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# Comparison of Analytical Methodologies Based on $^{1}H$ and $^{31}P$ NMR Spectroscopy with Conventional Methods of Analysis for the Determination of Some Olive Oil Constituents

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The present study was designed to assess the agreement between analytical methodologies based on $^{1}H$ and $^{31}P$ NMR spectroscopy and conventional analytical methods (titration, gas chromatography, and high performance liquid chromatography) for measuring certain minor and major constituents (free acidity, fatty acids, iodine value, and phenolic compounds) of olive oil. The standard deviations of the NMR method were comparable to those of the conventional methods, except perhaps those of the total hydroxytyrosol and total tyrosol. Linear regression analyses showed strong correlations between NMR and conventional methods for free acidity, total hydroxytyrosol, total tyrosol, total diacylglycerols, (+)-pinoresinol, (+)-1-acetoxypinoresinol, and apigenin; good correlations for linoleic acid, free hydroxytyrosol, and free tyrosol; and weak correlations for oleic acid, linolenic acid, saturated fatty acids, and luteolin. Furthermore, a method comparison study was conducted and the agreement between NMR and conventional methods was evaluated by using the Bland and Altman statistical analysis. The distribution of the data points in the bias plot showed that 96.4% and 100% of the measurements of free acidity and iodine value, respectively, were within the limits of agreement of the two methods. For the remaining constituents of olive oil, the percentage of measurements, located within the limits of agreement, ranged from 94% to 98.5%.

**KEYWORDS: Olive oil; quantitative NMR; phenolic compounds; free acidity**

## INTRODUCTION

In the past decade, high-resolution 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 in a NMR spectrum obtained in a fairly rapid manner combined with the easy sample preparation render this spectroscopic technique very attractive for the determination of the composition of olive oil. $^{1}H$ NMR spectroscopy has provided information (*1*, *3*−*6*) about lipid classes, fatty acid composition, unsaturation levels, and several minor compounds (sterols, squalene, terpenes, volatile compounds, and others), whereas $^{13}C$ NMR, among others, gave unique information about the positional distribution of fatty acids on glycerol and the stereochemistry of unsaturation (*1*, *2*).

Recently, $^{31}P$ NMR spectroscopy has been employed in olive oil analysis (*7*) supplementing $^{1}H$ NMR and $^{13}C$ NMR spectroscopy, especially in cases where 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 (*8*). This methodology is based on the derivatization of the labile hydrogen atoms of olive oil constituents bearing hydroxyl and/or carboxyl groups with the phosphitylating reagent 2-chloro-4,4,5,5-tetramethyldioxaphospholane (**1**) according to the reaction scheme illustrated in **Figure 1** and the use of the $^{31}P$ chemical shifts to identify the labile centers (compound **2**). Compound **1** reacts rapidly and

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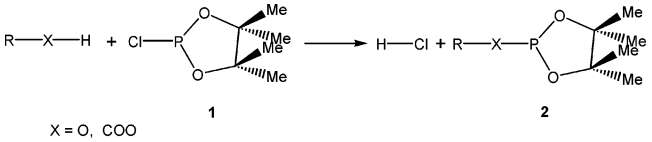


**Figure 1.** Reaction of hydroxyl and carboxyl groups of olive oil constituents with the phosphorus reagent 2-chloro-4,4,5,5-tetramethyldioxaphospholane (**1**).

quantitatively under mild conditions with the hydroxyl and/or carboxyl groups. This analytical approach has been used in the past (*7*−*9*) to detect and quantify minor constituents of olive oil, such as diacylglycerols, phenolic compounds, total free sterols, and free fatty acids (free acidity).

Despite the fact that NMR spectroscopy plays an ever-increasing role in the study of olive oil, comparison of the NMR results with those obtained by official and/or well-established (for phenolic compounds) analytical techniques is scarce in the literature and lacking any statistical analysis (*6*, *10*−*14*). Such a comparison would be a rigorous and efficient validation of $^{1}$H NMR and in particular $^{31}$P NMR spectroscopy as a quantitative analytical method for the quality control and authentication of extra virgin olive oil.

In the present study, we determined the amounts of certain constituents (fatty acids, iodine value, diacylglycerols, phenolic compounds, and free acidity) of a large number of olive oil samples by employing $^{1}$H NMR and $^{31}$P NMR spectroscopy, and we compared the NMR data with results obtained by independent laboratories using official and/or well-recognized methods of analysis. In particular, fatty acids concentration was determined by using $^{1}$H NMR spectroscopy and gas chromatography, free acidity by $^{31}$P NMR spectroscopy and titration, phenolic compounds by $^{31}$P NMR spectroscopy and high performance liquid chromatography, diacylglycerols by $^{31}$P NMR spectroscopy and gas chromatography, and iodine value by $^{1}$H NMR and titration. The statistical evaluation of the comparison was made according to Bland and Altman methodology (*15*).

## MATERIALS AND METHODS

**Olive Oil Samples**. A total of 145 olive oil samples collected from various regions of Greece and extracted from different olive varieties (koroneiki, athinolia, tsunati, kolovi, andramitiani, and two local varieties from kerkyra and Pilion) and years of harvesting (2002−2003, 2003−2004, 2004−2005, and 2005−2006) were used in the present study. A total of 111 samples were extra virgin olive oils with free acidity ≤0.8% in oleic acid, whereas the remaining 34 samples were lampante olive oils characterized by much higher free acidity (≫0.8% in oleic acid), especially those obtained from the Island of Kerkyra (Corfu). The choice of lampante olive oils was dictated by the need to widen the range of free acidity values in order to perform linear regression analysis, as will be shown later.

**Chemicals**. *NMR*. Pinacol, phosphorus trichloride, and protonated solvents (analytical grade) for synthesis of the phosphorus reagent, pyridine solvent (99%), and deuterated chloroform were purchased from Sigma-Aldrich (Athens, Greece).

*GC*. Analytical grade *n*-tetradecane, *n*-hexane, and the silylating agents trimethylchlorosilane and *N*,*N*-bis(trimethylsilyl)trifluoroacetamide, used for the analysis of diacylglycerols, were purchased from Labscan (Hasselt, Belgium). *n*-Triacodane, dilaurin, 1,3-diolein, 1,2-diolein, and 1-palmito-3-stearoylglycerol were purchased from Sigma Chemicals Co. (St. Louis, MO). For the analysis of fatty acids, analytical grade methanol, heptane, and potassium hydroxide were purchased from Sigma Chemicals Co. (St. Louis, MO).

*HPLC. Protocol A*. HPLC-grade acetonitrile, methanol, 2-propanol, and water were purchased from Merck (Darmstadt, Germany). Methanol, acetonitrile, and hexane used for phenolic extraction were proanalysis grade and purchased from Merck (Darmstadt, Germany). The standards used for the identification and quantification of phenolic compounds were tyrosol, apigenin, and luteolin purchased from Sigma-Aldrich (Germany); vanillic acid, *p*-coumaric acid, and ferulic acid purchased from Merck-Schuchardt (Hohenbrunn, Germany); oleuropein-7-glucoside purchased from Extrasynthese Co. (Genay, France), and (+)-pinoresinol purchased from Separation Research (Turku, Finland).

*Protocol B*. Methanol, *N*,*N*-dimethylformamide, hexane, water, and phosphoric acid were all of HPLC grade purchased from Teknokroma, S. L. (Barcelona, Spain). Tyrosol was purchased from Sigma Chemicals Co. (St. Louis, MO). Apigenin and luteolin were obtained from Extrasynthèse Co. (Genay, France).

*Titrations*. All reagents, solvents (analytical grade), and standards for the determination of free acidity (diethyl ether, 95% ethanol, 0.1 M potassium hydroxide, phenolphthaleine indicator) and iodine number (sodium iodide, starch indicator, 0.1 M sodium thiosulfate, cyclohexeneacetic acid) were purchased from Sigma-Aldrich (Athens, Greece).

**Preparation of the Phosphorus Reagent**. The derivatizing phosphorus reagent was synthesized from pinacol and phosphorus trichloride in the presence of triethylamine following the method described in the literature (*16*). However, to increase the yield of the reaction, we utilized *n*-hexane solvent and pyridine instead of benzene and triethylamine, respectively, suggested in the original method. This modification resulted in ∼45% yield of the product versus ∼20% obtained with the original method.

**Extraction of Phenolic Compounds**. For the NMR study and protocol A of the HPLC analysis, phenolic compounds were extracted following the method developed by Montendoro et al. (*17*) using a mixture of methanol−water (80:20 v/v). The phenolic extracts were dissolved in methanol for injection or used immediately for sample preparation prior to $^{31}$P NMR measurements. For protocol B of the HPLC analysis, phenolic extracts of olive oils were obtained following the procedure described elsewhere (*18*), using *N*,*N*-dimethylformamide.

**Standards for HPLC Analyses**. *Protocol A*. The standards used for quantification of phenolic compounds were oleuropein-7-glycoside for the quantification of hydroxytyrosol and hydroxytyrosol derivatives (dialdehydic form of elenolic acid linked to hydroxytyrosol, oleuropein aglycon); tyrosol for quantification of tyrosol and tyrosol derivatives (dialdehydic form of elenolic acid linked to tyrosol and the ligstroside aglycon); (+)-pinoresinol for quantification of the lignans (+)-pinoresinol and (+)-1-acetoxypinoresinol; and, finally, luteolin and apigenin for quantification of the flavonoids luteolin and apigenin, respectively. Identification of phenolic compounds was achieved by comparing their retention time with those of standards, from UV absorption and from GC−MS analysis of hydroxytyrosol and tyrosol derivatives as described elsewhere (*19*, *20*).

*Protocol B*. Apart from hydroxytyrosol, tyrosol, luteolin, and apigenin, the remaining phenolic compounds hydroxytyrosol acetate, 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 used as standards were obtained using a semipreparative 25 cm × 10 mm, i.d., 5 $\mu$m, Spherisorb ODS-2 (Teknokroma, Barcelona, Spain) HPLC column and a flow rate of 4 mL/min. The mobile phases and the gradient have been described previously (*21*). Individual phenols were quantified by a four-point regression curve on the basis of the standards obtained form commercial suppliers or from preparative HPLC as already described.

**HPLC Analysis of Phenolic Compounds**. HPLC equipments and conditions used to quantify phenolic compounds in olive oil with protocols A and B have been described in refs *19*, *20* and *18*, *21*, respectively.

**GC Analysis of Diacylglycerols and Fatty Acids.** The AOACS official method (*22*) developed by Firestone et al. (*23*) for the determination of mono- and diacylglycerols by capillary gas chromatography was utilized for the present analysis. A Shimadzu-17A gas chromatograph (Shimadzu Co., Kyoto, Japan) was fitted with a flame ionization detector (FID) and a column injection system. Separation was carried out on a fused-silica J&W capillary column (15 m × 0.32

mm id) (Agilent Technologies, Palo Alto, CA) coated with DB-5HT ((5% phenyl)−methylpolysiloxane) of 0.1 μm thickness. The European Union official method (*24*, *25*) for the determination of saturated and unsaturated fatty acids by capillary gas chromatography was utilized for the present analysis. The preparation of the fatty acid methyl esters was achieved via trans-esterification with a cold methanolic solution of potassium hydroxide according to the International Olive Oil Council official method (*26*). A Carlo Erba HRGC 5300 gas chromatograph (Rodano, Italy) equipped with a flame ionization detector (FID) and a split injection system was used. Separation was performed on a fused-silica capillary column (60 m, 0.25 mm id) (Thames Restek, Saunderton, UK) coated with Rtx-2330 (90% biscyanopropyl/10% phenylcyano propylpolysiloxane) of 0.2 μm thickness. The saturated fatty acids determined in the present study by GC were palmitic acid (16:0), heptadecanoic acid (C17:0), stearic acid (C18:0), arachidic acid (C20:0), behenic acid (C22:0), and lignoceric acid (C24:0).

**Determination of Free Acidity and Iodine Value**. These parameters were determined by employing the official methods of titration (*24*, *25*).

**Sample Preparation for Spectroscopic Analysis**. Sample preparation for the determination of olive oil constituents by employing $^{1}$H and $^{31}$P NMR experiments has been described in detail in previous publications (*7*−*9*). For the determination of free acidity and the diacylglycerol content, derivatization of the carboxyl and hydroxyl groups by the phosphorus reagent (*1*) was performed in olive oil without any posterior treatment (*7*, *8*), whereas the polar extract was phosphitylated for the quantification of phenolic compounds (*9*).

**NMR Experiments**. All NMR experiments were conducted on a Bruker AMX500 spectrometer operating at 500.1 and 202.2 MHz for proton and phosphorus-31 nuclei, respectively, at 30 ± 1 °C. Details of recording $^{1}$H and $^{31}$P NMR spectra and chemical shifts assignments can be found elsewhere (*7*−*9*, *27*).

**Statistical Analysis**. Basic statistics (mean, standard deviation, etc.), correlations between sets of data (linear regression), and scatter-plots (bias plots) were processed by using Statistica 7.1 for Windows (StatSoft Inc.).

## RESULTS AND DISCUSSION

The results of the analyses of the present olive oil samples by employing $^{1}$H and $^{31}$P NMR methodology and conventional analytical methods are summarized in three tables and are available as Supporting Information. These tables contain the results of free acidity for 137 olive oil samples determined by titration following the EC official method (*24*, *25*) and by $^{31}$P NMR spectroscopy according to references cited (*7*, *9*); phenolic compounds for 28 olive oil samples determined from the polar fraction of olive oils by HPLC using two experimental protocols (*18*−*21*) and by $^{31}$P NMR spectroscopy (*9*); 1,2- and 1,3-diacylglycerols for 26 olive oil samples determined by GC following the AOACS official method (*22*) and by $^{31}$P NMR spectroscopy (*7*, *8*); unsaturated and saturated fatty acid composition determined for 137 olive oil samples by GC according to the EC official method (*24*, *25*) and $^{1}$H NMR spectroscopy (*27*); and the iodine value determined for 38 olive oil samples by the EC official titration method (*24*, *25*) and $^{1}$H NMR spectroscopy (*27*). The composition of the unsaturated fatty acids (oleic acid, linoleic acid, and linolenic acid) and the saturated fatty acids (SFAs) in olive oils was calculated upon combining the various signal intensities in the $^{1}$H NMR spectra as shown elsewhere (*27*). Contrary to GC, the composition of each SFA could not be determined separately by $^{1}$H NMR spectroscopy (*1*, *5*, *27*). Instead, $^{1}$H NMR results reflect collectively the concentration of all SFAs in olive oils. Therefore, comparison of the $^{1}$H NMR and GC data for SFAs requires the summation of the individual SFA concentrations determined by the conventional GC method.

The concentration of phenolic compounds for 28 extra virgin olive oils was determined by liquid−liquid extraction followed by phosphitylation and recording the $^{31}$P NMR spectra of the phosphitylated compounds (*9*). The phosphitylated aromatic hydroxyl groups of tyrosol and hydroxytyrosol in their free and conjugated forms showed about the same chemical shifts in the $^{31}$P NMR spectrum. Therefore, the integrals of these two signals at $\delta$ 139.20 and 138.19 reflect the concentrations of total hydroxytyrosol and total tyrosol, respectively (*9*). On the other hand, HPLC was able to separate and quantify all forms of hydroxytyrosol and tyrosol (*18*−*21*). This means that the $^{31}$P NMR data for total hydroxytyrosol and tyrosol are to be compared with the summation of the individual concentrations of free hydroxytyrosol and tyrosol, hydroxytyrosol acetate, and the hydrolysis products of oleuropein and ligstroside, namely, 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 as determined by HPLC. Both HPLC protocols A and B used in this study were able to determine phenolic compounds in olive oil. However, phenolic compounds extracted from 18 out of 28 extra virgin olive oil samples and quantified by employing the HPLC protocol B took advantage of the appropriate standards used for the quantification of hydroxytyrosol acetate and the hydrolysis products of oleuropein and ligstroside (see above). For the remaining 10 olive oils samples analyzed using the HPLC protocol A, hydroxytyrosol acetate was not quantified, because its concentration was negligible. Moreover, the quantification of the hydrolysis products of oleuropein and ligstroside using the HPLC protocol A was made with oleuropein and tyrosol standards, respectively, which have much different absorption coefficient than the aforementioned hydrolysis products (*28*). Thus, comparison of the $^{31}$P NMR and HPLC data for total hydroxytyrosol and total tyrosol was made by using data from 18 olive oil samples analyzed by the HPLC protocol B. Further inspection of the data of analysis reveals that the percentages of (+)-pinoresinol and (+)-1-acetoxypinoresinol determined by the HPLC protocol B were systematically about four times higher than those obtained with $^{31}$P NMR methodology, whereas comparable values were observed while using the HPLC protocol A. A possible explanation of this discrepancy could be the different liquid−liquid extraction procedures used for the HPLC analysis following protocols A and B. It appears that the use of *N*,*N*-dimethylformamide as an extraction solvent in protocol B resulted in a more efficient recovery of lignans (*18*).

The NMR data were to be compared with those obtained by the conventional analytical methods. The normal plots of the differences between the NMR data and those of the conventional methods revealed one outlier in each of the data sets of total diacylglycerol, free hydroxytyrosol, apigenin, and luteolin that was removed in subsequent statistical analysis. One issue that should be discussed first is related to the repeatability of the measurements. **Table 1** summarizes the repeatability of the NMR results and those obtained by conventional methods. The repeatability was calculated by means of the following equation according to ISO 5725 (*29*):

$$\text{repeatability} = 2.8 \times \sqrt{\text{SD}^2} \qquad (1)$$

$SD^2$ is the variance of repeatability. The variance of repeatability for each compound in **Table 1** was calculated from the standard deviation of a number (8−10) of interday measurements using the same olive oil sample and the same protocol of the corresponding analytical method. The data in **Table 1** demonstrate that the repeatabilities associated with NMR and conventional measurements for most olive oil constituents are in general comparable, except perhaps those observed for total

**Table 1.** Repeatability ($r$)[a] for Measurements Using NMR Spectroscopy and Conventional Analytical Methods

| | NMR method | conventional method[b] |
|---|---|---|
| free acidity[c] | 0.07 | 0.06 |
| total diacylglycerols[c] | 0.13 | 0.11[f] |
| iodine value[d] | 3.72 | 2.69 |
| oleic acid[d] | 0.56 | 0.42 |
| linoleic acid[d] | 0.28 | 0.16 |
| linolenic acid[d] | 0.14 | 0.14 |
| SFA[d] | 0.28 | 0.22[d] |
| hydroxytyrosol[c,e] | 1.71 | 6.55 |
| tyrosol[c,e] | 10.79 | 2.18 |
| total hydroxytyrosol[c,e] | 14.47 | 123.00[f] |
| total tyrosol[c,e] | 11.01 | 79.04[f] |
| (+)-pinoresinol[c,e] | 1.22 | 7.06 |
| (+)-1-acetoxypinoresinol[c,e] | 2.19 | 10.14 |
| apigenin[c,e] | 0.32 | 0.59 |
| luteolin[c,e] | 3.44 | 2.88 |

[a] Calculated from eq 1. [b] The conventional methods are mentioned in text. [c] Determined by $^{31}$P NMR methodology. [d] Determined by $^{1}$H NMR methodology. [e] Includes extraction of phenolic compounds. [f] Calculated from pooled standard deviation.

**Table 2.** Correlation Coefficient ($R$), Intercept ($\alpha$), Regression Coefficient ($\beta$), 95% Confidence Interval for the Regression Coefficient, and Statistical Significance ($p$-value) of the Regression Analysis of the Dependent Variable (Determined by NMR Methods[a]) to the Independent Variable (Determined by Conventional Methods[a])

| | $R$ | a | $\beta$ | 95% CI of $\beta$ | $p$-value |
|---|---|---|---|---|---|
| free acidity (137)[b] | 0.994 | –0.056 | 0.978 | 0.960–0.996 | <0.001 |
| total diacylglycerols (25) | 0.972 | 0.046 | 0.966 | 0.865–1.067 | <0.001 |
| iodine value (38) | 0.528 | 40.933 | 0.481 | 0.219–0.743 | <0.001 |
| oleic acid (137) | 0.871 | 3.374 | 0.963 | 0.871–1.055 | 0.001 |
| linoleic acid (137) | 0.947 | 0.631 | 0.926 | 0.873–0.980 | <0.001 |
| linolenic acid (137) | 0.740 | 0.260 | 0.602 | 0.508–0.695 | <0.001 |
| SFA (137) | 0.802 | 3.790 | 0.719 | 0.780–1.007 | <0.001 |
| hydroxytyrosol[c] (27) | 0.945 | 3.664 | 0.896 | 0.811–1.080 | <0.001 |
| tyrosol[c] (28) | 0.937 | 5.708 | 0.877 | 0.795–1.009 | <0.001 |
| total hydroxytyrosol[d] (18) | 0.990 | –1.219 | 1.010 | 0.929–1.090 | <0.001 |
| total tyrosol[d] (18) | 0.968 | 27.107 | 0.958 | 0.827–1.090 | <0.001 |
| (+)-pinoresinol[d] (18) | 0.796 | –0.898 | 0.259 | 0.155–0.363 | <0.001 |
| (+)-pinoresinol[e] (10) | 0.991 | –0.360 | 0.829 | 0.737–0.920 | <0.001 |
| (+)-1-acetoxypinoresinol[d] (18) | 0.983 | –1.860 | 0.283 | 0.255–0.311 | <0.001 |
| (+)-1-acetoxypinoresinol[e] (10) | 0.736 | 7.379 | 0.660 | 0.165–1.156 | 0.015 |
| luteolin[c] (27) | 0.830 | 0.555 | 0.661 | 0.478–0.844 | <0.001 |
| apigenin[c] (27) | 0.982 | 0.385 | 0.921 | 0.847–0.995 | <0.001 |

[a] NMR and conventional methods are mentioned in the text. [b] Within parentheses is the number of samples analyzed. [c] Determined using both HPLC protocols A and B. [d] Determined using the HPLC protocol B. [e] Determined using HPLC protocol A.

hydroxytyrosol and total tyrosol. It should be noted that therepeatability of the latter compounds was calculated from the pooled standard deviations of the HPLC measurements for the free and conjugated hydroxytyrosol and tyrosol.

The common statistical approach to assess the degree of agreement between the NMR and conventional methods was based on regression analysis of the results obtained by the two methods and the use of the correlation coefficient as an indicator of agreement. **Table 2** contains the linear regression data of the dependent variable ($^{1}$H or $^{31}$P NMR methodology) with the independent variable (conventional method), i.e. correlation coefficients ($R$), intercept ($\alpha$), linear regression coefficient $\beta$ or slope, 95% confidence intervals (CIs) of $\beta$, and $p$-values. Strong correlations ($R = 0.968-0.994$) were observed between the NMR and conventional methods for free acidity, total hydroxytyrosol, total tyrosol, (+)-pinoresinol (data from HPLC protocol A), (+)-1-acetoxypinoresinol (data from HPLC protocol B), total diacylglycerols, and apigenin (data from both HPLC protocols A and B). Good correlations ($R = 0.933-0.947$) were observed for linoleic acid, hydroxytyrosol, and tyrosol (data from both HPLC protocols A and B). Weak correlations were observed for oleic acid, linolenic acid, SFAs, (+)-pinoresinol (data from HPLC protocol B), (+)-1-acetoxypinoresinol (data from HPLC protocol A), and luteolin, whereas iodine values showed the worst correlation of all (**Table 2**). The slopes are close to unity and the distances between lower and upper 95% confidence intervals of the slopes are smaller for the strong correlations, as expected. For weak correlations, the results obtained from the NMR methodology are lower than those determined from the conventional methods (**Table 2**). This observation is more pronounced for correlations between the two methods observed for the lignans (+)-pinoresinol and (+)-1-acetoxypinoresinol. The slopes of their regression lines for the data obtained from the HPLC protocol B are about four times smaller than unity (**Table 2**), reflecting the systematic error in the measured concentrations of the two lignans by NMR and HPLC methods, as discussed previously. Nevertheless, comparison of methods based on regression analysis appears to be inappropriate for several reasons (*30*, *31*). One problem was the dependence of the correlation on the range of the results in the samples; a wider range would result in a better correlation, but not necessarily to a better agreement. Indeed, linear regression of the free acidity data resulted in a very good correlation coefficient ($R = 0.994$), whereas this coefficient decreased to $R = 0.860$, when the lampante olive oils characterized by high free acidity values were removed from regression analysis. The bad correlation observed for the iodine values determined by titration and $^{1}$H NMR spectroscopy (**Table 2**) may be ascribed to the narrow data range of only 9 units.

An alternative approach to the use of linear regression and correlation was the difference or bias plot recommended by Bland and Altman (*15*). 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 of differences and displayed horizontal lines for the mean and for the mean $\pm 2 \times$ the standard deviation. The two horizontal lines corresponding to the mean $\pm 2 \times$ the standard deviation constitute the limits of agreement, which represent the 95% confidence interval for individual differences between the field and reference method. In summary, this plot allowed the assessment of how the differences differ systematically from zero (bias) and how much the difference varies (error). **Figure 2** summarizes the bias plots comparing the $^{1}$H and $^{31}$P NMR methodology with the conventional analytical methods of gas chromatography and titration for the determination of the total diacylglycerols, fatty acids, free acidity, and iodine value, while **Figure 3** shows the bias plots of the $^{31}$P NMR and HPLC methods used for the determination of phenolic compounds. **Table 3** contains the statistical parameters obtained from the Bland and Altman approach, i.e. the mean difference values, the upper and lower limits of agreements, and the 95% confidence intervals for the mean difference and the limits of agreement for the olive oil constituents determined in this study.The confidence intervals were calculated from the fol-

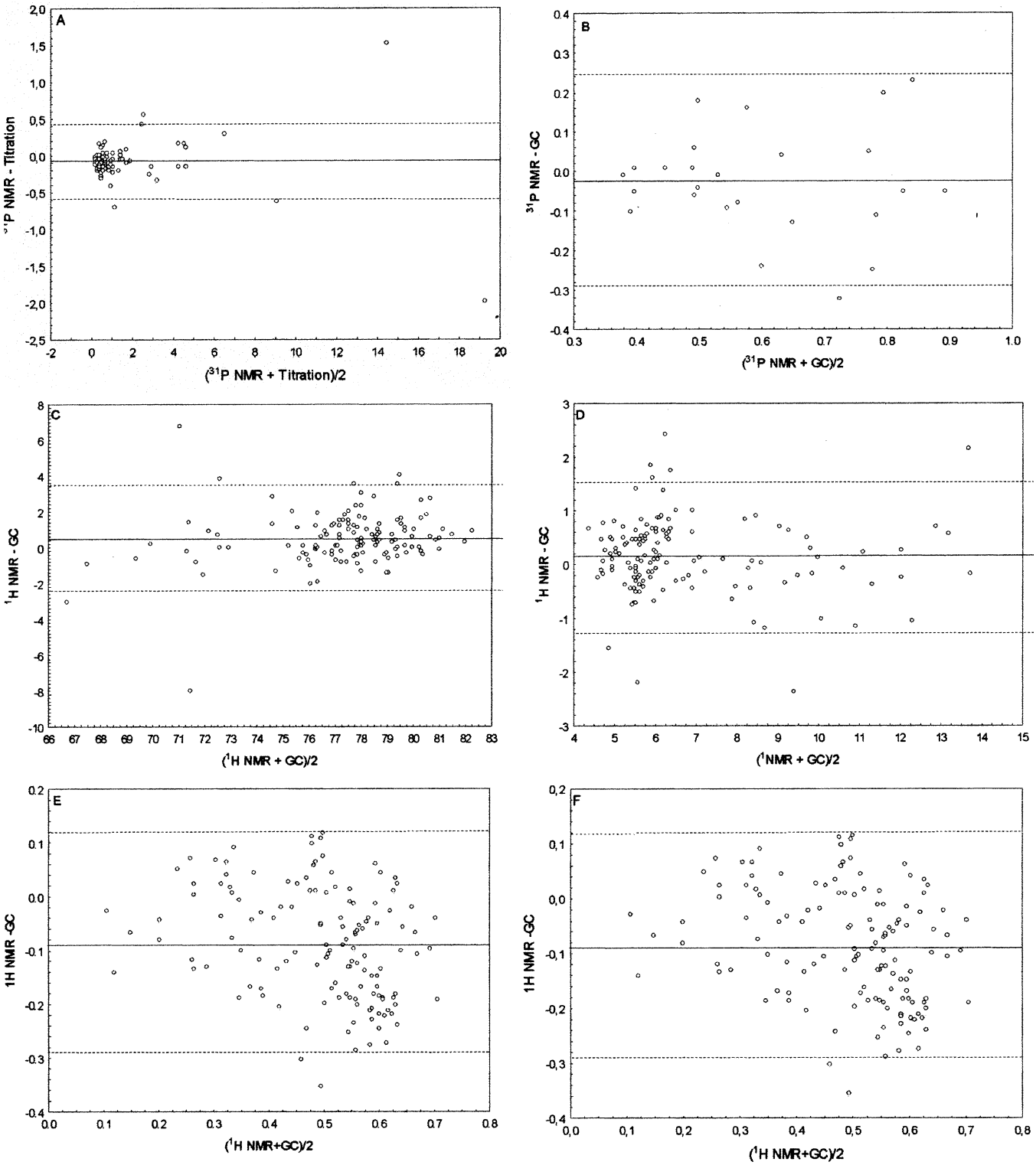


**Figure 2.** Overview of difference (bias) plots of olive oil samples measured by NMR spectroscopy and conventional methods against the average of measurements with the mean difference (solid lines) and limits of agreement (dotted lines) for (**A**) free acidity, (**B**) total diacylglycerols, (**C**) oleic acid, (**D**) linoleic acid, (**E**) linolenic acid, and (**F**) SFA. The NMR and conventional analytical methods used to determine these olive oil constituents are mentioned in **Table 1** and the text.

lowing formulas (*15*).

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

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

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

In these formulas, $\bar{d}$ is the mean value, $\text{SD}^2$ 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. Careful consideration of the statistical parameters summarized in **Table 2** and bias plots depicted in **Figures 2** and **3** led to the following conclusions regarding the agreement of the NMR and conven-

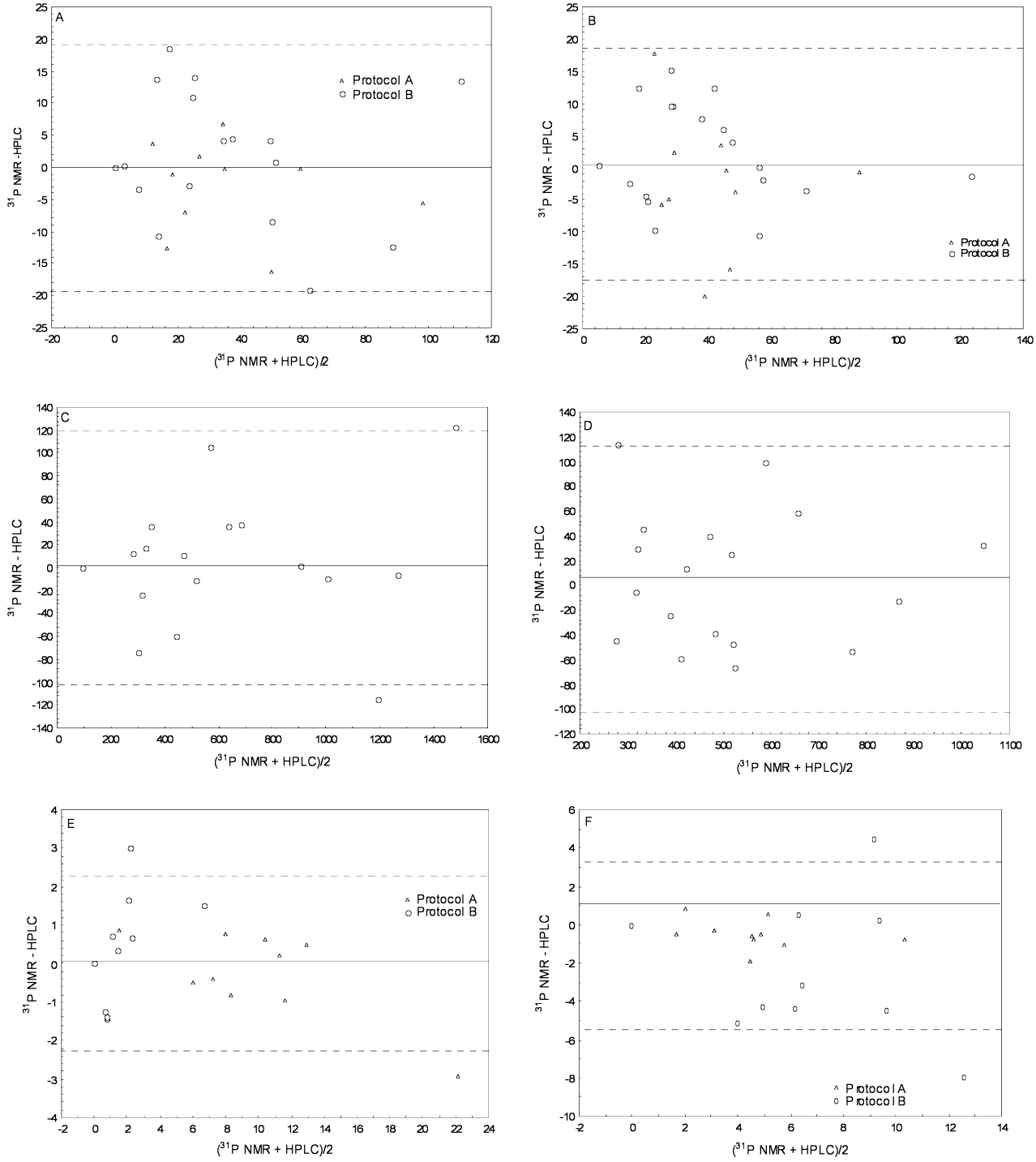


**Figure 3.** Overview of difference (bias) plots of olive oil samples measured by NMR spectroscopy and conventional methods against the average of measurements with the mean difference (solid lines) and limits of agreement (dotted lines) for (**A**) free hydroxytyrosol, (**B**) free tyrosol, (**C**) total hydroxytyrosol, (**D**) total tyrosol, (**E**) apigenin, and (**F**) luteolin. The NMR and conventional analytical methods used to determine these olive oil constituents are mentioned in **Table 1** and the text.

tional methods in measuring the present olive oil constituents.

**Free Acidity, Iodine Value, Fatty Acids, and Total Diacylglycerols**. The mean differences of the two methods for the measurements of free acidity, total diacylglycerols, iodine values, and the unsaturated and saturated fatty acids were slightly different from zero, indicating that there is at most a negligible systematic difference between measurements of these constituents of olive oil. In **Figure 2A**, which illustrates the distribution of the data points in the bias plot of free acidity, 132 measurements (96.4%) are located within the limits of agreement, leaving only three measurements (2.2%) near or on the limits, while only two measurements (1.5%) corresponding to the highest free acidity values are well outside the limits. Apart from one measurement of total diacylglycerols (**Figure 2B**), which is close to the lower limit of agreement, the remaining 24 measurements for total diacylglycerols, as well as the 38 measurements of iodine values (not shown), are within the limits of agreement in the respective bias plot. Five measurements (3.6%) for oleic acid, eight (5.8%) for linoleic acid, two (1.5%) for linolenic acid, and four (2.9%) for SFA out of a total of

**Table 3.** Mean Difference (Standard Deviation in Parentheses), Upper and Lower Limits of Agreement, and 95% Confidence Intervals (CI) for the Mean Difference and the Upper and the Lower Limits of Agreement for the Various Constituents of Olive Oil Determined by NMR Spectroscopy and Conventional Methods[a]

| | | limit of agreement | | 95% CI | | |
|---|---|---|---|---|---|---|
| | mean difference | upper | lower | for the mean difference | for the upper limit of agreement | for the lower limit of agreement |
| free acidity (137)[b] | –0.081 (0.254) | +0.427 | –0.589 | –0.124 to –0.038 | +0.353 to +0.501 | –0.663 to –0.515 |
| total diacylglycerol (25) | –0.026 (0.134) | +0.242 | –0.294 | –0.081 to +0.030 | +0.146 to +0.338 | –0.390 to –0.198 |
| iodine value (38) | –0.967 (2.295) | +3.623 | –5.557 | –1.721 to –0.213 | +2.316 to +4.930 | –6.864 to –4.250 |
| oleic acid (137) | +0.523 (1.478) | +3.479 | –2.433 | +0.273 to +0.773 | +3.046 to +3.912 | 2.866 to –2.000 |
| linoleic acid (137) | +0.144 (0.695) | +1.534 | –1.246 | +0.027 to +0.261 | +1.331 to +1.737 | –1.449 to –1.043 |
| linolenic acid (137) | –0.087 (0.102) | +0.117 | –0.291 | –0.070 to –0.104 | +0.087 to +0.147 | –0.321 to –0.261 |
| SFA (137) | +0.455 (0.913) | +2.281 | –1.371 | 0.301 to +0.609 | +2.014 to +2.548 | –1.638 to –1.104 |
| hydroxytyrosol[c] (27) | –0.127 (9.570) | +19.013 | –19.267 | –3.913 to +3.659 | +12.456 to +25.570 | –25. 824 to –12.710 |
| tyrosol[c] (28) | +0.410 (8.932) | +18.274 | –17.454 | –3.053 to +3.873 | +12.275 to +24.273 | –23. 453 to –11.455 |
| total hydroxytyrosol[d] (18) | +4.854 (57.600) | +120.054 | –110.345 | –23.790 to +33.498 | +70.442 to +169.666 | –159.958 to –60.734 |
| total tyrosol[d] (18) | +5.930 (53.942) | +113.814 | –101.954 | –20.895 to +32.755 | +67.353 to +160.275 | –148.415 to –55.493 |
| (+)-pinoresinol[c] (28) | –0.260 (1.444) | +2.628 | –3.148 | ––1.293 to +0.773 | +0.839 to +4.417 | –4.937 to –1.359 |
| (+)-1-acetoxypinoresinol[c] (28) | –0.407 (3.973) | +7.539 | –8.353 | –3.249 to +2.462 | +2.616 to +12.388 | –13.276 to –3.430 |
| luteolin[c] (27) | –1.072 (2.419) | +3.766 | –5.910 | –2.027 to –0.117 | +2.112 to +5.420 | –7.564 to –4.256 |
| apigenin[c] (27) | +0.045 (1.132) | +2.309 | –2.219 | –0.403 to +0.493 | +1.533 to +3.085 | –2.995 to –1.443 |

[a] NMR and conventional methods are mentioned in the text. [b] Within parentheses is the number of samples analyzed. [c] Determined using both HPLC protocols A and B. [d] Determined using the HPLC protocol B.

137 measurements are outside the limits of agreement, as shown in **Figure 2**, parts **C**, **D**, **E**, and **F**, respectively. In addition, the limits of agreement and the 95% confidence intervals depicted in **Table 3** for these constituents are small enough for us to be confident that the present $^{1}$H and $^{31}$P NMR methods can be used in place of the old methods of titration and gas chromatography.

**Phenolic Compounds**. The bias plots of total and free hydroxytyrosol, total and free tyrosol, apigenin, and luteolin are depicted in **Figure 3**. No such plots are presented for (+)-pinoresinol and (+)-1-acetoxypinoresinol due to the large discrepancies observed in their concentrations determined by $^{31}$P NMR and HPLC following protocols A and B (see above). The variability of the differences between the two analytical methods for the phenolic compounds is somewhat wide, reflecting mainly the small number of samples analyzed. Additional experimental data is expected to narrow the variability of differences and the limits of agreement. Moreover, total hydrotyrosol and total tyrosol concentrations show larger mean difference values and differences, relative to the other phenolic compounds (**Figure 3** and **Table 3**). This observation may be attributed in part to the poor repeatability of the HPLC measurements (**Table 1**). At any rate, almost all differences in the bias plots of **Figure 3** are within the limits of agreements for each phenolic constituent, indicating that the performance of NMR spectroscopy in determining phenolic compounds in olive oil is sufficient, provided that the same extraction and HPLC method will be used.

At this stage, it should be noted that the various NMR methodologies used in the present study show differences with respect to sample preparation and duration of analysis. In $^{1}$H NMR methodology, the NMR data were collected without sample pretreatment, thus rendering a simpler, faster, and probably lower-cost analysis than the conventional methods. In $^{31}$P NMR methodology for the determination of free acidity and diacylglycerols, the preparation of the phosphorus reagent and the phosphitylation reaction preceded the acquisition of the NMR spectrum. Although this method is less simple and requires more time than the previous $^{1}$H NMR method, it is much faster than the corresponding classical methods of titration and GC, because it determines several constituents (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 (*6*). Finally, the quantification of phenolic compounds, and in particular total tyrosol and total hydroxytyrosol, which contribute to the stability of extra virgin olive oil against oxidation (*32*), by the $^{31}$P NMR methodology 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 classical HPLC methods, inasmuch it does not require calibration with standards, which may not be available in the market.

**Supporting Information Available:** Tables A–C providing free acidity values, fatty acid composition, iodine values, diacylglycerols content, and concentrations of phenolic compounds for olive oil samples determined by NMR and conventional analytical methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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