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Influence of Ripe Table Olive Processing on Oil Characteristics and Composition As Determined by Chemometrics

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The changes in ripe olive fat produced by processing were studied according to cultivars using the general linear model, principal component analysis (PCA), predictive discriminant analysis (DA), and hierarchical clustering. Acidity, peroxide value, K_{270}, and ΔK increased during storage. Acidity also increased after sterilization, whereas K_{270} decreased after darkening; K_{232} showed a progressive decrease during processing. Fatty acids (except C17:0, C18:0, and C24:0), triacylglycerols (except PLLn, OOLn+PoOL, PLL+PoPoO, SOO, and POS+SLS), polar compounds, diacylglycerol, and monoacylglycerols also suffered statistically significant changes during processing. A PCA discriminated between cultivars and, within the same cultivar, among the raw materials from the rest of the treatments. Using fatty acid and triacylglycerol compositions, predictive DA discriminated between cultivars (100% correct), but high discriminant capacity among processing steps (95% correct assignation and 87% in cross-validation) was achieved only with fatty acids. A hierarchical clustering analysis successfully grouped cultivars and processing steps according to overall olive oil composition and quality.

KEYWORDS: Discriminant analysis; oil composition; principal component analysis; processing; ripe table olives

INTRODUCTION

Table olives constitute an important part of the Mediterranean diet, and their world production reached a total of 1,762,000 tons in the 2005−2006 season (*1*). One of the most common styles is ripe olives (California style). In brief, fruits for producing ripe olives (by alkaline oxidation) are previously preserved in an aqueous solution (brine or acidic water) and darkened throughout the year according to demand. Darkening consists of several treatments of dilute NaOH solutions and water washes (with aeration between them), immersion in a lactate or gluconate iron solution, and packing in a light brine (*2*). In specific circumstances, stronger conditions (oxygen and moderately high temperatures) can be used (*3*). Successive treatments may affect the composition of the olives.

Apart from moisture, oil is the major component of table olives (*4*). A comprehensive study of the fat content and its composition in the main commercial presentations of Spanish cultivars has been published recently (*4*). The effect of green table olive processing on the composition and nutritional value of olives in Turkish cultivars was reported by Ünal and Nergiz (*5*). Borzillo et al. (*6*) made a qualitative evaluation of the olive oil during ripening and processing by biomolecular components. However, there is no information on the changes that the olive fat suffers during the different processing steps used for producing ripe table olives.

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Differences in acidity in the oil extracted from olives by two different methods was the only significant effect noted by Vaz-Freire et al. (*7*). Kalua et al. (*8*) reported that the storage of fruits at ≈4 °C for 3 weeks increased the oil yield and moderated the sensory quality of the virgin olive oil obtained. Cold storage (5 °C) of olive fruits (between 4 and 8 weeks) before oil extraction reduced the bitterness in the extracted olives (*9*). Yousfi et al. (*10*) found that storing olives for 72 h in closed containers, in the presence or absence of 30 ppm ethylene, either at 20 or at 40 °C, caused significant effects on oil characteristics, whereas the use of modified atmospheres induced off-flavors and a reduction in the overall sensory quality. A delay in the olive extraction after harvesting caused detrimental changes in the physicochemical and nutritional parameters of the obtained oils (*11*). Changes in the olive oil composition during the refining process were studied in detail by Tiscornia et al. (*12*).

Chemometric studies related to olive oil are numerous. Among others, chemometrics has been used for the characterization of varietal olive oils based on their components (fatty acids, tocopherols, diacylglycerols, or triacylglycerols) (*13*), to differentiate French virgin olive oil RDOs by sensory characteristics, fatty acid and triacylglycerols composition, and chemometrics (*14*). A headspace−mass spectrometry coupling design for the sensory characterization and classification of extra virgin olive oil on the basis of its protected designation of origin, olive variety, and geographical origin has also been reported (*15*).

The aim of this work was to study the changes produced in the characteristics and quality (acidity, peroxide value, spectrophotometric

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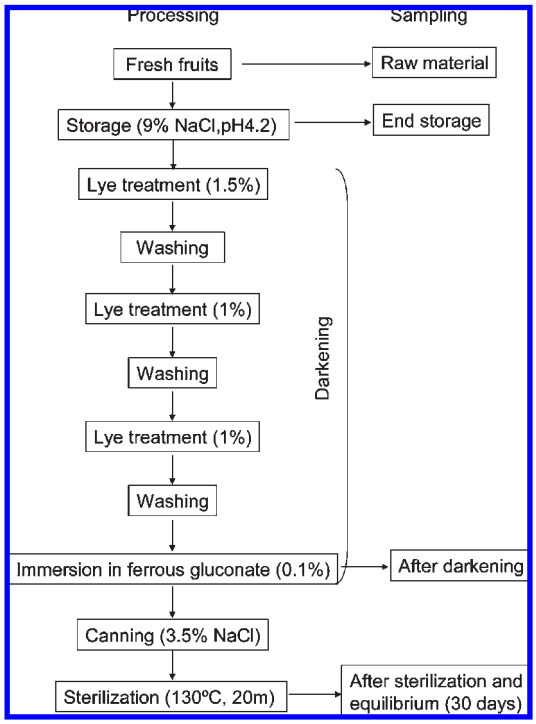

Figure 1. Diagram of the complete experimental design (ripe olive processing and sampling) followed in the work.

indices, and chemical composition) of oils during ripe olive processing. Particularly, this paper is devoted to the study of the characteristics and composition of the oils extracted from fruits at the following four steps: initial (raw material), end of storage, after darkening, and after sterilization. A general linear model, as well as unsupervised (principal components analysis and hierarchical clustering) and supervised (discriminant analysis) chemometric methods were used for quantifying and making such changes evident.

MATERIALS AND METHODS

A diagram of the complete experimental design (processing and sampling) is shown in **Figure 1**. A condensed description of the successive processing steps follows.

Cultivars. Olives were from the most popular Spanish cultivars for preparing ripe table olives: Hojiblanca (Cabra, Córdoba) and Manzanilla (Pilas, Sevilla).

Previous Storage. The storage process was carried out at pilot plant scale at room temperature. The olives (15 kg each cultivar) were stored in 25 L PVC fermenters for 6 months. Two containers for each cultivar were used. Initial brines consisted of an acidified solution (corrected to pH 4.2 with acetic acid) containing 9% NaCl. To compensate for salt absorption by the olives, the proportion of NaCl was raised periodically to reach equilibrium at 8% (w/v). Periodically (8 h/day), air was bubbled through the brine by means of a column introduced into the interior of the fermenters. Storage was controlled periodically according to pH, acidity, and NaCl concentrations in the brine as well as the microbial population. A more detailed description of the storage system and its control can be found elsewhere (*2*).

Darkening Process. Olives from each fermenter were treated in a cylindrical stainless steel container with successive lye solutions of 1.5, 1.0, and 1.0%, which progressively penetrated the flesh until the alkali reached the pit at the end of the last immersion (**Figure 1**). Between lye treatments, olives were suspended in tap water to remove the excess alkali and air was injected through the bottom of the container. When the skin was black, the washings were prolonged until the pH reached 8.0, and then a 0.1% ferrous gluconate solution with pH corrected to 4.5 was added to fix the color. The fruits were then canned in a 3.5% NaCl solution acidified with acetic acid to pH 4.5, subjected to sterilization for 20 min at 130 °C, and cooled to room temperature with tap water.

Equilibrium Period for the Packed Product. Sterilized olives were stored at room temperature for 30 days to permit equilibrium to take place before cans were opened and analyzed.

Fat Extraction. Oil was extracted from raw material olives (3 kg), at the end of storage, after darkening, and after sterilization (and equilibrium). There were true duplicates of each treatment (except for the raw material) because two storage fermenters were used in the design for each cultivar. Olives were manually pitted and mixed with a homogenizer Ultraturax T25 (IKA-Labortecnik, Staufen, Deutschland), and then 120 mL/kg boiling water (100 °C) was added to the paste to facilitate oil separation. The resulting suspension was subjected to malaxation (a process prior to extraction consisting of mixing the milled olives) in the ABENCOR thermobeater for 40 min at room temperature (22 ± 2 °C), and the liquid was removed by centrifugation using ABENCOR equipment (Abengoa, Madrid, Spain) similar to that used for the estimation of olive oil yield in olive mills (*16*). The liquid phase was allowed to decant, and the oil was obtained, filtered, and subjected to analysis. During extraction, one duplicate sample corresponding to Manzanilla raw material was irreversibly lost. All determinations in the oils from the raw material and from each true duplicate storage fermenter and according to processing steps were also made in duplicate.

To carry out the study, the oil was separated into nonpolar and polar fractions. Fatty acid and triacylglycerol compositions were determined in the first fraction, whereas oxidized triacylglycerols, diacylglycerols, and monoacylglycerols were analyzed in the second. Acidity, peroxide value, and spectrophotometric parameters were measured in the oil directly.

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

Peroxide value, expressed in milliequivalents of active oxygen per kilogram of oil (mequiv of O_2/kg), was determined by the reaction of a mixture of oil and chloroform/acetic acid with a solution of potassium iodine in the dark.

K_{270} and K_{232} extinction coefficients (related to the formation of conjugated dienes and trienes as well as other degradation products, respectively) were calculated from absorption at 270 and 232 nm, respectively, with a UV spectrophotometer (model Varian Cary 1E, Margrave, VIC, Australia), using a 1% solution of oil in cyclohexane and a path length of 1 cm. K_{266} and K_{274} were also estimated following the same methodology. Their values were used for estimating ΔK, according with the formula

$$\Delta K = K_{270} - \frac{K_{266} + K_{274}}{2}$$

This parameter is mainly used as a purity criteria for the detection of refined oils in virgin olive oils but was used in this work to detect possible changes during processing (mainly after sterilization) similar to those suffered by the olive oils during refining.

All of these quality and purity parameters were carried out following the analytical procedures described in Regulations (EEC) 2568/91 and (EC) 796/2002 (*17*, *18*).

Determination of the Unsaponifiable Fraction. The unsaponifiable matter was determined by saponification of the oil with potassium hydroxide in an ethanolic solution and extracted with diethyl ether, according to UNE 55004 standard method (*19*).

Separation of Polar and Nonpolar Compounds. The oils were fractioned using silica gel columns (*20*).

Triacylglycerol Composition. The analysis of triacylglycerols was performed, in the nonpolar fraction, according to the official chromatographic methods of the EEC Regulations 2568/91 and 2472/97 (*17*, *21*). A Waters 2695 separations module, a Waters 2414 refractive index detector, and a computer with Empower 2 software (Waters Associates, Milford, MA) were employed using a Lichrospher/Superspher RP18 column (250 × 4.0 mm, 4 μm particle size; Phenomenex, Torrance, CA), and the following settings were used: column oven, 30 °C; elution solvent, acetone/acetonitrile (1:1, v/v); flow rate, 1.5 mL/min. Theoretical and estimated by HPLC values of ECN42 as well as ΔECN42 (estimated by HPLC minus theoretical ECN42) were also calculated.

Fatty Acid Composition. The analytical methods for the determination of fatty acid composition are described in Regulation EEC 2568/91 (*17*). Fatty acids from the nonpolar fraction were converted to fatty acid methyl esters before analysis by shaking a solution of 0.2 g of oil and 3 mL of hexane with 0.4 mL of 2 N methanolic potassium hydroxide. The converted fatty acid methyl esters were analyzed with a Hewlett-Packard 5890 series II gas chromatograph, incorporating a fused silica capillary column Select FAME (100 m × 0.25 mm, 0.25 μm film thickness) (Varian, Bellefonte, PA), and a flame ionization detector was used for GC analysis. Hydrogen was used as the carrier gas at 1 mL/min. The injector (split 1:20) and detector temperatures were 250 °C. The operating conditions were as follows: the oven temperature was held at 120 °C for 5 min and then increased by 4 °C/min to 240 °C and held for 20 min at 240 °C.

Determination of Polar Compounds. The polar compounds were analyzed according to the method suggested by Dobarganes et al. (*20*). The conditions applied for high-performance size exclusion chromatography (HPSEC) analysis were as follows: sample solutions of 10−15 mg of polar compounds/mL in tetrahydrofuran were used for the analysis. An HP1050 system with a 10 μL sample loop and three 50, 100, and 500 Å Ultrastyragel columns (Waters Associates), 25 cm × 0.77 cm i.d., packed with a porous, highly cross-linked styrenedivinylbenzene copolymer (< 10 μm), connected in series and a refractive index detector (Hewlett-Packard), were used.

Chemicals. All reagents were of analytical grade, except acetone, acetonitrile, and tetrahydrofuran, which were of super purity grade from Romil (Cambridge, U.K.).

Reference standards for fatty acid determinations were saturated and unsaturated methyl esters (C4−C24) from Sigma (St. Louis, MO).

Reference triacylglycerols (LLL, OOO, PPP, SSS, LnLnLn, and PoPoPo) of > 98% purity were also purchased from Sigma. The abbreviations used for the fatty acids are Po for palmitoleic, L for linoleic, Ln for linolenic, O for oleic, P for palmitic, and S for stearic.

Statistical Analyses. Data from the previous determinations were arranged in a 28 × 44 matrix array, where rows were cases (cultivars × processing steps × replicates) and columns were variables (acidity, peroxide value, K_{232}, K_{270}, ΔK, fatty acids, triacylglycerols, and polar compounds).

Data were analyzed first using a GLM nested factorial design and later subjected to a chemometric analysis. To carry this out, standardized data (using the autoscale procedure) were successively studied by multiple analysis of variance (MANOVA) to test overall differences between groups across the different variables, principal component analysis (PCA), and discriminant analysis (DA). PCA was applied using a varimax rotation. For the selection of the number of principal components (PCs), only factors with eigenvalues of > 1.00 were retained. Then, the loadings of the original variables were projected onto the factorial plane formed by the first and second components.

The selection of variables containing the most powerful information for the correct classification was carried out on the basis of the canonical analysis of data, using the backward stepwise option. The values of probability to enter or to remove were fixed at 0.05 and 0.10, respectively. The number of steps was fixed at 100 and the minimum tolerance at 0.001, and no variable was forced to enter into any model. The scores of table olive samples were plotted on the canonical axes (discriminant coordinates or factors).

DA classification was achieved by means of the corresponding classification functions. Prior probabilities were established in proportion to the number of samples in each group. A leave-one-out cross-validation procedure was performed for assessing the performance of the classification rule. In this last step, the sample data minus one observation was used for the estimation of the classification functions, and then the omitted variable was classified from them. The procedure was repeated for all samples. Consequently, each sample was classified by classification functions, which were estimated without its contribution.

All collected data were also subjected to a hierarchical clustering analysis.

The different statistical techniques used in this work were implemented using Statistica, release 6.0 (GLM, PCA, and clustering analysis), and SYSTAT, release 10.2 (DA).

RESULTS AND DISCUSSION

Changes in Acidity, Peroxide Value, and Spectrophotometric Parameters. The effect of ripe olive processing on acidity is shown in **Figure 2a**. Initially, the oil present in the raw material had acidity slightly above 0.2 g of oleic acid/100 g of oil (usually expressed as %, w/w), regardless of cultivar, but was strongly affected by processing. The most influential step was storage, which significantly increased the acidity in both cultivars but mainly in the Manzanilla cultivar (about 1.2%). This acidity might have been produced by the action of lipases from the fruits themselves or, more likely, by those excreted by the microorganisms present in the storage solutions (*22*). In this case, strains of *Candida boidinii*, able to produce lipase, were isolated and identified from the storage brine (data not shown). The acidity produced during storage was markedly reduced, particularly in Manzanilla, during the darkening process, possibly due to the neutralization of the free fatty acids with the alkali used in this phase, and at the end, both cultivars had a very similar acidity. Later, there was a new increase during sterilization, which can be due to the hydrolysis of triacylglycerols at high temperatures (*23*). Overall, the process had produced a similar, significant increase in acidity, regardless of cultivar, with an average in the oils from the final products (sterilized olives) of ≤0.8 g of oleic acid/100 g of oil, which is the maximum limit established in the EC Regulations for extra virgin olive oil (*24*).

Peroxide value (similar in Manzanilla and Hojiblanca raw material) significantly increased during the storage phase; later, it showed a nonsignificant, slight decrease (**Figure 2b**). The formation of hydroperoxides during storage is due to autoxidation or to the action of lipoxygenase, which requires free fatty acids (preference: linolenic > linoleic > oleic) that are easily available in these olives due to the fatty acid increase during storage. Peroxide value from the final products (both cultivars) exceeded the limit of 20 mequiv/kg of oil, established by EC Regulation for virgin olive oils (*24*). This oil oxidation may occur even in anaerobic conditions because some lipoxygenases are able to oxidize fatty acids in the absence of oxygen (*25*).

K_{232} showed a progressive decreasing trend, which was significantly lower in the sterilized product than in the raw material (**Figure 2c**), showing significant differences between cultivars only after darkening. However, K_{270} was significantly higher in oils from Manzanilla with respect to those from Hojiblanca throughout most of the process, except in the sterilized product. K_{270} values increased after storage and sterilization but decreased with the darkening treatment (**Figure 2d**); its trend resembled the one followed by acidity (**Figure 2a**). Apparently, the darkening process did not cause an increase in conjugated dienes (in fact, there was a decrease in K_{232}) but led to the production of conjugated trienes and other secondary products of oxidation (nonanal, hexanal, and other volatile compounds) during storage and sterilization (increase of K_{270}) (*26*, *27*). However, there was a marked reduction in the presence of conjugated trienes and secondary products of oxidation during the darkening process. This decrease might be due to their degradation during alkali (NaOH) treatment or to the effect of Fe^{2+} (used to fix the color), which is oxidized to Fe^{3+} while reducing the oxidized oil compounds (*28*). K_{232} and K_{270} final indices have values below the limits established in the EU for extra or virgin olive oils (≤2.5 and ≤0.20 or 0.25), respectively (*24*).

The ΔK value was significantly different between cultivars throughout processing (so these differences may be due to the composition of the fruit in each cultivar) and showed trends similar to acidity (**Figure 2e**). Their values were always below the limit established in the EU for this parameter (≤0.01) (*24*).

Therefore, storage had a significant effect on all of the parameters studied in this section, which increased after this step, except K_{232}. Sterilization had a similar, but lighter, effect. However, darkening always decreased the values of all of the parameters

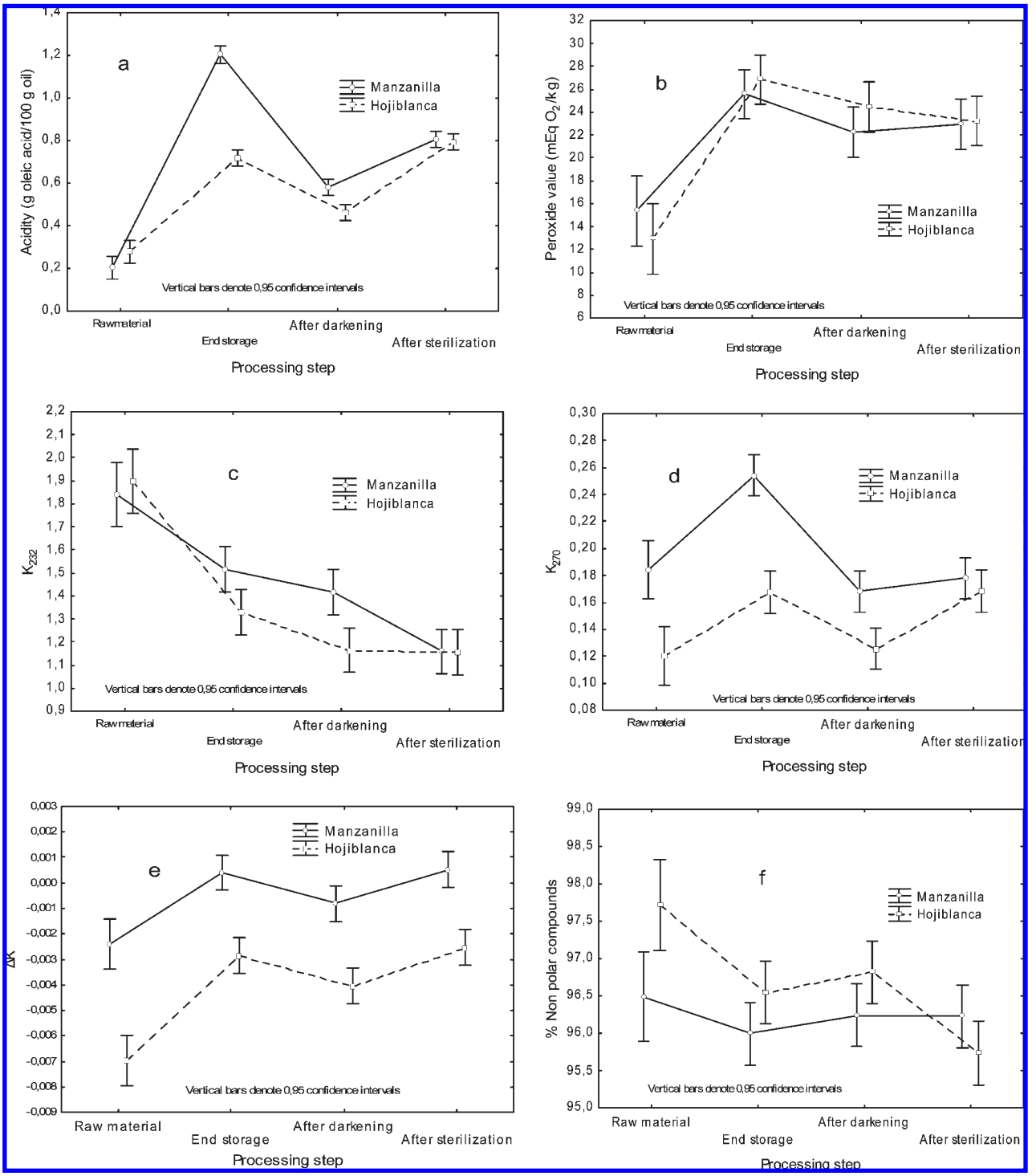

Figure 2. Changes in acidity, peroxide value, spectrophotometric characteristics, and percentage of nonpolar fraction during the processing of ripe olives, according to elaboration steps within cultivars.

(except peroxide value), having, apparently, a degrading effect on the primary oxidation products.

Changes in Nonpolar Compounds. Overall changes in nonpolar compounds are shown in **Figure 2f**. The percentage of this fraction was initially lower in Manzanilla than in Hojiblanca and significantly decreased during the storage phase in Hojiblanca. In this cultivar, there was also a nonsignificant increase after darkening, followed by a significant decrease (with respect to darkening) with sterilization. Nonpolar compounds only showed a nonsignificant, slight decrease after storage in Manzanilla. Overall, the nonpolar compounds did not change in Manzanilla, but there was a significant decrease with processing (raw material versus final product) in Hojiblanca.

Changes in Fatty Acid Composition. The averages of the fatty acid composition, expressed as percentage, according to processing steps within cultivars are shown in **Table 1**, and the results of the corresponding GLM analysis of variance for each acid, according to cultivars and processing steps within cultivars, are shown in **Table 2**. Bearing in mind their standard errors, the presence of C18:3n-6 and C20:3n-3+C20:4n-6 can be considered as not detected and will not be considered in further analysis. The most abundant acids were C18:1c, C16:0, C18:2n6, and C18:0, as

Table 1. Changes in Fatty Acid Composition (Mean ± Standard Error) According to Processing Steps within Cultivars[a]

	Manzanilla				Hojiblanca			
fatty acid (%)	raw material	end storage	after darkening	after sterilization	raw material	end storage	after darkening	after sterilization
C16:0	16.30 ± 0.04	16.20 ± 0.03	16.30 ± 0.03	16.41 ± 0.03	12.41 ± 0.04	12.39 ± 0.03	12.42 ± 0.03	12.48 ± 0.03
C17:0	0.16 ± 0.01	0.18 ± 0.01	0.18 ± 0.01	0.18 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.15 ± 0.01
C18:0	2.81 ± 0.01	2.81 ± 0.01	2.80 ± 0.01	2.80 ± 0.01	2.53 ± 0.01	2.51 ± 0.01	2.49 ± 0.01	2.53 ± 0.01
C20:0	0.39 ± 0.01	0.40 ± 0.01	0.39 ± 0.01	0.40 ± 0.01	0.37 ± 0.01	0.37 ± 0.01	0.37 ± 0.01	0.38 ± 0.01
C22:0	0.09 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
C24:0	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
C16:1	1.53 ± 0.01	1.51 ± 0.01	1.52 ± 0.01	1.52 ± 0.01	0.65 ± 0.01	0.66 ± 0.01	0.68 ± 0.01	0.68 ± 0.01
C17:1	0.31 ± 0.01	0.31 ± 0.01	0.34 ± 0.01	0.33 ± 0.01	0.24 ± 0.01	0.26 ± 0.05	0.27 ± 0.01	0.27 ± 0.01
C18:1c	69.81 ± 0.15	71.22 ± 0.10	71.01 ± 0.10	71.43 ± 0.11	75.72 ± 0.15	75.75 ± 0.11	75.80 ± 0.10	75.61 ± 0.10
C20:1	0.22 ± 0.01	0.23 ± 0.01	0.24 ± 0.01	0.23 ± 0.01	0.27 ± 0.01	0.28 ± 0.03	0.28 ± 0.01	0.28 ± 0.01
C18:2n-6	6.89 ± 0.07	6.20 ± 0.05	5.77 ± 0.05	5.78 ± 0.06	6.44 ± 0.07	6.50 ± 0.06	6.46 ± 0.05	6.55 ± 0.05
C18:3n-3	0.72 ± 0.01	0.74 ± 0.01	0.73 ± 0.01	0.73 ± 0.01	0.87 ± 0.01	0.87 ± 0.01	0.88 ± 0.01	0.90 ± 0.01
C18:3n-6	0.06 ± 0.06	0.02 ± 0.04	0.57 ± 0.04	0.01 ± 0.05	0.05 ± 0.06	0.04 ± 0.05	0.02 ± 0.04	0.01 ± 0.04
C20:3n-3 + C20:4n-6	<0.01 ± 0.01	<0.01 ± 0.01	<0.01 ± 0.01	<0.01 ± 0.01	<0.01 ± 0.01	<0.01 ± 0.01	<0.01 ± 0.01	<0.01 ± 0.01
C18:2t	0.64 ± 0.07	0.04 ± 0.05	<0.01 ± 0.05	<0.01 ± 0.05	0.16 ± 0.07	0.06 ± 0.05	<0.01 ± 0.05	0.05 ± 0.09
unsaponifiable g/100 g of oil	1.45 ± 0.09	1.11 ± 0.11	1.35 ± 0.05	1.14 ± 0.03	1.09 ± 0.03	1.37 ± 0.06	1.19 ± 0.06	1.36 ± 0.04

[a] Standard errors ≤0.01 are indicated as 0.01 to facilitate reading.

Table 2. *F* and *P* Values for the Nested Unvaried Analysis of Variance for Fatty Acid Composition[a]

	cultivar		processing step (within cultivar)	
fatty acid	*F* value	*P* value	*F* value	*P* value
C16:0	37256.00	0.000	9.00	0.000
C17:0	67.56	0.000	1.95	0.093
C18:0	1636.90	0.000	2.30	0.050
C20:0	208.20	0.000	10.30	0.000
C22:0	8.27	0.006	3.13	0.012
C24:0	8.86	0.005	1.92	0.099
C16:1	53177.30	0.000	5.40	0.000
C17:1	422.50	0.000	9.43	0.000
C18:1c	3494.00	0.000	15.00	0.000
C20:1	467.02	0.000	3.62	0.005
C18:2n-6	62.08	0.000	32.13	0.000
C18:3n-3	3659.10	0.000	11.70	0.000
C18:3n-6	16.97	0.000	19.92	0.000
C20:3n-3 + C20:4n-6	1.64	0.206	3.41	0.007
C18:2t	9.21	0.004	13.46	0.000
unsaponifiable	0.06	0.818	4.42	0.005

[a] Significance of the differences between cultivars or between processing steps within cultivars. Degree of freedom for cultivar, 1; processing steps within cultivar, 6; error, 46.

usual in olive oil (*29*). There were significant differences among cultivars for all of the fatty acids studied (**Table 2**). There were also significant differences between processing steps within cultivars for most of them, except C17:0, C18:0, and C24:0 (**Table 2**). Oleic (C18:1c), the most characteristic fatty acid in olives, was significantly higher in Hojiblanca, where it was scarcely affected by processing; but its proportion in the final product significantly increased with respect to the initial content in Manzanilla (**Table 1**). C16:0, the most abundant saturated fatty acid, was always higher in Manzanilla than in Hojiblanca, in which it was hardly affected by the process; however, in Manzanilla it reached its maximum after sterilization, but its lowest content after storage could have been due to readjustments of percentages or variation in analytical measure, given the high stability of this acid (**Table 1**). Linoleic acid (C18:2n-6) was significantly higher in raw Manzanilla, but was markedly reduced during processing, with the fatty acid being the most affected by processing. In Hojiblanca, on the contrary, it suffered practically no changes (**Table 1**). C18:3n-3 (linolenic acid) was higher in Hojiblanca, but its increase at the end of processing can be most likely due to readjustments of percentages or experimental error because it is unlikely that its content would increase during storage; C18:2t was initially higher in Manzanilla but, apparently, the *trans* fat was, in practice, degraded during the darkening process. Changes in other fatty acids can be observed directly in **Table 1**.

Table 3. Discriminant Analysis Based on Fatty Acids: Retained Variables and Canonical Discriminant Functions

fatty acid	*F* to remove	tolerance	function 1	function 2	function 3
C16:0	3.46	0.012	2.675	2.829	3.377
C18:0	16.59	0.003	−13.378	−2.368	5.260
C20:0	19.85	0.003	12.628	−6.013	−9.517
C24:0	6.62	0.181	−0.926	1.084	−0.805
C17:1	5.84	0.040	2.504	1.290	−1.436
C20:1	7.38	0.016	2.372	3.870	4.416
C18:2n-6	20.17	0.029	−4.756	0.988	1.139
C18:3n-6	6.05	0.534	0.348	0.630	0.725

The EU Regulations mention diverse limits for several fatty acids. They are ≤0.05, ≤1.0, ≤0.6, ≤0.4, ≤0.2, ≤0.2, ≤0.05, and ≤0.05 for C14:0, C18:3n-3, C20:0, C20:1, C22:0, C24:0, *trans*-oleic, and sum of *trans*-linoleic and linolenic isomers, respectively (*24*). The contents shown in **Table 1** were always below these limits except for the sum of the *trans*-linoleic isomers in the raw material; however, after processing, the contents of these were below the limits for Manzanilla and just around them for Hojiblanca. Therefore, overall, the oils in the final products were in agreement with the limits established for these fatty acids in the EC Regulations.

The application of PCA to the data from the diverse processing steps on the plane of the first two PCs did not lead to a clear differentiation among treatments, but predictive DA led to promising results. The retained variables that most contributed to discrimination and the coefficients of the canonical functions are shown in **Table 3**. The classification functions deduced in this predictive DA were able to determine a 100% correct classification of the samples according to cultivars and also led to a fairly good classification according to processing steps: 95% correct assignment and 87% in cross-validation (**Table 4**). Graphing the samples on the plane of the first two canonical functions led to a separation of the samples into separate regions (**Figure 3**). The misclassification was due to the inclusion of two storage samples into the after darkening and sterilization groups and one from sterilization into end storage. Then, although the differences among samples were not clearly revealed by PCA, the predictive

DA found enough differences to be able to differentiate not only between cultivars but also among processing steps.

Changes in Triacylglycerol Composition and ΔECN42. The triacylglycerol composition of the oils extracted from the different steps of ripe olive processing within cultivars is shown in **Table 5**. Their compositions were similar to those found in olive oil (*29*). The most abundant were those containing oleic acid because this was the fatty acid in the greatest proportion, particularly OOO+PLP (these are reported together because both overlap in the same peak) (34.7−44.3%), POO+SOL (25.2−30.5%), OOL+PoOO (10.5−12.0%). They were followed by SLL+POL (5.2−7.5%), POP+PLS (3.5−5.7%), and SOO (4.8−5.0%) (**Table 5**).

Table 4. Discriminant Analysis Based on Fatty Acids: Classification Matrix (Cases in Row Categories Classified into Columns)

	raw material	end storage	after darkening	after sterilization	% correct
raw material	8 (7)[a]	0 (1);	0 (0);	0 (0);	100 (88);
end storage	0 (1);	13 (12);	1 (1);	1 (1);	87 (80);
after darkening	0 (0);	0 (0);	16 (14);	0 (2);	100 (88);
after sterilization	0 (0);	1 (1);	0 (0);	15 (15);	94 (94);
total	8 (8);	14 (14);	17 (15);	16 (18);	95 (87);

[a] Jackknifed classification matrix in parentheses.

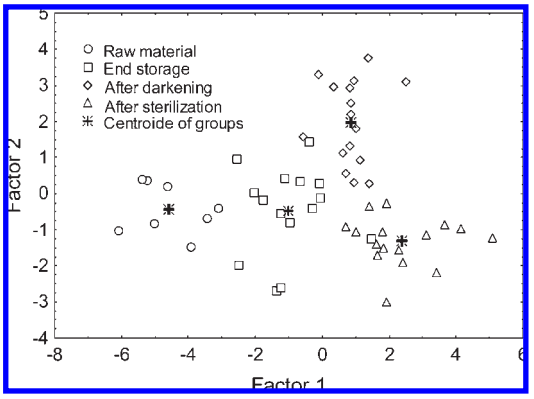

Figure 3. Distribution of cases (cultivar × processing steps) on the plane of the first two factors deduced from the canonical analysis of fatty acids in the nonpolar fraction during ripe olive processing.

Results from the nested ANOVA (effect of cultivar and processing step within cultivars) are shown in **Table 6**. There were no significant differences for PLLn, OLL, and POLn+PPoL among cultivars. The processing step, within cultivar, did not have a significant effect on PLLn, OOLn+PoOL, PLL+PoPoO, SOO, or POS+SLS. Changes in the other triacylglycerols can be observed in **Table 5** and the statistical significance of their differences in **Table 6**.

The composition of triacylglycerols has been used for grouping olive oils from diverse Tunisian olive cultivars or to differentiate registered designations of origin of French virgin olive oils (*14*).

The application of PCA to triacylglycerols did not lead to any clear differentiation according to processing steps within cultivars, but predictive DA led to 100% correct classification into cultivars; however, results were poorer for classification into elaboration step with an overall correct classification of around 60%. As a result, it was clear that triacylglycerol composition was different between cultivars but, overall, changes during

Table 6. *F* and *P* Values for the Nested Univaried Analysis of Variance for Triacylglycerols and ECN42 Parameters[a]

	cultivar		processing step (within cultivar)	
triacylglycerol	*F* value	*P* value	*F* value	*P* value
LLL	77.29	0.000	11.04	0.000
OLLn	37.36	0.000	4.62	0.004
PLLn	3.07	0.095	1.88	0.135
OLL	1.84	0.190	17.48	0.000
OOLn+PoOL	121.48	0.000	0.42	0.860
PLL+PoPoO	64.56	0.000	2.06	0.104
POLn+PPoL	0.68	0.420	4.22	0.007
OOL+PoOO	248.00	0.000	10.60	0.000
SLL+POL	320.78	0.000	5.68	0.001
OOO+PLP	5840.30	0.000	12.60	0.000
POO+SOL	85.25	0.000	12.00	0.000
POP+PLS	11533.30	0.000	3.10	0.028
SOO	17.20	0.000	2.50	0.055
POS+SLS	4691.30	0.000	1.90	0.132
ECN42 (theor)	0.15	0.708	3.65	0.013
ECN42 (exptl)	1.05	0.318	7.09	0.000
ΔECN	0.02	0.893	3.17	0.024

[a] Significance of the differences between cultivars or processing steps within cultivars. Degree of freedom for cultivar, 1; processing steps within cultivar, 6; error, 20.

Table 5. Changes in Triacylglycerol Composition (Mean ± Standard Error) According to Processing Steps within Cultivars[a]

	Manzanilla				Hojiblanca			
triacylglycerol	raw material	end storage	after darkening	after sterilization	raw material	end storage	after darkening	after sterilization
LLL	0.08 ± 0.01	0.06 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
OLLn	0.23 ± 0.01	0.23 ± 0.01	0.21 ± 0.01	0.20 ± 0.01	0.23 ± 0.01	0.25 ± 0.01	0.23 ± 0.06	0.25 ± 0.01
PLLn	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.01
OLL	1.37 ± 0.01	1.12 ± 0.03	0.98 ± 0.04	0.99 ± 0.04	1.08 ± 0.01	1.09 ± 0.02	1.08 ± 0.01	1.10 ± 0.01
OOLn+PoOL	1.38 ± 0.01	1.39 ± 0.02	1.36 ± 0.02	1.38 ± 0.01	1.57 ± 0.03	1.55 ± 0.03	1.54 ± 0.02	1.57 ± 0.03
PLL+PoPoO	0.44 ± 0.03	0.39 ± 0.02	0.35 ± 0.01	0.35 ± 0.01	0.28 ± 0.02	0.27 ± 0.03	0.26 ± 0.01	0.28 ± 0.02
POLn+PPoL	0.78 ± 0.01	0.78 ± 0.01	0.74 ± 0.01	0.76 ± 0.01	0.76 ± 0.01	0.76 ± 0.01	0.74 ± 0.01	0.77 ± 0.01
OOL+PoOO	11.64 ± 0.04	10.90 ± 0.11	10.59 ± 0.11	10.53 ± 0.13	11.91 ± 0.03	12.00 ± 0.06	12.03 ± 0.07	12.06 ± 0.01
SLL+POL	7.47 ± 0.02	6.94 ± 0.25	6.47 ± 0.12	6.55 ± 0.08	5.27 ± 0.01	5.22 ± 0.02	5.25 ± 0.03	5.36 ± 0.03
OOO+PLP	34.72 ± 0.02	36.09 ± 0.21	36.55 ± 0.21	36.38 ± 0.17	44.18 ± 0.02	44.31 ± 0.04	44.27 ± 0.05	43.80 ± 0.04
POO+SOL	29.67 ± 0.08	29.91 ± 0.13	30.37 ± 0.05	30.46 ± 0.08	25.17 ± 0.02	25.14 ± 0.04	25.26 ± 0.04	25.24 ± 0.02
POP+PLS	5.69 ± 0.06	5.60 ± 0.02	5.70 ± 0.03	5.71 ± 0.02	3.53 ± 0.08	3.47 ± 0.03	3.49 ± 0.03	3.55 ± 0.01
SOO	4.88 ± 0.05	4.93 ± 0.05	4.96 ± 0.02	4.97 ± 0.03	4.89 ± 0.02	4.86 ± 0.01	4.78 ± 0.02	4.87 ± 0.03
POS+SLS	1.67 ± 0.01	1.66 ± 0.01	1.67 ± 0.01	1.66 ± 0.02	1.09 ± 0.01	1.06 ± 0.01	1.06 ± 0.02	1.10 ± 0.01
ECN42 (theor)	0.38 ± 0.04	0.29 ± 0.01	0.31 ± 0.02	0.27 ± 0.01	0.31 ± 0.01	0.33 ± 0.03	0.31 ± 0.01	0.31 ± 0.01
ECN42 (exptl)	0.30 ± 0.01	0.28 ± 0.01	0.25 ± 0.01	0.25 ± 0.01	0.27 ± 0.01	0.28 ± 0.01	0.27 ± 0.01	0.29 ± 0.01
ΔECN	0.074 ± 0.040	0.006 ± 0.005	0.058 ± 0.011	0.019 ± 0.008	0.046 ± 0.005	0.048 ± 0.021	0.038 ± 0.007	0.020 ± 0.004

[a] Standard errors ≤0.01 are indicated as 0.01 to facilitate reading.

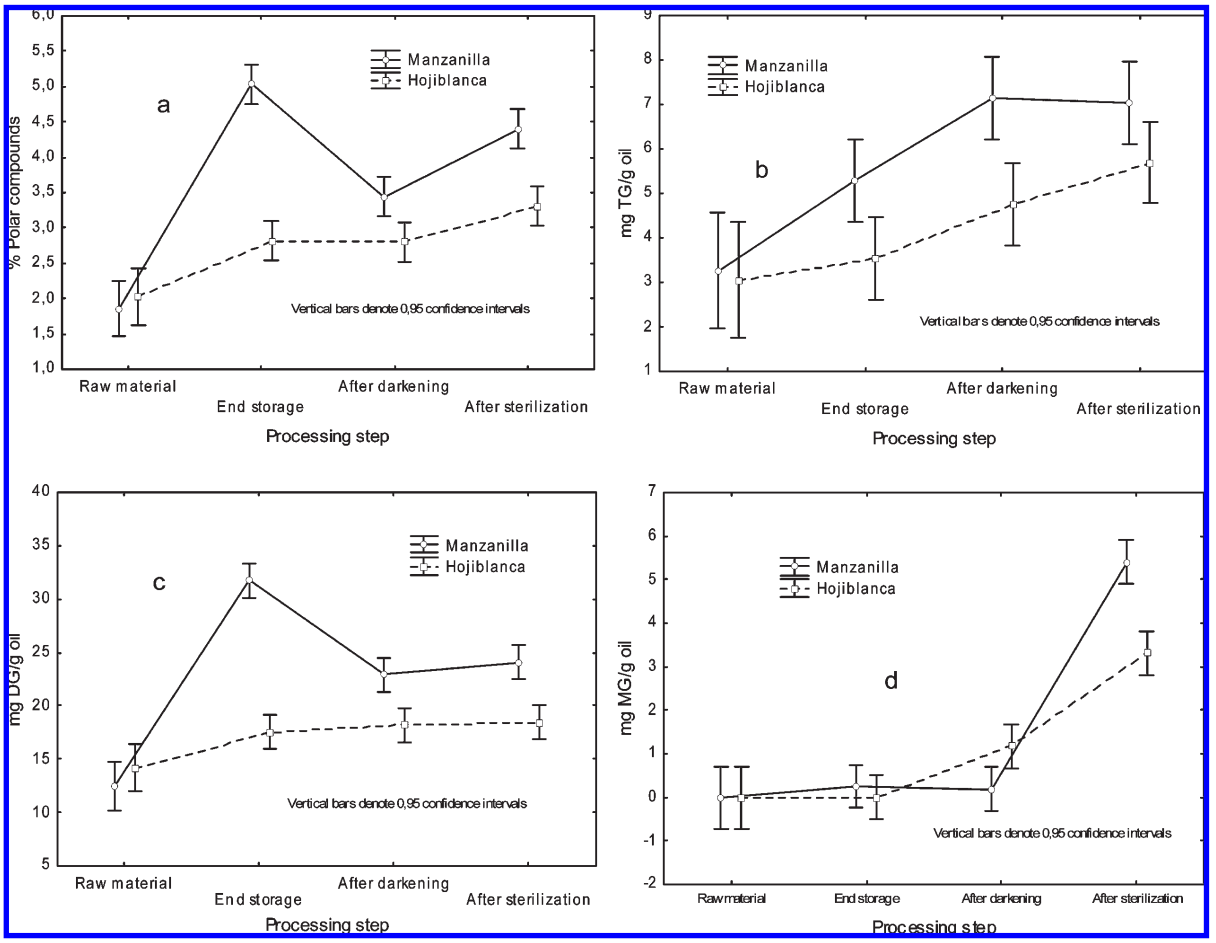

Figure 4. Changes in polar fraction and its components during ripe olive processing, according to elaboration within cultivars. Abbreviations: TG, oxidized triacylglycerols; DG, diacylglycerols; MG, monoacylglycerols.

processing were not enough to produce good discrimination among processing steps, possibly due to the diverse trends that each triacylglycerol followed in both cultivars.

ECN42 indices had relatively high variability and showed significant differences due to processing steps within cultivars (**Tables 5** and **6**). Both values decreased in Hojiblanca (but not in Manzanilla) due to processing. The difference between both, ΔECN42 (estimated by HPLC minus the theoretical one), was also significantly affected by processing (**Table 6**), but changes were always ≤0.2, the limit established by the CE Regulations (*24*). This parameter is used as a purity criterion to detect mixtures of olive oil and seed oils (which usually have high proportions of linoleic acid) but, in ripe olive processing, changes in polyunsaturated acids could also affect this parameter. However, ΔECN values found in this work indicate that it did not suffer a marked increase (≤0.2) in this case.

Changes in the Polar Fraction Composition. Polar compounds in olive oil are complex mixtures of degradation compounds originated during processing. It must be emphasized that while hydrolysis involves breakage of the ester bond with the formation of fatty acids, monoacylglycerols, and diacylglycerols (the normal compounds originating in the stages prior to the fat absorption in the intestine), oxidative degradation takes place in the unsaturated acyl groups of the triacylglycerols, modifying the nutritional properties of the fat (*30*). Then, the content of polar compounds in each phase can be an index of the degradation of the fat as ripe olive processing progresses. Initially, the polar compound levels were similar in both cultivars (**Figure 4a**). The largest increase was observed in Manzanilla after the storage process; then, darkening caused a marked decrease, possibly due to the effect of the NaOH solution, but sterilization again increased the concentration of their contents. This behavior is usual, and it is well documented that heat treatments increase the presence of polar compounds (*28*,*30*). In Hojiblanca, the polar compound increases were always below those observed in Manzanilla. These trends mean that there is a progressive degradation in the oil quality, which was higher in Manzanilla than in Hojiblanca. Oxidized triacylglycerols (**Figure 4b**) increased progressively throughout processing, leading to the same concentrations at the end of processing in both cultivars, although their formation was lower (especially at the end of storage and after darkening) in Hojiblanca. The changes in diacylglycerols resembled those of total polar compounds (**Figure 4c**), indicating that most of the changes observed in the polar fraction were due to the formation of these substances. On the contrary, monoacylglycerols (**Figure 4d**) increased mainly with sterilization. The heat treatment was then the most aggressive step for the degradation of triacylglycerols. In both cultivars, a marked decrease in the presence of free fatty acids due to the darkening process was observed (**Figure 2a**). Therefore, there is a partial neutralization of free fatty acids during the treatment with NaOH solution. This is, then, proof that the lye treatment may affect olive fat despite the fact that it is protected in the interior of the cells. Some of the changes observed (acidity production, oxidation of polyunsaturated fatty acids, and formation of secondary oxidation products) can be related with a partial deterioration of the oils and a slight loss of the nutritional value of ripe table olives during processing. Possibly, similar results can also occur in other table olive processing.

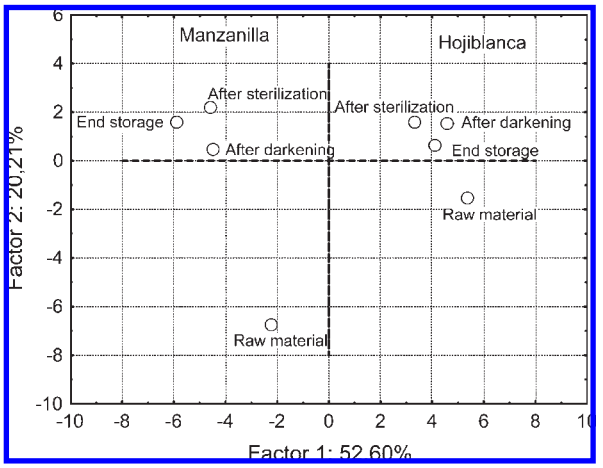

Figure 5. Distribution of cases (cultivar × processing steps) on the plane of the first two factors deduced after PCA of all variables studied.

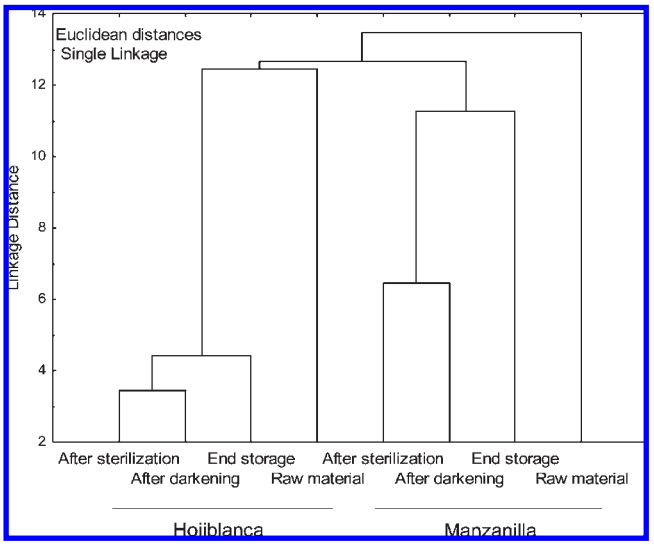

Figure 6. Results of the hierarchical clustering analysis of cases (cultivar × processing step), using all of the studied variables.

Overall Changes in the Olive Fat during Processing. Conclusions regarding overall changes, taking into account all of the variables studied, were made possible by means of the PCA and clustering analysis. PCA results showed eight eigenvalues of >1, which accounted for 98.96% of the original variance; so, theoretically, the original 44 variables studied could be reduced to only 6, with a reduced loss of variability (variance). Most of the variance was explained by the first (factor 1) (52.60%), followed by the second (factor 2) (20.21%) and the third (13.56%). There were no clear derived variables that could be related to each of these factors, but the projection of the cases on the factor 1 versus factor 2 plane led to a clear segregation of the cases (cultivars × processing steps) (**Figure 5**). Manzanilla raw material was characterized by negative values of both factors (left bottom quadrant), whereas the processed olives from this cultivar, regardless of the processing stage, were grouped above it, with positive values for factor 2. Hojiblanca olives were situated on the opposite side (they were always characterized by positive values for factor 1, right quadrant). The situation was similar to that observed for the Manzanilla cultivar, with the only difference being that the distance between the raw material and the processed fruits was less. As a result, it can be deduced that the olive fat was affected by processing and that Manzanilla fat was more deeply modified (greater distance).

All of the collected data were also submitted to a hierarchical clustering analysis, which was capable of distinguishing among the eight different treatments (two cultivars × four processing steps). This technique produced a hierarchy of partitions such that any cluster of a partition is fully included in one of the clusters of the later partitions. Such partitions are best represented by a tree dendogram (binary tree).

The dendogram produced by the hierarchical clustering analysis is shown in **Figure 6**. The distribution of the cluster is very similar within cultivars, but it can easily be noted that the effects caused by processing in Manzanilla cultivar were stronger than those occurring in Hojiblanca. At a linkage distance of about 13, it was possible to distinguish only two clusters: Manzanilla raw material and the rest of the treatments (including Hojiblanca raw material). Decreasing the degree of difference (lower linkage distance) to 7−11, the samples are distributed into five major clusters. The first one corresponded to the Manzanilla raw material; the second one was the sample from the same cultivar after storage (meaning that storage caused great changes in the fat characteristics of this cultivar), but there were no differences between samples after darkening or sterilization. Therefore, overall, most of the changes in the olive fat in Manzanilla cultivar were produced by storage. The third one included oxidized and sterilized Manzanilla. The fourth corresponded to Hojiblanca raw material, and the fifth included all processed Hojiblanca olives. Overall, **Figure 6** gives a clear image of the influential steps and the quantitative effects.

It has been shown that the characteristics of the fat from different table olive cultivars were different not only in the raw material but also in the processed fruits. Many of the parameters used to characterize olive fat (the olive oil extracted) were affected by processing, particularly by the storage process. Acidity, peroxide value, and ΔK increased significantly. Most of the fatty acids, except C17:0, C18:0, and C24:0, were significantly affected by some processing step. Similarly, the triglyceride compositions were also modified by the processing treatments, except PLLn, OOLn+PoOL, PLL+PoPoO, SOO, and POS+SLS, but, as result of these changes, ΔECN42 never showed values above the limits established in the EC Regulations. There was also a significant increase in the polar compounds and all of their components; the most significant changes were related to the increase in diacylglycerols and free fatty acids due to hydrolysis during the storage process, although increases in monoacylglycerols and free fatty acids were also observed in the sterilization process because of thermal degradation. The presence of oxidized triacylglycerols, on the contrary, followed a steady increase throughout processing.

The application of chemometric analysis was able to detect such differences. Using fatty acid composition, predictive DA was able to discriminate between cultivars (100% correct) and had a high discriminant capacity among processing steps (95% correct assignation and 87% in cross-validation). However, triacylglycerol composition, apparently, suffered lighter modifications than fatty acids and only showed good discriminant efficiency for cultivars. Using the 44 variables studied, PCA did not lead to a reduction in them but clearly separated the groups corresponding to different cultivars and, within these, the raw material from the rest of the treatments. The hierarchical clustering analysis was an unsupervised technique able to produce good discrimination among cultivars and treatments within cultivars. At a linkage distance of 7−11, five clusters were clearly distinguishable: raw Manzanilla and Hojiblanca cultivars, stored Manzanilla, oxidized and sterilized Manzanilla, and processed Hojiblanca. As a result, hierarchical clustering analysis was able to produce a clear

intuitive visual map of the effects of cultivars and processing steps on the composition and quality of olive oil.

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