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

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A method for moisture determination in olive oil using ^{31}P NMR spectroscopy is developed. This method is based on the replacement of the hydrogen atoms of water molecules with the tagging agents 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane and diphenylphosphinic chloride. Both reagents were successful in determining moisture in olive oil. However, only the second reagent provided a clean and instantaneous reaction under mild condition with no side reactions as observed with the first reagent. A study comparison was made to assess the agreement between the present analytical NMR method and the well-established methods of Karl Fischer titration.

KEYWORDS: Moisture; olive oil, ^{31}P NMR spectroscopy; Karl Fischer titration

INTRODUCTION

Water content has long been recognized as an important factor determining the quality of olive oil. Small quantities of water in olive oil are responsible for the creation and persistence of the suspended and dispersed material that constitutes the so-called "veiling" of extra virgin olive oil (EVOO) (*1, 2*). Although there are conflicting experimental results in the literature (*1–5*) concerning the oxidative stability of veiled EVOO relative to filtered ones, the former is not attractive to the consumer mainly due to its appearance.

Several methods for moisture determination in lipids including olive fruits and oils have been elaborated in the past relying on Karl Fischer titration (*6*), IR and Raman spectroscopy (*7, 8*), low-resolution time-domain NMR spectroscopy (*9*), dry-oven method with continuous weighing (*10*), and azeotropic distillation with an immiscible organic solvent such as toluene or xylene (*10*). These approaches are designed as rapid commercial methods to monitor moisture with reasonable accuracy. In particular, the dry-oven technique is accompanied by several advantages such as easy performance speed and good reproducibility. Nevertheless, measurements of this type include the volatile compounds of olive oil in addition to moisture. The possibility of olive oil oxidation constitutes another disadvantage of heating, because this process is usually conducted in open air. Distillation methods show difficulties associated with low precision of the measuring device and the codistillation of water-soluble components leading to erroneous results.

In our laboratory, we have utilized intensively high-resolution ^{31}P NMR spectroscopy for the qualitative and quantitative determination of minor constituents (mono- and diacylglycerols, polyphenols, total free sterols, free fatty acids, glycerol, D-glucose, and maslinic acid) of olive oil after derivatization of hydroxyl and carboxyl groups with the phosphitylating agent 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (compound **1** in reaction **Scheme 1**) (*11, 12*). In these studies (*11, 12*), the ^{31}P chemical shifts of the various signals of the phosphitylated minor constituents were reported relative to a signal at δ 132.20, which has been assigned to the respective anhydride depicted as compound **2** in reaction **Scheme 1**.

We thought that the same reaction could be used for the in situ determination of water content in olive oil. This methodology has been used earlier (*13*) for the moisture determination in coals after extracting the water with a suitable solvent. In this paper we report the results of our attempt to perform moisture determination of several olive oil samples by employing ^{31}P NMR spectroscopy. Also reported is the successful moisture determination by using an alternative phosphorus reagent, diphenylphosphinic chloride (**3**), which appears to be superior to **1** (*13*) for reasons to be discussed later. This methodology for moisture measurements in olive oil will be compared with the conventional method of the Karl Fischer (KF) titration.

MATERIALS AND METHODS

Olive Oil Samples and Reagents. Fourteen extra virgin olive oil (EVOO) samples (years of harvesting 2004–2005) and one sample of olive-pomace oil (sample 15) were used in the present study. The EVOOs obtained from Zakynthos (samples 1–3), Messinia (samples 4–6), Lakonia (sample 7), and Heraklion (samples 8–10) were extracted from the olive variety Koroneiki, whereas those from Lesvos originated from two different olive varieties, that is, Andramitini (samples 11–13) and Kolovi (sample 14).

Protonated solvents and reagents for synthesis (reagent or analytical grade), deuterated chloroform, and pyridine were purchased from Sigma-Aldrich (Athens, Greece). The Karl Fischer reagents (HYDRANAL-composite 5 and HYDRANAL methanol dry) were purchased from Hydranal (Riedel-de-Haan, Seelze, Germany).

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Scheme 1

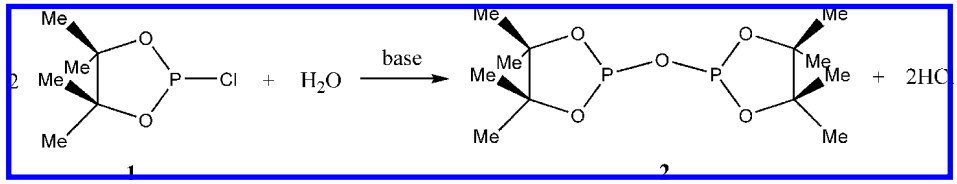


Table 1. Calibration of Karl Fischer Titration: Comparison of Titration Results with Known Weights of Water

weighed (% w/w)	measured (% w/w)
0.300	0.419
0.500	0.575
0.700	0.684
1.000	1.017
1.300	1.453
	$R = 0.984 \pm 0.041$
	slope $= 0.960 \pm 0.040$
	intercept $= -0.047 \pm 0.034$

Preparation of Compounds. The derivatizing reagent **1** was synthesized from pinacol and phosphorus trichloride according to the published procedure (*14*), with a slight modification by us (*11*) to increase the yield of the reaction. The phosphorus reagent **3** was prepared from dibutylphosphite and the Grignard reagent phenyl magnesium chloride as described in the literature (*15*); the yield was 65% against 75% for the literature value. The internal standard methyltriphenylphosphonium iodide (**4**) was synthesized from triphenylphosphine and methyl iodide following the literature procedure (*16*); the product yield was 55% (literature value, 65%). Compound 2-chloro-4,4,5,5-tetramethyldioxaphospholane-2-oxide (**5**) was obtained upon photo-oxidation of reagent **1** with singlet oxygen as follows: In a test tube containing 5 mL of toluene solvent and 100 μL of **1**, a preweighed amount of the photosensitizer tetraphenylporphyrin (TPP) was added. The concentration of the photosensitizer was 10^{-4} M. The test tube was placed on an ice bath and irradiated by a Cermax 300 W xenon lamp. Oxidation was carried out by bubbling pure oxygen through the solution for 4 h under continuous stirring.

The purity of the various reaction compounds mentioned above was checked by ^{1}H and ^{31}P NMR spectroscopy.

Karl Fischer Titration. KF titrations were carried out with a TURBO2 blending titrator (Thermo Orion) applying the one-component technique using the titrant HYDRANAL-composite 5 and the solvent HYDRANAL methanol dry. Titrations were carried out at room temperature using 800 mg of olive oil. Samples were introduced into the titration cell by means of a 5 mL disposable syringe without needle. Calibration of this method was performed by titrating known weights of water. The good correlation between the KF results and the weights of water is reflected in the correlation coefficient and the slope of the linear regression shown in **Table 1**.

Sample Preparation for Moisture Determination in Olive Oil with Reagents 1 and 3. A stock solution was prepared by dissolving 0.6 mg of chromium acetylacetonate, $Cr(acac)_3$ (0.165 μM), and 13.5 mg of the internal standard cyclohexanol (13.47 mM) in 10 mL of a mixture of pyridine and $CDCl_3$ solvents (1.6:1.0 volume ratio) and protected from moisture with 5A molecular sieves. The required volume of the stock solution (0.4 mL) and the reagent **1** (100 μL) were placed in a 5 mm NMR tube, and the mixture was left for about 5 min at room temperature to allow reagent **1** to react with the moisture of the medium. The ^{31}P NMR spectrum was then recorded to determine the background moisture content. After the determination of the basal moisture, 100 mg of olive oil was added; the reaction mixture was left to react for about 15 min at room temperature, and upon completion of the reaction the solution was used to obtain the ^{31}P NMR spectra.

The same solvent mixture of pyridine and $CDCl_3$ (10 mL) was used for the water derivatization with reagent **3**, with a slightly different volume ratio (2:1), in which 22.3 mg/mL of $Cr(acac)_3$ and 0.2 g of the internal standard methyltriphenylphosphonium iodide were added. The stock solution was protected from moisture with 5A molecular sieves. The required volume of the stock solution (0.4 mL) and the reagent **3** (100 μL) were placed in a 5 mm NMR tube, and the mixture was left for about 5 min at room temperature to allow reagent **3** to react with the moisture of the medium. The ^{31}P NMR spectrum was then recorded to determine the background moisture content. After the determination of the basal moisture, 100 mg of olive oil was added; the reaction mixture was left to react for about 15 min at room temperature, and upon completion of the reaction, the solution was used to obtain the ^{31}P NMR spectra.

Tests of Reagents 1 and 3 with Known Water Concentrations. Reagents **1** and **3** were added in excess (150 μL each) in 400 mL of the standard solutions prepared as described above. After determination of the basal moisture of the mixture of solvents and the reagent, water in different portions in 5 mL of dry pyridine was injected, and the ^{31}P NMR spectrum was obtained after each addition. Pyridine was dried by distillation over barium oxide before use.

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 26 $\pm$ 1 °C.

One-dimensional ^{13}P NMR spectra were recorded by employing the inverse gated decoupling technique to suppress NOE effects. Typical spectral parameters for quantitative studies using reagent **1** were as follows: 90°; pulse width, 12.5 μs; sweep width, 55 kHz; relaxation delay, 25 s; memory size, 32K. To ensure quantitative spectra, the magnitude of the relaxation delay adopted was >5 times the relaxation time (T_1 = 4.6 s) of the phosphitylated cyclohexanol; 32 transients were accumulated for each spectrum.

The spectral parameters for obtaining the ^{31}P NMR spectra by employing reagent **3** were modified somewhat, because the relaxation time measured for the internal standard methyltriphenylphosphonium iodide (T_1 = 0.1 s) was >1 order smaller than that of the phosphitylated cyclohexanol. Typical spectral parameters for quantitative studies using reagent **3** were as follows: 90°; pulse width, 12.5 μs; sweep width, 3 kHz; relaxation delay, 0.5 s; memory size, 32K; 32 transients were accumulated for each spectrum. For all FIDs, 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 control experiments, each FID was processed three times for each spectrum, and the average value was taken for the calculation of the moisture content.

One-dimensional ^{1}H NMR spectra were acquired with the following acquisition parameters: time domain, 64K; 90°; pulse width, 9.3 μs; spectral width, 12 ppm; relaxation delay, 2 s; 32 scans and 8 dummy scans were accumulated. Baseline correction was performed carefully by applying a polynomial fourth-order function to achieve a quantitative evaluation of all signals of interest. The spectra were acquired without spinning the NMR tube to avoid artificial signals, such as spinning side bands of the first or higher order.

IR Experiments. The IR spectra were recorded on a FTIR Spectrum BX (PerkinElmer). The preparation of the sample for the IR measurements was as follows: 100 μL of reagent **1** was added in 1 mL of the mixture pyridine/chloroform (1.6:1 v/v). After completion of the reaction of the reagent with background moisture, 200 μL of the mixture was transferred to a NaCl cell of 0.8 mm path length. The solvents were removed by a stream of nitrogen gas, and the IR spectrum was recorded (range of 400–4000 cm^{-1}) at room temperature with 20 scans and a resolution of 1 cm^{-1}. The IR spectrum of the mixture was recorded again after the addition of 5 mg of water.

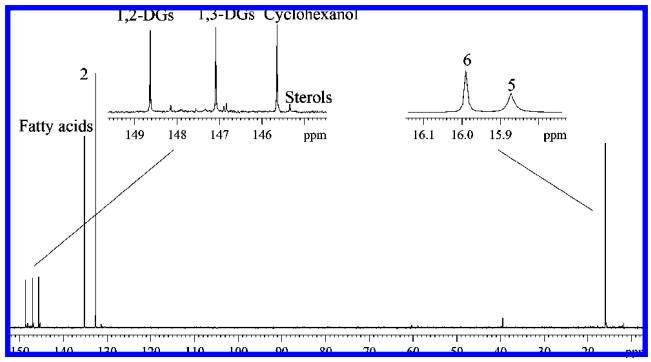


Figure 1. 202.3 MHz ^{31}P NMR spectrum of a phosphitylated sample of olive oil with reagent **1** in pyridine/chloroform solution. The left-hand-side inset shows the region where the signals of the phosphitylated diacylglycerols, total free sterols, appear. The right-hand-side inset illustrates the signals of the reaction products of reagent **1** with the water content in olive oil.

RESULTS AND DISCUSSION

^{31}P NMR Methodology. *Reaction of the Phosphorus Reagents* **1** *with Water.* In a previous paper (*11*), we reported the usefulness of reagent **1** in the quantitative analysis of olive oil, as well as its application to the quality control and authentication of EVOO. A number of minor compounds bearing hydroxyl and carboxyl groups were identified. The inset in **Figure 1** shows the well-documented ^{31}P NMR spectrum (*11, 12, 17*) of an EVOO sample in the region where the signals of the phosphitylated compound with the reagent **1** monoacylglycerols, the two diacylglycerol isomers, total free sterols, and free fatty acids are observed. Expansion of this spectrum to low frequencies reveals three more signals. The signal at low magnetic field strength (δ 132.20) has been assigned already to the phosphitylation product **2** of water with reagent **1** according to reaction 1, in which the phosphorus nucleus is in oxidation state +3. This signal has been used as a reference signal for the ^{31}P chemical shifts of the various phosphitylated compounds in the corresponding ^{31}P NMR spectra (*11*). The ^{31}P chemical shifts of the other two signals at δ 15.99 and 15.87 indicate the formation of two additional products, assigned tentatively to compounds 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane-2-hydride (**6**) and 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane-2-oxide (**5**), respectively, in which the phosphorus nucleus is in oxidation state +5. It is known (*18*) that the phosphorus nucleus in an oxidation state of +3 resonates at higher frequencies than the same nucleus in an oxidation state +5. The relative proportion of compounds **2**, **5**, and **6** was dependent on the water content.

To examine the influence of water content on the formation of the three products, the concentration of the reagent **1** was kept in excess while small amounts of water were added to pyridine/chloroform solutions (1.6:1 volume ratio). As shown in **Table 2**, the amount of compound **5** remained practically constant, whereas the amounts of both compounds **2** and **6** increased continually, with compound **2** being the predominant entity in solution.

Identification of Compounds **5** *and* **6.** By recording a coupled ^{31}P NMR spectrum (not shown), we were able to confirm immediately that one of the two byproducts at low frequency had a hydrogen attached directly to the phosphorus nucleus, inasmuch only the signal at δ 15.99 was split into a doublet with a characteristic one-bond P−H coupling constant of 710.13 Hz (*19*). Further support for this conclusion was derived from

Table 2. Concentration (Percent w/w) Changes of the Reaction Products of Reagent **1**[a] with Water upon Addition of Known Quantities of Water

water added (mg)	**2**	**5**	**6**
0.0	0.028	0.009	0.003
0.1	0.034	0.010	0.004
0.5	0.046	0.010	0.008
0.7	0.057	0.009	0.012
1.0	0.081	0.010	0.022
1.0	0.099	0.009	0.034
1.0	0.105	0.010	0.048

[a] In excess; see Material and Methods.

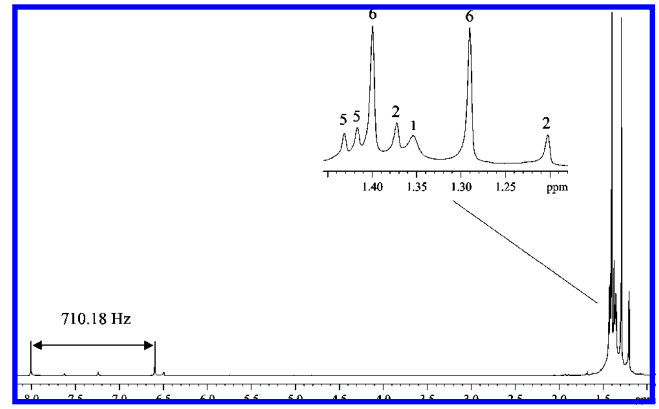


Figure 2. 500 MHz ^{1}H NMR spectrum of the reaction products of reagent **1** with water. The inset is an expansion of the region where the signals of the methyl protons of the various phosphitylated compounds appear.

the 500 MHz ^{1}H NMR spectrum of the mixture of the three products shown in **Figure 2**. The doublet at δ 7.25 ($J_{H,P}$ = 710.18 Hz) and the two strong singlets at δ 1.37 and 1.47 with relative intensities of 1:6:6 confirmed the formation of a P−H bond and the presence of two pairs of nonequivalent methyl groups. The geminal methyl groups at positions C-4 and C-5 of the phospholane ring are diastereoscopic and mutually equivalent by symmetry due to the presence of a plane of symmetry bisecting the C−C bond of the five-membered phospholane ring and passing though the H−P═O bond. The geometry of **6** was confirmed by performing DFT quantum mechanical calculations. The phosphorus atom in an oxidation state of +5 has a tetrahedral arrangement surrounded by four atoms: three oxygens and one hydrogen. Therefore, the signal at δ 15.99 in the ^{31}P NMR spectrum has been definitely assigned to the phosphorus nucleus of compound **6**. Moreover, to prove that the proton nucleus in compound **6** originated from water, the reaction 1 of reagent **1** was carried out in D_2O. The ^{31}P NMR spectrum of the reaction product **6** showed a triplet ($J_{P,D}$ = 106.81 Hz) of relative intensities 1:1:1 (not shown) indicative of the presence of a P−D bond (*19*).

A careful scrutiny of the spectrum in **Figure 2** reveals the presence of five more singlets of much lower intensity (indicated by numbers) than those observed for compound **6**. The two pairs of signals, each with relative intensity 1:1, were ascribed to the nonequivalent geminal methyl protons of compound **2** and to those of the unknown compound **5**. The broad singlet at δ 1.35 was assigned to the four methyl groups of reagent **1**. The lack of observation of two separate signals for each nonequivalent pair of geminal methyl groups for **1** was attributed to solvent effects (mixture of pyridine/chloroform), because the ^{1}H NMR spectrum of **1** in pure chloroform-*d* solutions showed clearly two signals for the methyl protons (not shown). It should be noted that no coupling between the phosphorus nuclei with

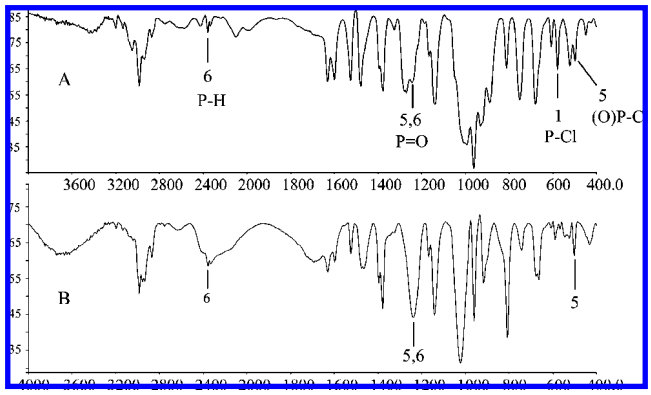


Figure 3. (**A**) IR spectrum of the reaction products of reagent **1** with water; (**B**) the same IR spectrum, but after the addition of an excess of water.

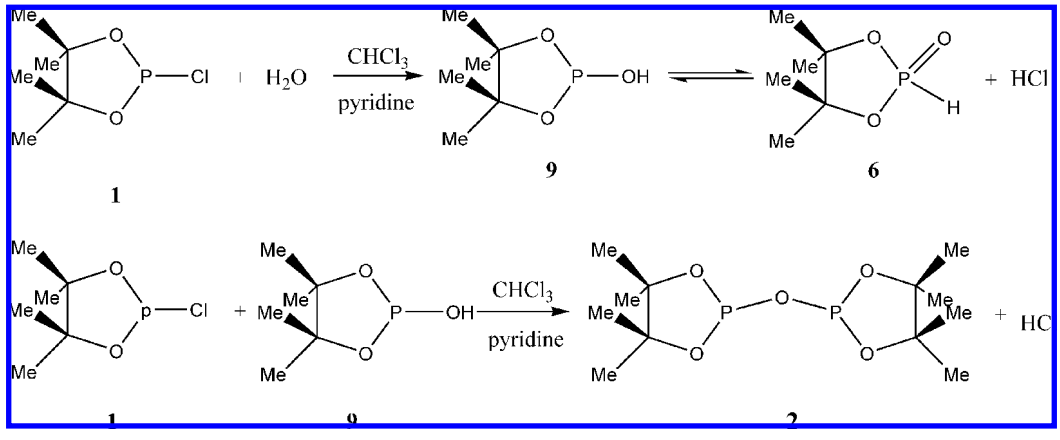
methyl protons of compounds **1**, **2**, **5**, and **6** was observed in their coupled ^{1}H and ^{31}P NMR spectra.

Compound **5** has a structure rather similar to that of compound **6**, bearing instead a directly attached chlorine atom to the phosphorus nucleus. Its minimum energy geometry calculated by DFT was similar to that of compound **6**. The phosphorus atom has a tetrahedral arrangement surrounded by three oxygens and one chlorine atom. There are several reasons favoring this structure: first, the 2-fold increase in the linewidth (~8 Hz) of the signal at δ 15.87 in the ^{31}P NMR spectrum relative to those measured for the other two signals (~4 Hz). This observation may be attributed to the presence of the quadrupolar chlorine nucleus in compound **5**. Second, the IR spectrum of the reaction products of reagent **1** with water, depicted in **Figure 3A**, shows a band at 501 cm^{-1} ascribed to the bond formed by the pentavalent (V) phosphorus atom with the chorine atom, P(V)−Cl. It is known (*20*) that the P−Cl bond of trivalent (III) and pentavalent (V) phosphorus atoms absorbs in the IR spectrum between 500 and 650 cm^{-1} and that bond P(III)−Cl absorbs at longer wavelengths than bond P(V)−Cl. Moreover, methyl substitution at the phospholane ring shifts the IR band of the P−Cl bond to shorter wavelengths. For instance, bond P(III)−Cl of the compound 2-chloro-1,3,2-dioxaphospholane (**7**) absorbs at 599 cm^{-1} (*21*). This absorption band shifts to shorter wavelengths by 21 cm^{-1} for compound **1** (578 cm^{-1}), which is the tetramethyl derivative of **7** (*22*). Accordingly, the absorption band of bond P(V)−Cl of compound **5** is expected to appear at shorter wavelengths by about 20 cm^{-1} relative to that of bond P(V)−Cl of compound 2-chloro-1,3,2-dioxaphospholane-2-oxide (**8**). The bond P(V)−Cl of compound **8** absorbs at 520 cm^{-1} (*23*); therefore, the band at 501 cm^{-1} in the IR spectrum of the reaction mixture with reagent **1** (**Figure 3A**) was correctly attributed to bond P(V)−Cl of compound **5**. The IR spectrum in **Figure 3A** shows a few more characteristic bands of compounds **1**, **2**, **4**, and **5**. A third and more convincing piece of evidence supporting the structure of **5** was its synthesis by photo-oxidation of reagent **1** with reactive singlet oxygen. Photo-oxidation of **1** produced a product that had the same ^{1}H and ^{31}P chemical shifts in pyridine/chloroform solution as compound **5**.

Mechanism of Reaction 1. The reaction pathway, according to the aforementioned transformations upon addition of water in the mixture of pyridine/chloroform (1.6:1 volume ratio) containing reagent **1**, can be described by the reaction in **Scheme 2**. In pyridine/chloroform solution, the chlorine atom of reagent **1** is replaced by a hydroxide ion by a simple nucleophilic substitution affording the intermediate 2-hydroxy-4,4,5,5-tetramethyldioxaphospholane (compound **9**). The short-lived intermediate cannot be detected by NMR and either isomerizes to compound **6** via a self-redox reaction or reacts with a second molecule of reagent **1** to produce compound **2**. At the same time, in both reaction steps, the released HCl gas is captured by the pyridine base. Compound **5** is not included in the above reaction scheme, because it does not constitute a byproduct of the reaction of water with reagent **1**; it is rather produced by direct photo-oxidation of the phosphorus reagent **1**. This conclusion is supported by the IR spectrum of the reaction products of reagent **1** with an excess of water as seen in **Figure 3B**. The spectrum shows that the intensity of the band due to the P(V)−Cl bond of compound **5** at 501 cm^{-1} does not change appreciably, whereas the consumption of **1** is reflected in the complete disappearance of the band of bond P(III)−Cl at 578 cm^{-1}. The constancy of the concentration of compound **5** regardless of the amount of the added water in the reaction advocates for this conclusion (**Table 2**). Therefore, the calculation of water content in test samples and olive oil samples was based only on the integration of the signals of compounds **2** and **6** in the ^{31}P NMR spectra.

Reaction of the Phosphorus Reagent **3** *with Water.* The next choice for the olive oil moisture determination was the less reactive diphenylphosphinic chloride **3** used by Verkade and co-workers (*13, 24*) for moisture determination in coals and by Dadey et al. (*25*) for the quantification of total phenol content in various coal-derived materials. ^{31}P NMR spectra of the reaction of **3** with water in pyridine/chloroform solutions (reaction **Scheme 3**) showed that the anhydride **10** was produced without the formation of any other byproduct. Such a spectrum is shown in **Figure 4**; the sole reaction product, **10**, gives a signal at δ 28.05.

Scheme 2



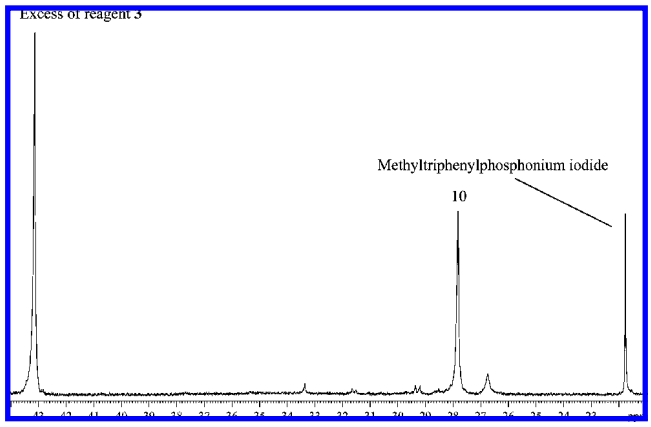


Figure 4. 202.3 MHz ^{31}P NMR spectrum of a phosphitylated sample of olive oil with reagent **3** in pyridine/chloroform solution. Methyltriphenylphosphonium iodide is the internal standard.

Scheme 3

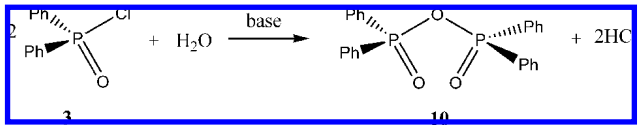


Table 3. ^{31}P NMR Spectroscopic Moisture Determination of Known Percentage Content of Water with Reagents **1** and **3**

water added	water calcd (%) with reagent **1**	water calcd (%) with reagent **3**
0.594	0.486	0.559
1.000	1.026	1.026
2.000	2.016	2.080
3.000	2.665	2.808
4.000	4.320	3.888
5.000	5.130	4.986
9.000	8.880	9.140
	$R = 0.999 \pm 0.005$ slope $= 0.006 \pm 0.034$ intercept $= 0.988 \pm 0.095$	$R = 0.999 \pm 0.003$ slope $= -0.048 \pm 0.017$ intercept $= 1.020 \pm 0.002$

The reactivity of reagent **3** against the hydroxyl groups of organic materials was found to be inferior compared to that of reagent **1**. Therefore, the use of the internal standard cyclohexanol for the quantification of compound **10** was avoided, because the complete phosphitylation of cyclohexanol with **3** lasted about an hour, lengthening thus considerably the duration of the analysis. After several attempts with other phosphorus compounds, we chose methyltriphenylphosphonium iodide (**4**) that gave a well-separated signal at δ 22.00 (**Figure 4**).

Validation of the ^{31}P NMR Methodology. The capability of reagents **1** and **3** for moisture determination was tested by injecting known amounts in the reaction medium for each reagent as described under Materials and Methods. After the addition of each new portion of water, a ^{31}P NMR spectrum was recorded. Comparison of the known amount of water and that calculated from the ^{31}P NMR spectra is shown in **Table 3**. Good correlation was obtained between the ^{31}P NMR method by using both reagents and the known weight of water. Linear regression of the data in **Table 3** resulted in good correlation coefficients (R) and a regression coefficient close to unity. The repeatability and reproducibility of our ^{31}P NMR method for moisture determination using reagents **1** and **3** were also examined (**Table 4**). The repeatability for both reagents was calculated by recording five consecutive spectra on the same day (intraday experiments), and using the same solution containing 0.2% w/w water, whereas the reproducibility was estimated by performing measurements on different days (interday experiments) on five different water samples (0.2% w/w) and using the same experimental protocol for each measurement. Statistical analysis of the data in **Table 4** shows that the performance of both reagents for moisture determination is equally successful.

Table 4. Repeatability[a] and Reproducibility[a] of the ^{31}P NMR Methodology by Using Reagents **1** and **3**

	calcd water content (% w/w)			
	reagent **1**		reagent **3**	
A/A	repeatability	reproducibility	repeatability	reproducibility
1	0.203	0.203	0.202	0.201
2	0.205	0.194	0.199	0.205
3	0.198	0.196	0.201	0.196
4	0.202	0.204	0.203	0.193
5	0.195	0.197	0.196	0.203
av	0.201	0.199	0.200	0.200
SD	0.003	0.005	0.003	0.005
%CV	1.493	2.513	1.500	2.500

[a] Calculated using solutions containing 0.2% w/w of water.

Moisture in Olive Oils. For a more rigorous comparison test with our ^{31}P NMR results, the moisture contents of a number of EVOO samples and one olive-pomace oil sample were determined by employing the KF titration. The results of the moisture determinations by KF titration and by the present ^{31}P NMR methodology using both reagents **1** and **3** are collected in **Table 5**. Linear regression of the data in **Table 5** provides very good correlation coefficients (0.980 for reagent **1** and 0.993 with reagent **3**) and slopes close to unity (0.998 for reagent **1** and 1.006 with reagent **3**). However, a more rigorous treatment of the data in **Table 5** should take into consideration the errors of the various experimental methods. These errors are reflected in the variances of the residuals (observed values − predicted values) (*26*). Because these variances are usually unknown, we used the loss function in eq 1, which appears to be more appropriate because it reflects the proportionality of the inverse of the variances of the residuals to the values of the independent variables (*26*).

$$\text{loss function} = (\text{obsd} - \text{pred})^2 \times \frac{1}{\text{KF}^2} \qquad (1)$$

The KF titration is considered to be an independent variable. The new regression data given in **Table 5** indicate that inclusion of errors in regression analysis weakens somewhat the correlation between the two experimental analytical techniques. It should be noted at this stage that regression as a statistical approach for comparison studies appears to be inappropriate for several reasons (*27, 28*). For instance, linear regression correlates simply the data of two methods, but does not prove the existence of any agreement between methods.

An alternative approach to the use of linear regression and correlation was the difference or bias plot recommended by Bland and Altman (*29*). On the abscissa they used the mean value of the methods to be compared, and on the ordinate they plotted the calculated difference between measurements by the two methods. They further estimated the mean and standard deviation (SD) of differences and displayed horizontal lines for the mean and for the mean ± 2SD. The two horizontal lines corresponding to the mean ± 2SD constitute the limits of agreement, which represent the 95% confidence interval for individual differences between the field and reference method.

Table 5. Moisture in Olive Oils Determined by ^{31}P NMR Spectroscopy Using Reagents **1** and **3** and by Karl Fischer Titration

origin	NMR (reagent **1**)	NMR (reagent **3**)	Karl Fischer
Zakynthos	0.274	0.309	0.284
Zakynthos	0.230	0.291	0.277
Zakynthos	0.252	0.288	0.266
Messinia	0.750	0.771	0.769
Messinia	0.228	0.259	0.259
Messinia	0.264	0.271	0.260
Lakonia	0.287	0.267	0.287
Heraklion	0.272	0.249	0.245
Heraklion	0.202	0.267	0.243
Heraklion	0.247	0.213	0.204
Lesvos	0.572	0.552	0.603
Lesvos	0.191	0.270	0.253
Lesvos	0.310	0.335	0.329
Lesvos	0.257	0.300	0.289
Heraklion[a]	0.372	0.397	0.419
R	0.980 ± 0.055	0.993 ± 0.033	
slope	0.998 ± 0.056	1.006 ± 0.033	
intercept	−0.015 ± 0.020	0.008 ± 0.012	
R^b	0.977 ± 0.023	0.980 ± 0.020	
slope[b]	0.992 ± 0.021	1.090 ± 0.023	
intercept[b]	0.056 ± 0.005	−0.028 ± 0.008	

[a] Olive-pomace oil sample. [b] Obtained by using weighted least-squares (see text).

In summary, this plot allowed the assessment of how the differences differ systematically from zero (bias) and how much the difference varies (error). **Figure 5** illustrates the bias plot comparing the ^{31}P NMR methodology with the KF conventional analytical method for moisture determination. **Figure 5A** shows the plot of the difference [^{31}P NMR(R_1) − KF] versus the average values of the two methods, [^{31}P NMR(R_1) + KF]/2, using phosphorus reagent **1** (R_1), whereas **Figure 5B** illustrates the difference [^{31}P NMR(R_3) − KF] versus [^{31}P NMR(R_3) + KF] utilizing reagent **3** (R_3). As shown in **Figure 5**, the mean differences of the two methods for the measurements of moisture were slightly different from zero (−0.015 for reagent **1** and 0.010 for reagent **3**), indicating that there is at most a negligible systematic difference between measurements of moisture in olive oil. In **Figure 5A**, which illustrates the distribution of the data points in the bias plot of moisture determined by using reagent **1**, all measurements are located within the upper (+0.0445) and lower (−0.0749) limits of agreement. Apart from 1 measurement of moisture using reagent **3** (**Figure 5B**), which is close to the upper limit of agreement (+0.0459), the remaining 14 measurements are within the limits of agreement (lower limit, −0.0254) in the respective bias plot. Moreover, no any obvious relationship between the difference and the average are observed in either bias plot; that is, the differences do not vary systematically over the range of measurements.

Because results in this study furnish only the sample statistics, it is necessary for the generalization of the results to other populations to report confidence intervals (CIs). Within a specified probability (95%), CIs show a range of values based on the observed data within which the population value lies (*30*).

$$95\%\ \text{CI for mean bias} = \bar{d} \pm t \times \sqrt{\text{SD}^2 / n} \qquad (2)$$

$$95\%\ \text{CI for upper limit} = (\bar{d} + 2\text{SD}) \pm t \times \sqrt{3\text{SD}^2 / n} \qquad (3)$$

$$95\%\ \text{CI for lower limit} = (\bar{d} - 2\text{SD}) \pm t \times \sqrt{3\text{SD}^2 / n} \qquad (4)$$

The ±95% CIs calculated from the formulas reported in ref *28* were found to be −0.03173−0.000133 and 0.00039−0.020143 for the mean differences [^{31}P NMR(R_1) − KF] and [^{31}P NMR(R_3) − KF], respectively. The ±95% CIs for the upper and lower limits of agreement were estimated to be 0.0159−0.0732

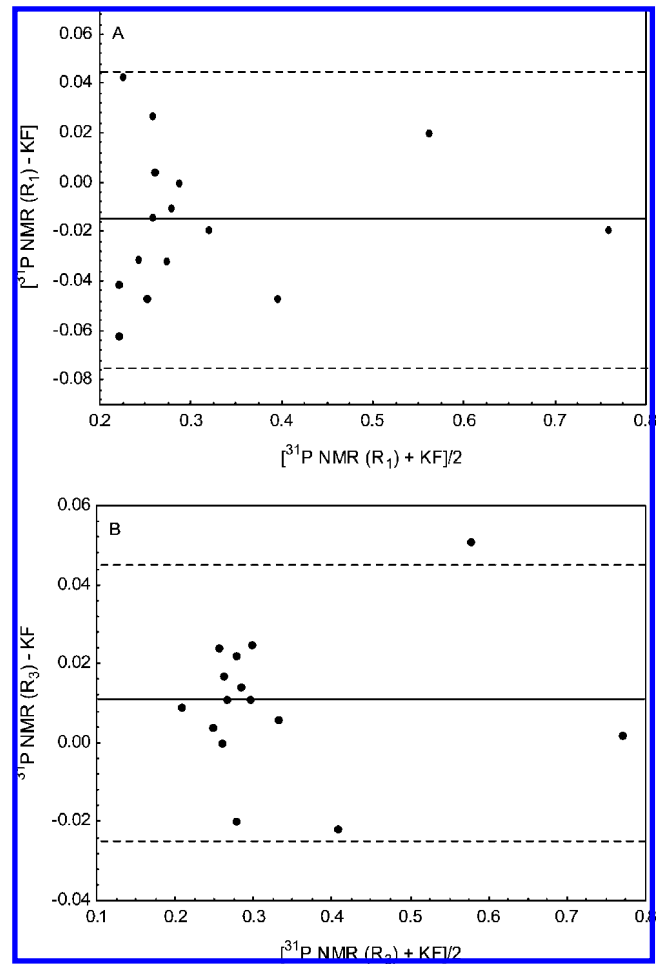


Figure 5. Difference (bias) plots of olive oil samples measured by ^{31}P NMR spectroscopy and Karl Fischer titration against the average of measurements with the mean difference (solid lines) and limits of agreement (dotted lines) by using (**A**) phosphorus reagent **1** and (**B**) phosphorus reagent **3**.

and −0.0463 to 0.1036 for the NMR methodology using reagent **1**, respectively, whereas those for the NMR methodology using reagent **3** were found to be 0.0288−0.0630 and −0.0083 to 0.0425, respectively. In eqs 2−4 d is the mean value, SD^2 is the variance of the difference, and t is the critical value for the 5% two-sided test drawn from tables of t distribution with $n - 1$ degrees of freedom (df), where n is the sample size.

In summary, this study proposes a new methodology for moisture determination in olive oil based on ^{31}P NMR spectroscopy. This methodology can be extended to other edible oils. Two phosphorus reagents were used, both reacting with water molecules quantitatively under mild conditions. Measurements with phosphorus reagent **1** require two integrations, and therefore they are somewhat less precise than those performed with reagent **3**; the latter gives a sole product with water and thus a single resonance in the corresponding ^{31}P NMR spectrum. Nevertheless, the use of reagent **1** allows the simultaneous quantification of several olive oil constituents in a single experiment.

Supporting Information Available: Coupled ^{31}P NMR spectra of the reaction product **6** of reagent **1** with H_2O and D_2O, respectively; ^{1}H NMR signals of the nonequivalent methyl protons of reagent **1** in a mixture of deuterated pyridine/chloroform solvents, and in chloroform-*d* solvent. This material is available free of charge via the Internet at http://pubs.acs.org.

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