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# NMR and statistical study of olive oils from Lazio: A geographical, ecological and agronomic characterization

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**Abstract**

NMR and statistical procedures were used to analyse olive oils obtained from trees grown in different areas of Lazio, an Italian region, under different irrigation conditions. In order to obtain information on "real" commercial olive oils and to study the effects of some agronomical and ecological factors on the olive oil composition, we studied commercial multi-varietal olive oils, all produced in well-characterized areas of Lazio. $^{1}H$ and $^{13}C$ NMR techniques, coupled to a suitable multivariate statistical procedure, were used to analyse 72 multi-varietal extra virgin and PDO (Protected Denomination of Origin) olive oils harvested in 2003, from the northern area, the centre and the southern area of Lazio. The intensity of selected $^{1}H$ and $^{13}C$ NMR variables were submitted to three different statistical methods, namely, analysis of variance (ANOVA), principal component analysis (PCA) and linear discriminant analysis (LDA). $^{1}H$ and $^{13}C$ NMR spectroscopy allowed us to obtain a good chemical characterization of the samples, giving information on major and minor compounds with an experimental error exactly the same and always extremely low for all the analyzed components. As a result of the statistical analysis, olive oils from the same geographical areas were well grouped. Since the amounts of some minor volatile components, such as aldehydes, terpenes and squalene, as well as, the content of β-sitosterol, the most important sterol present in olive oils, are sensitive to the pedoclimatic conditions, the intensity of the corresponding NMR signals turned out to be the most discriminating factors in the geographic classification. Moreover, the NMR and statistical protocol allowed us to investigate the roles of irrigation and altitude on the olive oil composition: the contents of oleic and saturated fatty acids turned out to be strongly influenced by the irrigation practice, whereas the content of volatile compounds was sensitive to the altitude of the olive trees. As a result of our study, olive oils were well grouped according to the irrigation practice as well as to the altitude at which olive trees were grown.


*Keywords:* NMR; Statistical analysis; Olive oils; Altimetry; Irrigation

## 1. Introduction

In recent years many studies have been performed to characterize and classify olive oils using different techniques (Armanino, Leardi, Lanteri, & Modi, 1989; Baleстrieri, Bucci, Magrì, Magrì, & Marini, 2004; Boggia, Zunin, Lanteri, Rossi, & Evangelisti, 2002; Mannina, Sobolev, & Segre, 2003; Salter et al., 1997).

It has been previously demonstrated that NMR spectroscopy is a powerful tool for characterizing olive oils according to geographical origin (Mannina, Fontanazza, Patumi, Ansanelli, & Segre, 2001; Mannina, Patumi, Proietti, Bassi, & Segre, 2001; Sacchi et al., 1998) and the PDO (Mannina, D'Imperio, Lava, Schievano, & Mammi, 2005). Moreover, using $^{13}C$ data a perfect

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separation of mono-varietal olive oils from a restricted geographical area has been achieved (Mannina, Dugo et al., 2003).

It has also been reported (Mannina, Fontanazza et al., 2001) that NMR data are useful in the resolution of particular agronomical problems; for instance, $^{13}C$ NMR data, coupled to a suitable statistical analysis, allow the choice of the Italian cultivars more suitable to the extreme pedoclimatic conditions present in Catamarca, a wild region of Argentina.

The olive tree (*Olea Europea*) is a drought-resistant plant, usually grown in areas with limited water resources. In Mediterranean areas characterized by scarce rainfalls, irrigation could influence both the olive oil production and olive oil quality. However, controversial results are reported on the effects of different irrigation regimes on olive oil composition and yield (Motilva, Tovar, Romero, Alegre, & Girona, 2000; Patumi et al., 2002; Romero, Tovar, Girona, & Motilva, 2002; Tovar, Motilva, & Romero, 2001; and references quoted therein).

Other factors, such as the altitude, may cause variation of the olive oil composition. Mono-varietal olive oils, from the same cultivar, grown at different altitudes, 100 and 400 m, are reported to exhibit small variations in acidity and phenol content, both of which are higher in olive oils obtained from plants grown at lower altitudes (Osman, Metzidakis, Gerasopoulos, & Kiritsakis, 1994).

Other studies, again on mono-varietal olive oils, claim that hill-grown trees produce olive oils with a higher oleic acid content than do the same trees grown at lower altitudes (Paz, Beltrn, Ortega, Fernandez, & Jimenez, 2005). It must be noted, however, that, in this specific study, the used cultivars are not native; therefore climate conditions may play a fundamental role in determining the olive oil composition.

It is also reported (Ravalli, 2004) that *Dacus Oleae* infestation is often present at an altitude below 400 m, giving rise to olive oils with a high acidity value and sensorial defects.

We have previously demonstrated that, using a suitable set of NMR resonances, it is possible to discriminate among mono-varietal olive oils grown in the same geographic area (Mannina, Dugo et al., 2003) whereas, using another set of NMR resonances, it is possible to classify and characterize olive oils produced in different pedoclimatic areas (Mannina, Fontanazza et al., 2001; Mannina, Patumi, Proietti, & Segre, 2001; Sacchi et al., 1998) and the PDO (Mannina et al., 2005).

In the present paper, we have used NMR and statistical methodology to analyze commercial multi-varietal olive oils from well-defined areas of Lazio, not only to group the olive oils according to their geographical areas, but also to explore the effects of altitude and irrigation on the olive oil composition and therefore on the olive oil classification.

## 2. Materials and methods

### 2.1. Sampling

Multi-varietal virgin and PDO olive oils (72 samples, mixtures of 22 cultivars) harvested in 2003, from different geographical areas of Lazio, see Fig. 1 and Table 1, were analyzed.

In Table 1, information regarding the altitude and irrigation is also shown. In Table 2, the mean altitudes and the rain fall data, averaged over 25 years and relative to the southern area, the centre and the northern area of Lazio, are listed.

### 2.2. $^{13}C$ spectra

$^{13}C$ NMR analyses were performed on a Bruker ARX 250 spectrometer equipped with a 5 mm probe operating at the $^{1}H$ frequency of 250.13 MHz ($B_o = 5.8$ T). Olive oil samples (100 μl) were dissolved in $CDCl_3$ (600 μl), directly in the 5 mm NMR tube.

$^{13}C$ NMR spectra were acquired using the following conditions: number of scans 512; $\pi/2$ pulse 8.3 μs; time domain (TD) 256 K data points; relaxation delay plus acquisition time 12 s; digital resolution 0.22 Hz; spectral width 200 ppm; the WALZ16 sequence was applied during the whole sequence for proton decoupling; the temperature of the sample in the probe was set at 300 K.

$^{13}C$ NMR spectra were obtained by the Fourier transformation (FT) of the FID (free induction decay), applying a Gaussian multiplication with a negative line-broadening factor of 0.1 Hz and a Gaussian position of 0.2 and using a zero filling (Size = 256 K) procedure. The resulting $^{13}C$ NMR spectra were manually phased. Chemical shifts were reported with respect to the signal due to α-methylenic protons of the glycerol moiety set at 62.36 ppm. The baseline was corrected automatically.

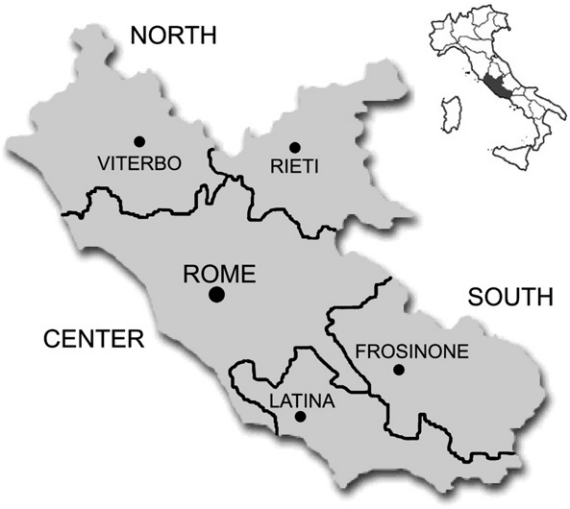


Fig. 1. Map of Lazio. The three geographical areas (north, centre and south) and the five provinces (Viterbo, Rieti, Rome, Frosinone and Latina) discussed in the text are shown.

Table 1
Geographical origin, ecological factor (altimetry) and agronomical factor (irrigation) of 72 multi-varietal extra virgin and PDO olive oils from Lazio and harvested in 2003

| Sample | Geographical factor | | | Ecological factor | | Agronomical factor |
|---|---|---|---|---|---|---|
| | Town[a] | Province[b] | Geographical area | Altitude (m) | Altitude class[c] | Irrigation |
| 1 | Canino | VT | NORTH | na | na | na |
| 2 | Fara Sabina | RI | CENTRE | 360 | A | YES |
| 3 | Nerola | RM | CENTRE | 400 | B | NO |
| 4 | *Sonnino* | *LT* | *SOUTH* | 500 | B | YES |
| 5 | Nerola | RM | CENTRE | na | na | NO |
| 6 | Fara Sabina | RI | CENTRE | 200 | A | YES |
| 7 | Fondi | LT | SOUTH | na | na | na |
| 8 | Montopoli in Sabina | RI | CENTRE | 336 | A | NO |
| 9 | *Serrone* | *FR* | *SOUTH* | na | na | na |
| 10 | Bolsena | VT | NORTH | 350 | *A* | NO |
| 11 | Fara Sabina | RI | CENTRE | 250 | A | NO |
| 12 | Montorio Romano | RM | CENTRE | na | na | na |
| 13 | Nerola | RM | CENTRE | na | na | na |
| 14 | Anagni | FR | SOUTH | 350 | A | YES |
| 15 | Cervaro | FR | SOUTH | 300 | A | na |
| 16 | Nerola | RM | CENTRE | na | na | na |
| 17 | Castelnuovo di Farfa | RI | CENTRE | 350 | A | na |
| 18 | *S. Donato Val di Comino* | *FR* | *SOUTH* | na | na | NO |
| 19 | Torri in Sabina | RI | CENTRE | na | na | na |
| 20 | Cori | LT | SOUTH | na | na | na |
| 21 | Montopoli in Sabina | RI | CENTRE | na | na | na |
| 22 | Anagni | FR | SOUTH | 500 | B | NO |
| 23 | Alatri | FR | SOUTH | 475 | B | NO |
| 24 | Tivoli | RM | CENTRE | 300 | A | na |
| 25 | Fara Sabina | RI | CENTRE | 300 | A | NO |
| 26 | Aprilia | LT | SOUTH | na | na | na |
| 27 | Gradoli | VT | NORTH | na | na | NO |
| 28 | Nerola | RM | CENTRE | 400 | B | NO |
| 29 | Montelibretti | RM | CENTRE | 450 | *B* | NO |
| 30 | Nerola | RM | CENTRE | 300 | A | NO |
| 31 | Roma | RM | CENTRE | na | na | na |
| 32 | Anagni | FR | SOUTH | na | na | na |
| 33 | Piglio | FR | SOUTH | na | na | na |
| 34 | Vetralla | VT | NORTH | na | na | na |
| 35 | Blera | VT | NORTH | na | na | na |
| 36 | Cervaro | FR | SOUTH | 400 | B | NO |
| 37 | Moricone | RI | CENTRE | na | na | na |
| 38 | Canino | VT | NORTH | 250 | *A* | na |
| 39 | Fara Sabina | RI | CENTRE | 300 | A | NO |
| 40 | *Cori* | *LT* | *SOUTH* | na | na | na |
| 41 | *Anagni* | *FR* | *SOUTH* | 400 | B | NO |
| 42 | Canino | VT | NORTH | na | na | na |
| 43 | Canino | VT | NORTH | na | na | na |
| 44 | Cellere | VT | NORTH | na | na | na |
| 45 | Sonnino | LT | SOUTH | na | na | NO |
| 46 | Montelibretti | RM | CENTRE | na | na | na |
| 47 | Alatri | FR | SOUTH | na | na | na |
| 48 | *San Giovanni Campano* | *FR* | *SOUTH* | 300 | A | NO |
| 49 | Viterbo | VT | NORTH | na | na | na |
| 50 | Montelibretti | RM | CENTRE | na | na | NO |
| 51 | Arpino | FR | SOUTH | 540 | B | na |
| 52 | Montefiascone | VT | NORTH | 450 | B | YES |
| 53 | Roccamassima | LT | SOUTH | 375 | *A* | NO |
| 54 | Nerola | RM | CENTRE | 400 | B | NO |
| 55 | Fara Sabina | RI | CENTRE | na | na | na |
| 56 | Tivoli | RM | CENTRE | na | na | na |
| 57 | Fara Sabina | RI | CENTRE | 300 | A | NO |
| 58 | Palombara Sabina | RM | CENTRE | 250 | A | NO |
| 59 | Fara Sabina | RI | CENTRE | na | na | na |
| 60 | Roma | RM | CENTRE | 450 | B | NO |

(*continued on next page*)

Table 1 (*continued*)

| Sample | Geographical factor | | | Ecological factor | | Agronomical factor |
|---|---|---|---|---|---|---|
| | Town[a] | Province[b] | Geographical area | Altitude (m) | Altitude class[c] | Irrigation |
| 61 | Frascati | RM | CENTRE | 200 | A | na |
| 62 | Castelnuovo di Farfa | RI | CENTRE | 350 | A | na |
| 63 | Cantalupo in Sabina | RI | CENTRE | na | na | na |
| 64 | Castelnuovo di Farfa | RI | CENTRE | 350 | A | na |
| 65 | Fara Sabina | RI | CENTRE | 300 | A | NO |
| 66 | Montorio Romano | RM | CENTRE | na | na | na |
| 67 | Fara Sabina | RI | CENTRE | na | na | na |
| 68 | Fara Sabina | RI | CENTRE | 250 | A | NO |
| 69 | Nerola | RM | CENTRE | 400 | B | NO |
| 70 | Nerola | RM | CENTRE | na | na | na |
| 71 | Palombara Sabina | RM | CENTRE | 250 | A | NO |
| 72 | *Castelnuovo di Farfa* | *RI* | *CENTRE* | na | na | na |

na = not available.
[a] Anomalous samples are reported in italic characters.
[b] LT = LATINA; RM = ROMA; VT = VITERBO; FR = FROSINONE; RI=RIETI.
[c] Altitude <400 m; B = Altitude ⩾400 m.

In order to perform the statistical analysis, the intensity of 10 selected resonances (see Table 3 and Fig. 2) was measured; the semi-automatic peak-picking routine in the Bruker TOPSPIN software was used. The intensity of the 10 signals was measured with respect to that of the signal at 62.36 ppm normalized to 100 (signal A in Fig. 2) due to α-methylenic protons of the glycerol moiety.

### 2.3. $^1H$ spectra

$^1$H NMR analyses were performed on a Bruker AVANCE AQS600 instrument equipped with a 5 mm probe operating at the $^1$H frequency of 600.13 MHz ($B_o = 14.3$ T).

Olive oil samples (20 μl) were dissolved in DMSO (20 μl) and $CDCl_3$ (700 μl) directly in the 5 mm NMR tube.

The $^1$H NMR spectra were acquired using the following conditions: number of scans 1024; π/2 pulse ~8 μs; time domain (TD) 64 K data points; relaxation delay plus acquisition time 3.5 s; spectral width 18.5 ppm; the temperature of the sample in the probe was set at 300 K.

$^1$H NMR spectra were obtained by the Fourier transformation (FT) of the FID (free induction decay), applying an exponential multiplication with a line-broadening factor of 0.3 Hz and a zero filling (Size = 64 K) procedure. The resulting $^1$H NMR spectra were manually phased. Chemical shifts were reported with respect to the residual $CHCl_3$ signal set at 7.26 ppm. For ensuring a better quantitative comparability of the spectra, the baseline was corrected using a multi-point correction. In particular, the Cubic Spline Baseline Correction routine in the Bruker TOPSPIN software was used.

In order to perform the statistical analyses, the intensities of 17 signals, see Table 3 and Fig. 2, 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 2.251 ppm (signal B in Fig. 2) due to α-carboxyl protons of all acyl chains, normalized to 1000.

### 2.4. Statistical analysis (*Brereton, 2003; Siegel, 1988*)

The statistical elaboration of the NMR data was performed using two programmes: SPSS for Windows (version 6.0; 1993) and STATISTICA package for Windows (version 5.1, 1997).

The statistical analyses previously reported (Brereton, 2003; Fauhl et al., 2000; Lavine and Workman, 2004; Mannina, Patumi, Proietti, Bassi et al., 2001; Mannina, Dugo et al., 2003; Mannina et al., 2005) namely analysis of variance (ANOVA), principal component analysis (PCA), linear discriminant analysis (LDA), were preceded by an explorative analysis.

The explorative analysis was performed to remove anomalous samples and therefore to reduce the initial data matrix. A box plot, based on the median and the

Table 2
Mean altitude and climate parameters averaged over 25 years relative to the southern, the centre and the northern areas of Lazio

| | Geographical areas of Lazio | | |
|---|---|---|---|
| | South (13)[a] | Centre (16) | North (8) |
| Mean altitude (m) | 433 ± 252 | 328 ± 124 | 356 ± 109 |
| Mean temperature each year (°C) | 14.5 ± 1.1 | 13.7 ± 1.0 | 14.3 ± 0.4 |
| Mean value of the rainfall per year (mm) | 1205 ± 121 | 1044 ± 134 | 899 ± 102 |
| Mean number of rainy days per year | 96 ± 9 | 86 ± 6 | 80 ± 5 |

The standard deviation is also shown.
[a] In brackets, the number of data for each geographical area is shown.

Table 3
ANOVA applied to the intensity of 27 NMR resonances of 65 olive oil samples

| *N* | NMR resonances (ppm) | Geographical classification | | Altimetry | | Irrigation | | Shapiro–Wilk's *W* test (*p* level) | Skewness's index | Kurtosis's index |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *F* (Fisher) | *p* Level | *F* (Fisher) | *p* Level | *F* (Fisher) | *p* Level | | | |
| 1 | 173.27 | 2.5360 | 0.0869 | 0.3483 | 0.5589 | 2.0380 | 0.1631 | 0.0830 | 0.1035 | −0.6359 |
| 2 | 173.26 | 0.3745 | 0.6891 | 0.0247 | 0.8760 | 0.1142 | 0.7376 | 0.4625 | 0.2810 | −0.3781 |
| 3 | 173.24 | 4.1565 | 0.0199 | 0.9139 | 0.3456 | 0.3525 | 0.5569 | 0.1862 | −0.1518 | −0.5051 |
| 4 | 173.23 | 18.8063 | 0.0000 | 0.4836 | 0.4914 | 0.0390 | 0.8447 | 0.5768 | 0.1318 | −0.4741 |
| 5 | 172.83 | 1.3915 | 0.2559 | 0.0576 | 0.8117 | 0.6638 | 0.8023 | 0.9343 | −0.0891 | −0.0726 |
| 6 | 172.82 | 14.5610 | 0.0000 | 2.9094 | 0.0969 | 0.3180 | 0.5767 | 0.0558 | −0.5344 | −0.0609 |
| 7 | 14.14 | 1.999 | 0.1435 | 1.3251 | 0.2575 | 13.2723 | 0.0009 | 0.1478 | −0.6348 | −0.2874 |
| 8 | 14.13 | 4.2280 | 0.0187 | 0.6944 | 0.4103 | 8.9533 | 0.0053 | 0.0553 | 0.4776 | −0.4109 |
| 9 | 14.12 | 0.9303 | 0.3995 | 2.8782 | 0.0987 | 0.0013 | 0.9712 | 0.2392 | 0.4370 | 0.7863 |
| 10 | 14.09 | 17.3643 | 0.0000 | 1.9062 | 0.1761 | 1.1256 | 0.2967 | 0.3931 | −0.2300 | −0.1313 |
| 11 | 9.704 | 1.6283 | 0.2041 | 4.3477 | 0.0444 | 5.3772 | 0.0269 | 0.0000 | −1.7885 | 5.2339 |
| 12 | 9.454 | 3.3559 | 0.0409 | 2.2802 | 0.1400 | 0.3728 | 0.5458 | 0.0546 | −0.7579 | 1.3498 |
| 13 | 4.885 | 1.2872 | 0.2829 | 0.0235 | 0.8790 | 0.0990 | 0.7550 | 0.0000 | 1.7968 | 5.6545 |
| 14 | 4.661 | 9.8195 | 0.0002 | 6.3314 | 0.0166 | 0.4424 | 0.5107 | 0.0914 | 0.1816 | −0.6283 |
| 15 | 4.609 | 10.0609 | 0.0002 | 7.9349 | 0.0079 | 0.2595 | 0.6140 | 0.5863 | 0.1159 | −0.4385 |
| 16 | 4.541 | 2.7255 | 0.0729 | 18.8734 | 0.0001 | 0.8609 | 0.3604 | 0.5381 | −0.2226 | −0.0256 |
| 17 | 3.988 | 2.2172 | 0.1170 | 0.3300 | 0.5693 | 0.0158 | 0.9008 | 0.1064 | 0.5787 | 1.2908 |
| 18 | 3.636 | 5.6380 | 0.0055 | 1.6735 | 0.2043 | 0.4042 | 0.5294 | 0.1181 | 0.0711 | 0.9809 |
| 19 | 2.746 | 5.3939 | 0.0068 | 0.0216 | 0.8841 | 0.4696 | 0.4981 | 0.0563 | 0.8653 | 1.1418 |
| 20 | 2.710 | 50.8026 | 0.0000 | 2.1571 | 0.1508 | 1.2295 | 0.2758 | 0.3898 | −0.2446 | −0.1498 |
| 21 | 1.620 | 24.4395 | 0.0000 | 36.3714 | 0.0000 | 0.2777 | 0.6365 | 0.1074 | 0.5153 | −0.3956 |
| 22 | 1.244 | 4.8626 | 0.0107 | 0.4489 | 0.5072 | 0.2186 | 0.6433 | 0.0371 | 0.9526 | 1.0097 |
| 23 | 1.197 | 10.0595 | 0.0002 | 8.1899 | 0.0071 | 0.0670 | 0.7974 | 0.0000 | −1.7965 | 4.7379 |
| 24 | 0.978 | 28.9421 | 0.0000 | 7.9322 | 0.0079 | 0.0003 | 0.9865 | 0.3673 | 0.0246 | −0.2405 |
| 25 | 0.910 | 0.7817 | 0.4618 | 1.0644 | 0.3093 | 0.3639 | 0.5506 | 0.0458 | 0.6533 | 1.7969 |
| 26 | 0.843 | 33.5942 | 0.0000 | 1.0717 | 0.3077 | 0.1419 | 0.7089 | 0.9133 | −0.1643 | 0.1529 |
| 27 | 0.623 | 12.0265 | 0.0000 | 6.3480 | 0.0165 | 0.2235 | 0.6396 | 0.8054 | 0.1139 | −0.3889 |

The chemical shift (ppm) of the 27 NMR resonances is shown. The distribution of the data was tested by means of three tests shown in the last three columns.

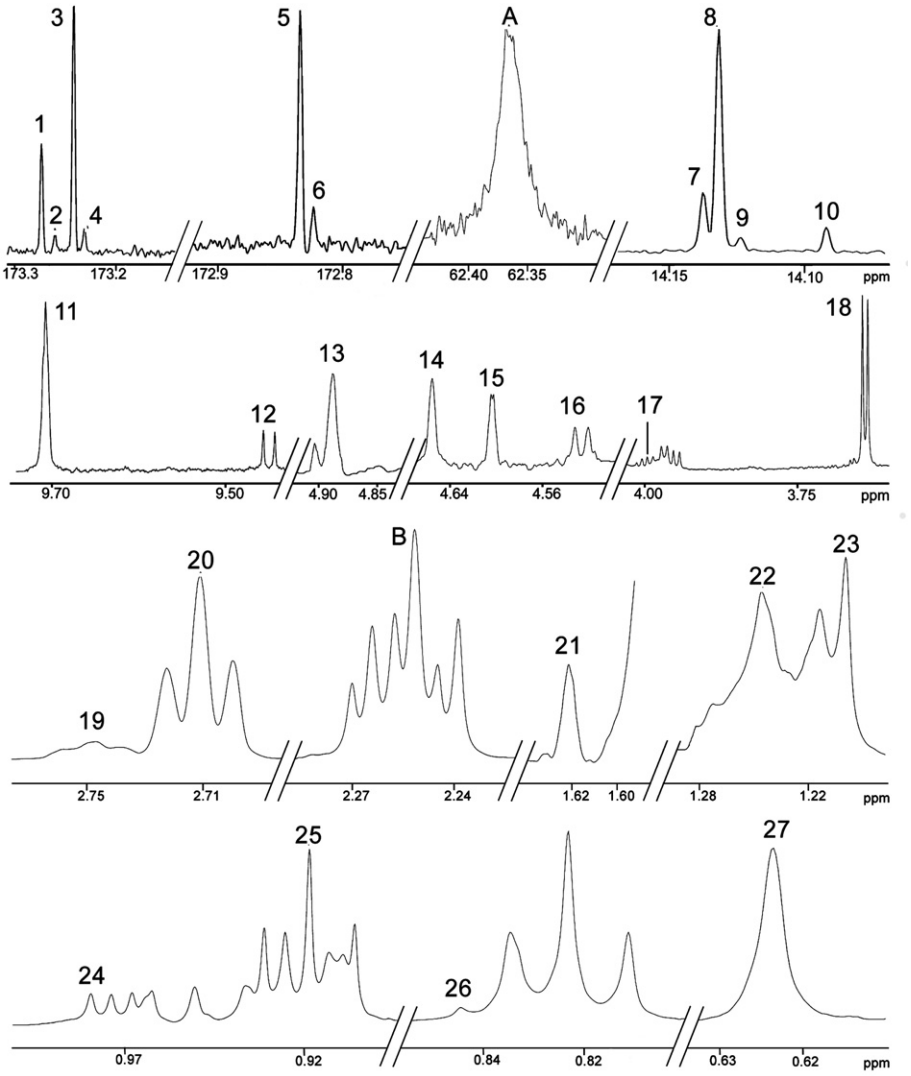


Fig. 2. $^{13}C$ and $^{1}H$ NMR signals used for the statistical analyses. 1: carbonyl signal of *sn* 1,3 saturated fatty chain; 2: carbonyl signal of *sn* 1,3 eicosen-11-oic and vaccenic fatty chains; 3: carbonyl signals of *sn* 1,3 oleic fatty chains; 4: carbonyl signals of *sn* 1,3 linoleic fatty chains; 5: carbonyl signals of *sn* 2 oleic fatty chains; 6: carbonyl signals of *sn* 2 linoleic fatty chains; A: reference peak ($^{13}C$ NMR spectra) due to α-methylenic protons of glycerol moiety normalized to 100; 7: methyl of palmitic and stearic fatty chains; 8: methyl of oleic fatty chains; 9: methyl of eicosenoic and vaccenic fatty chains; 10: methyl of linoleic fatty chains; 11: hexanal; 12: *trans* 2-hexanal; 13: terpene 4; 14: terpene 3; 15: terpene 2; 16: terpene 1; 17: methylenic protons in α glycerol moiety of *sn* 1,3 diglycerides; 18: methylenic protons in α glycerol moiety of *sn* 1,2 diglycerides; 19: diallylic protons of linolenic fatty chains; 20: diallylic protons of linoleic fatty chains; B: reference peak ($^{1}H$ NMR spectra) due to methylenic protons bound to C2 normalized to 1000; 21: squalene; 22: methylenic protons of all unsaturated fatty chains; 23: methylenic protons of palmitic and stearic fatty chains; 24: wax; 25: methyl of linolenic fatty chains; 26: methyl of linoleic fatty chains; 27: methyl-18 of β-sitosterol.

quartiles, was used: it allows the anomalous samples which are in extreme and outlier areas of the box plot to be removed.

In the case of box plot and ANOVA, the normal distribution of the data set was tested using specific tests, namely, the Shapiro Wilk's test, the Skewness index and Kurtosis index. The Shapiro Wilk's test indicates the state of the distribution using a $W$ statistic parameter. Small values of $W$ indicate an evident distance from a normal distribution. Kurtosis index measures the "peakedness" of a distribution. If the Kurtosis index is clearly different from 0, then the distribution is either flatter or more peaked than normal; the *kurtosis* of the normal distribution is 0. Skewness index measures the deviation of the distribution from the symmetry. If the Skewness index is clearly different from 0, then that distribution is asymmetric: a normal distribution is, in fact, perfectly symmetric.

In the case of PCA, the percentage of variance, for each specific principal component, was reported. The PCA results are shown as biplots involving the superimposition of the scores of the principal component scores on the highest variance and the variable loadings.

For each test, a $p$ level $<0.05$ (5% probability of error) was treated as a borderline acceptable error level.

## 3. Results and discussion

### 3.1. Geographical characterization

"Lazio" is an Italian region, see Fig. 1, which can be divided into three well-defined geographical areas, namely, the northern area, the centre, and the southern area, characterized by different pedoclimatic conditions. In Table 2, the mean altitude, the mean temperature each year, the mean rainfall per year and the mean number of rainy days per year relative to the three areas of Lazio averaged over the 25 years are shown. It must be noted that the southern area is characterized by wetter weather than the other two areas.

All 72 olive oils, namely 11 samples from the northern area, 41 samples from the centre and 20 samples from the southern area, were submitted to the NMR analysis. In previous papers (Mannina, Fontanazza et al., 2001; Mannina, Patumi, Proietti, Bassi et al., 2001; Mannina, Patumi, Proietti, Segre et al., 2001; Mannina et al., 2003, 2005), the NMR resonances, ten $^{13}C$ resonances and seventeen $^{1}H$ resonances, more sensitive to the geographical origin and/or to the cultivars and/or to the shelf life of the olive oils, have been reported; it is important to note that, depending on the specific problem, these 27 NMR variables have different discriminating powers.

The spectrum of these 27 NMR resonances, relative to an olive oil from Lazio, and its labelling, is shown in Fig. 2, whereas the relative chemical assignment and chemical shifts are given in the captions of Fig. 2 and in Table 3, column 1, respectively.

The normalized intensities of the 27 selected resonances (Mannina, Fontanazza et al., 2001) were submitted to a suitable statistical analysis. The normal distribution of the 27 variables was tested by means of specific tests of normality shown in Table 3. The Shapiro Wilk's test, the Skewness index and Kurtosis index indicated that only variables 11, 13 and 23 (see Fig. 2) have a significant deviation from the normal distribution. However, these variables turn out not to have a significant rule in the PCA and LDA analyses.

The box plot analysis (Brereton, 2003; Lavine & Workman, 2004) allowed us to sort out seven anomalous samples shown in Italic characters in Table 1. These samples showed anomalous resonance intensities, possibly due to anomalous agronomical practices or bad extraction procedures.

The ANOVA was then performed, on the intensity of the selected resonances of the remaining 65 olive oil samples, to find a possible clustering of olive oil samples from the northern area, the centre and the southern area of Lazio. The results are shown in Table 3, columns 3 and 4. Variables with a $p$ level higher than 0.05 and a low $F$ value were sorted out and the remaining 17 variables were submitted to the PCA.

The PCA map, labelled according to the three geographical areas of Lazio, is shown in Fig. 3. The PC1 and PC2 scores, explaining 51% of the total variance, allow a good separation of the three geographical areas to be observed.

PC1 (explaining 35% of the total variance) contributes mainly to the discrimination between olive oils from the south and the centre. Linonenic acid, β-sitosterol, oleic acid and squalene are the most discriminant parameters: in fact, samples from the centre have a high value of linolenic acid and β-sitosterol, as shown by the high value of the corresponding loadings lined along PC1 (linolenic acid: 4, 6,

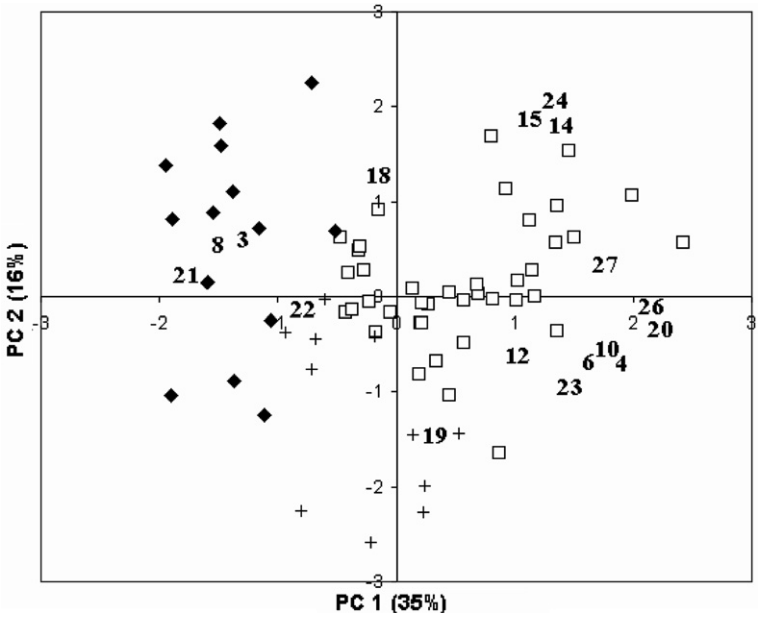


Fig. 3. PCA performed on the intensities of the variables 3, 4, 6, 8, 10, 12, 14, 15, 18, 19, 20, 21, 22, 23, 24, 26 and 27 (see Fig. 1). Olive oil samples (65) are distributed in three categories: the northern area (11) (+), the centre (40) (□) and southern area (14) (♦). Loadings are represented by the corresponding variable numbering.

Table 4
Mean values and standard deviation of the intensity (arbitrary units) of the selected NMR variables

| | Samples | 3[a] | 4 | 6 | 8 | 10 |
|---|---|---|---|---|---|---|
| NORTH | 11 | 42.98 ± 3.67 | 4.45 ± 0.74 | 4.09 ± 0.80 | 68.51 ± 3.31 | 7.77 ± 0.64 |
| CENTRE | 40 | 42.78 ± 3.17 | 4.92 ± 0.83 | 4.40 ± 0.58 | 67.67 ± 3.48 | 8.27 ± 1.01 |
| SOUTH | 14 | 45.79 ± 4.30 | 3.35 ± 0.44 | 3.43 ± 0.59 | 70.77 ± 3.82 | 6.45 ± 0.88 |
| | | 12 | 14 | 15 | 18 | 19 |
| NORTH | 11 | 0.23 ± 0.09 | 0.26 ± 0.09 | 0.18 ± 0.07 | 10.81 ± 1.07 | 9.50 ± 0.37 |
| CENTRE | 40 | 0.23 ± 0.08 | 0.37 ± 0.08 | 0.28 ± 0.06 | 11.99 ± 1.20 | 9.13 ± 0.43 |
| SOUTH | 14 | 0.17 ± 0.03 | 0.32 ± 0.08 | 0.24 ± 0.08 | 12.22 ± 0.92 | 8.86 ± 0.68 |
| | | 20 | 21 | 22 | 23 | 24 |
| NORTH | 11 | 74.86 ± 5.25 | 11.62 ± 1.49 | 2552 ± 56 | 3030 ± 125 | 0.39 ± 0.11 |
| CENTRE | 40 | 83.79 ± 6.57 | 12.88 ± 2.27 | 2544 ± 54 | 3006 ± 125 | 0.89 ± 0.19 |
| SOUTH | 14 | 63.28 ± 4.59 | 17.63 ± 3.58 | 2602 ± 88 | 2826 ± 214 | 0.70 ± 0.25 |
| | | 26 | 27 | | | |
| NORTH | 11 | 87.46 ± 4.15 | 4.74 ± 0.22 | | | |
| CENTRE | 40 | 96.44 ± 6.72 | 4.96 ± 0.39 | | | |
| SOUTH | 14 | 80.29 ± 5.01 | 4.43 ± 0.37 | | | |

[a] Variables are labelled according to Fig. 1.

10, 20, 26; β-sitosterol: 27), whereas samples from the south show a high value for oleic acid (3, 8) and squalene (21).

PC2 (explaining 16% of the total variance) contributes mostly to the separation between the northern area and the centre. The most discriminant parameters correspond to terpenes (14, 15), wax (24), having a high value in the samples from the centre and to linolenic acid (19) having a high value in samples from the north (see the corresponding loadings shown in Fig. 3).

The mean value of the NMR variable intensities corresponding to the olive oils from the three areas of Lazio is summarized in Table 4. Olive oils from the northern area are poorer in terpenes, resonances 14 and 15; olive oils from the centre are richer in linoleic acid, resonance 26, and in terpenes, resonances 14 and 15; olive oils from the southern region are richer in squalene, resonance 21, and poorer in linoleic, signal 26. Finally, the oleic acid content decreases slightly from south to north; this observation might be related to the presence of different cultivars, to the agronomical practice or to the rainfalls which give water to the soils.

Using the same 17 variables, an LDA was performed, giving the map shown in Fig. 4a. Obviously, the group separation is improved with respect to the PCA map; the LDA map, in fact, improves the group separation and, being *per se* a classification model, confirms the pedoclimatic differences among the three areas, suggested also by the rainfall parameter previously discussed and shown in Table 2.

Now, we can discuss all the 27 variables given in Table 3. The irrelevance of the resonance peak due to *sn* 1,3 diglycerols, signal 17, is due to the fact that diglycerols are sensitive to the shelf-life and not to pedoclimatic factors. Actually, this resonance was included in order to discard, as anomalous, those samples in which *sn* 1,3 diglycerol intensity is too high. Resonances sensitive to the geographic area, that is to pedoclimatic effects, are due to volatile compounds, such as aldehydes, terpenes, and the squalene and other minor compounds, such as β-sitosterol and linolenic acid. This observation is in agreement with previously reported NMR data (Mannina, Fontanazza et al., 2001), as well as observations based on sensorial analysis (Aparicio, Calvente, & Morales, 1996).

As stated earlier, the three areas of Lazio were chosen for their intrinsic different pedoclimatic conditions, and do not take into account political geographical divisions present in Lazio. The LDA map of Fig. 4b shows an LDA study identical with Fig. 4a, with the samples labelled according to the five political divisions, into which Lazio is divided (five provinces, as described in Fig. 1). It is possible to observe that within the ellipse corresponding to the southern area in Fig. 4a, samples from Frosinone are slightly separated from samples from Latina. Since, in these two provinces, the agricultural practices are slightly different, and the cultivars are not the same, we performed an LDA using all five political divisions as *a priori* information. The result of this procedure is shown in Fig. 5.

Here, the three geographical areas are still well observable but, within this pattern, samples from the political areas appear quite well selected for the Frosinone/Latina area, whereas samples from the Rome/Rieti areas appear partly overlapped. However, it must be taken into account that the observed behaviour might be due to the presence of different cultivars or to different agronomic practices.

### 3.2. Altitude characterization

An interesting point, regarding the global pedoclimatic effect, is the possible effect of altitude on the olive oil composition. In order to evaluate this, we used a data-base including only those olive oils for which the altitude of the olive trees was known. The purpose is to distinguish a possible effect on olive oils originating from olive trees grown on hills,that is higher than 400 m, with respect to

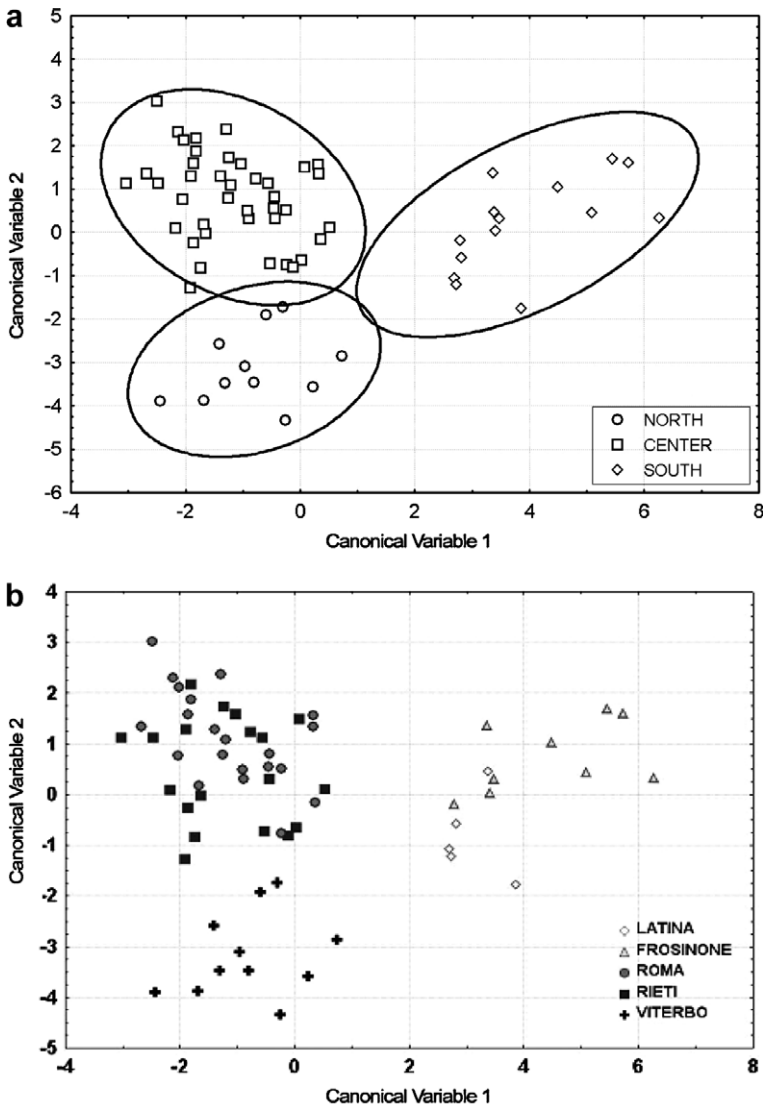


Fig. 4. LDA performed on the intensities of the variables 3, 4, 6, 8, 10, 12, 14, 15, 18, 19, 20, 21, 22, 23, 24, 26 and 27 (see Fig. 1). a: olive oil samples from the northern (11), the centre (40) and the southern (14) areas of Lazio are differently labelled. The ellipses represent 95% confidence regions for each group. b: olive oil samples from the five provinces (see Fig. 1) are differently labelled.

olive oils originating from lower altitudes. Only 36 samples were suitable for this analysis; in fact, half of our samples had to be discarded because the altitude of the olive trees was not known. After an explorative analysis, four other samples were discarded; therefore, only 32 samples were submitted to the ANOVA using the intensities of the 27 NMR resonances. The ANOVA allowed the variables with the highest discriminating power to be selected (see Table 3, columns 5 and 6). The eight discriminant variables are again related to volatile compounds (variable 11 related to hexane, variables 14, 15, 16 to terpenes, variable 21 to squalene), to some minor compounds (variables 24 and 27 related to a wax and β-sitosterol) and to saturated fatty acid (variable 27).

A PCA was performed on the intensity of the eight selected discriminating resonances: the PCA map shown in Fig. 6, clearly shows two separated zones; on the left side, all samples from hill-top cultivations, that is higher than 400 m, are present, whereas, on the right side of the map, only samples from lower altitudes are present.

The observed separation is present only in the axis corresponding to PC1, explaining 58% of the total variance. Again, terpenes (14–16) and wax (24), with high values in samples from low altitudines, and squalene (21) with a high value in samples from hill-top cultivations, contribute efficiently to discriminate samples from the two areas, as shown by the corresponding loadings.

In Table 5, the mean values of the intensities of the eight selected resonances are shown. The hill-top olive oils have a higher amount of squalene (resonance 21), and lower amounts of terpenes (resonances 14–16), and waxes (resonance 24).

These data agree well with previous studies (Paz et al., 2005) on the conservation capability of the hill top olive oils containing a larger amount of squalene, a well-known

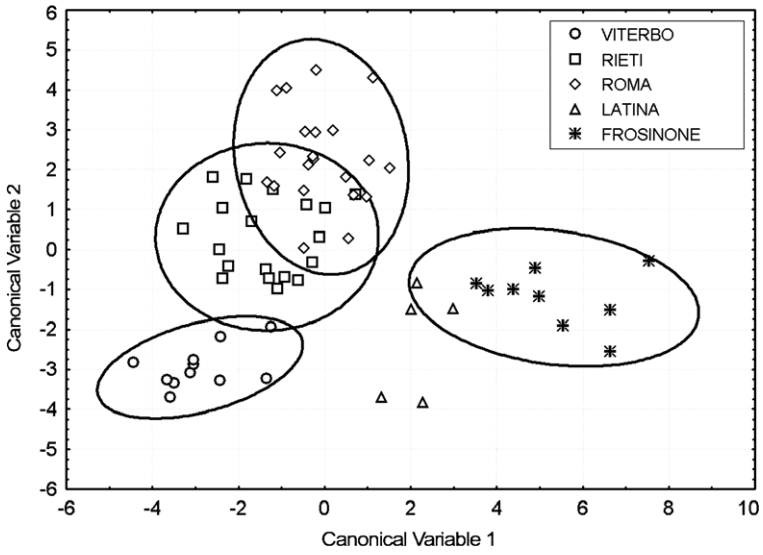


Fig. 5. LDA performed on the intensities of the variables 4, 6, 8, 10, 11, 12, 14, 15, 16, 18, 19, 20, 21, 22, 23, 24, 25, 26 and 27 (see Fig. 1). Olive oil samples from the five political provinces of Lazio (see Fig. 1) are differently labelled. The ellipses represent 95% confidence regions for each group. The ellipse is not reported for Latina olive oils, due to the small number of samples.

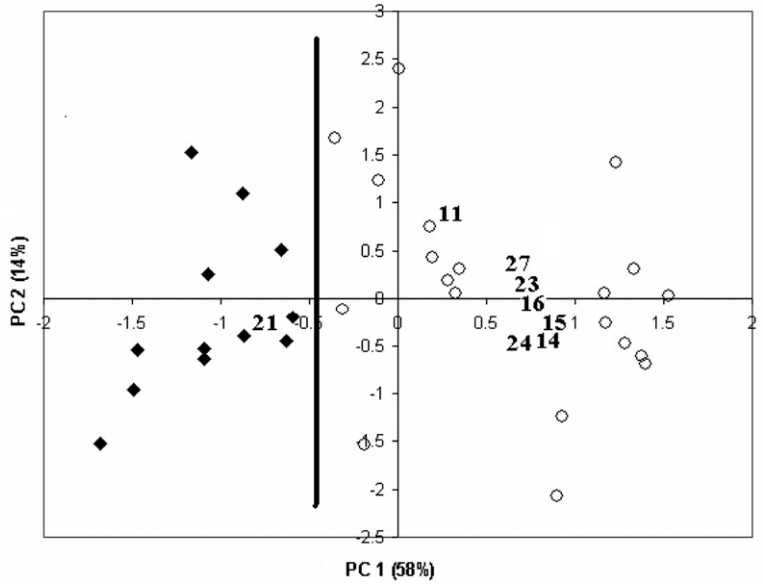


Fig. 6. PCA performed on the intensities of the variables 11, 14, 15, 16, 21, 23, 24 and 27 (see Fig. 1). Olive oil samples (32) from olive trees at an altitude <400 m are labelled with a ○ whereas samples from olive trees at an altitude >400 m are labelled with a (♦). Loadings are represented by the corresponding variable numbering.

Table 5
Mean values and standard deviation as a function of the altitude for the variables (arbitrary units) used in the multivariate statistic analysis

| | *N* | 11* | 14 | 15 | 16 | 21 | 23 | 24 | 27 |
|---|---|---|---|---|---|---|---|---|---|
| <400 | 20 | 0.29 ± 0.07 | 0.43 ± 0.08 | 0.33 ± 0.07 | 0.40 ± 0.06 | 11.54 ± 1.67 | 3045 ± 95 | 1.02 ± 0.18 | 5.04 ± 0.38 |
| >400 | 12 | 0.25 ± 0.07 | 0.31 ± 0.04 | 0.22 ± 0.04 | 0.28 ± 0.04 | 16.95 ± 3.43 | 2863 ± 146 | 0.67 ± 0.15 | 4.57 ± 0.34 |

* Variables are labelled according to Fig. 1.

antioxidant (Owen et al., 2000; Psomiadou & Tsimidou, 1999, 2002a, 2002b; Rastrelli, Passi, Ippolito, Vacca, & De Simone, 2002; Villén, Blanch, Ruiz del Castillo, & Herraiz, 1998).

### 3.3. Agronomic characterization: the irrigation factor

An analogous procedure was followed for analyzing irrigation effects. Only 33 samples were considered (see Table

Table 6
Mean values and standard deviation as a function of the hydro-regime for the variables (arbitrary units) used in the multivariate statistic procedure

| | Samples | 7[a] | 8 | 11 |
|---|---|---|---|---|
| Irrigated | 5 | $14.73 \pm 1.16$ | $72.46 \pm 1.66$ | $0.29 \pm 0.09$ |
| Not irrigated | 28 | $19.71 \pm 2.45$ | $67.30 \pm 3.33$ | $0.26 \pm 0.05$ |

[a] Variables are labelled according to Fig. 1.

1). Only three discriminant variables, namely 7, 8 and 11, were selected by ANOVA (see Table 3, columns 7 and 8); the mean values of the intensities of these variables are shown in Table 6. A careful observation of the data shows that the major effect of the irrigation procedure is to increase the amount of oleic acid, signal 8, and to decrease the amount of saturated fatty acids, signal 7. The major effect of the irrigation procedure is therefore to increase the amount of oleic acid and to decrease the amount of saturated fatty acids.

The intensities of these three resonances were submitted to PCA. Fig. 7 shows a PCA map obtained on irrigated and non-irrigated cultivations, respectively. The interpretation of this map requires a careful data analysis. At first sight, an oblique line separates samples from irrigated soils from samples from non-irrigated ones; this separation depends mostly on PC1 (64% of the total variance). A high value of oleic (acid) and a low value of saturated acid characterize olive oils from trees grown on irrigated soils, as shown by the corresponding loadings.

A careful analysis of the origin of samples allowed us to include samples 3, 5, 16 and 28 in a small well-defined triangle shown in Fig. 7; in fact, all these samples, come from Nerola (RI) where a particular soil situation is present. These samples derive from cultivations on a soil where a prevailing clay matrix is present. In fact, since clays tend to absorb water and to release it slowly, the hydro-stress normally present in the olive tree cultivation (Hamdy, Ragab, & Scarascia-Mugnozza, 2003; Mubeen, 2005) is reduced. It is common knowledge that clay soils are not suitable for growing olive trees. (Fontanazza, 1996). Nevertheless, this assessment is only true for soils saturated with water and therefore not permeable to the air. However, when the clay granulometry is sufficiently coarse, the penetration of the air is good enough to allow a good growth of olive trees. This is the case for the Nerola area, where samples 3, 5, 16 and 28 come from. These samples can be considered as grown on irrigated soils, and again a vertical dotted line (see Fig. 7), separates samples from irrigated and non-irrigated cultivations. PC1 is responsible for the discrimination and again the most important variable is the content of oleic acid.

In conclusion, the above results show that the NMR and statistical procedure is an important tool for the characterization of olive oils allowing detection (in the same experiment) of minor compositional changes not obtainable with any other single analytical procedure. The NMR technique, in fact, allows us to determine different major or minor compounds of an olive oil in the same experiment, analyzing the sample directly without any work-up or extraction procedure; moreover, since the experimental error for all the analyzed components is exactly the same and always extremely low, the multivariate statistical analysis turns out to be much improved.

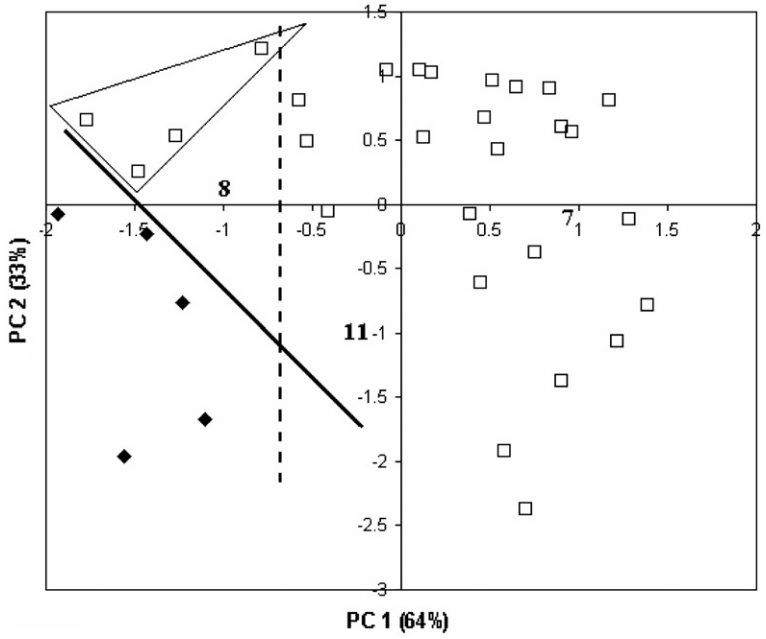


Fig. 7. PCA performed on the intensities of the variables 7, 8 and 11 (see Fig. 1). Olive oil samples (33) from irrigated (♦) and non-irrigated (□) soils are separated by an oblique line. Olive oil samples from a soil with a prevailing clay matrix are inside a triangle. Loadings are represented by the corresponding variable numbering.

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