148.0		
n-octadecanol	147.1		
n-hexanol	147.1		
tyrosol	138.3 (phenolic)		
	146.9 (aliphatic)		
β-sitosterol	145.0		
syringic acid	141.9	135.2	
stearic acid		134.8	
oleic acid		134.8	
linoleic acid		134.8	
linolenic acid		134.8	
hexanal			147.7
p-hydroxybenzophenone	137.7		

RESULTS AND DISCUSSION

Table 1 summarizes the ^{31}P chemical shifts of the phosphorylated derivatives of model compounds used in this study from the peak of the reaction product with water. The assignment of the peaks for the primary and secondary hydroxyl derivatives of the mono- and diglycerides has been verified by recording ^{1}H-coupled ^{31}P NMR spectra, which showed the expected triplets for the primary hydroxyl and doublets for the secondary hydroxyl derivatives ($^{3}J_{P,H} = 10-11$ Hz). Inspection of Table 1 reveals a number of interesting features. First, the hydroxyl groups in aliphatic and benzyl alcohols are clearly distinguishable from the aromatic hydroxyls in the spectra of the phosphorylated derivatives of the model compounds. This is best illustrated in the chemical shifts of the two phosphorylated hydroxyl groups of tyrosol. The phosphorylated phenolic hydroxyl appears at δ 138.3 and that of the aliphatic one at δ 146.9. Second, the ^{31}P chemical shift is sensitive to the position of the hydroxyl in mono- and dioleins. This observation facilitates a clear distinction of these compounds in olive oils, which is not feasible using proton NMR spectroscopy at fields lower than 600 MHz without prior derivatization (Sacchi et al., 1997). Third, the ^{31}P chemical shifts of the phosphorylated free saturated and unsaturated carboxylic acids used in this study appear at higher fields than the other model compounds. However, no effect of the degree of unsaturation of the aliphatic chains of fatty acids on the ^{31}P chemical shifts was observed. Fourth, as expected, the chain length of the saturated alcohols does not affect the ^{31}P chemical shifts of these compounds.

The applicability of this method to the quantitative determination of the olive oil constituents bearing active functional groups is demonstrated in Figures 1 and 2. Figure 1 illustrates the ^{31}P NMR spectrum of a mixture of mono- and diglycerides. The peak at δ 145.2 belongs to the internal standard (cyclohexanol, CH). Well-separated peaks for each component of the mixture are observed. Figure 2 depicts the correlation of the measured amount of each model compound through integration of the corresponding phosphorus signal to that of the weighed amount and to the amount obtained from integration of the corresponding peaks in their proton NMR spectra. The correlation is linear with a correlation coefficient of $r = 0.998$, slope $= 0.06 \pm 0.17$, and intercept $= 1.00 \pm 0.01$, indicating that this method is reliable for quantitative analysis.

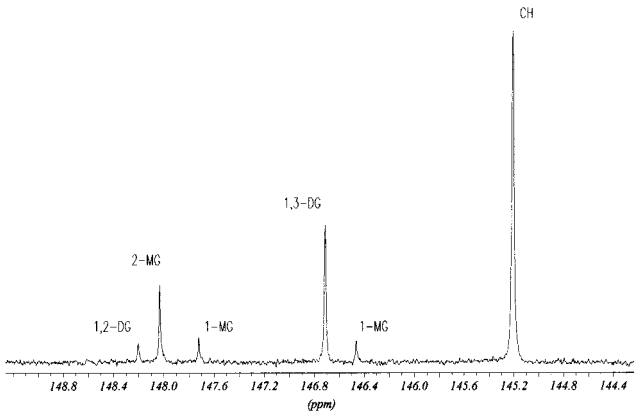

Figure 1. 202.2 MHz ^{31}P NMR spectrum of a mixture of 1-monoglyceride (1-MG), 2-monoglyceride (2-MG), 1,2-diglyceride (1,2-DG), and 1,3-diglyceride (1,3-DG). The peak denoted CH belongs to the internal standard cyclohexanol.

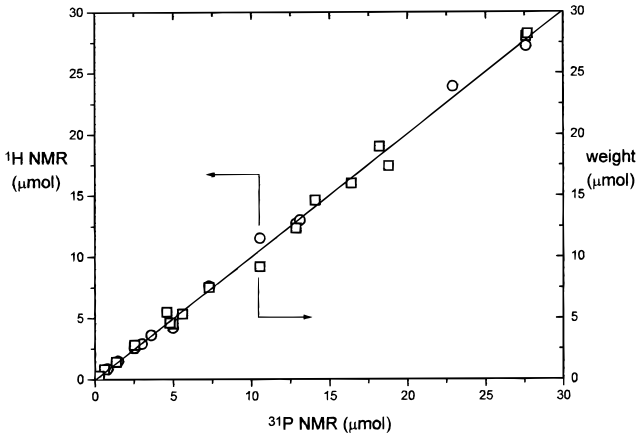

Figure 2. Correlation of the measured amount for each model compound from integration of the corresponding phosphorus signal to that of the weighed amount and to the amount obtained from integration of the corresponding peaks in its proton NMR spectrum.

Several olive oil samples from different regions of Greece and of different years of production were derivatized with reagent **I**, and, subsequently, their ^{31}P NMR spectra were recorded. A typical spectrum is shown in Figure 3a. The excellent resolution between the ^{31}P chemical shifts of the mono- and diglycerides permits a reliable quantification of the mono- and diglycerides in the olive oil. This spectrum can be compared to the 500 MHz proton NMR spectrum of the same olive oil (see Figure 3b) in the region where mono- and diglycerides appear. No monoglyceride peaks appear in the spectrum, whereas the extensive overlap of the diglycerides with those of the α,α' protons of triglycerides and their ^{13}C satellites does not permit a reliable quantitative analysis. The peak overlap is expected to be more severe at lower fields.

In the ^{31}P NMR spectrum (Figure 3a), the two more intense peaks at δ 146.7 and 148.2 are attributed to 1,3- and 1,2-diglycerides, respectively. The phosphorylated 1-monoglycerides exhibit signals of low intensity close to those observed for their model compounds (Figure 3 and Table 1). However, no peaks for the 2-monoglycerides were observed in the ^{31}P NMR spectra of the olive oil samples used in this study. Smaller peaks in the spectrum have been assigned to aliphatic alcohols, tyrosol, and sterols (see Figure 3a). Two peaks remain unidentified at present.

Table 2. Mono- and Diglyceride Contents in Olive Oils[a]

sample	origin	year of production	variety	1-MG (%)	1,3-DG (%)	1,2-DG (%)	1,2-DG/total DG
1	Iraklion	1994−1995	Koroneiki	0.05	0.99	0.81	0.47
2	Ilia	1994−1995	Koroneiki	0.07	1.11	0.73	0.40
3	Ilia	1995−1996	Koroneiki	0.09	0.67	1.29	0.66
4	Magnisia	1995−1996	Amphissis	0.10	0.65	1.47	0.69
5	Leykada	1995−1996	Lianolia	0.05	1.25	1.52	0.55
6	Lesvos	1995−1996	Kolovi	0.11	1.26	1.42	0.53
7	Lasithion	1997−1998	Koroneiki	0.05	0.40	1.26	0.76
8	commercial	unknown	unknown	0.06	2.48	1.31	0.34

[a] Five to six ^{31}P NMR experiments were carried out for each sample. The precision of the measurements ranged from ±0.07 to ±0.01 for the different samples.

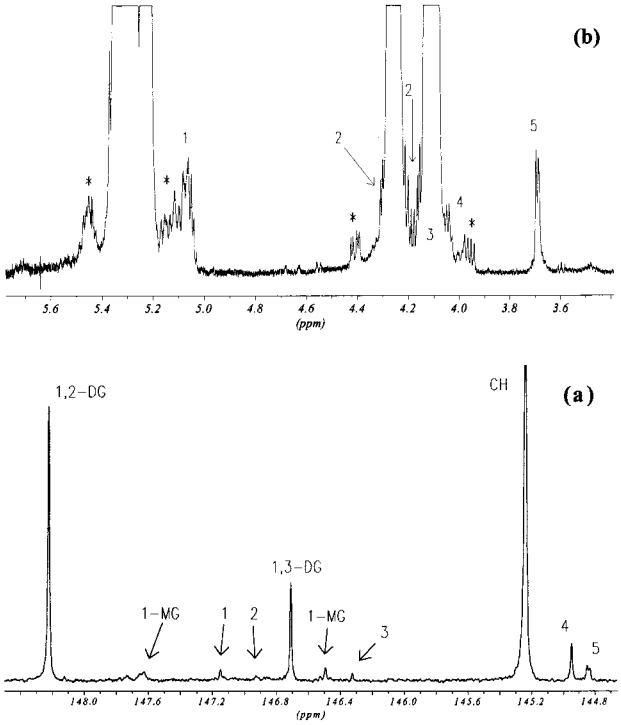

Figure 3. (a) ^{31}P NMR and (b) ^{1}H NMR of an olive oil sample. The region where mono- and diglycerides absorb is illustrated in both spectra. In the ^{31}P spectrum the peaks with Arabic numbers are assigned as follows: 1, aliphatic alcohols; 2, tyrosol (aliphatic hydroxyl); 4, sterolic hydroxyl; 3 and 5, unknown. In the proton spectrum the peaks are assigned as follows: 1, CH; 2, α-CH_2; 5, α′-CH_2 of 1,2-diglycerides; 3, CH; 4, CH_2 of 1,3-diglycerides; *, ^{13}C satellites of triglyceride signals. The peak denoted CH belongs to the internal standard cyclohexanol.

The integrals of these peaks are used to determine quantitatively 1-monoglycerides (1-MG), 1,2-diglycerides (1,2-DG), 1,3-diglycerides (1,3-DG), the 1,2-diglycerides/1,3-diglycerides (1,2-DG/1,3-DG) ratio, and the 1,2-diglycerides/total diglycerides (1,2-DG/total DG) ratio. The results of such an analysis for various virgin olive oil samples are summarized in Table 2. It is worth mentioning that an overnight ^{13}C NMR experiment performed for sample 8 gave a value of 0.35 for the 1,2-DG/total DG ratio, which is in excellent agreement with the value of 0.34 obtained from the ^{31}P NMR experiment in 30 min.

It has been suggested (Sacchi et al., 1997) that the ratio of 1,2-DG to the total amount of diglycerides (1,2-DG/total DG) plotted against the total amount of diglycerides is a useful index of the quality of olive oils. Refined and pomace oils tend to fall to the lower right of this plot, whereas virgin olive oils tend to gather at the upper left of the same plot. Such a plot can be obtained from the data of Table 2 and is presented in

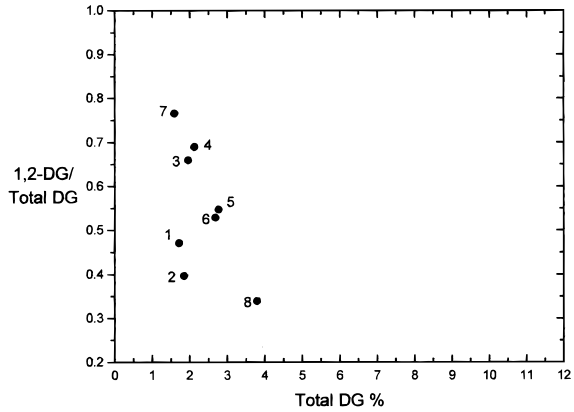

Figure 4. Plot of the ratio 1,2-diglycerides/total diglycerides (1,2-DG/total DG) to the total diglycerides (total DG) of olive oil samples.

Figure 4. Inspection of the plot reveals that seven olive oil samples (samples 1−7) examined (Table 2) can be considered as virgin, except perhaps sample 8. The lower 1,2-DG/total DG ratio observed for samples 1−6 relative to sample 7 (Table 2) may be explained partially on the basis of their older year of production. Samples 1−6 are 2−3 years older than sample 7 and thus contain a lower amount of 1,2-diglycerides. Nevertheless, other factors such as olive variety, olive ripeness, and geographic origin are essential in determining the decrease of 1,2-diglycerides in olive oils (Sacchi et al., 1993).

CONCLUSION

^{31}P NMR spectroscopy appears to be a facile quantitative technique for the analysis of mono- and diglyceride content in virgin olive oils, especially at low magnetic fields. Its excellent resolution and high sensitivity to the environment of labile protons may be exploited to determine quantitatively other constituents of the olive oil, such as saturated alcohols, polyphenols, sterols, free fatty acids, and volatile compounds. This study is in progress in our laboratory.

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