Assessment of the Minor Component Transformations in Fat during the Green Spanish-Style Table Olives Processing

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Running title: Transformations in green olive fat minor components during processing

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ABSTRACT

There is an increasing interest of consumers for natural and healthy products. This work assesses the transformations that green Spanish-style processing of Manzanilla and Hojiblanca table olives produce on the minor components of its fat. Discriminant Analysis showed that most of the variability was not due to processing (24.4%) but to differences between cultivars (59.2%). Therefore, the final products have a similar quality than the original olive fat; that is, the quality of the fat was scarcely affected. The only systematic trends observed were the decrease in hexacosanol, tetracosanol and octacosanol (fatty alcohols) and C46 (wax), after lye treatment, and the high levels of alkyl esters in the packaged product. Thus, minor component levels in green table olives are, in general, within the limits established for extra virgin olive oil since the alkyl esters should be considered habitual products of fermentation and not as an alteration as in olive oil.

KEYWORDS: green Spanish-style table olives, processing, Manzanilla cultivar, Hojiblanca cultivar, olive fat, minor olive fat components, chemometrics.
INTRODUCTION

The production of table olives was 2,650,000 tonnes in the 2015/2016 season. The European Union produced 868,000 tonnes, with Spain being the main contributor (~550,000 tonnes). The so-called green Spanish-style table olive is the most popular and accounts for about 60% of the total consumption. Processing always includes several treatments to reduce the natural bitterness of olives. The green Spanish-style consists of an immersion in lye to degrade the bitter compounds, followed by washing, brining, fermentation, conditioning and packaging, all of them performed using aqueous solutions.

Fat is the primary nutrient in table olives. The study of numerous table olive commercial presentations showed important differences in the concentrations of their oil components, with triacylglycerols and their associated fatty acids constituting most of the olive lipids. However, many other minor components (sterols, fatty alcohols, triterpenic dialcohols, waxes, fatty acid esters and products of degradation) play essential roles in oil quality characteristics. In this way, several sterols, triterpenic dialcohols, waxes, and alkyl esters are subjected to constraints in olive oil, according to the European Union Legislation and the Standard issued by the International Olive Council. Furthermore, their levels are determinant for olive oil classification. Therefore, when investigating the effect of processing on the table olive fat, the study of the changes in the minor component profiles is essential.

Due to the low concentration of NaOH used for debittering, the exclusive application of aqueous solutions during the elaboration, and the localisation of the oil droplets in the interior of the cell, it was considered for decades that the olive fat was not affected by the green Spanish-style processing. However, recent studies have shown that ripe olive preparation may affect the olive fat composition, including the minor components.
Most of such transformations were produced during the previous storage/fermentation process and were associated with the presence of a lipolytic microflora during this phase; among the species isolated, *Candida boidinii* showed the strongest activity.\(^\text{10}\) Despite it, individual yeasts have shown a limited effect on the oil quality parameters of green Spanish-style table olives.\(^\text{11}\) On the contrary, the pitting and pitting and stuffing processes of ripe and green Spanish-style table olives, respectively, produced significant modifications on the minor components of the released oils.\(^\text{12,13}\) Other cultivars and processing have also been studied by different authors. Sakouhi et al.\(^\text{14}\) reported the changes in \(\alpha\)-tocopherol and fatty acids after processing as green Spanish-style Tunisian autochthonous cultivars, finding important differences among them. Interestingly, the presence of \(\alpha\)-tocopherol was positively related to the content of unsaturated fatty acids. No changes in tocopherols during different processing conditions (Spanish-style, short process, and “Picholin” style) whereas their contents decreased in the packaged products, with significant losses after 12 months storage.\(^\text{15}\) Bianchi\(^4\) has published a review on the changes in phenols and lipids during processing. However, in mature Oinotria table olive processing (blanching, salting, and drying), biophenols decreased while the fatty acid composition remained without deterioration.\(^\text{16}\) Also, Lanza et al.\(^\text{17}\) has related the presence of high proportions of alkyls esters in Kalamata and Moresca cultivars with the use of low concentrations of brines in Greek style olives while Giarraffa and Nocellara del Belice cultivars produced only ethyl esters.

The combination of analytical techniques and chemometrics can be an excellent strategy for detecting changes in the fat and oil compositions.\(^\text{18}\) The chemometric analysis was useful to detect changes in the oil characteristics of ripe olives during processing\(^\text{8,9}\) and to segregate the various oils released during the table olive conditioning.\(^\text{12,13}\) Furthermore, the same techniques were also applied to study the
effects of green Spanish-style processing on the quality parameters and fatty acid compositions of Manzanilla and Hojiblanca olive fat;\textsuperscript{19} however, the effects on the minor components still require research.

This work aimed to study the minor component (polar compounds, sterols, fatty alcohols, triterpenic dialcohols, waxes, and alkyl esters) profiles of the green Manzanilla and Hojiblanca fat throughout Spanish-style processing. General Linear Model, unsupervised (Principal Component Analysis and Hierarchical Clustering), and supervised (Discriminant Analysis) chemometric techniques were used for the characterisation, identification of transformations, and grouping the corresponding fat samples according to cultivars and production phases.

**MATERIAL AND METHODS**

**Olives.** Fruits were of the Manzanilla and Hojiblanca cultivars harvested at the so-called green maturation stage. They were provided by a local processor (JOLCA S.L., Huevar del Aljarafe, Sevilla, Spain).

**Processing.** Olives of each cultivar were processed in duplicate according to the green Spanish-style. It consisted of treating, in a 50 L container, 30 kg Manzanilla and Hojiblanca olives with 20 L of NaOH solutions at 25 and 30 g L\textsuperscript{-1} concentrations, respectively, until the alkali reached 2/3 of the flesh. Then the olives were washed with tap water for 18 h and brined in 35 L PVC containers (20 kg olives, 15 L brine), using a 90 g L\textsuperscript{-1} NaCl solution where they followed a spontaneous lactic fermentation. After equilibrium olive-flesh/brine, the NaCl concentration was \textasciitilde55 g L\textsuperscript{-1} and increased progressively to reach \textasciitilde60 g L\textsuperscript{-1} NaCl due to the evaporated brine replacement during the fermentation period. At the end of the fermentation (7 months), 10 kg olives from the two cultivars were packed (separately for each replicate) in glass containers. The
physicochemical conditions of brines were fixed to reach the equilibrium at 5 g L\(^{-1}\) lactic acid and 55 g L\(^{-1}\) NaCl. Both processing and packaging were intended to closely mimic the standard procedures usually followed for the elaboration of these cultivars at industrial scale.

**Samples and Fat Extraction.** Olive samples from each cultivar and replicate were withdrawn from the fresh fruits, lye-treated olives, the fermented fruits, and the final product (after two months of packaging). The samples were coded as M (Manzanilla) and H (Hojiblanca), followed by T0, T1, T2, or T3, respectively, to identify the successive processing phases.

The samples were extracted using the ABENCO system (Abengoa, Madrid, Spain). In short, the fruits were pitted and mixed with a homogeniser Ultraturrax T25 (IKA-Labortecnik, Staufen, Deutschland); then, hot water was added to the paste to reach about 30 °C in the suspension. The resulting mixture was subjected to malaxation for 40 min at room temperature (22 ±2 °C), and the liquid was removed by centrifugation using an ABENCO equipment. The liquid phase was allowed to decant, and the oil was obtained, filtered and subjected to analysis. The process was similar to that used for the estimation of olive oil yield\(^{20}\) and the efficient extraction of table olive fat\(^{3,5,8,9,10}\)

**Separation of Polar and non-Polar Compounds.** The oils were fractioned using silica gel columns, according to the procedure developed by Dobarganes et al.\(^{21}\)

**Determination of Polar Compounds.** The total polar compounds (PC) and their components (polymerized triacylglycerols (PTG), oxidised triacylglycerols (OxTG), diacylglycerols (DG), and free fatty acids plus traces of unsaponifiable matter (FFA)) were analysed according to the method developed by Dobarganes et al.\(^{21}\)
Determination of Sterols and Triterpenic Dialcohols. This analysis was performed according to the method described by the EU 1348/2013.\textsuperscript{22}

Determination of Fatty Alcohols. This analysis was carried out according to the method outlined by the EU 2015/1833.\textsuperscript{23}

Determination of Waxes and Alkyl Esters Contents. This analysis was performed according to the method described by EU 1348/2013.\textsuperscript{22}

Apparatus and Reagents. 

Apparatus. The determinations were carried out using an equipment HP1050 system (HPLC) equipped with a refractive index detector (Hewlett-Packard, CA, USA) and an HP 5890 Series II gas chromatograph (Hewlett-Packard, Minnesota, USA) fitted with a flame ionization detector. Reagents. All reagents were of analytical grade and chromatographic grade.

Statistical analysis. For statistical analysis, data were structured in a matrix array where rows were cases (treatments), and columns were variables (the contents of the minor components). The effect of processing phases on the minor components was studied by GLM and chemometric techniques (after data standardisation according to variables).\textsuperscript{13}

The chemometric analysis comprised the first survey of correlation, followed by Principal Component Analysis (PCA), Hierarchical Clustering (HC), and Discriminant Analysis (DA). For PCA only eigenvalues $\geq 1$ (according to the Kaiser criterion) were retained.\textsuperscript{24} DA was achieved by using the complete matrix of components, but for the deduction of the canonical and discriminant functions, only those variables containing the most powerful information were retained. The selection of variables was made by the backwards stepwise procedure, with 0.05 and 0.10 probability values for entering
and removing them. The minimum tolerance was fixed at 0.00001 and the number of steps at 100. A leaving-one-out cross-validation procedure was used for assessing the performance of the classification rule.¹²

Statistica software version 7.0 (StatSoft Inc., Tulsa, USA), XLSTAT (Addinsoft, 2017), and IBM SPSS Statistics Version 22 (IBM Corporation, Spain) was used for data processing.

RESULTS AND DISCUSSION

The samples of this study were subjected to the usual green Spanish-style table olive processing and packaging.² Therefore, the transformations observed in their olive fat mimic those expected at industrial scale. It should be pointed out that this work complements the previous research carried out on Manzanilla and Hojiblanca to study the effects of green Spanish-style processing on the quality parameters and fatty acid and triacylglycerols of olive fat. Also, green Spanish-style processing may introduce important changes on other fat/olive characteristics like α-tocopherols or pigment (chlorophylls and carotenoids).¹⁴,²⁵ However, this work is exclusively focussed on the changes that this processing style may produce on the olive fat minor components included in the olive oil legislations.⁶,⁷

Polar Compound Profiles. A previous description of the diverse polar compounds in diverse table olive processing was reported by Bianchi.⁴ Most of the polar compounds (PC) correspond to degradation products. The absence of polymerized triacylglycerol in the fats from all samples may indicate a limited secondary oxidation.²⁶ The concentrations of total PC were always higher in Manzanilla than in Hojiblanca, but processing did not produce systematic effects within cultivar (Table 1). The levels in Hojiblanca were reduced; however in Manzanilla were comparable to those observed
in recently extracted extra virgin olive oil (EVOO) (~25 mg/g) but lower than those found after 12 month olive oil storage (33 mg/g). The lowest values observed during ripe olive processing (18-50 mg/g) were similar to those reported here for Manzanilla but higher than those for Hojiblanca. Therefore, green Spanish-style processing is, apparently, less aggressive with the fat than Californian style processing (ripe olive); furthermore, its packaged products may have total polar compounds comparable to EVOO. In Italian cultivars, the initial contents of PC in the fresh fruit fats were of the same order than in this work; but, after fermentation as natural green olives, the contents were higher, regardless of cultivar. Similarly, the levels (27-37 mg/g) in the oils released from pitting of green Spanish-style or ripe olives were higher than those found in this work (Table 1).

Oxidized triacylglycerols (OxTG) constituted the major fraction of the polar compounds and were higher in Manzanilla than in Hojiblanca (Table 1). Due to the high stability and low volatility of OxTG, these compounds are considered as an index of the secondary oxidation level of oils, usually correlated with K270. However, the progression of these compounds into further oxidation products, like polymerised triacylglycerols, during green Spanish-style Manzanilla and Hojiblanca processing was not observed due to the reduced absolute changes observed in OxTG. This result is in contrast to the changes observed in K270 which had a marked decrease after lye treatment, followed by an increase in the fermented product but without influence of packaging. In ripe olives, the OxTG increased progressively throughout processing, leading to the similar concentrations in the packaged fruits of both cultivars (5.8 and 7.3 mg/g oil in Hojiblanca and Manzanilla, respectively), with levels below most of the values in Table 1. In the table olives conditioning operations, the lowest proportions of OxTG were observed in oils from the ripe olive pitting operation while the highest was
observed in oils from green olive pitting.\textsuperscript{12} The OxTG contents in green Spanish-style processing are higher (Table 1) than those found in green, directly brined, Italian cultivars, possibly because the fat oxidation was stronger and progressed further into PTG.\textsuperscript{26}

Diacylglycerols (DG) are usually considered as a useful index for oil quality and are produced by the hydrolysis of triacylglycerols.\textsuperscript{28,29} In this work, Manzanilla and Hojiblanca had low and similar contents at the end of processing (3.75-4.15 mg/g oil) (Table 1). The results are in contrast with the higher concentrations found in the fats from ripe olives (17-24 mg/g oil) or in oils released during the pitting and stuffing green table olives with vegetable products (15-30 mg/g oil).\textsuperscript{8,12} The difference may be attributed to the higher lipolytic activity in ripe olives or the environmental conditions, in the case of the released oils during the conditioning operations. The presence of DG was elevated in the fresh fruits of Italian cultivars which content significantly increased as the natural processing progressed, possibly because of the lack of inactivation of the lipolytic enzymes in this elaboration.\textsuperscript{26}

The free fatty acids (FFA) are also produced by hydrolysis of triacylglycerols.\textsuperscript{26,29} Their concentrations in this work did not have a clear trend during processing and, overall, the changes were limited (Table 1), although their highest concentrations were found in the packaged products in agreement with the acidity observed when studying the quality parameters of these olives.\textsuperscript{19} FFA was particularly high (~45 mg/g oil) in oils released from the pitting and stuffing with vegetable origin material green Spanish style table olives.\textsuperscript{12} Furthermore, there was also an evident increase of FFA during the natural processing of Italian cultivars, in agreement with the DG formation and the apparent higher enzymatic lipolytic activity in the non-lye treated olives.\textsuperscript{26}
Therefore, the overall hydrolytic degradation during processing green Spanish-style olives was limited and moderate in comparison with the changes in DG observed in natural fermentation.\textsuperscript{26} This reduced activity could be due to the partial inactivation of the enzymatic hydrolytic process in the lye-treated fruits and the low contribution of lipolytic yeasts to this degradation during fermentation since, at the moment, their effects are questionable as recently demonstrated in individually yeast inoculated processed olives.\textsuperscript{11} Shimizu et al. also found a good correlation between FFA and DG and demonstrated that the hydrolytic degradation in olive oil occurred during the previous to extraction storage.\textsuperscript{30}

**Unsaponifiable Fraction Profiles. Sterols.** Sterols are important non-glyceridic compounds usually related to the olive oil authenticity in the EU.\textsuperscript{22} Approximately, 10-15\% are esterified, but most of them (85-90\%) are free.\textsuperscript{31} Regardless of cultivar, sterol concentrations are remarkable (Table 2) and, therefore, green Spanish-style table olives may contribute to the beneficial effects of phytosterols as blood cholesterol-lowering agent.\textsuperscript{32} Total sterols, β-sitosterol, and campesterol concentrations were always higher in Hojiblanca than in Manzanilla (Table 2), and processing hardly affected their concentrations. Among components, β-sitosterol was the most abundant followed by Δ\textsuperscript{5}-avenasterol, campesterol and clerosterol (Table 2). The cholesterol content, regardless of cultivar, was higher in the fresh fruits than after processing but no systematic effects were detected in the other sterols (Table 2). Sterols contributed to discrimination among oils from diverse geographic regions in Apulian.\textsuperscript{33} Sterolic composition, with sitostanol, campestanol, and campesterol as the major contributors played an essential role in the characterisation of oils from some Tunisian olive cultivars.\textsuperscript{34} Oils from the same cultivar but different geographical areas were correctly classified using their sterol profiles.\textsuperscript{35} In Spanish commercial table olives, the sterol
contents were significantly different between cultivars but not between processing styles.\textsuperscript{5} In ripe olive, subjected to more degrading processing than the green Spanish-style products, $\beta$-sitosterol, $\Delta^5$-avenasterol and cholesterol significantly contributed to segregation according to elaboration phases.\textsuperscript{9}

Some of the sterols (in percentages) are subjected to constraints in the EU\textsuperscript{22} and the IOC\textsuperscript{7} for olive oil. Their proportions are relevant concerning olive oil classification with thresholds shown in parenthesis in the last row of Table 2, when appropriate. As observed, the percentages found for them in this work were mostly below their limits (Table 2, in brackets).

\textit{Triterpene Dialcohols.} These compounds are triterpenic pentacyclic dialcohols identified by Fedeli\textsuperscript{36} and are currently considered for the classification of olive oils according to the EU\textsuperscript{22}. Usually, the proportion of erythrodiol is higher in oils extracted by solvents than in those obtained by the press.\textsuperscript{22} In this work, the levels of erythrodiol were greater than those of uvaol and this, in turn, was higher in Hojiblanca than in Manzanilla (except for MTO). However, processing did not produce any systematic trend (Table 3). Also, the erythrodiol contents were close to the upper range of those observed in ripe olives (6-40 mg/kg oil in Hojiblanca; 54-87 mg/kg, in Manzanilla), but higher than in the oils released during the conditioning process of table olives (not detected-15 mg/kg oil) (Table 3).\textsuperscript{9,13} Uvaol was absent in oils from several steps of ripe olive processing and in oils from table olives conditioning operations.\textsuperscript{9,13} The erythrodiol+uvaol percentages (EU regulation) were higher in Manzanilla than in Hojiblanca but were not affected by treatments, regardless of cultivar (Table 3). Their levels were always below the limit established in the EU legislation for EVOO (4.5\%).\textsuperscript{22}
**Fatty Alcohols.** In olive oil, fatty alcohols with an even number of carbon atoms are abundant while those with odd are only found as traces. The total concentration of these compounds in fresh fruits was higher in Manzanilla (305±7 mg/kg oil) than in Hojiblanca (200±7 mg/kg oil). Tetracosanol (C_{24}) (except in Hojiblanca), hexacosanol (C_{26}) and octacosanol (C_{28}) markedly decreased after lye treatment (Figure 1). Docosanol (C_{22}) increased during processing in Hojiblanca but was stable in Manzanilla (Figure 1). Fatty alcohol levels increased with the olive oil extraction by solvent. In oils from seven autochthonous cultivars (Calabria, South of Italy) was found significant differences among cultivars and a general decline as harvesting time progressed.

**Wax and Alkyl Ester Profiles.** The waxes are esters of fatty alcohols and fatty acids. Most waxes in olive oils have an even number of carbons (C_{36}-C_{46}). Hydrolysis of triacylglycerols favours the formation of waxes. Wax content in pomace oil is usually higher than in EVOO; therefore, the wax level in oils is used to detect fraudulent mixtures. Waxes can also be formed spontaneously during olive oil storage, at rates depending on the proportions of reactants and environmental conditions. A detailed description of the wax composition, alkyl esters and methyl phenyl esters and their changes in table olives were reported by Bianchi. In this work, total waxes (C_{40}+C_{42}+C_{44}+C_{46}) and sum waxes (C_{42}+C_{44}+C_{46}) were always higher in oils from Manzanilla than in Hojiblanca but were not affected by processing (Table 3). In the EU regulation, the sum waxes for EVOO and virgin olive oil (VOO) should be ≤150 mg/kg; however, the threshold of total waxes affects only to lampante (≤300 mg/kg) or lower categories of olive oils (≤350 mg/kg). In this study, the sum waxes (Table 3) was always below the limit established in the EU for EVOO; furthermore, the total waxes were also far below the limits for lampante oil. Therefore, the wax content of the green Spanish-style table olives fat is comparable to those in EVOO. Concerning the
individual components, the levels of C_{40} and C_{42} were always significantly higher in Manzanilla (Figure 2, panel a and b) but C_{44} and C_{46} had greater contents in Manzanilla only after fermentation and packaging (Figure 2, panels c and d). The trends of C_{40}, C_{42}, and C_{44} were scarcely, or not, affected by processing, but C_{46} significantly decreased after lye treatment. The low proportion of waxes in oils from green Spanish-style olives (very far below the limits for EVOO) is a good indicator of the soft processing of this product.\textsuperscript{6} On the contrary, these compounds suffer a sharp increase in hard extraction conditions or careless oil storage.\textsuperscript{40}

Alkyl esters are produced by the reaction of free fatty acids and ethanol or methanol and are associated with fermentative deterioration of olives before extraction. Low levels indicate oils from recently picked olives and, indirectly, of good quality since their presence leads to sensory defects in olive oil.\textsuperscript{41} In olive processing, the formation of methanol during lye treatments is well known.\textsuperscript{42} Also, production of ethanol, methanol and other volatile during all table olive processing is common.\textsuperscript{2, 43} However, excessively high levels (450-550 mg kg\textsuperscript{-1} oil) in directly brined olives have been related to abnormal fermentation and poorly conducted technological treatments.\textsuperscript{17} In this work, lye treatment produced a significant increase of methyl esters regardless of cultivar but formed ethyl esters only in Manzanilla (Figure 3). However, fermentation significantly increased the ethyl esters in Hojiblanca and only ethyl oleate contents in Manzanilla (Fig. 3, panel b and c).

The limit for total ethyl esters established in the EU regulation for EVOO is ≤35 mg/kg.\textsuperscript{6} The ethyl ester concentrations in oils from fermented and packaged Manzanilla were below it and, on the contrary, those from Hojiblanca were above (Table 3); consequently, the fats from the final products of the green Spanish-style Hojiblanca table olives should not be considered as EVOO according to this criterion. However, the
direct application of the olive oil legislation\(^6\)\(^,\)\(^7\) to these fats, in this case, can be misleading because ethyl alcohols have not any unfavourable connotation in table olive storage/fermentation.\(^4\) Consequently, the use of ethyl ester as a quality criterion for table olive fat will be questionable. Furthermore, only oils with very high levels of ethyl esters (which is not the case) have been sensory assessed as defective (with fermentative notes like fusty, winery, vinegary, muddy sediment, or musty).\(^4\) Therefore, the levels reached in green Spanish-style table olives could hardly produce the unpleasant sensations found in oils from non-brined fusty/muddy olives.\(^4\)

Nevertheless, the formation of these volatile compounds during processing should not be underestimated. The activity of yeasts during fermentation should eventually be controlled by inoculation of lactic acid bacteria or by the use of yeast starter cultures with limited production of methyl or ethyl esters. They could prevent excessive formation of esters and preserve the processed olive fat quality in levels comparable to EVOO.

In general, ethyl esters and waxes were the minor components which showed the most consistent trend during processing, although their roles in the interpretation of the processing effects on table olive fat or sensory characteristics will still require further clarification.

**Segregation of Overall Minor Component Profiles throughout Processing by Multivariate Analysis.** The presence of several significant correlations among compounds qualified the data for multivariate analysis.

The PCA of data extracted five factors with eigenvalues >1. The overall variance explained by the two first Factors was 57.58% (Figure S1, a). Factor 1 was responsible for differentiation between cultivars (variance explained 36.69%) while Factor 2 was
expected to segregate according to treatments (21.09%). Oils from the fresh fruits of both cultivars were in the 2\textsuperscript{nd} quadrant well separated between them and from the processed olives. Oils from the different processing phases of Hojiblanca were situated on the right of the graph with lye treated olives being on the upper-left but without any trend among fermented and packaged olives (Figure S1, panel a). All Manzanilla samples, except the fresh fruit, were grouped on the 3\textsuperscript{rd} quadrant with scarce differences among them. Therefore, apparently, processing had a lower effect on the minor components of Manzanilla than on the Hojiblanca fat (somewhat dispersed in the graph).

Another approach for studying dissimilarities among treatments is cluster analysis, using the interaction cultivar·treatment as the grouping variable. Three clusters were obtained (Figure S1, panel b). The most considerable dissimilarity was also observed between cultivars. The technique segregated Hojiblanca from Manzanilla. However, the Hojiblanca cluster did not distinguish between fresh fruit and processing phases, grouping all the samples in the same cluster. Samples from Manzanilla were grouped into two closely related clusters, but significantly different. One consisted of fresh and lye treated fruits (plus one fermented sample) while the other was composed of fermented and packaged olives. Therefore, clustering was not efficient in segregating the fresh fruits but confirmed that the highest dissimilarity corresponded to cultivars; on the contrary, PCA was more consistent and did not lead to any wrong assignation.

A further study to differentiate among treatments was attempted by DA. The preliminary ANOVA showed that there were significant differences among treatments for most of the variables, except for $\Delta^5$-avenasterol and $\Delta^7$-stigmasterol (data not shown) and, therefore, DA application was viable. The most powerful discriminating variables were chosen by applying the backwards elimination (Table S1). The major
contributions to discrimination, as assessed by their standardized coefficients, were FFA (1.674, Function 1), ethyl oleate (1.790, Function 2), methyl oleate (-1.347, Function 1) and OxTG (-1.265, Function 1), C_{24} (-0.700, Factor 5), C_{26} (0.918, Function 3), C_{22} (0.947, Function 4), and β-sitosterol (0.931, Function 1) (Table S1). In this case, the first two functions accounted for high cumulative variance (83.6% vs only 57.58% in the PCA analysis), making the DA analysis reliable. In the plane of the two first function axes, the treatments are represented by their centroids while the samples within them are indicated by circles (Figure S2). All samples were correctly grouped. The overall situation was similar to that observed in the PCA but clearer. Segregation between cultivars was based on Function 1 (Manzanilla treatments are situated on the left of the similar Hojiblanca counterparts) and explained most of the variance (59.2%). Regardless of cultivars, the fresh fruits are situated at the bottom of the graph while the samples from the processed olives are placed above them. Therefore, Function 2 segregates among treatments, and the various processing phases are well differentiated within cultivar. However, Function 2 only accounts for 24.4% of the total variance; that is, for less than half of the cultivar variance (Function 1). Therefore, the DA has confirmed the differences between cultivars above commented and has also disclosed scarce but noticeable differences between processing phases, only partially revealed by PCA and clustering. The higher overall segregation capacity of DA (based on the high variance explained) was also reaffirmed by the confusion matrix which showed, even in the validation process, 100% correct assignations of samples to treatments.

In summary, the most affected compounds during processing were fatty alcohols and alkyl esters. In general, the contents of minor components were below or close to the limits established for EVOO by EU and IOC. Only the ethyl esters in fermented and packaged Hojiblanca were above them, due to their generation during fermentation.
Therefore, based on their composition, the fats from Manzanilla and Hojiblanca green Spanish-style table olives may have fat qualities comparable to EVOO. However, these transformations were scarce since, overall, the DA assigned most of the variability to cultivars (~59.2%) and only a limited proportion (~24.4%) to processing. That is, the differences in minor components between cultivars are more relevant than the changes introduced by processing. This result is in agreement with the results obtained for quality parameters and fatty acids and triacylglycerols, whose chemometric analysis also showed that most of the variance was due to differences between cultivars and only a reduced proportion to processing. In addition, the parameters with restrictions on the European or IOC legislation were within the limits established for them. On the contrary, Pasqualone et al. reported significant increase of all the indexes of oxidative and hydrolytic degradation of lipids; mainly the hydrolytic degradation was found higher than that reported in the literature for Californian-style (ripe) olives. Therefore, in general, the green Spanish-style table olive fat in Manzanilla and Hojiblanca cultivars preserves their original natural nutritive and healthy characteristics.

ASSOCIATED CONTENT

Supporting Information

Segregation as projections of the cultivar-treatment interactions on the plane of the first two Principal Component Factors and cluster dendrogram, standardized canonical discriminant function coefficients, and the corresponding projection of the cultivar-treatment interactions on the plane of the first two canonical discriminant functions (PDF).

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Notes

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FIGURE CAPTIONS

Figure 1. Fatty alcohol profiles (unweighted means) of Manzanilla and Hojiblanca olive fat throughout green Spanish-style processing. Vertical bars denote 0.95 confidence intervals.

Figure 2. Waxe profiles (unweighted means) of Manzanilla and Hojiblanca olive fat throughout green Spanish-style processing. Vertical bars denote 0.95 confidence intervals.

Figure 3. Alkyl ester profiles (unweighted means) of Manzanilla and Hojiblanca olive fat throughout green Spanish-style processing. Vertical bars denote 0.95 confidence intervals.
Table 1. Polar compound profiles of Manzanilla and Hojiblanca olive fat throughout green Spanish-style processing.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Treatment</th>
<th>Total polar compounds (mg/g oil)</th>
<th>Polar compound components (mg/g oil)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>OxTG</td>
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<tr>
<td>Manzanilla</td>
<td>Fresh fruit</td>
<td>20.91\textsuperscript{a}</td>
<td>14.14\textsuperscript{a}</td>
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<td></td>
<td>After lye</td>
<td>19.36\textsuperscript{ab}</td>
<td>13.37\textsuperscript{a}</td>
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<tr>
<td></td>
<td>Fermentation</td>
<td>17.85\textsuperscript{c}</td>
<td>11.61\textsuperscript{b}</td>
</tr>
<tr>
<td></td>
<td>Packaging</td>
<td>18.27\textsuperscript{bc}</td>
<td>11.42\textsuperscript{bc}</td>
</tr>
<tr>
<td>Hojiblanca</td>
<td>Fresh fruit</td>
<td>15.63\textsuperscript{d}</td>
<td>10.18\textsuperscript{c}</td>
</tr>
<tr>
<td></td>
<td>After lye</td>
<td>9.11\textsuperscript{e}</td>
<td>5.74\textsuperscript{e}</td>
</tr>
<tr>
<td></td>
<td>Fermentation</td>
<td>14.38\textsuperscript{d}</td>
<td>8.55\textsuperscript{d}</td>
</tr>
<tr>
<td></td>
<td>Packaging</td>
<td>14.44\textsuperscript{d}</td>
<td>7.71\textsuperscript{d}</td>
</tr>
</tbody>
</table>

Pooled Standard Error: 0.51 0.41 0.17 0.27

Notes: OxTG, oxidised triacylglycerols; DG, diacylglycerols; FFA, free fatty acids+residual unsaponifiable matter. Data in each treatment are the average (unweighted mean) of two samples per replicate, except fresh fruits (two samples per cultivar). Values within columns followed by different letters are different at p≤0.05.
Table 2. Sterol profiles (mg/kg oil or percentages, in parenthesis) of Manzanilla and Hojiblanca olive fat throughout green Spanish-style processing.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Treatment</th>
<th>Cholesterol</th>
<th>Campesterol</th>
<th>Stigmasterol</th>
<th>Clerosterol</th>
<th>β-sitosterol (apparent)</th>
<th>Sitostanol</th>
<th>Δ^5^-Avenasterol</th>
<th>Δ^5,24^-Stigmastadienol</th>
<th>Δ^7^-Stigmastenol</th>
<th>Δ^5^-Avenasterol</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manzanilla</td>
<td>Fresh fruit</td>
<td>8.21 b (0.8)</td>
<td>27.6 c (2.5)</td>
<td>14.78 b (1.4)</td>
<td>19.9 b</td>
<td>907 b (94.0)</td>
<td>22.8 a</td>
<td>52.8 a</td>
<td>18.6 b</td>
<td>7.0 abc (0.5)</td>
<td>7.95 abc (94.0)</td>
<td>1087 cd</td>
</tr>
<tr>
<td></td>
<td>After lye</td>
<td>5.74 c (0.6)</td>
<td>28.3 cd (2.8)</td>
<td>13.07 c (1.3)</td>
<td>14.5 c</td>
<td>864 c (94.0)</td>
<td>8.9 bc</td>
<td>43.4 bc</td>
<td>9.0 c</td>
<td>4.8 c (0.4)</td>
<td>6.25 bcd</td>
<td>998 d</td>
</tr>
<tr>
<td></td>
<td>Fermentation</td>
<td>5.83 cd (0.5)</td>
<td>34.9 b (2.9)</td>
<td>16.82 b (1.4)</td>
<td>15.7 c</td>
<td>1037 cd (94.0)</td>
<td>10.0 bc</td>
<td>50.5 a</td>
<td>8.6 bc</td>
<td>6.6 abc (0.4)</td>
<td>5.56 cd</td>
<td>1192 c</td>
</tr>
<tr>
<td></td>
<td>Packaging</td>
<td>5.64 cd (0.5)</td>
<td>31.5 bc (2.6)</td>
<td>19.16 a (1.6)</td>
<td>16.8 e</td>
<td>1079 c (94.6)</td>
<td>15.3 b</td>
<td>46.8 ab</td>
<td>8.6 bc</td>
<td>5.3 bc (0.4)</td>
<td>5.18 d</td>
<td>1233 c</td>
</tr>
<tr>
<td>Hojiblanca</td>
<td>Fresh fruit</td>
<td>11.52 a (0.6)</td>
<td>47.3 a (2.4)</td>
<td>15.93 b (0.8)</td>
<td>24.4 ab</td>
<td>1820 a (95.5)</td>
<td>8.7 bc</td>
<td>38.3 bc</td>
<td>7.7 bc</td>
<td>7.3 abc (0.4)</td>
<td>6.67 bcd</td>
<td>1988 ab</td>
</tr>
<tr>
<td></td>
<td>After lye</td>
<td>6.69 c (0.4)</td>
<td>41.7 a (2.3)</td>
<td>12.49 a (0.7)</td>
<td>26.4 a</td>
<td>1660 b (95.7)</td>
<td>7.9 c</td>
<td>44.5 abc</td>
<td>12.5 b</td>
<td>9.0 abc (0.4)</td>
<td>8.19 ab</td>
<td>1831 b</td>
</tr>
<tr>
<td></td>
<td>Fermentation</td>
<td>4.79 d (0.2)</td>
<td>45.3 a (2.3)</td>
<td>15.62 b (0.8)</td>
<td>24.6 ab</td>
<td>1795 a (95.7)</td>
<td>5.5 c</td>
<td>44.7 abc</td>
<td>5.2 c</td>
<td>9.9 ab (0.4)</td>
<td>9.79 a</td>
<td>1967 a</td>
</tr>
<tr>
<td></td>
<td>Packaging</td>
<td>5.84 cd (0.2)</td>
<td>45.6 a (2.3)</td>
<td>16.18 b (0.8)</td>
<td>24.1 ab</td>
<td>1817 a (95.7)</td>
<td>7.1 c</td>
<td>45.0 abc</td>
<td>6.9 bc</td>
<td>11.0 a (0.4)</td>
<td>9.72 a</td>
<td>1996 a</td>
</tr>
</tbody>
</table>

Pooled Standard Error 0.45 2.4 0.84 1.8 45 2.1 2.6 1.3 1.7 0.74 50

Limits EU and IOC (≤0.5) (≤4.0) (<camp.) (≥93.0) (≤0.5) ≥1000

Notes: Data in each treatment are the average (unweighted mean) of two samples per replicate, except fresh fruits (two samples per cultivar). Values within columns followed by different letters are different at p ≤ 0.05. Brassicasterol was not detected in these olive cultivars; apparent β-sitosterol is the sum of Δ^5,23^-stigmastadienol, clerosterol, β-sitosterol, sitostanol, Δ^5^-avenasterol, and Δ^5,24^-stigmastadienol. Values in parenthesis are the concentrations of sterols subjected to constraints and in the last row are the limits established for them in Olive Oil.16,6
Table 3. Triterpene dialcohols (erythrodiol, uvaol, erythrodiol+uvaol, erythrodiol+uvaol estimated according to the EU regulation), total waxes, sum waxes and total ethyl ester profiles of Manzanilla and Hojiblanca olive fat throughout green Spanish-style processing. Concentrations expressed as mg/kg oil, except for erythrodiol+uvaol EU in percentages.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Treatment</th>
<th>Erythrodiol</th>
<th>Uvaol</th>
<th>Erythrodiol+uvaol</th>
<th>Erythrodiol+uvaol EU</th>
<th>Total waxes (C40+C42+C44+C46)</th>
<th>Sum waxes (C42+C44+C46)</th>
<th>Total ethyl esters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manzanilla</td>
<td>Fresh fruit</td>
<td>34.0&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>7.64&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>41.6&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>3.69&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>63.74&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45,25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.21&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>After lye</td>
<td>26.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.48&lt;sup&gt;c&lt;/sup&gt;</td>
<td>31.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.97&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>63.97&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45,61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.35&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Fermentation</td>
<td>43.4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>4.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>47.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.81&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>63.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45,83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.05&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Packaging</td>
<td>44.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.27&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>50.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.90&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.27&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hojiblanca</td>
<td>Fresh fruit</td>
<td>22.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.2&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>1.54&lt;sup&gt;d&lt;/sup&gt;</td>
<td>39.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30,58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.56&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>After lye</td>
<td>31.0&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>9.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.7&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.13&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>37.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33,58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.89&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
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<td>Fermentation</td>
<td>21.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.47&lt;sup&gt;d&lt;/sup&gt;</td>
<td>36.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31,29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43.50&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Packaging</td>
<td>26.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.40&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>37.1&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>1.83&lt;sup&gt;d&lt;/sup&gt;</td>
<td>32.85&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29,38&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41.02&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Pooled Standard Error</td>
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<td>4.8</td>
<td>0.94</td>
<td>5.2</td>
<td>0.33</td>
<td>2.02</td>
<td>1.76</td>
<td>1.31</td>
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<tr>
<td>Limits EU and IOC</td>
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<td></td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

Notes: Data in each treatment are the average (unweighted mean) of two samples per replicate, except fresh fruits (two samples per cultivar). Values within columns followed by different letters are different at p≤0.05. Data in the last row are the limits established in Olive Oil <sup>5,6</sup>; the limit for the sum waxes applies to Extra Virgin Olive Oil (EVOO) and Virgin Olive Oil (VOO) while the total wax is for lampante, and total ethyl esters are for EVOO.
Figure 1
Figure 2
Figure 3