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Review

^{31}P NMR spectroscopy in the quality control and authentication of extra-virgin olive oil: A review of recent progress

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This review is a brief account on the application of a novel methodology to the quality control and authentication of extra-virgin olive oil. This methodology is based on the derivatization of the labile hydrogens of functional groups, such as hydroxyl and carboxyl groups, of olive oil constituents with the phosphorus reagent 2-chloro-4,4,5,5-tetramethyldioxaphospholane, and the use of the ^{31}P chemical shifts to identify the phosphitylated compounds. Various experimental aspects such as pertinent instrumentation, sample preparation, acquisition parameters and properties of the phosphorus reagent are reviewed. The strategy to assign the ^{31}P signals of the phosphitylated model compounds and olive oil constituents by employing 1D and 2D NMR experiments is presented. Finally, the capability of this technique to assess the quality and the genuineness of extra-virgin olive oil and to detect fraud is discussed. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: ^{31}P NMR spectroscopy; extra-virgin olive oil

INTRODUCTION

In the past decade, high-resolution ^{1}H as well as ^{13}C NMR spectroscopy has emerged as a potential analytical tool for the analysis of vegetable oils and in particular olive oil.[1–4] The amount of information contained in an NMR spectrum obtained fairly rapidly, combined with easy sample preparation, renders this spectroscopic technique very attractive for the determination of the composition of olive oil. ^{1}H NMR spectroscopy has provided valuable information[1,3,4] about lipid classes, fatty acid composition, unsaturation levels and several minor compounds (sterols, squalene, terpenes, volatile compounds, etc.), whereas ^{13}C NMR, among others, has given unique information[2] about the positional distribution of fatty acids on the glycerol moiety and the stereochemistry of unsaturation.

This review describes an alternative nuclear magnetic resonance method that supplements ^{1}H NMR and ^{13}C NMR spectroscopy, especially in cases in which strong signal overlap and dynamic range problems in ^{1}H NMR spectra and/or long relaxation times of the insensitive ^{13}C nuclei render the analysis of olive oil a difficult task. This method[5] is based on the derivatization of the labile hydrogens of functional groups, such as hydroxyl and carboxyl groups, of olive oil constituents with the phosphorus reagent 2-chloro-4,4,5,5-tetramethyldioxaphospholane (**1**) according to the reaction scheme illustrated in Fig. 1, and the use of the ^{31}P chemical shifts to identify the labile centers (compound **2**). The present review is focused on the basic aspects underlying the ^{31}P NMR methodology, including instrumentation, preparation of samples and the phosphorus reagent, experimental conditions, chemical shifts of the phosphitylated compounds and finally its application to authentication and quality assessment of extra-virgin olive oil (EVOO).

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WHY ^{31}P NMR SPECTROSCOPY?

It was mentioned that ^{1}H and ^{13}C NMR spectroscopies have been used previously in the analysis of EVOO. Although these magnetic resonance methods are quantitative and do not require any sample pretreatment, they are not so effective under certain circumstances. For instance, the unambiguous identification and quantification of certain minor constituents of olive oil, such as mono- and diacylglycerols, demand ^{1}H NMR spectrometers operating at rather high magnetic field strengths ($\geq$14.1 T or 600 MHz in terms of the proton Larmor frequency). At lower magnetic field strengths, the diacylglycerol resonances are overlapped by the strong resonances of the triacylglycerols,[1] questioning therefore the ability of this magnetic resonance method for reliable quantitative determination of these minor components at lower

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magnetic field strengths.[6] On the other hand, the large range of chemical shifts (~1000 ppm) reported for the ^{31}P nucleus[7] ensures a good separation of the diacylglycerol signals. This is shown in Fig. 2, which depicts the ^{1}H and ^{31}P NMR spectra of an EVOO sample at two magnetic field strengths.[6] The excellent resolution between the ^{31}P chemical shifts allows a reliable detection of the phosphitylated mono- and diacylglycerols, even at a magnetic field strength of 9.4 T or 162.0 MHz (Fig. 2(b)). Apart from mono- and diacylglycerol signals, the ^{31}P NMR spectra in Fig. 2(a) and (b) show one additional signal at δ 144.96 attributed to the phosphitylated total free sterols (STE). The signal at δ 145.13, labeled as CH, is due to the internal standard (IS) cyclohexanol, whereas the phosphitylated free fatty acids, which represent the free acidity of olive oil, give a single signal at δ 134.80 (not shown). No monoacylglycerol resonances could be detected in the proton spectra (Fig. 2(c) and (d)). An extensive overlap of the diacylglycerol resonances with those of the strong α, α' proton resonances of the triglycerides and their ^{13}C satellites is observed even at 11.74 T or 500 MHz (Fig. 2(c)). The situation becomes worse at lower magnetic field strengths (compare spectra (c) and (d) of Fig. 2). The study of diacylglycerols (but not monoacylglycerols due to their low concentration in olive oil, ≤0.3%) in olive oil by ^{31}P NMR spectroscopy was feasible at even lower magnetic field strengths.

Apart from the wide range of ^{31}P chemical shifts, the 100% natural abundance of the ^{31}P nucleus and its high sensitivity, which is only ~15 times less than that of the proton nucleus, make the ^{31}P NMR experiments a reliable analytical tool to determine amounts of the order of micromolar, or lower, depending on the available instrumentation. These properties of the ^{31}P nucleus should be contrasted with the low natural abundance and sensitivity of the ^{13}C nucleus, which, in addition, is characterized by long relaxation times. Thus, quantitative ^{13}C NMR experiments require lengthy accumulations and long relaxation delays to achieve a satisfactory signal-to-noise (S/N) ratio. It is worth mentioning that an overnight ^{13}C NMR experiment performed on an olive oil sample gave a value of 0.77 for the ratio D of the 1,2-diacylglycerol concentration over the total amount of diacylglycerols (1,2-diacylglycerol + 1,3-diacylglycerol), which is in excellent agreement with the value 0.78 obtained from a ^{31}P NMR experiment in ~30 min. Another advantage of the ^{31}P NMR method is the introduction of an IS of known amount (usually cyclohexanol) in the reaction mixture, which allows the determination of the absolute concentration of the phosphitylated product **2**, avoiding thereby normalization conditions.

Limitations of the present methodology may be the preparation of the phosphorus reagent and the

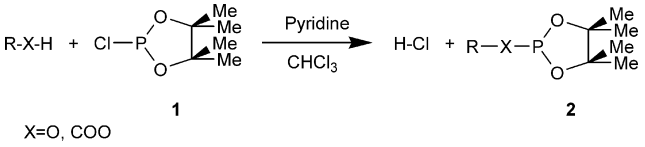

Figure 1. Reaction of hydroxyl and carboxyl groups of olive oil constituents with the phosphorus reagent 2-chloro-4,4,5,5-tetr -amethyldioxaphospholane (**1**).

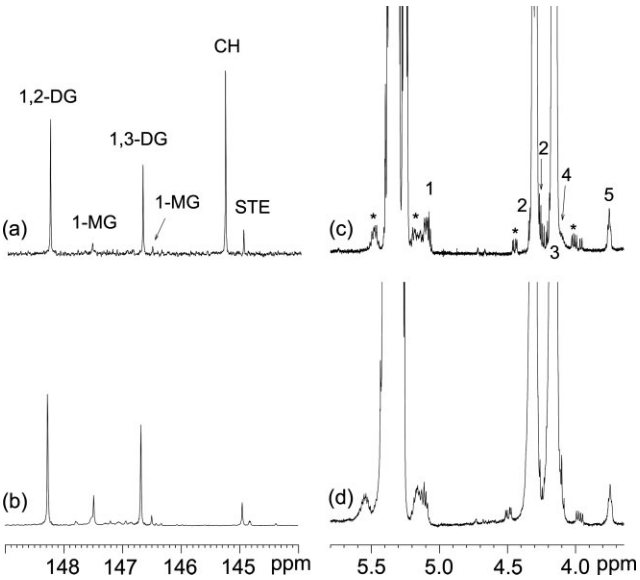

Figure 2. Expansions of the diacylglycerols region of (a) 202.3 MHz and (b) 162.0 MHz ^{31}P-NMR spectra; (c) 500 MHz and (d) 400 MHz ^{1}H NMR spectra of an EVOO sample. The ^{31}P NMR spectra show peaks that belong to 1,2-diacylglycerol (1,2-DG), 1,3-diacylglycerol (1,3-DG), 1-monoacylglycerol (1-MG), and total free sterols (STE). The peak denoted as CH is due to the IS cyclohexanol. In the proton spectrum the peaks are assigned as follows: 1, CH; 2, α-CH_2; 5, α'-CH_2 of 1,2-DG; 3, CH; 4, CH_2 of 1,3-DG. The asterisks represent the ^{13}C satellites of triglyceride signals.

phosphitylation reaction preceding the acquisition of the ^{31}P NMR spectrum, lengthening the duration of the analysis relative to ^{1}H NMR spectroscopy. Although this method is less simple and requires more time than the ^{1}H NMR method, it is much faster than the corresponding classical methods of titration and gas chromatography (GC) because it determines several constituents (e.g. monoacylglycerols, diacylglycerols, total free sterols and free acidity) in a single spectrum. Moreover, it avoids several problems, such as lipid oxidation, involved in the traditional GC analysis.[8] Finally, the quantification of phenolic compounds and, in particular, total tyrosol and total hydroxytyrosol that contribute to the stability of EVOO against oxidation[9] involves one more step, i.e. extraction, which lengthens further the duration of this NMR method. Nevertheless, it can be considered as a valuable alternative to the conventional high-performance liquid chromatography (HPLC). The HPLC method, although accurate with low detection and quantification limits, when applied to olive oil extracts requires calibration with standards that may not be available commercially. In addition, analytical results depend on the mobile phase used, whereas difficulties arise in the interpretation of the chromatograms whenever unknown substances with the same retention time as those of EVOO constituents are present.

EXPERIMENTAL

NMR instrumentation

The magnetic field strength of the NMR spectrometer to be used depends on the type of study being made. For

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the detection and quantification of the diacylglycerols, total free sterols and acidity, which give well-separated signals, relatively low magnetic field strengths from 7.05 to 9.4 T (Larmor frequencies 121.5 and 162.0 MHz for the ^{31}P nucleus) are adequate.[5,6] However, the detection of phenolic compounds in the polar fraction of olive oil may require higher magnetic field strengths up to 11.74 T (202.3 MHz) to improve the sensitivity and resolution of the experiment.[10]

Phosphorus reagent

The phosphorus reagent **1** was first prepared[11] in 1967 from pinacol and phosphorus trichloride in the presence of triethylamine and purified by distillation under vacuum. Since then, this reagent has been used as a derivatizing agent in several applications related to the analysis of lignin by Argyropoulos' group,[12,13] coal materials by Verkade's group[14] and polymeric materials.[15,16] Nevertheless, the yield of the produced phosphorus reagent following the original method was rather low (~20%). To increase the yield of the reaction, we utilized hexane instead of benzene and pyridine base instead of triethylamine suggested in the original method. This modification resulted in ~45% yield of the product. The present phosphorus reagent does not form isomeric products or side reactions upon derivatization of OH and COOH groups.[5,6,10,17] For instance, phenol-containing olive oil model compounds bearing hydroxyl and carboxyl groups remained stable for more than 2 weeks after derivatization.[17] Additional advantages offered by phosphorus reagent **1** relative to other tagging reagents have been described in Refs 12,14,18 and 19.

Sample preparation

A stock solution was prepared by dissolving 0.6 mg of chromium acetylacetonate [$Cr(acac)_3$, (0.165 μM)] and 13.5 mg 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 5 Å molecular sieves. Each model compound (0.1–3.0 mg depending on its molecular weight and the number of hydroxyl groups) and 100 to 150 mg of olive oil were dissolved in 0.4 ml of the stock solution in a 5-mm NMR tube. For the determination of phenolic compounds, the polar extract obtained from 35 g of olive oil was dissolved in 0.4 ml stock solution. Predetermined amounts of reagent **1** (5–30 μl for model compound and olive oil, depending on the number of the functional groups, and 50 μl for the olive oil extract) were added. The mixture was left to react for about 15 min at room temperature. Upon completion of the reaction, the solution was used to obtain the ^{31}P NMR spectra.

Pyridine was selected as the second component of the solvent mixture because it captures immediately the hydrogen chloride gas liberated during the phosphitylation reaction forming the pyridine hydrochloride salt. This drives the overall phosphitylation reaction (Fig. 1) to total conversion. Pyridine solvent should be in excess relative to the phosphorus reagent, since the latter is still a strong derivatizing reagent and the HCl liberated in further derivatization is capable of inducing decomposition of the derivatized compounds.[19] The role of the chloroform solvent is twofold: first it ensures the dissolution of olive oil, and second it does not allow the precipitation of the pyridine-HCl salt

Quantitative one-dimensional ^{31}P NMR spectra

To obtain reliable quantitative data by using one dimensional (1D) ^{31}P NMR spectra, several criteria must be considered: (i) Complete derivatization with the tagging reagent **1** must be achieved, as was shown in earlier publications;[5,10,12,14,17] (ii) Thermal equilibrium must be reached by the phosphorus nuclei before the pulse sequence repetition. This was guaranteed by using repetition time at least 5 times greater than the longest spin–lattice relaxation time (^{31}P-T_1). The measured ^{31}P spin–lattice relaxation times for several phosphitylated model compounds in the mixture of pyridine and chloroform solvents, including the phosphitylated IS (cyclohexanol) were very long (5–10 s),[10,20,21] lengthening considerably the duration of the experiment; (iii) Addition of paramagnetic $Cr(acac)_3$ lowers the spin-lattice relaxation times of the phosphorus nuclei, shortening thus the duration of the measurements significantly. For instance, the largest ^{31}P spin–lattice relaxation time (4.6 s) measured in the presence of $Cr(acac)_3$ was found to be that of the phosphitylated IS cyclohexanol.[10] Therefore a repetition time of 25 s is considered the most appropriate for the present quantitative ^{31}P NMR experiments; (iv) To eliminate NOE effects, the inverse gated decoupling technique was used; (v) The S/N ratio of the ^{31}P NMR experiments depends on the concentration of the constituent in EVOO, the number of the functional groups in the molecule to be derivatized and the chemical nature of the functional group and its environment. A reasonable S/N ratio was achieved by optimizing the number of scans to 16 for diacylglycerols and model compounds and to 32 scans for the derivatized phenolic compounds in EVOO extracts. The suggested spectral parameters for quantitative studies are: 90° pulse width, 10 kHz sweep width; 25 s repetition time; 16–32 K memory size (zero-filled to 32–64 K, respectively). For processing the NMR spectra, line broadening of 1 Hz and a drift correction are recommended prior to Fourier transformation, as well as application of a polynomial fourth-order baseline correction before integration. All chemical shifts are reported relative to the product of the reaction of **1** with water (sample moisture), which has been observed to give a sharp and stable signal in pyridine–$CDCl_3$ at 132.20 ppm. Finally, spectroscopic acquisition is carried out at room temperature, mainly because decomposition of the phosphitylated compounds was observed at high temperatures. At any rate, no sensitivity or resolution enhancement was observed when recording spectra at higher temperatures.

Two-dimensional ^{31}P NMR spectra

Apart from coupled and/or decoupled 1D ^{31}P NMR spectra, two-dimensional (2D) gradient selected homonuclear ^{31}P–^{31}P (gradient ^{31}P–^{31}P homonuclear spectroscopy, *g*-P-P-COSY) and heteronuclear ^{1}H–^{31}P experiments provide the experimentalist with additional means for clarifying assignments in 1D ^{31}P NMR spectra. Heteronuclear ^{1}H–^{31}P spectra were obtained either with magnetization transfer from protons to phosphorus-31 nuclei through chemical

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bonding (gradient $^1H-^{31}P$ heteronuclear multiple quantum coherence, *g*-H-P-HMQC), or through-space ($^1H-^{31}P$ heteronuclear NOE, H-P-HOESY).

g-P-P-COSY spectra were obtained basically by using 128 increments of 1K data points. Eight scans and four dummy scans were accumulated for each increment with a recycle delay of 1.0 s. The data set was zero-filled to a 1 K × 1 K matrix prior to Fourier transformation. A squared sinusoidal window function was used in both dimensions. Two sinusoidal-shaped field gradients of strength ratio $G_1 : G_2 = 1 : 1$ and 2 ms duration each were used.

g-H-P-HMQC spectra were usually acquired with 128 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 $J_{H,P} \sim 10$ Hz. Optimized experiments for couplings of $J_{H,P} \sim 3$ Hz resulted in identical spectra. No proton decoupling was performed during acquisition. Sixteen scans and eight dummy scans were accumulated for each increment, with a recycle delay of 1.0 s. The data set was zero-filled to a 1 K × 1 K matrix prior to Fourier transformation. A squared sinusoidal window function was used in both dimensions. Three sinusoidal-shaped field gradients, G_1, G_2, G_3 of 2 ms duration were used with the strength ratio $G_1 : G_2 : G_3 = 50 : 30 : 52.4$ in order to satisfy the relationship

$$G_1(\gamma_H + \gamma_P) + G_2(-\gamma_H + \gamma_P) + G_3(-\gamma_H) = 0$$

It should be noted that the *g*-H-P-HMBC experiment to obtain long-range H–P couplings was not applicable, inasmuch the phosphitylated compounds had no direct H–P couplings to be suppressed.

$^1H-^{31}P$ heteronuclear NOE spectra were recorded with 128 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. Forty-eight scans and four dummy scans were accumulated for each increment with a recycle delay of 1.0 s. The data set was zero-filled to a 1 K × 1 K matrix prior to Fourier transformation. A squared sinusoidal window function was used in both dimensions. No phase correction was applied to all 2D spectra, which were displayed in magnitude mode.

CHEMICAL SHIFTS

Chemical shift ranges

A large number of olive-oil-related model compounds bearing aromatic and aliphatic hydroxyl groups and carboxyl groups were derivatized with **1**, and their chemical shifts were recorded.[5,10,17] Well-separated ranges of ^{31}P chemical shifts for the three classes of phosphitylated functional groups were observed. The ^{31}P chemical shifts for all phosphitylated aromatic hydroxyl groups cover a rather wide range (δ 136.4–142.9 ($\Delta\delta = 6.5$)), whereas the aliphatic hydroxyl groups cover a narrower range (δ 145.7–149.6 ($\Delta\delta = 3.9$)). The ^{31}P chemical shift range of carboxyl groups is quite narrow (δ 134.6–135.3 ($\Delta\delta = 0.7$)). Monosubstituted phenols at *para* position show small deviations (±0.5 ppm) relative to simple phenol (δ 128.02), whereas the introduction of substituents at *ortho* or *meta* position resulted in significant shifts at higher frequencies, depending on the nature and the number of the substituents. For instance, the ^{31}P chemical shift of the phosphitylated hydroxyl group of *p*-coumaric acid (**3**), bearing the substituent at *para* position relative to the hydroxyl group, was found at δ 137.82, not very different from that of phenol. The ^{31}P chemical shift of the phosphitylated hydroxyl changed to δ 138.63 for caffeic acid (**4**) bearing a second *o*-hydroxyl group, to δ 139.46 for ferulic acid (**5**) bearing an *o*-methoxy group and to δ 142.23 for sinapic acid (**6**) bearing two methoxy groups at the *ortho* position.[17] The same trend was observed for lignans.[10] The phosphitylated hydroxyl group of (+)-pinoresinol (**7**) with one methoxy group at the *ortho* position exhibited one signal at δ 139.84, whereas the signal of the phosphitylated hydroxyl group of syringaresinol (**8**), flanked by two *o*-methoxy groups, was shifted to δ 142.89.

Concentration and temperature effects on the ^{31}P chemical shifts were minimal as demonstrated elsewhere by conducting appropriate experiments with model compounds.[10] Also, no significant chemical shift change was observed for each polyphenol in a mixture of polyphenol model compounds in pyridine/chloroform relative to that of the individual polyphenol in its own solution.[10] Nevertheless, very small changes (up to 0.05 ppm) were observed in olive oil extracts. These changes may be attributed to the higher polarity of the medium resulting from the olive oil extraction.

Model compounds

^{31}P NMR spectra are often obtained by proton decoupling. This means that the multiplicity of the ^{31}P NMR signals is lost, and by implication all inherent structural information. Nevertheless, proton decoupling results in single resonance lines for each magnetic nonequivalent phosphorus nucleus, facilitating thereby spectroscopic assignment and quantitative measurements. Spectroscopic parameters, such as signal intensities, chemical shifts δ and $^{31}P-^1H$ and $^{31}P-^{31}P$ coupling constants are readily measurable from the decoupled and coupled spectra, respectively, although assignment of the chemical shifts is not always an easy task. More than 30 model compounds possessing structures likely to be present in olive oil have been used, and a series of 2D NMR techniques were applied in order to assign unambiguously the phosphorus signals and unravel the identity and structure of the phosphitylated constituents of olive oil.[5,10,17]

The ^{31}P chemical shifts assignment for model compounds bearing a single functional group, such as free fatty acids, and diacylglycerols presented no problem. Also, compounds with one, two or even three different functional groups, such as tyrosol (**9**) bearing one saturated and one aromatic hydroxyl groups, can be easily detected by ^{31}P NMR spectroscopy. Another example is the assignment of the three phosphitylated hydroxyl groups (two of them are equivalent) of gallic acid (**10**) based on the relative signal intensities.[17] Nevertheless, the chemical shift assignment for more complex molecules, in particular, for the substituted diphenols, demands the use of 2D NMR experiments.[10,17] Our general strategy to assign the ^{31}P signals of complex phosphitylated compounds involves the following steps:[10,17] First,

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elucidation of the splitting patterns observed in 1D ^{31}P NMR spectra. Second, assignment of proton chemical shifts and, if necessary, carbon chemical shifts of the phosphitylated compounds by employing pertinent homonuclear ^{1}H–^{1}H and heteronuclear ^{1}H–^{13}C 2D NMR experiments. Third, assignment of the phosphorus signals by employing NMR experiments connecting signals via H–P and/or P–P long-range scalar interactions or via through-space H–P dipole–dipole connectivities. This strategy has been applied for the assignment of the ^{31}P chemical shifts of a large number of phosphitylated model compounds.[10,17] Examples of ^{1}H–^{31}P and ^{31}P–^{31}P chemical shift correlated spectra, and ^{1}H–^{31}P HOESY spectra are shown in Figs 3, 4 and 5 for the phosphitylated homoprotocatechuic acid (**11**), gallic acid (**10**) and protocatechuic acid (**12**), respectively. It is interesting to note that application of the ^{31}P–^{31}P chemical shift correlated technique has revealed five- and six-bond coupling pathways usually observed for the disubstituted *ortho* and *meta* phenols in olive oil. Depending on the $\Delta\nu/J$ ratio of the ^{31}P resonances, A_2, AB and AX types of ^{31}P NMR spectra were obtained (Fig. 6).[22]

Phenolic compounds in olive oil

Figures 7(a) and (b) show the ^{31}P NMR spectrum of the polar part of an EVOO sample from Messinia in the regions where the aromatic and aliphatic phosphitylated hydroxyl groups of phenolic compounds are resolved, respectively. The assignment of the ^{31}P chemical shifts reported in Figs 7(a) and (b) was based on the chemical shifts of the appropriate model compounds that were purchased, synthesized or extracted from olive oil, and by spiking the sample with pure compounds, when necessary.[10] Table 1 compares the ^{31}P NMR chemical shifts of the model compounds with those observed in the ^{31}P NMR spectrum (Figs 7(a) and (b)). The strong signals at δ 138.19 and δ 139.20 reflect the total amount of tyrosol and hydroxytyrosol contained in olive oil, respectively, since the phosphitylated aromatic hydroxyl groups of these compounds in their free and esterified forms are expected to show about the same chemical shifts. The

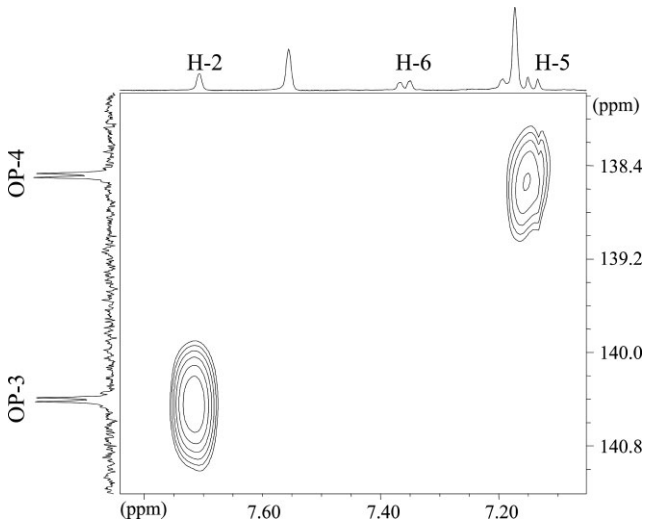

Figure 3. 500 MHz *g*-H-P-HMQC spectrum of the phosphitylated hydroxyl groups of homoprotocatechuic acid (**11**).

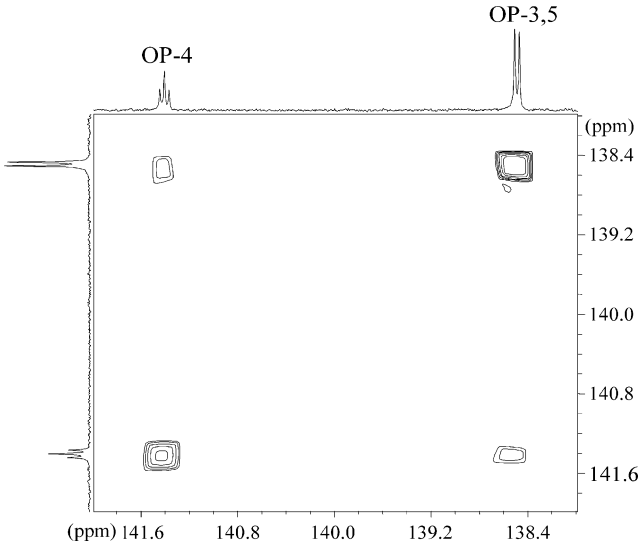

Figure 4. 202.3 MHz *g*-P-P-COSY spectrum of the phosphitylated hydroxyl groups of gallic acid (**10**).

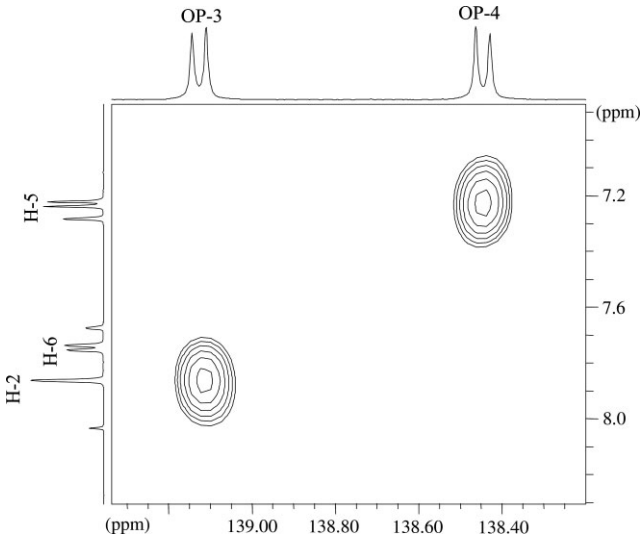

Figure 5. 500 MHz H-P-HOESY spectrum of the phosphitylated hydroxyl groups of protocatehuic acid (**12**).

esterified hydroxytyrosol and tyrosol involve their acetate derivatives and the hydrolysis products of oleuropein and ligstroside (the dialdehydic form of elenolic acid linked to hydroxytyrosol, the dialdehydic form of elenolic acid linked to tyrosol, the oleuropein aglycon and the ligstroside aglycon), respectively, formed during olive oil extraction from olive fruits and/or during storage.[23] Free and esterified hydroxytyrosol constitute an important class of phenolic compounds that contributes to the stability of EVOO against oxidation[9] and benefits human health.[24] The complete assignment of phenolic compounds, as well as for those compounds contained in the polar fraction of olive oil, has been described in detail in Ref. 10.

Olive oil moisture

Moisture in olive oil is an important factor in determining its quality. It has been suggested[25] that moisture (water in olive oil) and small particles suspended and dispersed in

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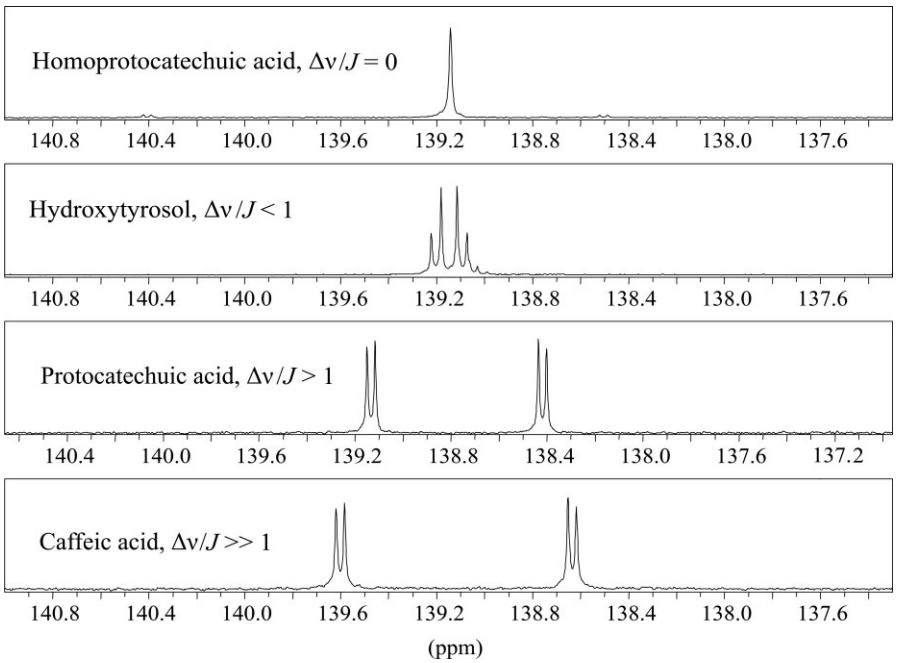

Figure 6. Experimental splitting pattern of the homoallylic coupling between two ^{31}P nuclei in four phenolic compounds for various ratios of $\Delta\nu/J$. A_2 for homo protocatechuic acid (**11**), AB for hydroxytyrosol (**13**) AX for protocatechuic acid (**12**) and caffeic acid (**4**) types of spectra are observed.

olive oil can induce upon storage cloudiness, characterizing the so-called *veiled* (cloudy) olive oil. This phenomenon, which varies with the extraction system used, results in different appearances and settling times. Although there are conflicting experimental results regarding the stability of veiled olive oil relative to that of filtered olive oil, it appears that veiled olive oil does not meet the consumer preference mainly because of its appearance. It was thought that the present ^{31}P NMR methodology could give quantitative information about the moisture content in an easy way. Our preliminary results indicate that reagent **1** may not be the appropriate reagent for moisture determination since it forms three products with moisture in the presence of pyridine. Nevertheless, the sum of integrals of the corresponding signals in the ^{31}P NMR spectrum in combination with the IS gives quantitative results for moisture. At any rate, it appears to be quite annoying to integrate three resonance lines for determining one quantity. Moreover, the result would contain the cumulative error of three measurements. Further work is in progress in our laboratory for exploring the possibility of using other phosphorus reagents that may react more cleanly with water.

SENSITIVITY, REPEATABILITY, REPRODUCIBILITY AND VALIDATION OF THE ^{31}P NMR METHODOLOGY

The applicability of the ^{31}P NMR method to quantitative analysis has been tested several times against ^{1}H NMR spectroscopy and of known weight standard.[5,10,17] Linear relationships were observed, with very good correlation coefficients (R = 0.998–0.999) between the reference concentrations and those obtained from ^{31}P NMR spectra. Nevertheless, a comparison of the ^{31}P NMR results with those obtained by official and/or well-recognized analytical methods presents a more rigorous and efficient validation test for the ^{31}P NMR methodology. Such a comparison has been made in a recent publication[26] with results obtained by independent laboratories using official and/or well-recognized conventional methods of analysis. In particular, free fatty acids concentration (free acidity) was determined by using ^{31}P NMR spectroscopy and titration; phenolic compounds by ^{31}P NMR spectroscopy and HPLC; and diacylglycerols by ^{31}P NMR spectroscopy and gas chromatography. A method comparison study was conducted and the agreement between NMR and conventional methods was evaluated by using the Bland and Altman statistical analysis.[27] The distribution of the data points in the bias plots showed that 96.4, 96 and 100% of the measurements of free acidity, diacylglycerols and phenolic compounds were within the limits of agreement of the two methods.

The repeatability and reproducibility of the method have been tested several times in the past and were found to be 1.0–1.5% and 2.2–2.5%, respectively.[5,10,17,28] The repeatability was measured on the basis of several consecutive ^{31}P NMR spectra recorded for the same model compound (e.g. phosphitylated hydroxytyrosol), whereas the reproducibility was tested by performing measurements on several different solutions of model compounds and using the same experimental protocol for each measurement. It is interesting to note that the repeatability of the whole methodology for the determination of phenolic compounds (extraction–phosphitylation–spectrum) was estimated to be 3.9%.[10] This analysis demonstrated that the ^{31}P NMR methodology is accurate and precise.

The minimum detectable amount of the EVOO minor constituents was calculated on the basis of measurements for phenolic compounds.[10] Assuming a minimum S/N ratio of 3

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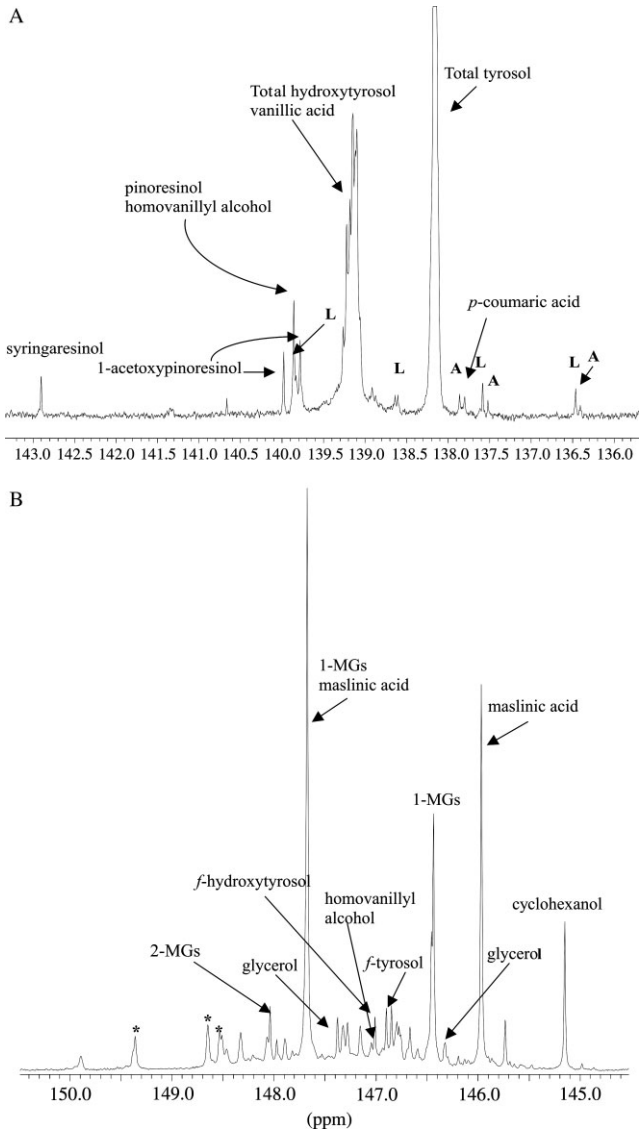

Figure 7. 202.3 MHz ^{31}P NMR spectrum of the phosphitylated polar fraction of an EVOO sample from Messinia in a chloroform/pyridine solution. (A) Aromatic region; (B) aliphatic region. **A**, apigenin; **L**, luteolin; 1-MGs, 1-monoacylglycerols; 2-MGs, 2-monoacylglycerols; *f*-hydroxytyrosol, free hydroxytyrosol; *f*-tyrosol, free tyrosol. Unidentified signals of the hydrolysis products of oleuropein-7-*O*-glucoside (and ligstroside) are denoted by asterisks.

and the expected molecular weights of phenolic compounds, which lie between 150 and 500 Da, the detection limit ranges from 0.26 to 0.85 mg/kg for the present instrumentation.

QUALITY ASSESSMENT AND AUTHENTICATION OF EVOO

Analyses for the quality control and authentication of EVOO are usually carried out by using conventional analytical techniques, including chromatographic methods (GC and HPLC), of several fractions of this product. The usual approach to detect EVOO adulteration is to compare the chemical composition of the suspect olive oil with limits for several of its constituents and/or physical constants imposed by the European Union and other official food organizations. Any adulterant addition is expected to modify the concentration of these constituents, or at least it will indicate an anomaly in its chemical composition. Nevertheless, there exist two major problems associated with this procedure; inherent physical variation of olive oil characteristics influenced by extraneous factors is often observed. Strong rainfall or snowfall may alter the composition of certain constituents beyond the official limits, making thereby the olive oil in question suspect of adulteration. A second problem appears when a foreign oil has very similar chemical composition to that of EVOO (e.g. refined hazelnut oil). As a result, the development of novel and more effective analytical techniques to detect EVOO adulteration at concentrations of interest (5–20% or lower), and, if possible, the use of markers that differentiate EVOO from foreign oils, is highly desirable. In our laboratory, we have developed a novel analytical methodology for the quality control and authentication of EVOO based on ^{31}P NMR spectroscopy in combination with chemometrics using as markers the diacylglycerols and the phenolic compounds.

Quality control of EVOO

Evaluation of EVOO quality can be made by employing ^{31}P NMR spectroscopy and using the information it provides on minor compounds. In particular, the minor constituents 1,2-diacylglycerols (1,2-DGs) (**23**) and 1,3-diacylglycerols (1,3-DGs) (**24**) accompanying the major triacylglycerol (TG) components have been proven to be useful indices to assess the quality of EVOO.[6] There are two sources generating 1,2-DGs: incomplete biosynthesis of triacylglycerols,[29] and the limited hydrolysis of TGs during EVOO storage.[30] On the other hand, 1,3-DGs are considered to be secondary products resulting from the isomerization of 1,2-DGs during the extraction process and continued during the storage of the olive oil.[30–32] Therefore, freshly made EVOO from healthy olive fruits is expected to contain almost solely 1,2-DGs, the concentration of which decreases during storage, while the 1,3-DG content and the total DG concentration increase. In this respect, the concentration levels of both 1,2-DGs and 1,3-DGs are indicative of EVOO freshness. From these facts, the ratio D of 1,2-diglycerides to the total amount of diacylglycerols [D = 1,2-DGs/(1,2-DGs + 1,3-DGs)] has been considered as a potential index to assess the freshness of EVOO.[6]

The previous investigation considers the problem of EVOO quality from a qualitative point of view since it is unable to specify the duration of storage of EVOO reflected on the diacylglycerols concentration. Evaluation of the storage time of EVOO is important for the olive oils suppliers, who deal with tons of olive oil, and, of course, for the consumers. What was really needed was a mathematical expression connecting the storage time or age of olive oil with the diacylglycerol concentration. This expression has been derived[31] through a kinetic study of the diacylglycerol formation upon hydrolysis of TGs, and the subsequent isomerization reaction catalyzed by free fatty acids, following the kinetic scheme:[31]

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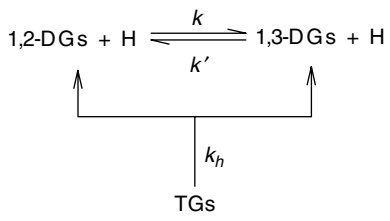
Table 1. Compilation of ^{31}P NMR Chemical Shifts and multiplicities (in parentheses) of polyphenol model compounds and polyphenols in EVOO[a]

Compound	OP of model compounds	Chemical shifts in EVOO
p-Coumaric acid (**3**)	137.82 (s)	137.80
Free tyrosol (**9**)	146.91 (s)	146.89
Total tyrosol	138.20 (s)	138.19[b]
Free hydroxytyrosol (**13**)	146.99 (s)	146.96
Total hydroxytyrosol	139.25 (d), 139.13 (d)	139.23–139.14[c]
(+) Pinoresinol (**7**)	139.84 (s)	139.84
Syringaresinol (**8**)	142.89	142.89
(+)1-Acetoxypinoresinol (**14**)	139.86 (s), 140.03 (s)	139.78, 139.97
Apigenin (**15**)	136.46 (s)	136.42
	137.44 (s)	137.52
	137.88 (s)	137.86
Luteolin (**16**)	136.47 (s)	136.46
	137.52 (s)	137.56
	138.60 (d)	138.62
	139.80 (d)	139.82
Oleuropein-7-*O*-glucoside (**17**)	139.18 (d), 139.11 (d)	139.23–139.14[c]
Homovanillyl alcohol (**18**)	139.84 (s), 147.00 (s)	139.84, 147.00
1-Monoacylglycerols (**19**)	146.45 (s), 147.64 (s)	146.47, 147.66
2-Monoacylglycerols (**20**)	148.00 (s)	148.05
Glycerol (**21**)	146.32 (s), 147.37 (s)	146.33, 147.38
α-D-Glucopyranose[d]	146,70, 146.79, 147.28, 147.91, 148.08	146.66, 146.76, 147.28, 147.86, 148.04
β-D-Glucopyranose[d]	145.71, 147.67, 147.28, 147.16, 148.32	145.69, 147.65, 147.24, 147.22, 148.28
Maslinic acid (**22**)	145.97 (s), 147.67 (s)	145.95, 147.66

[a] Obtained from Refs 10,17.
[b] Free and esterified tyrosol (ligstroside and its hydrolysis products) contribute to this strong signal.
[c] Free and esterified hydroxyrosol (oleuropein and its hydrolysis products) contribute to this strong signal.
[d] Singlets.



Here, k and k' are the rate constants of the forward and backward reaction during the isomerization process and k_h is the rate constant of hydrolysis. By fitting the differential equations describing the above kinetic scheme to the experimental data describing the evolution of 1,2-DGs, 1,3-DGs and free acidity with time spanning 18 months in light and dark at ambient temperature, we arrived at a mathematical expression relating the storage time, t_A, or age of olive oil with the diacylglycerol concentration in terms of the ratio D and free acidity, H:

$$age = t_A = \frac{-\ln\left(\frac{D - 0.32}{D_0 - 0.32}\right)}{0.0165 \times H} \quad (1)$$

Details of derivation, validation of this simple equation can be found in the published work.[31] Calculations were performed by using Eqn 1 and a value of $D_0 = 0.94$, which is typical for freshly extracted virgin olive oils. Very good agreement was observed between the calculated age and the actual storage time for olive oil samples up to 10–12 months at ambient temperature. The slight underestimation of the true storage time for $t_A > 12$ months sets an upper limit of the applicability of Eqn (1). This discrepancy was expected, because at very long storage ($t > 12$ months) the D values are very close and sometimes smaller than the calculated equilibrium value $D_{eq} = 0.32$, making thus the difference $(D_0 - 0.32)$ in Eqn (1) zero or even negative. Therefore, for DG isomerization that has reached the equilibrium ($D \sim D_{eq}$), the calculated storage time of the respective olive oil by employing Eqn (1) is only indicative, leading to the conclusion that the olive oil is more than one year old. Application of Eqn (1) to several EVOO samples obtained from olive oil companies gave good agreement (±10 days) between the calculated age and the actual storage time.

The concentration changes of DGs upon storage do not have an immediate effect on the organoleptic properties of olive oil. However, they do reflect in a quantitative manner the aging of olive oil. This is quite important since aging is accompanied by degradation of the natural antioxidants of olive oil, such as α-tocopherol and phenolic compounds, thereby downgrading the quality of olive oil. After long

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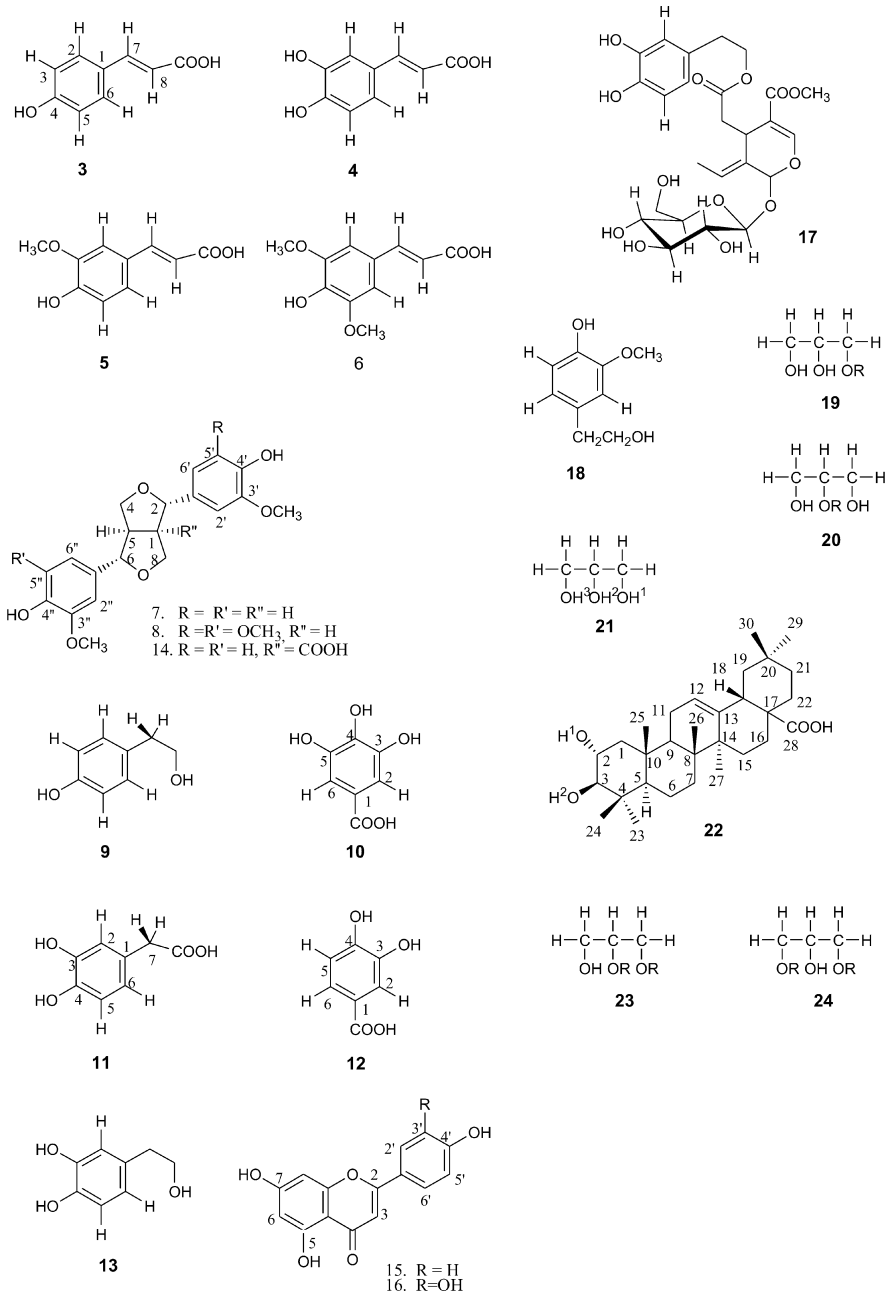

Scheme 1

storage (one year or more), olive oil becomes rancid with poor nutritional and organoleptic characteristics.

Detection of EVOO fraud

EVOO is a fine product with high nutritional value and significant health benefits.[24,33] It is known for its superior organoleptic characteristics (aroma and taste) and its remarkable antioxidant properties.[9,33] Cultivation of olive trees, harvest of olive fruits and extraction of olive oil is a hard and time-consuming work, which adds to EVOO's relatively high commercial value. Therefore, attempts to adulterate this commodity with less expensive materials, such as seed oils and/or olive oils of lower quality (refined olive oil) are by no means rare. Needless to say, that this practice deteriorates EVOO's quality and nutritional value, and causes major economic losses and mistrust for the olive oil market. In this respect, it is important to distinguish between EVOO and foreign oils, such as hazelnut oil and refined olive oil (ROO), and mixtures of EVOO with hazelnut oil and EVOO with ROO.

Contrary to the natural content of total diacylglycerols in fresh EVOOs, which does not exceed 1–3%, olive oils of lower quality contain higher levels of total diacylglycerols.[30–32] For instance, ROO contains 4–5% of total diacylglycerols (mainly 1,3-DGs), which go up to 15–20% in olive–pomace oils.[30,34] The isomerization of 1,2-DGs to 1,3-DGs in refined olive oils reaches very rapidly its equilibrium state, where the ratio D assumes its equilibrium value, $D_{eq} \sim 0.33$. Therefore, diacylglycerols can be used to detect EVOO adulteration with olive oils of lower quality, such as lampante olive oils (LOO) and ROO.

Statistical treatment of the diacylglycerol content and other constituents determined by ^{31}P NMR spectroscopy for a

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total of 54 samples allowed the discrimination of EVOO from olive oils of lower quality, such LOO and ROO, and detection of EVOO adulteration. On the basis of the Fisher *F*-ratio, determined by one-way ANOVA, the following constituents (variables) for which $F > 1$ were selected: 1,3-DGs, total DGs, *D*, log of free acidity and TDG-free acidity with probability $p < 0.000001$. The application of the statistical method of hierarchical cluster analysis (HCA), and in particular discriminant analysis (DA), to these parameters allowed a good separation of the olive oil samples among these three grades of olive oil.[35] By using the same methodology, mixtures of EVOO with LOO and, in particular, EVOO with ROO can be detected whenever the proportion of LOO and ROO are equal or higher than 5% for both adulterants.[35] In addition, the measurement of the compositional parameters of commercial blended olive oils (legal mixtures of EVOO with ROO) by ^{31}P NMR spectroscopy, and the calculation of Mahalanobis distances from DA, which measures the distances between each point (sample) and the centroid of each group in a multidimensional space defined by the variables, have led to a semiquantitative estimation of the proportion of added refined olive oil.[35]

A further application of the NMR methodology to the EVOO authentication is the classification of several edible oils and the detection of EVOO adulteration with refined seed oils.[28] For this purpose, 192 samples of 13 types of edible oils including EVOO were analyzed by employing NMR spectroscopy. Total free sterols, free acidity, 1,2-DGs, 1,3-DGs, total DGs and the ratio *D* were determined with ^{13}P NMR spectroscopy. In addition, ^{1}H NMR spectroscopy has been employed for determining the fatty acid composition and iodine value,[1,6,28] in order to increase the number of variables for statistical analysis. Selection of variables with the highest discriminatory power was made as before by employing one-way ANOVA. According to this statistical test, 8 variables out of 11 were selected and used to classify the 192 samples of 13 types of edible oils (including EVOO and hazelnut oil) by using DA. The use of 2 of the 8 canonical functions or roots of the model obtained by DA with the highest discriminating power succeeded in classifying the 192 oil samples into 13 different groups.[28]

For adulteration studies, a series of artificial mixtures of EVOO–hazelnut oil, EVOO–corn oil, EVOO–sunflower oil and EVOO–soybean oil were prepared at concentrations ranging from 5 to 50% w/w and analyzed by ^{1}H NMR and ^{31}P NMR spectroscopy. Subsequent DA of the data allowed detection of adulteration as low as 5% w/w for each seed oil including hazelnut oil.[28] This finding is very interesting since, as was mentioned, adulteration with hazelnut oil is one of the most difficult to detect with conventional analytical techniques because of the similar composition of hazelnut oil and olive oil.

Varietal classification of EVOO

Phenolic compounds and free acidity were determined by ^{31}P NMR spectroscopy, and the data were combined with a multivariate statistical procedure in order to classify monovarietal Greek EVOO belonging to particular cultivars. A selection of phenolic compounds (total hydroxytyrosol, total tyrosol, apigenin, luteolin) based on their sensitivity to different cultivars was performed by using one-way ANOVA. Then, DA was applied to the analytical data of 110 olive oil samples, and the results are shown in Fig. 8. Grouping of EVOO according to three Greek cultivars, koroneiki, athinolia and kolovi, was obtained. Of the total, 97.5%, 95% and 75% of EVOO samples from koroneiki, kolovi, and athinolia, respectively, were correctly classified.

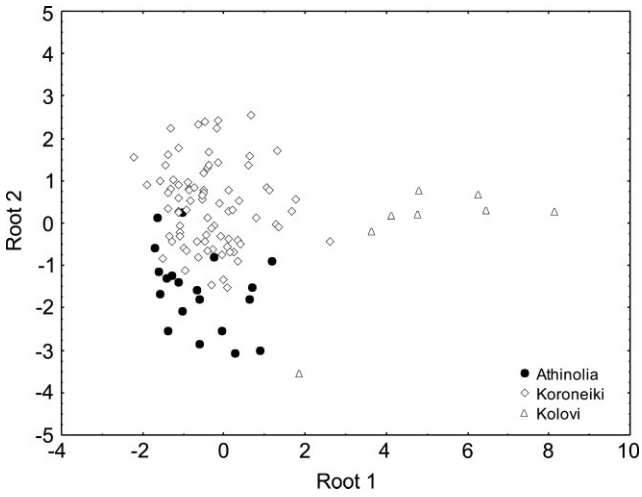

Figure 8. Linear discriminant analysis plot of 110 Greek EVOO samples based on phenolic compounds and free acidity determined by ^{31}P NMR spectroscopy.

CONCLUSIONS AND FUTURE PERSPECTIVES

The ^{31}P NMR methodology presented briefly in this review offers new opportunities for the analysis of edible oils, and in particular for the quality control and authentication of EVOO. Although this technique is considered to be more expensive than conventional methods of analysis, it has a number of advantages that compensate the rather high cost of an NMR spectrometer. A single run detects in a rapid way all the phosphitylated minor compounds present in the olive oil sample and provides signals, the intensities of which reflect the number of magnetically equivalent phosphorus nuclei. The assignment of the chemical shifts of the various functional groups is well documented, making thus this technique very appropriate for the screening of a large number of samples, and a valuable tool for the quality control and authentication of EVOO. Its application can be extended to the quality control and authentication of other foods and beverages, such as wines and honey. As a matter of fact, it has been employed to detect and quantify glycerol in wines from various region of Greece.[36] Moreover, chemical shift assignments of ^{31}P NMR spectra of a series of mono- and oligosaccharides by using 2D NMR techniques is currently in progress in our NMR laboratory. These substances will be used as model compounds for the detection and quantification of sugars in honey.

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