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Influence of Harvest Method and Period on Olive Oil Composition: an NMR and Statistical Study

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The influence of harvest period and harvest method on olive oil composition was investigated by nuclear magnetic resonance (NMR) spectroscopy and by some quality parameters such as free acidity, peroxide value, and UV spectrophotometric indices. This work focuses on two secondary factors (harvest period and harvest method) and investigated their interactions with primary (genetic and pedoclimatic) and secondary (agronomic practices and technological procedures) factors. To avoid misinterpretation, the general linear model analysis (GLM) was used to adjust the result obtained from the analysis of variance (ANOVA). In this way, the effect of the factor of interest was corrected for the effects of the other factors that might influence the variable under investigation. The weight of each factor was evaluated by the variance component analysis (VCA). Finally, multivariate statistical analyses, namely, principal component analysis (PCA) and linear discriminant analysis (LDA), were applied. Samples were grouped according to the harvest period and harvest method. Volatile compounds, that is, hexanal and *trans*-2-hexenal, as well as the *sn*-1,3-diglycerides and squalene, significantly decreased during the ripening. The relative value of the ΔK parameter and the hexanal amount were higher in the olive oils obtained from olives harvested by one type of hand-held machine (shaker), whereas the unsaturated fatty chains in the olive oils were higher when another type (comb) was used.

KEYWORDS: Olive oil; harvest period; harvest method; NMR; quality parameters; ANOVA; general linear model analysis (GLM); variance component analysis (VCA); principal component analysis (PCA); linear discriminant analysis (LDA)

INTRODUCTION

Olive oil plays an important role in the modern diet, especially in the Mediterranean area, where olive cultivation has a strong historical, cultural, and economic value. Olive oil has long been studied by different analytical methodologies to evaluate the factors that might influence its chemical composition.

The quality and uniqueness of extra virgin olive oils are primarily determined by genetic and pedoclimatic factors (*1*), which can be defined "principal factors". The influence of the cultivar on the olive oil profile depends on the activity of enzymes involved in several pathways, mostly the lipoxygenase cascade, which is genetically determined (*2*). The pedoclimatic factors depend on environmental conditions such as the type and structure of the soils (structural factors) and/or climatic conditions (i.e., temperature, rainfall), which vary over the year ("effect of crop year") (*3*).

In addition to the principal factors, other factors also influence olive oil composition. These factors are mainly governed by farmers and thereby can be defined as "secondary". For instance, agronomic practices such as irrigation, fertilization, and harvest method might affect the fruit physiology, whereas technological procedures such as processing and storage might alter the olive oil composition (*4*).

One of the most investigated effects is the influence of olive ripening on olive oil composition. During the ripening process, several changes affecting weight, pulp/stone ratio, color, chemical composition, oil accumulation, and enzyme activity can occur in the fruit, which can all influence the organoleptic and chemical properties of the final olive oil (*5*).

Various authors have studied these structural changes (*1*) focusing on different aspects such as phenolics composition, fatty acid or volatile profile (*6*), protein amount and antioxidant

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activity (7), sugar and mineral content (8), enzymatic activities (5), olive fruit weight (9), and organic acids (10).

The oxidative stability and organoleptic properties of olive oils have also been studied in relation to the ripening degree (1, 4). Differences in phenolic profile and other antioxidant (α-tocopherol, β-carotene) or volatile compounds during the ripening process have been also observed (2, 5, 11, 12). In general, antioxidants, volatile compounds, and related parameters decrease during ripening (3). Fatty acids have been also investigated, showing that the amounts of palmitic, stearic, and linolenic fatty acids decrease during fruit ripening, whereas oleic and linoleic acid amounts increase (13). Moreover, the level of some metals in olive oil composition has been studied (14).

The choice of time and method of harvest can greatly affect the quality and quantity of olive oil, the production in the following year, and the economic return. The period chosen for harvesting also affects the length of time needed to detach the olives (because olive detachment time decreases during ripening). On the other hand, the length of time needed to detach the olives influences the effectiveness of manual and mechanical harvesting (15). The time and harvest methods are, therefore, strictly correlated.

The method used to harvest olives depends on cultural techniques, size and shape of the tree, and soil conditions of the orchard. Manual harvest and harvesting olives on the ground are now in disuse.

Mechanical methods can be grouped into two categories: (i) harvesting from the tree by tractor shakers, which are attached to the principal trunk or lateral branches; and (ii) harvesting from the tree by combs or whippers. The latter are composed of a stake (2−3 m long) with a head that might be a comb, whipper, or small pincer for shaking the lateral branches of the tree.

Few studies have investigated the effect of harvesting method on olive oil composition. Some authors have analyzed many samples of olive oils and showed that those picked manually had lower metals than those mechanically harvested (14). Other authors have investigated the effect of damage incurred during harvest; for example, the oil quality of hand-picked fruit has been compared with that of mechanically harvested olives (16). Oil from hand-picked fruits had lower free acidity, higher polyphenol content, and slightly lower peroxide levels than the oil of mechanically harvested olives. The harvest method also shown has a decisive influence on the herbicide concentrations found in olives (17).

The effects of harvest periods and methods on olive oil have been investigated using different methodologies such as spectrophotometric analysis, titration, gas chromatography, and, mainly, high-performance liquid chromatography (12).

Several studies (18−20) have shown that NMR spectroscopy is a powerful tool for characterizing olive oils according to cultivar, geographical characterization, quality, and genuineness. To our knowledge, no previous studies have used this technique to characterize olive oils in relation to the harvest period and method.

In this study, we have investigated the influence of harvest period and method on olive oil composition and their interactions with other primary and secondary factors. This aspect is important because the primary factors could obscure or, in the worst case, distort the effect of the secondary factors or vice versa. In addition, univariate and multivariate statistical analyses have been performed to minimize the misinterpretation of results. The influence of harvest period and method on olive oil composition has been investigated by ^{1}H NMR techniques and using quality parameters such as free acidity, peroxide value, and UV spectrophotometer index.

MATERIALS AND METHODS

Sampling. Olive oils were collected in an area of the Adriatic district in the Molise region (southern central Italy). The pedoclimatic unit was characterized by an elevation between 200 and 700 m above sea level, a substrate composed of Miocene and Pleistocene clayey sediments, an annual mean rainfall of between 680 and 1040 mm, an annual mean temperature range of 13.0−15.5 °C, and soils that are calcareous, of low depth, well drained, and characterized by fine texture and low stoniness.

This study was carried out in two consecutive crop years (2007/2008 and 2008/2009). The olive orchards consist of 10−50-year-old and 2−5 m high olive trees not submitted to any irrigation procedure. The collected olive oils were not monovarietal, but were obtained by mixing olives from typical Italian cultivars (Leccino, Moraiolo, Frantoio and Coratina) and from some local cultivars (Oliva nera di Colletorto, Noccioluta, Rosciola di Rotello, Gentile di Larino and Rumignana (21).

Information about the primary (genetic and pedoclimatic) and secondary factors (i.e., agronomic practices and technological procedures) that could influence olive oil composition (**Table 1**) was collected from standardized farmer interviews. Olive oil samples were grouped in two subsets. The first subset, composed by 45 samples, was used to study the effect on the olive oil composition of two harvest periods: the first period (from September 15 to October 31) is characterized by olives with ripening stages of "green" or "yellow" reported for simplicity as green; the second period (from November 1 to December 15) is characterized by olives with ripening stages of "purple" or "black" reported for simplicity as ripe). The second subset, composed by 34 samples, with information about the harvest method, was used to investigate the effect on olive oil composition of two types of hand-held tools, namely, combs and shakers.

Analytical Determination. *Analytical Indices* (*Quality Parameters*). Free acidity, peroxide value, and UV spectrophotometric indices (K_{232}, K_{270}, ΔK) were evaluated according to the official methods described in Regulation EC 2568/91 of the Commission of the European Union. For each sample all parameters were determined in triplicate.

Acidity, expressed as grams of oleic acid per 100 g of olive oil, was determined by the titration of a solution of oil dissolved in ethanol/ether (1:1, v/v) with 0.1 M potassium hydroxide in ethanol.

Peroxide value, given in milliequivalents of active oxygen per kilogram of oil, was determined as follows: a mixture of olive oil and acetic acid/chloroform acid (3:2, v/v) was left to react in darkness with saturated potassium iodide solution. The free iodine was then titrated with a sodium thiosulfate solution.

K_{270}, K_{232}, and ΔK extinction coefficients were measured with a UV spectrophotometer (Lambda E2-150, PerkinElmer) using a 1% (v/v) solution of olive oil in isooctane and a path length of 1 cm.

NMR Measurements. ^{1}H NMR experiments were recorded at 300 K on a Bruker AVANCE AQS600 spectrometer operating at the proton frequency of 600.13 MHz (B_0 = 14.09 T). Olive oil sample (20 μL) was dissolved in DMSO (20 μL) and $CDCl_3$ (700 μL) directly in a 5 mm NMR tube.

The ^{1}H spectrum was acquired using the following conditions: number of scans, 1024; π/2 pulse, ∼8 μs; time domain (TD), 64K data points; relaxation delay plus acquisition time, 3.5 s; spectral width, 18.5 ppm. ^{1}H NMR spectra were obtained by the Fourier transformation (FT) of the free induction decay (FID) by applying an exponential multiplication with a line-broadening factor of 0.3 Hz and zero-filling (size = 64K) 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. To ensure a better quantitative comparability of the spectra, the baseline was corrected using a multipoint correction. In particular, the cubic spline baseline correction routine in the Bruker Topspin software was used. To compare the spectra, the signal intensity was measured using the semiautomatic peak-picking routine of the Bruker Topspin software. The intensity of selected signals was measured with respect to the resonance at 2.251 ppm, due to the all methylenic protons bound to C2 (acyl group), normalized to 1000 (**Figure 1**). This signal can be assumed to be constant in all olive oils because the acyl group is present in all fatty chains regardless of fatty chain length or position on the glycerol moiety.

Statistical Analysis. All variables had an almost normal distribution except the free acidity and K_{270} in the data set used to investigate the effect of the harvest period and only the K_{270} in the data set used to investigate the effect of the harvest method. For free acidity and K_{270} variables logarithmic transformations were applied.

Table 1. Factors That Can Influence Olive Oil Composition

	factor investigated[a]	level of each factor
1	**harvest period**	1.1 = from September 15 to October 31 (olives green or yellow = ripening stage "green") 1.2 = from November 1 to December 15 (olives purple or black = ripening stage "ripe")
2	**harvest method**	2.1 = hand-held machines with combs 2.2 = hand-held machines with shakers
3	crop year	3.1 = 2007/2008 3.2 = 2008/2009
4	localization zone (village)−(pedoclimatic condition)	4.1 = San Giuliano di Puglia 4.2 = Colletorto 4.3 = Santa Croce di Magliano 4.4 = Bonefro
5	altitude	5.1 ≤ 400 m above sea level 5.2 > 400 m above sea level
6	fertilizer type	6.1 = minerals 6.2 = organic 6.3 = mixing of two type
7	grass-covered	7.1 = coverage of 100% 7.2 = coverage of 50% 7.3 = coverage of 0%
8	time before olive milling	8.1 ≤ 24 h 8.2 > 24 h
9	olive oil mill type	9.1 = continuous system (two or three phases) 9.2 = traditional olive oil extraction (pan crusher and a hydraulic press)
10	temperature of mixing phase	10.1 ≤ 25 °C 10.2 > 25 °C

[a] In bold are the factors that have been investigated in this work.

Some univariate procedures were used for data pretreatment before a multivariate unsupervised procedure which was applied to the variables selected in the data pretreatment step.

At the beginning, the analysis of variance (ANOVA) univariate was applied to all variables to detect discriminant variables among the harvest period and method ($p < 0.05$).

Then, we performed the general linear model (GLM). The GLM procedure provides an analysis of variance for one dependent variable by one or more independent factors. The factors (categorical) divide the population into groups (with two or more levels) such as harvest period (green versus ripe), harvest method (comb versus shaker), and crop year (2008 versus 2009). By GLM analysis we investigated interactions between factors as well as the effects of individual factors. On our data, all factors that had an influence on the dependent variable in the univariate analysis (ANOVA), with a less restrictive p value ($p < 0.1$), were inserted into the full model of the GLM. In this way, we obtained a p value adjusted (or corrected) for the factors that might influence the dependent variable. Generally, the p value corrected by GLM is higher than the initial value obtained by a simple ANOVA (*22*).

Finally, variance component analysis (VCA) was applied to estimate the contribution of each factor present in the model on the total variance of the dependent variable. The VCA is based on the GLM, in which factors are assumed to have a linear relationship to the dependent variable. This analysis provides a way to assess the amount of variation in a dependent variable that is associated with one or more random-effects factors. The factors are the same as analyzed in the GLM. The minimum norm quadratic unbiased estimator (MINQUE) method was used. MINQUE is robust and does not require normality assumptions (*23*).

Multivariate statistical procedures were also performed on the variables selected by the GLM procedure; these analyses were applied under the strictest conditions to avoid the possibility of hyperoptimistic results. Principal component analysis (PCA), without any a priori hypothesis, provided a global overview of the compositional variability in the samples through the projection of the data into hyperspaces defined by linear combinations, that is, the principal components (PCs) of the variables. The PCs were calculated to represent the maximum of variance in our data set. The percentage of the variance for each specific factor provided the contribution of the factor to the grouping, whereas the variables loading provided the weight of each variable toward discrimination.

Linear discriminant analysis (LDA) was also performed. The discriminant functions deduced with an a priori hypothesis were calculated. The relative contribution of the variables toward discrimination can be explained by the standardized discriminant function coefficients, whereas the positive or negative sign of the coefficient indicated either a positive or negative contribution, respectively. The results were the linear combinations of dependent variables that predict the membership of each sample to the corresponding group.

The models were validated by the leave-one-out method, which is a form of cross-validation. In this method, each case is classified using a discriminant function based on all cases except the given case. This is thought to give a better estimate of what classification results would be in the population. The percentage of olive oil samples correctly identified was also evaluated by the parameters of *recognition* (the percentage of samples in the training set correctly classified) and *prediction* (the percentage of samples in the test set correctly classified) (*24*).

SPSS software package for Windows (version 15.0; 2006) was used to perform the pretreatment and compute the ANOVA, GLM, and VCA, whereas the Statistica software package for Windows (version 6.0, 1997) was used to compute the PCA and LDA.

RESULTS AND DISCUSSION

For all olive oil samples taken into consideration in this work, the analytical parameters, that is, acidity value, peroxide value, and UV spectrophotometric index, are widely within the limits of

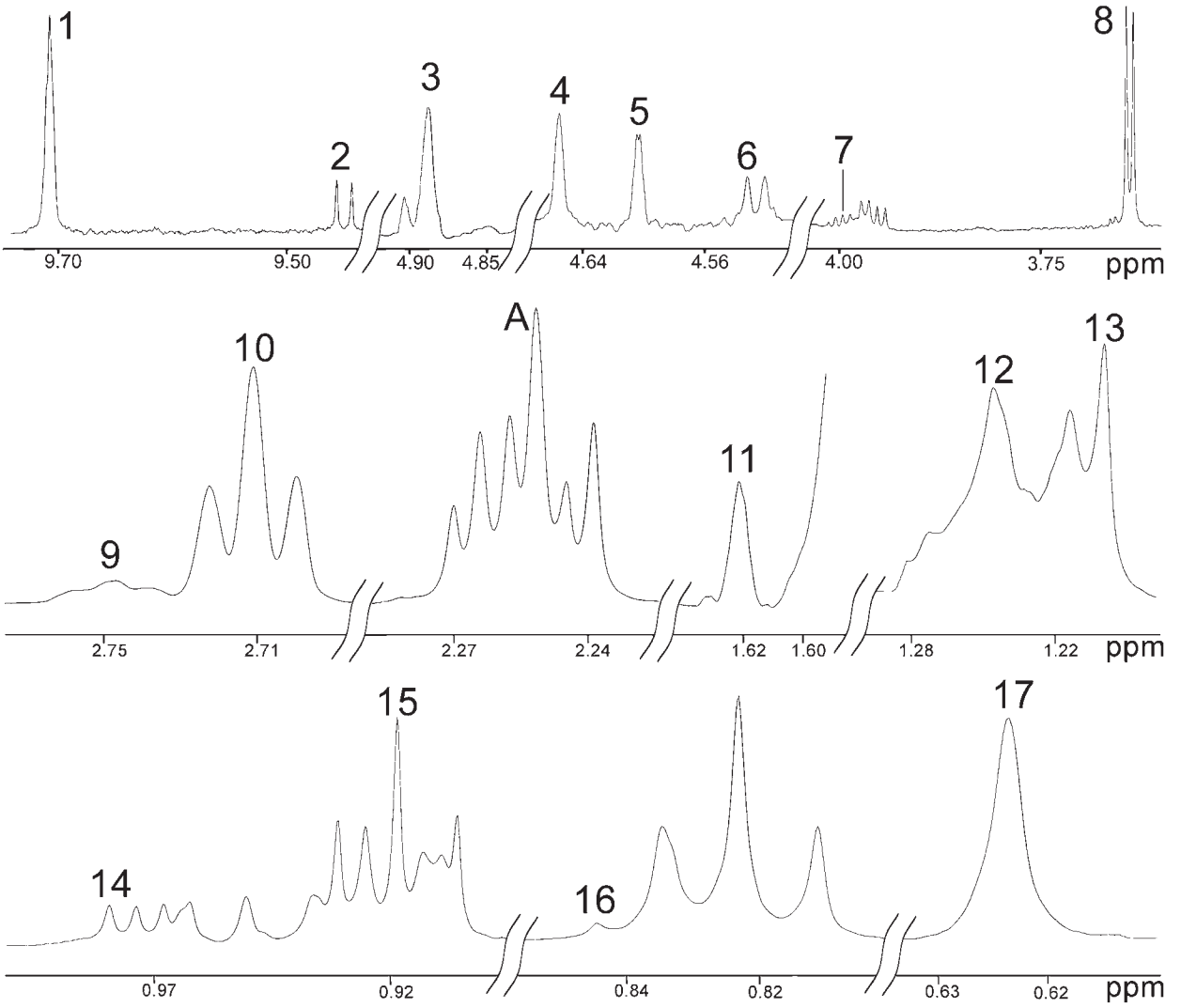

Figure 1. 600.13 MHz ^{1}H NMR signals of extra virgin olive oil. Peaks: 1, hexanal (9.704 ppm); 2, *trans*-2-hexenal (9.454 ppm); 3, terpene 4 (4.885 ppm); 4, terpene 3 (4.661 ppm); 5, terpene 2 (4.609 ppm); 6, terpene 1 (4.541 ppm); 7, methylenic protons in α-glycerol moiety of *sn*-1,3-diglycerides (3.988 ppm); 8, methylenic protons in α-glycerol moiety of *sn*-1,2-diglycerides (3.636 ppm); 9, diallylic protons of linolenic fatty chains (2.746 ppm); 10, diallylic protons of linoleic fatty chains (2.710 ppm); A, reference peak due to methylenic protons bound to C2 normalized to 1000 (major peak at 2.251 ppm); 11, squalene (1.620 ppm); 12, methylenic protons of all unsaturated fatty chains (1.244 ppm); 13, methylenic protons of palmitic and stearic fatty chains (1.197 ppm); 14, wax (0.978 ppm); 15, methyl of linolenic fatty chains (0.910 ppm); 16, methyl of linoleic fatty chains (0.843 ppm); 17, methyl-18 of β-sitosterol (0.623 ppm).

Regulation 2568/91. For this reason, all olive oil samples could be labeled "extra virgin" according to European Union rules.

Harvest Period. The influence of the harvest period on olive oil composition could produce a strong variability in several compounds, as reported in previous studies (*3*, *13*). Here, the influence of the harvest period was investigated by univariate analysis (**Table 2**). All conventional parameters showed no significant differences between the olive oils obtained from green olives and olive oils obtained from ripe olives. Although our data showed a slight increase in free acidity and a slight decrease in peroxide value during ripening, these differences were not significant. The trend of these variables during ripening is in accordance with other studies (*4*).

The K_{232} value slightly decreased, whereas the K_{270} value slightly increased, but not significantly, in the olive oils from olives processed at a later stage of ripeness. The results of many works disagree about the evolution of these parameters during ripening (*1*, *4*).

Four variables from the ^{1}H NMR spectra showed significant differences by ANOVA analysis: hexanal, *trans*-2-hexenal, *sn*-1,3-diglycerides, and squalene signals (**Table 2**). To be sure that the results obtained by ANOVA were really due to the influence of the harvest period, a GLM analysis was performed. The four variables previously selected again showed significant differences in the new analysis. Hexanal was adjusted for the crop year and pedoclimatic condition (localization zone). However, the *p* value remained significant (adjusted value = 0.0011), meaning that the effect of the harvest period is slightly obscured by the crop year and pedoclimatic conditions. VCA showed that the main contribution of the variance is given by the harvest period (42.9% of the total variance explained), whereas the crop year and pedoclimatic conditions explain 19.6 and 13.7% of the variance, respectively. The remaining variance was attributable to the other factors reported in **Table 1** and to statistical error (random effects).

The *p* value of *trans*-2-hexenal, adjusted for the crop year, was 0.0003. In this case, the crop year was the principal effect because it explained 41.5% of the total variance, whereas the harvest period explained only 26.9%. The means reported in **Table 2** show that hexanal and *trans*-2-hexenal decreased remarkably

Table 2. ANOVA, GLM, and VCA Values for the Variables Measured on the Harvest Period

	harvest period				ANOVA	GLM		VCA			
	green (19)[a]		ripe (26)[a]					contribution of each effect (%)			
variable	mean ± SD	min/max	mean ± SD	min/max	p[b]	p[b]	adjusted for[c]	1[c]	3[c]	4[c]	others + error
Conventional parameters											
free acidity	0.20 ± 0.05	0.14/0.29	0.2 ± 0.2	0.08/1.16	0.7447[d]						
peroxide value	8 ± 1	5.0/11.0	7 ± 2	4.8/14.5	0.1209						
K_{232}	2.2 ± 0.5	1.56/3.18	2.0 ± 0.3	1.55/2.77	0.2183						
K_{270}	0.13 ± 0.03	0.08/0.20	0.2 ± 0.2	0.11/1.14	0.1065[d]						
ΔK	−0.002 ± 0.001	−0.004/−0.001	−0.002 ± 0.002	−0.006/0.002	0.7757						
NMR signals (normalized intensities)											
1[e]: hexanal	0.16 ± 0.04	0.10/0.24	0.09 ± 0.03	0.03/0.14	**<0.0001**	**0.0011**	3 + 4	**42.9**	**10.6**	**13.7**	**32.8**
2: *trans*-2-hexenal	0.16 ± 0.04	0.10/0.24	0.10 ± 0.04	0.04/0.18	**<0.0001**	**0.0003**	3	**26.9**	**41.5**		**31.5**
3: terpene 4	0.17 ± 0.05	0.11/0.28	0.16 ± 0.05	0.07/0.24	0.6010						
4: terpene 3	0.5 ± 0.1	0.32/0.75	0.6 ± 0.1	0.24/0.87	0.3006						
5: terpene 2	0.4 ± 0.1	0.23/0.68	0.5 ± 0.1	0.20/0.76	0.1297						
6: terpene 1	0.36 ± 0.06	0.27/0.47	0.33 ± 0.07	0.19/0.54	0.2593						
7: diglycerides *sn* 1,3	0.5 ± 0.2	0.15/0.98	0.3 ± 0.1	0.10/0.67	**0.0005**	**0.0280**	3	**9.0**	**56.8**		**34.2**
8: diglycerides *sn* 1,2	12 ± 2	9.48/13.79	11 ± 2	7.63/14.63	0.8022						
9: linolenic (C18:3) (diallylic)	9.1 ± 0.9	7.96/11.15	9.4 ± 0.9	7.91/11.04	0.2274						
10: linoleic (C18:2) (diallylic)	90 ± 10	72.8/125.1	100 ± 10	79.9/116.3	0.0842						
11: squalene	13 ± 2	8.28/16.19	12 ± 2	8.31/15.09	**0.0278**	**0.0278**	NC[f]				
12: unsaturated	2500 ± 100	2297/2723	2500 ± 70	2346/2630	0.8688						
13: palmitic (C16:0) + stearic (C18:0)	3300 ± 300	2822/3778	3400 ± 100	3174/3775	0.0665						
14: wax	1.3 ± 0.4	0.71/2.01	1.5 ± 0.5	0.55/3.47	0.0714						
15: linolenic (C18:3) (methyl)	17 ± 2	13.29/21.30	17 ± 2	14.80/20.99	0.3767						
16: linoleic (C18:2) (methyl)	120 ± 10	97.4/142.6	120 ± 10	102.1/147.7	0.0937						
17: β-sitosterol	4.5 ± 0.6	2.87/5.57	4.6 ± 0.5	3.81/5.78	0.5245						

[a] Number of samples. [b] In bold are significant p values. [c] Adjusted for (effects): 1, harvest period; 2, harvest method; 3, crop year; 4, localization zone; 5, altitude; 6, fertilizer type; 7, grass covered; 8, time before olive milling; 9, olive oil mill type; 10, temperature of mixing phase. [d] After logarithmic transformation. [e] The NMR peaks are indicated also by numbers reported in **Figure 1**. [f] NC, not conditioned by other factors.

during the ripening process. These results are in agreement with other authors (*11*). The trend of these two volatile compounds analyzed during the ripening is probably related to the amount of lipoxygenase pathway and to several factors that might influence its activity such as the presence of a specific substrate or compounds, such as phenols, that inhibit lipoxygenase activity (*6*, *25*).

The concentration of *sn*-1,3-diglycerides was influenced not only by the harvest period but also by crop year, as shown by the high value of the variance explained by the latter factor (56.8% of the total variance). The strong effect of crop year increased the p value. The *sn*-1,3-diglycerides also decreased during the ripening process as shown in **Table 2**. This trend is probably due to the incomplete triglyceride biosynthesis at the first stage of the ripening process. In fact, in the synthesis of triglycerides, the enzyme initially acts on the 1- and 3-positions along the glycerol chain and then reacts on the fatty acids in position 2 (*26*).

Finally, the squalene concentration showed significant differences in the olive oils obtained from green and ripe olives. Significant differences are related only to the harvest period. The means reported in **Table 2** show that squalene decreased during the ripening process. This result is in agreement with the conclusions reported by other authors (*4*). This decrease during the season is probably due to the sacrificial role of squalene in some oxidative processes more active in mature olives (*27*).

Unlike the results reported by some authors (*13*), our results did not show any differences in the amount of saturated fatty chains. This is probably due to the strong influence of crop year on the concentration of these compounds. All of the other factors reported in **Table 1** did not influence the four variables under investigation.

The minimum and maximum values of the four variables, selected by GLM (**Table 2**), showed an overlapping of the two groups; for this reason it is difficult to distinguish the two olive oil groups using the univariate analysis only. Then, the variables that showed significant differences were used in the multivariate statistics. Initially, PCA was performed (**Figure 2**). A good separation of the olive oil samples according to harvest period of olives was obtained. The first two PCA factors together were responsible for 77.2% of the total variance. The separation of the two groups was well evident along factor 1, which explained a larger part of the variance (52.6%). The largest contribution to the separation, in factor 1, was given by the *sn*-1,3-diglycerides (loading +0.853), hexanal (loading +0.823), and *trans*-2-hexenal (loading +0.793), whereas the contribution of squalene was less significant (loading +0.264). The positive sign of hexanal, *trans* 2-hexenal, *sn*-1,3-diglycerides, and squalene loadings for factor 1 is consistent with the expected higher concentration of these compounds in green olives. LDA was also performed. The standardized coefficients (sc) of the four variables in the classification function is given: hexanal sc = 0.696; *trans*-2-hexenal sc = 0.668; squalene sc = 0.470; *sn*-1,3-diglycerides sc = −0.042. The two volatile compounds, hexanal and *trans*-2-hexenal, had the highest discriminating power.

The recognition ability of the model is good: 96.2 and 89.5% of the olive oils from ripe and green olives, respectively, were correctly identified. Overall, 93.3% of the original group of olive oil samples were correctly identified. The prediction ability of the model calculated by leave-one-out (cross-validation) methods gives the following results: 96.2 and 89.5% of the olive oils from ripe and green olives, respectively, were correctly identified (the same as for the recognition values). Overall, 91.1% of the original group of olive oil samples were correctly identified.

Harvest Method. The influence of the harvest method on olive oil composition (**Table 3**) was also investigated. In particular, we focused our attention on two particular methods of mechanical

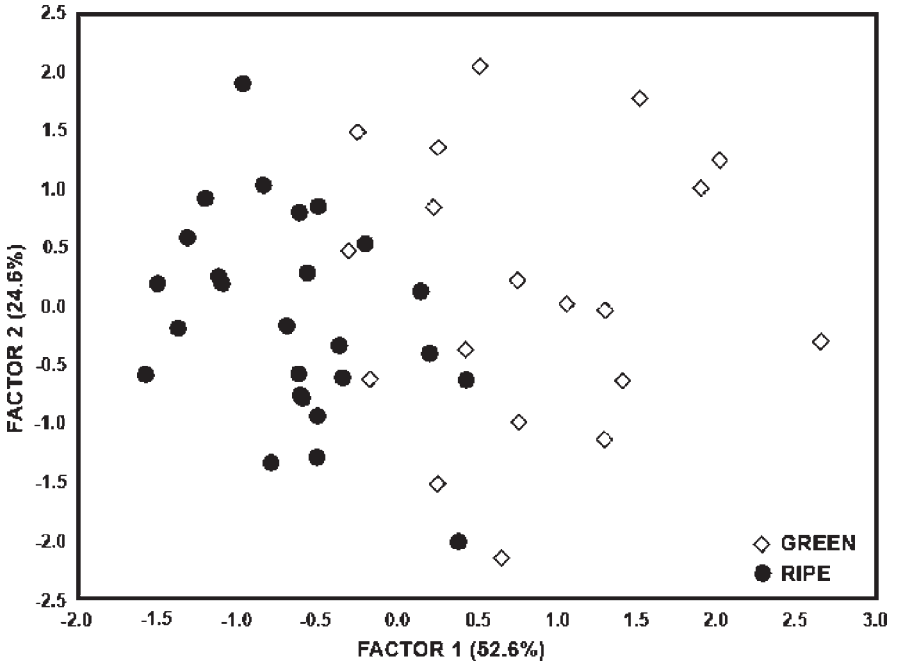

Figure 2. PCA performed on the variables selected by ANOVA and GLM analyses: hexanal, *trans* 2-hexenal, *sn*-1,3-diglycerides, and squalene (**Figure 1**). Olive oil samples were distributed into two categories: olive oils from green olives (19 samples labeled ◇) and olive oils from ripe olives (26 samples labeled ●).

Table 3. ANOVA, GLM, and VCA Values for the Variables Measured on the Harvest Method

	harvest method				ANOVA	GLM		VCA			
	combs (24)[a]		shakers (10)[a]					contribution of each effect (%)			
variable	mean ± SD	min/max	mean ± SD	min/max	p[b]	p[b]	adjusted for[c]	2[c]	1[c]	6[c]	others + error
Conventional parameters											
free acidity	0.19 ± 0.04	0.14/0.25	0.17 ± 0.04	0.10/0.25	0.2143						
peroxide value	7 ± 2	5.0/14.5	8 ± 2	6.0/11.0	0.4106						
K_{232}	2.1 ± 0.3	1.61/2.79	2.5 ± 0.4	2.21/3.18	**0.0010**	0.6140	3 + 1 + 7				
K_{270}	0.2 ± 0.2	0.11/1.14	0.15 ± 0.02	0.12/0.18	0.5369[d]						
ΔK	−0.003 ± 0.002	−0.006/0.001	−0.001 ± 0.001	−0.003/0.001	**0.0311**	0.0135	6	**28.5**		**15.7**	**55.8**
NMR signals (normalized intensity)											
1[e]: hexanal	0.09 ± 0.03	0.03/0.14	0.16 ± 0.05	0.09/0.24	**<0.0001**	**0.0001**	1	**60.8**	**2.6**		**36.7**
2: *trans*-2-hexenal	0.11 ± 0.04	0.07/0.2	0.19 ± 0.03	0.16/0.24	**<0.0001**	0.9797	3 + 1 + 7				
3: terpene 4	0.20 ± 0.07	0.10/0.45	0.16 ± 0.04	0.11/0.22	0.1466						
4: terpene 3	0.5 ± 0.1	0.24/0.87	0.65 ± 0.09	0.46/0.75	**0.0141**	0.9864	3				
5: terpene 2	0.5 ± 0.1	0.20/0.76	0.56 ± 0.09	0.39/0.68	**0.0467**	0.7883	3				
6: terpene 1	0.32 ± 0.06	0.19/0.42	0.38 ± 0.06	0.28/0.44	**0.0128**	0.7664	3 + 1 + 5				
7: diglycerides *sn* 1,3	0.3 ± 0.2	0.10/0.82	0.7 ± 0.2	0.26/0.98	**<0.0001**	0.5017	3				
8: diglycerides *sn* 1,2	12 ± 2	8.47/15.04	11 ± 1	8.88/12.87	0.1232						
9: linolenic (C18:3) (diallylic)	9.7 ± 0.9	8.02/11.15	9 ± 1	7.11/10.28	**0.0165**	0.2958	3				
10: linoleic (C18:2) (diallylic)	100 ± 10	79.9/116.3	100 ± 10	87.3/125.1	0.5472						
11: squalene	12 ± 2	9.44/21.53	13 ± 2	10.05/16.19	0.4178						
12: unsaturated	2530 ± 80	2346/2723	2380 ± 50	2297/2450	**<0.0001**	**<0.0001**	6	**71.2**		**9.2**	**19.5**
13: palmitic (C16:0) + stearic (C18:0)	3400 ± 200	2822/3778	3400 ± 200	3127/3764	0.8884						
14: wax	1.3 ± 0.6	0.55/3.47	1.7 ± 0.2	1.11/1.92	0.1160						
15: linolenic (C18:3) (methyl)	18 ± 2	15.05/20.99	18 ± 2	16.53/21.30	0.3345						
16: linoleic (C18:2) (methyl)	120 ± 10	102.1/149.6	120 ± 10	110.3/142.6	0.6699						
17: β-sitosterol	4.6 ± 0.5	3.14/5.78	4.9 ± 0.3	4.42/5.57	0.0568						

[a] Number of samples. [b] In bold are significant p values. [c] Adjusted for (effects): 1, harvest period; 2, harvest method; 3, crop year; 4, localization zone; 5, altitude; 6, fertilizer type; 7, grass covered; 8, time before olive milling; 9, olive oil mill type; 10, temperature of mixing phase. [d] After logarithmic transformation. [e] The NMR peaks are indicated also by numbers reported in **Figure 1**.

harvest using hand-held machines that require the presence of a worker. The hand-held machines comprise a stake (2−3 m long) with a head that consists of combs or pincers which shake the lateral branches of the tree. In this paper, the effects of these two hand-held machines on olive oil composition are discussed. To our knowledge, no other works have examined this issue.

The ANOVA shows that 10 variables are significantly different in the olive oils from the two different harvesting methods (**Table 3**). Nevertheless, when we performed the GLM analysis, the results were completely different. Only three variables, namely, the ΔK parameter, hexanal, and unsaturated fatty chains, remained significant, which turned out to be significant

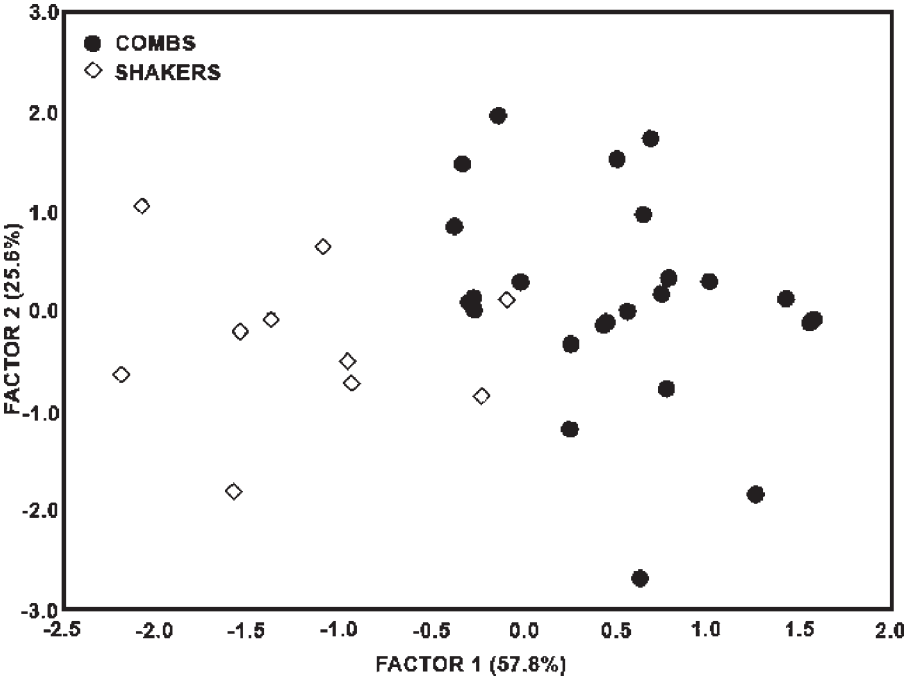

Figure 3. PCA performed on the variables selected by ANOVA and GLM analyses: hexanal and unsaturated fatty chains (**Figure 1**) and the ΔK parameter. Olive oil samples were distributed into two categories: olive oils from olives harvested by combs (24 samples labeled ●) and olive oils from olives harvested by shakers (10 samples labeled ◇).

for the discrimination. Therefore, the GLM adjusts the result obtained from ANOVA, correcting the factor of interest from the effects of the other factors. This means that significant variables in the ANOVA analysis, such as *trans*-2-hexenal and linolenic fatty acid, become not significant in the specific case of olive oils from the two harvesting methods. For this reason, in the following analyses only the three variables that remain significant after GLM analysis were taken into consideration.

Among the conventional analyses, only the ΔK parameter showed significant differences between the olive oils obtained from olives harvested by combs or shakers. The p value of the ΔK parameter adjusted for the fertilizer type used during the crop season was 0.0135, which decreased compared with the ANOVA. The effect of fertilizer type explained 15.7% of the total variance, whereas the harvest method explained 28.5% of the total variance. This variable was strongly affected by the other factors reported in **Table 1** and by the random effects that explain 55.8% of the total variance. The means reported in **Table 3** show that the relative value of the ΔK parameter was higher in the olive oils obtained from olives harvested by shakers.

Two variables from the NMR analyses showed significant differences: hexanal and unsaturated fatty chains (**Table 3**). Hexanal was only adjusted for the harvest period. VCA showed that the variance was mostly explained by the harvest method (60.8% of the total variance). The harvest period explained only 2.6% of the variance, and for this reason the adjusted p changed very little (0.0001). The remaining 36.7% of the variance was attributable to the other factors reported in **Table 1** and to random effects. The amount of hexanal increased when the shakers were used.

In addition, the unsaturated fatty chains were influenced by the harvest method. A small influence of fertilizer type was also shown (9.2% of the total variance); however, the adjusted p value remained almost the same (< 0.0001). The harvest method explained 71.2% of the variance, whereas the other factors reported in **Table 1** and the random effects were responsible for 19.5% of the total variance. The means reported in **Table 3** show that the unsaturated fatty chains in the olive oils decreased when the shakers were used.

The remaining factors reported in **Table 1** did not influence (or influence only partially) the three variables under investigation.

These results show that an increase of hexanal is usually accompanied by a decrease in unsaturated fatty chains. On the other hand, the ΔK parameter is influenced by changes in the concentration of unsaturated fatty chains, which are rich in double bonds.

However, at the current state of knowledge, it is difficult to explain how these trends are related to the harvest method. Only some hypotheses can be suggested.

In general, the mechanical harm of fruits during harvest can increase several autoxidative processes, which are detrimental to olive oil quality and can lead to an increase in acidity and peroxide values and a decrease in total polyphenols and can also influence the concentration of unsaturated fatty chains and volatile compounds (*28*). The higher quality found for hand-picked harvest further supports this hypothesis (*16*). For this reason, the olive oils obtained using combs should be of poor quality because the olives can be damaged during harvesting. This does not happen using shakers because they do not touch directly the olives. However, our data do not show evident differences in the acidity and peroxide qualitative parameters. On the other hand, the shakers might break the branches of the tree and affect the tree vigor and physiological conditions leading, in the long term, to a change in quality and quantity production (*29*, *30*). Under these conditions, plants undergo mechanical stress. It has been reported that, in some plant species subjected to some stress, the level or activity of lipoxygenase (LOX) increases (*31*, *32*).

In our case, the decrease of unsaturated fatty acids and the increase of hexanal in olive oil obtained from olives harvested by shakers can be explained by considering the lipoxygenase pathway. This pathway, which involves the activation of different enzymes, gives rise to different amounts of aldehydes, alcohols, and hexyl acetates, all of which have sensory properties and contribute to the overall flavor (*6*, *11*, *33*). In particular, hexanal is biosynthesized from C18 unsaturated fatty acids in plants.

The minimum and maximum values of the three variables, selected by GLM (**Table 2**), show an overlapping in the two groups. Therefore, as in the previous case, a PCA (**Figure 3**) was performed. Again, a good separation of the olive oil samples according to harvest method was obtained. The first two PCA factors together were responsible for 83.4% of the total variance. The separation of the two groups of samples is well observable along factor 1, which explains a larger part of the variance (57.8%). The loadings of unsaturated fatty chains (+0.794), hexanal (−0.825), and the ΔK parameter (−0.651) were high, and all variables contributed to the separation.

The negative loadings for hexanal and the ΔK parameter, for factor 1, are in agreement with the lower values of these two parameters in the olive oils obtained from olives harvested by combs. We can observe relatively higher values of the same parameters for the olive oils obtained from olives harvested by shakers.

LDA was also performed, giving the following standardized coefficients in the classification: unsaturated sc = −0.707; hexanal sc = 0.625; ΔK parameter sc = 0.219. The unsaturated fatty chains and hexanal had the highest discriminating power. The recognition ability of the model shows that 100.0 and 90.0% of the olive oils from olives harvested by combs and shakers, respectively, were correctly identified. Overall, 97.1% of the original group of the olive oil samples were correctly identified. The prediction ability of the model calculated by leave-one-out (cross-validation) methods shows that the 95.8 and 80.0% of the olive oils from olives harvested by combs and shakers, respectively, were correctly identified. Overall, 91.2% of the original group of the olive oil samples were correctly identified.

In conclusion, we investigated the interactions between two secondary factors, namely, harvest period and harvest method, with other primary and secondary factors on extra virgin olive oil composition. The study has been conducted mainly by ^{1}H NMR technique, which, again, proved to be an excellent tool to study olive oils.

The most interesting results obtained in this work are as follows. (I) hexanal, *trans*-2-hexenal, *sn*-1,3-diglycerides, and squalene significantly decreased during ripening. (II) The relative values of the ΔK parameter and hexanal are higher in the olive oils obtained from olives harvested by shakers, whereas the unsaturated fatty chains are higher when the comb hand-held machines are used. To our knowledge, this is the first study to examine the affects of these harvest methods on olive oil composition.

Furthermore, we used a new integrated statistical methodology to avoid misinterpretation, using the GLM to adjust the result obtained from ANOVA. Hence, the effect of the factor of interest was corrected for the effects of the other factors that might influence the variables under investigation. The weight of each factor was evaluated by VCA. This methodology provides an important contribution to the study of olive oils. Multivariate statistical analyses such as PCA and LDA were also performed, showing that samples are well grouped on the basis of the harvest period and method.

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