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Piedmont olive oils: Compositional characterization and discrimination from oils from other regions

Costanza Aghemo[1], Andrea Albertino[1], Roberto Gobetto[1], Carola Lussiana[2], Antonino De Maria[3], Deborah Isocrono[3]

[1]Dipartimento di Chimica I.F.M., Università di Torino, Via Giuria 7, 10125 Torino, Italy
[2]Dipartimento di Scienze Zootecniche, Università di Torino, Via L. da Vinci, 44 Grugliasco, Italy
[3]Dipartimento di Colture Arboree, Università di Torino, Via L. da Vinci, 44 Grugliasco, Italy

Running title: Characterization and discrimination of Piedmont olive oils

Correspondence: Roberto Gobetto, Università di Torino, Via Giuria 7, 10125 Torino, Italy
e-mail: roberto.gobetto@unito.it
tel: +39 011.6707520
fax: +39 011.6707855

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Abbreviations: SFA, saturated fatty acids, **PCA**, principal component analysis, **^{1}H-NMR**, ^{1}H-nuclear magnetic resonance, **S**, saturated, **O**, oleic acid, **L**, linoleic, **Ln**, linolenic acid

Summary

Piedmont olive oils collected in 2010 were characterised, for the first time, in terms of their fatty acid profile using GC and ^{1}H-NMR and compared to other oils from five Italian regions. Applying NMR spectroscopy on the olive oil samples, without manipulation, it is possible to calculate the proportion of the different acyl groups in the oil samples. As the area of the signals is proportional to the number of each type of proton in the sample, saturated, monounsaturated (oleic acid) and polyunsaturated (linoleic and linolenic acids) fatty acids were determined. All analyzed samples can be categorized as virgin olive oil extra quality according to the oleic/linoleic ratio. Based on a preliminary geographical investigation, olive oils produced in the North of Italy show a good separation from those from Central and Southern regions.

Practical applications: Oil characterization of new products is the basis for further nutritional and food technological investigations and the quality of edible oils is of great concern especially for products available on the market. The two adopted techniques show a remarkable agreement in the evaluation of fatty acid composition of oil samples. Also, this research, by means of ^{1}H-NMR, provides information on geographical origin of the olive oils of Northern Italian regions with respect to Central and Southern regions.

1 Introduction

The olive tree (*Olea europaea* L.) is one of the most ancient cultivated fruit tree in the Mediterranean basin where, according to recent studies, its domestication may have occurred [1].

Only few vegetable oils can be consumed in their natural state and olive oil is one of these. Nowadays, the Mediterranean basin is the area where almost the 80% of the global production of olive oil takes place. In recent years, olive oil nutritional quality and healthy value have contributed to increase the oil consumption and have promoted cultivation of olives outside the Mediterranean basin.

Apart from well-characterized small production - as the Garda lake oil distinguished with a European Protected Denomination of Origin trademark since 1998 - Northern Italy is not usually known as an area suitable for olive tree culture; nevertheless, in recent years, this cultivation raises a new interest in this area.

The Piedmont region (North Western Italy) comprises some areas characterized by a microclimate that allows olive cultivation. Most of the 250 estimated hectares of oil olive orchards in Piedmont have been planted in the last ten years and at present over 95000 trees have been cultivated. Studying olive oil obtained in this part of Italy is of great interest due to the extreme, for the species, environmental conditions and for the peculiarities of the product.

The large increase in demand for high-quality and healthy products makes virgin olive oil authenticity of prime importance in food industry. Numerous are the criteria which define the authenticity or genuineness of a food product; moreover authenticity issues of food products such as olive oil, wine or coffee can be associated with a geographical origin, nature of the soil, climatic conditions or variety [2]. At the same time, due to its higher commercial value extra virgin olive oil is often illegally adulterated with other less expensive vegetable oils. For this reason the authentication of virgin olive oil became a

nodal point at the beginning of 90's for both producers and customers. New analytical methods to control olive oil purity have been developed by European Union [3-5].

Triglycerides, differing in their substitution patterns in terms of length, degree and kind of insaturation of the acyl groups, are the main constituent of olive oils, which are also characterized by minor compounds such as mono- and diglycerides, sterols, aliphatic alcohols, fatty acids and phenolic compounds.

Olive oil chemical composition can be investigated by means of several techniques; among them Proton Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy, coupled with chemometrics studies, allows the identification of behaviours related to specific production areas, vintages and variety. ^{1}H NMR spectroscopy is a very suitable technique for the analysis of complex matrix because it allows to obtain, in a single experiment, the whole fingerprint of an edible and liquid sample as it is without extensive manipulations.

Several papers have been published so far concerning the study of the authentication of olive oil by means of fingerprint techniques, namely NMR [6-12] and chromatographic techniques, mainly GC [13-18] which is the most common method used for the determination of fatty acid composition in food. Moreover, both NMR and gas chromatography, based on the analysis of fatty acid profile, have been proposed for the detection of illegal additions of hazelnut or sunflower oil to olive oil [19, 20].

The present paper aims to characterize olive oils from Piedmont, a new product never analysed before, and to verify its geographical peculiarities by discriminating it from other Italian olive oil samples.

The preliminary characterization has been performed by GC analysis in order to obtain total fatty acid composition. The main fatty acid profile was compared to that determined by ^{1}H NMR. Moreover, the origin discrimination has been investigated on selected ^{1}H NMR resonances which were submitted to multivariate statistical methods.

2 Materials and methods

2.1 Samples, standards and reagents

All solvents used were of analytical reagent grade. Potassium hydroxide, sodium hydrogen sulphate, methanol, heptane purchased from Sigma–Aldrich (Milan, Italy), sodium-3-trimethylsilylpropionate-d_4 (TSP-d_4), was purchased from Cambridge Isotope Laboratories Inc. All solvents were used as received without further purification.

Forty-four independent oil samples of different geographical origin were investigated in a preliminary way: oils from Piedmont and Friuli-Venezia Giulia were obtained by producers involved in University projects; and oils from Tuscany, Sardinia, Apulia, and Sicily were obtained from local olive oil producers.

Distribution of samples in the Italian regions follows a latitude gradient; the main characteristics of analyzed olive oil samples are reported in Table 1. All the samples are blends of distinctive varieties of each selected region, coming from 2010 crop season. Each sample underwent the same extraction system (cold-extraction) and the same storage conditions.

2.2 GC analysis

Fatty acid methyl esters (FAMEs) of 0.1 g of olive oil were prepared by using a solution of KOH in methanol and 0.5 g of $NaHSO_4*H_2O$ [21] and were separated and quantified by gas chromatography. A Shimadzu instrument (GC17A, Shimadzu Corporation Analytical Instruments Division, Kyoto, Japan) equipped with a CP-Sil 88 capillary column (100 m × 0.25 mm ID, 0.20 μm film thickness; Varian Inc., Lake Forest, CA) was used. The column temperature was held at 45°C for 5 min, then raised 20°C min^{-1} up to 195°C and maintained for 28 min. The temperature of the injector and the flame-ionization detector was maintained at 250°C and 280°C, respectively; the

injection volume was 0.1 μl; nitrogen constant linear flow rate was set at 40 ml min^{-1}. Peaks were identified by comparison of retention times with FAME standards (Matreya and Restek Corporation). Results were expressed as a percentage of each individual FA per total FAs detected. All analyses were done in duplicate. The obtained results are expressed as mean ± standard deviation of three replicates.

2.3 NMR analysis

All the samples were stored at low temperature and the internal atmosphere was pumped out using a vacuum pump. All solvents used were of analytical reagent grade and were purified according to literature [22].

20 μl of olive oil were dissolved in 700 μl of chloroform-d and 20 μl of DMSO-d_6. As olive oil minor polar compounds had a low solubility in chloroform, the addiction of DMSO was necessary for enhancing the sample solubility. Then, 550 μl of the solution, poured in a little cone, were transferred into a 5 mm NMR tube. A slight modification of the ^{1}H NMR procedure reported by D'Imperio *et al.* [23] has been applied.

^{1}H NMR spectra were recorded at 300 K using a Bruker Avance 600 spectrometer operating at 600.13 MHz for ^{1}H and equipped with a 5 mm broad-band probe (Bruker, Milan, Italy). To acquire the ^{1}H spectra of oil samples, the following acquisition parameters were used: each spectrum was acquired with 2048 scans of 64 K data points with a spectral width of 18.43 ppm, an acquisition time of 3 s and a relaxation delay of 0.5 s. Experiments were carried out at 300 K. Prior to Fourier Transformation the FID was apodized using a Lorentzian line broadening of 0.5 Hz. In order to achieve a quantitative evaluation of all peaks of interest the baseline was corrected using a multi-point correction (Cubic Spline Baseline Correction routine). Each oil sample was analysed four times and values were averaged. Chemical shift were referenced to the residual signal of chloroform at 7.26 ppm.

In order to perform the statistical analysis, the intensities of 16 signals were measured using the semi-automatic peak-picking routine present in the Bruker Topspin software. The intensities of the selected signals were compared with that of the resonance at 1.56 ppm, due to methylene protons in β position, in relation to carboxyl group, normalized to 1000. As reported in literature [24], this procedure gives an index proportional to the molar ratio between each compound and the total amount of fatty chains.

Moreover, in order to perform the evaluation of fatty acid composition, area values of ^{1}H NMR spectra signals of olive oil samples were obtained by fitting total area to 100. From the ^{1}H NMR spectrum, the determination of the main fatty acids in olive oil can be made in several ways [25, 26]. Best results were obtained with the method by Guillèn *et al.* [27] who determined saturated (S), monounsaturated (oleic acid, O) and polyunsaturated fatty acids (linoleic and linolenic acids, L and Ln respectively) according to the following equations:

$$Ln\ (\%) = 100\ [B/(A+B)] \quad (1)$$

$$L\ (\%) = 100\ [(E/D) - 2\ [B/(A+B)]] \quad (2)$$

$$O\ (\%) = 100\ [(C/2D) - (E/D) - 2\ [B/(A+B)]] \quad (3)$$

$$S\ (\%) = 100 - [1 - [(C/2D)] \quad (4)$$

where A is the area of the signal 1 (Figure 1), corresponding to methyl protons in all acids except linolenic, B is the area of signal 2, corresponding to protons of linolenic acyl groups, C is the area of signal 3, related to protons in allyl methylene groups, D is the area of signal 4, corresponding to methylenic protons in α position, in relation to the carboxyl group and E in the area of the signal 5, related to bis-allylic protons.

2.4 Statistical analysis

GC and NMR data concerning main fatty acid composition were analysed with General Linear Model (GLM) Procedure of SAS (2004) [28] according to the following model:

$Y_{ijk} = \mu + R_i + M_j + (R*M)_{ij} + \varepsilon_{ijk}$,

where Y_{ijk} = mean of response variable, μ = population mean, R_i = effect of origin (Italian region), M_j = effect of analyses method, $(R*M)_{ij}$ = effect of interaction between origin and method, and ε_{ijk} = experimental error.

Principal Component Analysis (PCA), an unsupervised pattern recognition method that reduces data dimensionality by performing a covariance analysis between factors, was used to analyse the samples geographical distribution on data-sets autoscaled and mean centred. Multivariate analysis was performed by using the chemometrical package LATENTIX 2.0 (Latent5, Denmark, www.latentix.com).

3. Results and discussion

3.1 Fatty Acid Composition by GC and ^{1}H NMR

In Figure 1 ^{1}H-NMR signals relative to the different proton types in the spectrum for a virgin olive oil, recorded at 600 MHz, are reported. General assignment of the main signals is well-known [29-31].

All analyzed samples can be categorized as extra virgin olive oil according to the oleic/ linoleic ratio [32]. In particular, it is worth noting that the higher oleic/ linoleic ratio values occurs for northern samples (Piedmont oil: 14.78 and 14.95 for GC and NMR respectively; Friuli-Venezia Giulia oil: 13.73 and 13.54 for GC and NMR respectively) than values related to other regions (Table 2).

All samples are characterized by levels of oleic acyl groups between 70 and 80% of total fatty acid contents and by levels of linolenic acid lower than 1%. These results suggest the absence of any addition of edible oils other than olive oils in the samples under investigation [12]. As can be seen (Table 2), the composition of fatty acid chains varied noticeably between samples coming from different regions. Concerning Piedmont olive oil, the C18:1 values were only significantly different from Sicilian ones. Piedmont samples are

characterized by the lowest linoleic acid values (5.36% and 5.34% for GC and NMR method respectively). Similar linolenic acid contents were observed only in olive oils collected in Friuli-Venezia Giulia.

This parameter represents a significant tool for the discrimination of geographical origin of Italian oils since Northern extra-virgin olive oils are significantly different from oils produced in Southern regions.

The monounsaturated fatty acids (MUFAs) contribute to limit cardiovascular risk by reducing the amount of cholesterol in blood [33] and they are less vulnerable to lipid peroxidation, and consequently to rancidification than polyunsaturated fatty.

Samples coming from Northern Italy were characterized by MUFA/PUFA ratios higher than 11, whereas samples from Central and Southern Italy (Tuscany, Sicily, Sardinia and Apulia) showed values in the range 7-9. In particular, concerning the Northern olive oils, the Piedmont samples showed higher ratios (13.17 and 13.04 respectively for NMR and GC) than Friuli-Venezia Giulia ones (12.30 and 12.45 respectively for NMR and GC). On the other hand, samples coming from Sicily showed lower ratio values (7.54 and 7.62 respectively for NMR and GC) than other Italian samples.

The differences observed for oleic acid and linoleic acid values, as well as MUFA/PUFA and oleic/linoleic ratio were highly significant ($P<0.001$) between regions using both GC and NMR method, but were not completely discriminant between the established classes

Concerning saturated fatty acid (SFA) determined using GC method, the higher and so negative healthier value was observed for Piedmont oils (16.15%) and it was significant different from saturated fatty acid in Sardinian and Apulian samples. It’s worth noting that a high variability occurs in the samples coming from the Northern regions of Italy.

On the basis of SFA composition, NMR method was not able to differentiate olive oils coming from different Italian regions (P=0.54; not significant), while GC method showed significant differences (P< 0.01) between regions.

These results were expected because, as reported by Knothe *et al.* [26], the quantification of the saturated fatty acids can be theoretically obtained from the ^{1}H NMR spectrum assuming that there are only two major saturated present in the samples. This approach is possible only in the case of prepared mixtures and not in the case of real samples, as those analysed in the work.

Nevertheless, comparing the NMR results with those obtained by gas chromatography, it is worth noting the good agreement between the two data sets as previously reported by Dias *et al.*, which highlighted that the standard deviations of the NMR method are comparable to those of conventional methods, except those of total hydroxytyrosol and total tyrosol [34].

3.2 Geographical investigation by NMR

Experimental data obtained by NMR, coupled with chemometrics analysis, were used to verify if the geographical origin of Piedmont olive oils could be considered a peculiar feature compared to other Italian regions.

Some literature data [35] show that fatty acid composition is an important parameter for the discrimination of the monovarietal olive oils according to cultivars whereas it is less related to the geographical origin of the samples. Since our samples are blends of typical varieties in each region, the fatty acid composition data were not considered as relevant discriminat parameter.

Previous studies have demonstrated that, a suitable set of ^{13}C NMR resonances can easily discriminate among mono-varietal olive oils grown in the same geographic area, [36] whereas another set of NMR resonances, among which β-sitosterol, squalene, terpenes, trans-2-hexanal and hexanal, is useful for classifying olive oils produced in different pedoclimatic areas [37, 38]. Therefore, a set of NMR resonances (Figure 2) evaluated by Mannina *et al.* and

by D'Imperio *et al.* [23, 38, 39] as more sensitive to the geographical origin of the olive oil have been used in this work.

Sacchi and co-workers [40] showed that specific resonances due to minor components, such as sterols, n-alkanals, trans-2-alkanals and other volatile compounds are important parameters for the determination of geographic features. Moreover the pedoclimatic conditions (climate and soil) have an important influence in the enzymatic component responsible for the production of volatile compounds well-known as flavors. The antioxidant component, probably also related to these conditions, can affect the synthesis of volatile compounds labelled as off-flavors [41]. Squalene and linolenic acid are also related to the production of volatile compounds. Squalene is a known antioxidant compound of olive oil therefore a high production of volatile compounds during decomposition could be related to its quantity in the analysed sample [42]. Furthermore, the squalene is an acyclic terpene and it can produce, during its decomposition, some of the terpenes in the set of NMR resonances [43]. The fatty chains of linolenic acid play the role of substrate during the synthesis of trans-2-hexanal [41].

Figure 2 showed the spectrum of these 16 resonances relative to samples from Piedmont, with the relative chemical assignment. The normalized 16 resonances were submitted to the chemometric analysis.

Three components were calculated and the model corresponding to the three PCs explains 60.0% of the total variance. Data scores and loadings have been shown in Figure 3 and Figure 4 respectively A clear separation between samples from the six Italian regions was observed. The PC1 axis, explaining 25% of the total variance, contributed mainly to the discrimination between Northern olive oils coming from Piedmont and Friuli-Venezia Giulia and Central and Southern samples. These differences could be due to particular climatic conditions of northern regions, as Piedmont and Friuli-Venezia Giulia, which are characterized by cooler climate than other regions. The associated

loadings (Figure 4) showed the spectral features contributing to the discrimination of olive oil samples. Concerning the observed separation of olive oils coming from North and South of Italy, squalene, *trans*-2-hexanal, β-sitosterol and terpenes were the most discriminating resonances. In particular, samples from Piedmont and Friuli-Venezia Giulia had a high value of squalene (11), *trans*-2-hexanal (2) and terpenes (4, 5 and 6) along PC1, whereas central and southern Italy samples were characterized by higher values of β-sitosterol (16), aldehydes (1, and 3), linolenic and linoleic acid (9, 10, 14 and 15). No variable used for the multivariate data analysis was completely discriminant between the Northern oils and the Centre and Southern oils.

Along PC2 and PC3, explaining 18.9% and 15.5% of the total variance respectively, Apulian samples were more enriched in two unknown aldeydes (1 and 3) compared to samples coming from Sicily and Sardinia. On the other hand, Sicilian and Sardinian oils showed higher level of linolenic acid (9 and 14).

4 Conclusions

Piedmont olive oils were analysed for the first time by NMR and GC. The results of this preliminary work have led to some useful conclusions about the quality and the fatty acid composition of Piedmont olive oils. On the basis of the results, all the Piedmont samples were classified as extra virgin olive oil, with high levels of monounsaturated acids. Generally, the results of ^{1}H NMR and GC determination of the unsaturated fatty acids were in good agreement. Moreover, discrimination of Northern and Southern Italian olive oil samples was obtained by NMR coupled with chemometric analysis.

Despite the limited number of samples, this work revealed some interesting trends which, in our opinion, justify further in-depth examinations.

An interesting preliminary separation was obtained between olive oils from Piedmont and olive oils from other regions. The present results suggest that the

most discriminating resonances were squalene, trans-2-hexanal, β-sitosterol and terpenes. This observation is in agreement with previously reported NMR data, as well as observations based on sensorial analyses

In order to better analyze and valorize the product “Piedmont olive oil” the extension of the sampling to different Italian regions is needed, as is the validation of the method in different years of harvesting.

Piedmont is an Italian region not usually known as olive oil producer but in which *Olea europaea* L. culture is nowadays getting an arising importance. Thus, our first characterization is of great interest due to the peculiarities of the product and to the possibility of a market expansion.

Conflict of interest statement

The authors have declared no conflict of interest.

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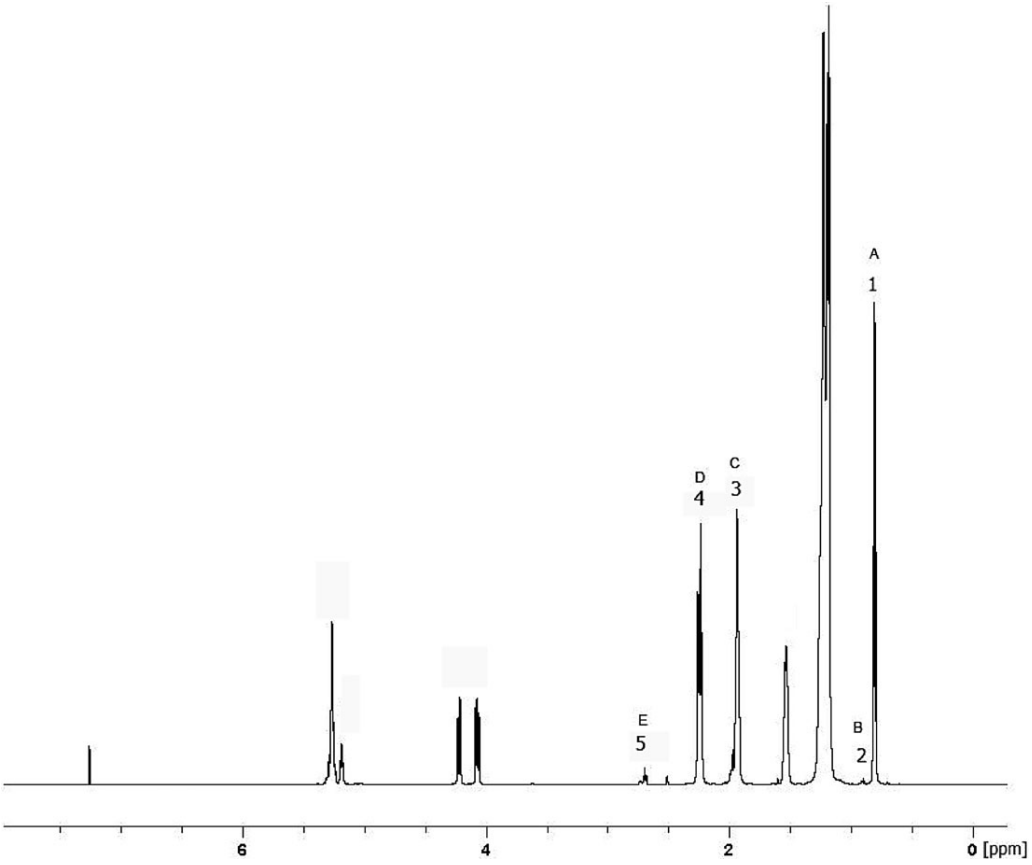

Figure 1. 1HNMRspectra signals for the evaluation of fatty acid composition. (A 1): methyl protons in all acids except linolenic; (B 2): protons of linolenic acyl groups; (C 3): protons in allyl methylene groups; (D 4): methylenic protons in a position, in relation to the carboxyl group; (E 5): bis-allylic protons.

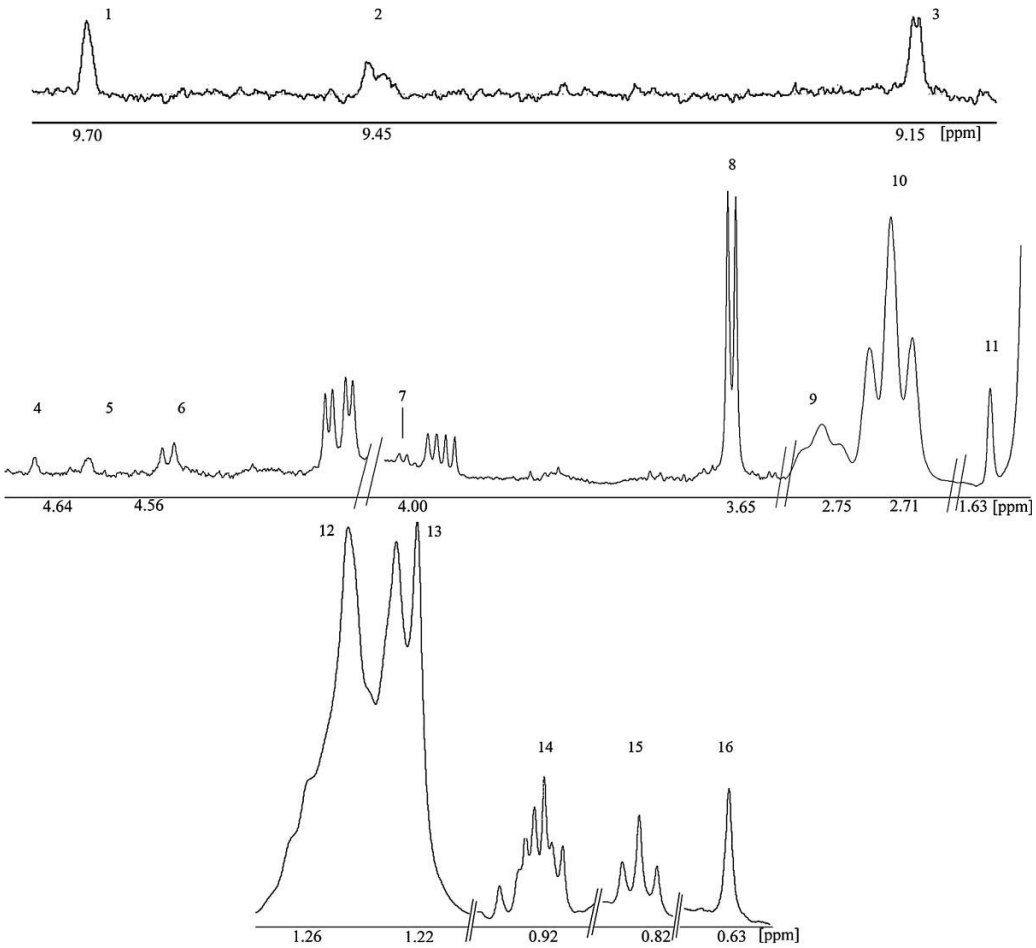

Figure 2. 1H NMR signals used for the statistical analysis. 1:hexanal, 2: unknown aldehyde, 3: trans-2-hexanal, 4:terpene 3, 5:terpene 2, 6:terpene 3, 7: methylenic proton in a glycerol moiety of sn 1,3 diglycerides, 8: methylenic proton in a glycerol moiety of sn 1,2 digkycerides, 10: diallylic protons of linolenic fatty chains, 11: diallylic protons of linoleic fatty chains, 11: squalene, 12: methylenic protons of all unsaturated fatty chains, 13: methylenic protons of saturated fatty chains, 14: methylic protons of linolenic fatty chains, 15: methylic protons of linoleic fatty chains, 16: methyl-18 of b-sitosterol.

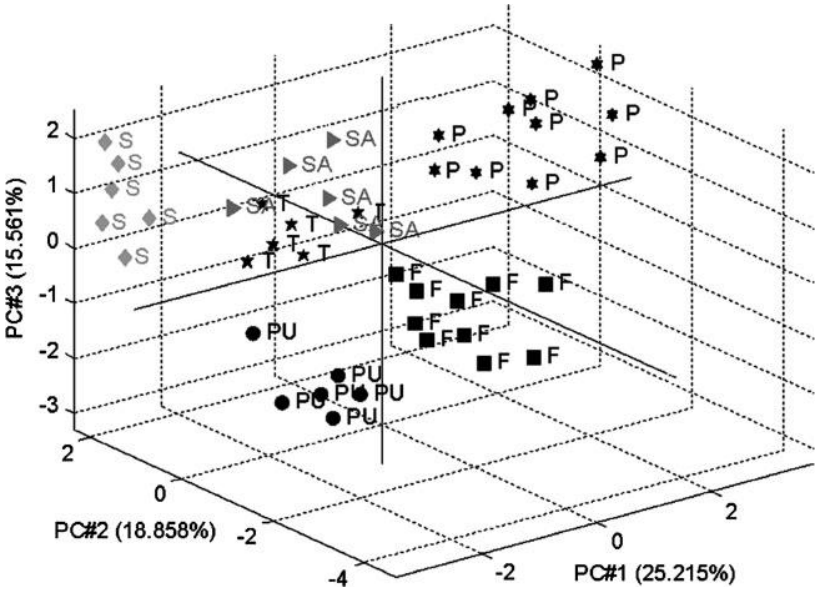

Figure 3. 3D representation of PCA calculated from 1H NMR data: (P) Piedmont, (SA), Sardinian, (F) Friuli, (S), Sicilian, (T) Tuscan, (PU) Apulian samples. Scores are reported.

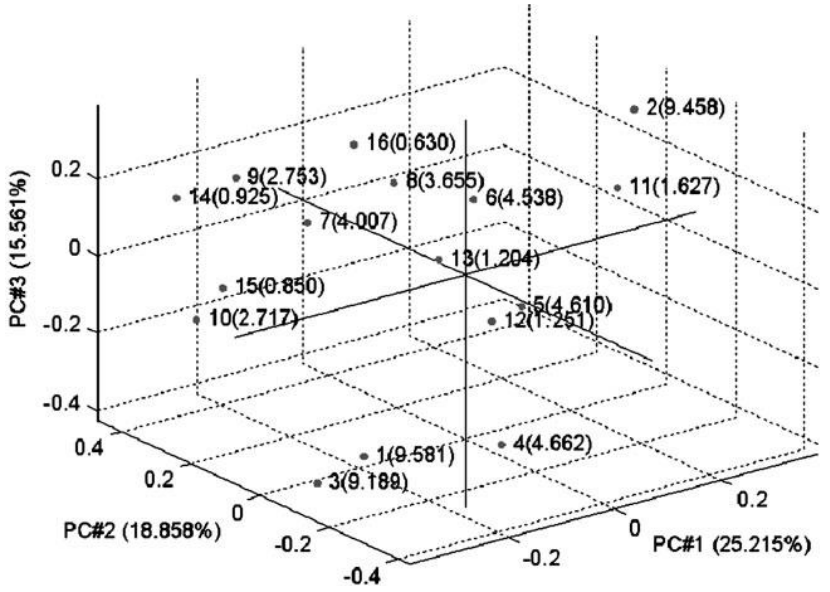

Figure 4. 3D representation of PCA calculated from 1H NMR data.oadings are reported.