Andalusian Protected Designations of Origin of Virgin Olive Oil: The role of chemical composition in their authentication.

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ABSTRACT

The latest regulation to protect unique virgin olive oils that have particular properties is called protected designations of origin (PDOs), and it has proven to be a successful way to protect quality and geographical authenticity. Nevertheless, sometimes the registration of new PDOs associated to new demarcations of geographic areas are almost based on administrative aspects rather than objective chemical data. In this study we analyse the ability of chemical compounds of virgin olive oils with PDO that are not labile and their concentrations do not vary over time - fatty acids, alcohols, hydrocarbons, methyl-sterols and sterols - to differentiate them from those produced in their neighbouring geographical areas. Three cases in Andalusia region (Southern Spain) that combine pedoclimatic characteristics and variations of cultivar have been studied (9 areas with PDO and 3 non-PDO areas). With a modified procedure of ANOVA analysis - the Brown-Forsythe test, chemical compounds that showed good characteristics for the classification of samples were selected, and this ability was later checked by applying the unsupervised algorithm of Principal Component Analysis. Differences in their chemical composition were found when comparing the PDO oils with those produced in the neighbouring olive tree groves despite chemical criteria are not among the most decisive factors for registering PDOs.

Keywords: Virgin olive oil; Protected Designation of Origin; Chemical characterization; Minor compounds; Geographical traceability.

1. INTRODUCTION

The European Community established the controlled labelling of food products, including olive oil among others, based on geographical indications such as Protected Designation of Origin (PDO) to satisfy the increasing consumer demand on geographical identification.^[1] This legal framework keeps pace with consumer expectations and improves the protection of virgin olive oils (VOOs) with particular botanical, chemical and sensory characteristics. Thus, this protection is considered as a plus to guarantee quality, authenticity, tradition and safety of VOOs.^[2] Thus, VOOs being protected under well-defined requirements of a PDO should be explained by unique characteristics regarding their chemical composition and sensory properties. Analytical data could support the distinctive properties of oils under PDOs so allowing consumers to differentiate them from other PDOs. Additionally, chemical characterization of PDO oils also allows to preserve them from fake copies, this latest meaning a high potential risk. Therefore, if characteristics of PDOs are based on somewhat vague and imprecise information about VOO chemical content (e.g., such as content of phenols between 400 and 600 ppm) and sensory descriptors (e.g., “fresh grass”, “almond”, “light in the mouth”, “medium fruitiness”, “well-balanced and rounded”), soil characteristics of PDO geographical area (e.g., regosol), or even the historical references, then, the assumed singularities might not be sufficient to reveal a distinction from other PDOs and non PDO oils. A lack of chemical characterization may have authenticity implications since they might be vulnerable to fraud. On the other hand, simple chemical information such as the composition of fatty acids is not enough to characterize VOOs from PDOs.

The empirical fact is that most of PDOs are not based on particular measurable scientific characteristics of their VOOs. Instead, their adoption is related to varying characteristics of mill enterprises, managerial and supervisory staff, and differences and similarities between the practices implemented by PDO when compared with the rest.^[3] But we cannot rule out that, in

some cases, the decision to include or not a municipality in a PDO can be based almost exclusively in local policies.

This work investigates the possible singularities of VOOs produced by PDOs located in Andalusia - the first region in the rank of olive oil production in the world - by the analysis of chemical variables which concentrations do not change during their shelf-life (e.g. sterols and hydrocarbons). In this study, the analysis of information of particular characteristics that may vary over time - like sensory descriptors or the concentrations of phenols and pigments – has been avoided. These changing variables strongly depends on the time of VOO extraction or bottling.^[4-6] Thus, ripeness and shelf-life are involved in their concentration or values in addition to the geographical influence and they do not provide any guarantee when determining the genuineness of VOOs bottled with a PDO label.

The aim of this work is centred on the VOOs from Andalusian PDOs approved by the European Commission. There are twelve PDOs at the current time: “Antequera, Baena, Estepa, Lucena, Montes de Granada, Montoro-Adamuz, Poniente de Granada, Priego de Córdoba, Sierra de Cádiz, Sierra de Cazorla, Sierra de Segura, and Sierra Mágina” (Figure 1). Nine of these twelve PDOs have been selected to be characterized chemically and the current problems in the chemical characterization of potential PDOs were illustrated by analysing three different cases. The first one analyses two PDOs with the same main cultivar (var. Hojiblanca) and partially surrounded by orchards with this cultivar and identical climate and soil. The second case, however, studies three PDOs with several percentages of their cultivars and which VOOs are distinguished, or partially distinguished, from its neighbourhoods (PDO or non-PDO) probably due to the percentages of the cultivars in the production of their VOOs. The third example is much more complex as the geographical zone is the Spanish province of Jaén, where there are three PDOs surrounded by non-PDOs with the same main cultivar (var. Picual). Climate, soil and altitude of the orchards in the province of Jaén are not homogenous but diverse

and olive grove is mostly monoculture in the entire province. It is of importance to remark that this province contributes with more than 20% to the World production. Considering all this complexity in olive oil production, this study describes the differences in the concentrations of 64 chemical compounds in the VOOs produced by the co-operative societies of each one of the PDOs and their neighbouring producing zones. The objective is to achieve a complete characterization of the PDOs with minor compounds that may inform on slight differences between them. In order to have a realistic approach, the results were examined in terms of the whole production at regional level (PDOs in different provinces) and at smaller level (PDO with boundaries producing areas). The information of the inclusion of each municipality in each PDO was also considered when analysing the chemical differences.

2. MATERIALS AND METHODS

2.1. Samples

The VOO samples were supplied by the cooperative societies located in the Andalusian provinces of Córdoba, Jaén, Málaga and Sevilla. This procedure prevented getting doubtful samples that were mixtures of oils from different geographical origins or unknown olive grove.

Thus, 12 VOO samples were from the olive mills of PDO “Estepa” (coded as P1), which olive tree groves are mainly in the province of Sevilla, while 28 VOO samples (coded as P2 and P6) were from its neighbouring olive mills in the provinces of Málaga and Córdoba. Hojiblanca is the major cultivar though var. Arbequina is gaining importance over the years due to intensive plantations in P1 (around 10%),^[7] while var. Picual means more than 20% of the plantations in P6.^[8] Geographical location appears in Figure 1 while Table 1 shows information of samples and cultivars.

In the province of Córdoba, the PDO “Montoro-Adamuz” (P3) produces VOOs from two major cultivars: Nevadillo Negro and Picual (also known as Nevadillo Blanco). VOOs from

neighbouring cooperatives are located in the area of “Los Pedroches” (E1) in the North while the PDOs “Baena” and “Priego de Córdoba” are in the Southern part of the province, and the area “Campiñas” (E2) is in-between the former and the two latter PDOs; more information in Figure 1 and Table 1.

Jaén, the main worldwide producer area (province), has three PDOs named “Sierra Mágina” (P7), “Sierra del Segura” (P8) and “Cazorla” (P9). The rest of samples from this province are non-PDO oils although a PGI at European level has been applied for the entire province of Jaén. These VOOs produced by cooperative societies near the three PDOs in Jaén were clustered as E3 (Table 1). They are located in the areas named “La Loma” (J3), “El Condado” (J2) and “Sierra Sur” (J1) as shown in Figure 1.

2.2. Analytical Methods

Table 2 shows the chemical compounds identified and quantified for this study which have been clustered into fatty acids, hydrocarbons (n-alkanes and n-alkenes), alcohols (linear, terpenic and diterpenic) and sterols and methyl-sterols. All the samples were analyzed in duplicate.

The determination of fatty acid methyl esters (FAME) was performed according to the official method of the International Olive Council.^[9] Chromatographic analyses were carried out on a Varian 3900 chromatograph (Varian, Walnut Creek, CA) equipped with a flame ionization detector (FID).

The determination of alkanes and alkenes was performed following the method described by Aparicio and Alonso^[10]. Thus, 1 mL of standard solution of n-eicosane was added to 20 g of the oil sample. The mixture was then saponified with 75 mL of 10 % ethanolic potassium hydroxide for 30 min, and next distilled water (100 mL) was added and the solution was extracted twice with hexane (100 mL). The extracts were washed three times with a volume

of 100 mL of a mixture of ethanol-water (1:1); the water was removed with anhydride sodium sulphate. The solution was evaporated to dryness in a rotary evaporator at 30 °C under reduced pressure to reduced volume (1 mL). The hydrocarbons were separated from other components by column chromatography (50 cm x 1.5 cm i.d., with a Teflon stopcock, and filled with 15 g of silica gel slurried with hexane). The total unsaponifiable matter (0.2 g approx.) was dissolved in 1 mL of hexane and the solution was poured into the column head. The flask was rinsed three times with hexane; approximately 1 mL each time. These rinses were added to the column head, waiting for the previous addition to pass completely into the column. The sample was then eluted with hexane at a rate of about 1 mL/min. An aliquot of 60 mL was collected and then concentrated to approximately 1 mL in the rotary evaporator at 30 °C under reduced pressure, and the resulting sample was analyzed by gas chromatography. A fused silica column HP-5 (30m x 0.32mm i.d x 0.25 µm film thickness) (Agilent Technologies, Madrid, Spain) was used. Hydrogen was employed as carrier gas with a head pressure of 10 psi. The temperatures of the injector and detector (FID) were set at 290 °C and 320 °C, respectively. The oven temperature was held at 110 °C for 6 min and programmed to rise to 4 °C/min to a temperature of 300 °C, where it was held for 8 minutes. An injection volume of 1 µL was used.

The method for the saponification was carried out according to the IOC method^[11] while the isolation of the non saponifiable fraction and the separation of the sterols of this fraction in olive oil samples was implemented as described by Aparicio and Alonso^[10]. The bands of sterols and alcohols were, however, independently isolated and extracted with diethyl ether (30 mL) and evaporated to dryness again in the rotary evaporator. Next, the flasks were dried with N₂ for 10 minutes and silanized with 200 µL of a mixture of hexamethyldisilazane (HMDS), pyridine and trimethylchlorosilane (9:3:1) and then 1 µL of each solution was injected into the gas chromatograph. A fused silica column HP-5 (30m x 0.32mm i.d x 0.25 µm film thickness) was also used for the analyses of sterols and alcohols. Hydrogen was employed as carrier gas

with a head pressure of 10 psi. The temperatures of the injector and detector (FID) were set at 290 °C and 320 °C respectively. For alcohol separation the oven temperature was held at 200 °C for 8 min and programmed to rise 5 °C/min up to a temperature of 210 °C, where it was held for 15 min. The oven temperature was held at 263 °C for the separation of sterols.

2.3. Statistical Analysis

The data matrix of chemical compounds was analysed by uni-and multi-variate mathematical procedures. The Brown-Forsythe test was used to determine the homogeneity of the variances and selection of variables (chemical compounds) with univariate discriminate ability. The Brown-Forsythe test is a modification of the ANOVA and t-tests for mean differences in which the homogeneity of variance assumption is less determinant. This procedure gives quite accurate error rates even when the underlying distributions for the raw scores deviate significantly from the normal distribution. Principal Component Analysis (PCA) was used to determine whether the set of selected variables was able to discriminate the production of PDOs from their neighbouring olive oil producers. The statistical analyses were carried out with Statistica 6.0 (Statsoft Inc., Tulsa, OK).

3. RESULTS AND DISCUSSION

The regulations on the protected designations of origin (PDO) have had an important impact on the olive oil sector from the economic point of view. It has meant that the number of PDOs has increased up to a point that implementing methodologies that can control the incorrect labelling of the geographical origin of VOOs (for example, false PDOs) - which is one of the main priorities of producers and anti-fraud agencies - is currently the greatest challenge for the researchers in the context of geographical traceability. It is well-known the impossibility of finding a single marker of VOO that may be directly related to a single

geographical origin. However, the concentrations of some chemical compound, which are partially modulated by the pedoclimate characteristics of the olive grove and, obviously, the olive tree cultivar, can help though not without problems.

Table 2 describes the selection of the chemical series, which are invariant during olive oil shelf-life but are related to different factors like, for example, cultivar and pedoclimate variables. Those factors conform to the chemical profile of the olive oils produced in particular geographical origins. Thus, olive oils from orchards located in different hemispheres usually show different profiles of polyunsaturated fatty^[12-13] but also inside the same country^[14-15] while olive oils from orchards located above 1000 m have a profile of sterols and terpenic alcohols different from orchards located in valleys.^[16] The characterization of oils from different geographical origins is, however, mainly due to cultivars^[17-19] but also to climate, soil and agricultural practices.^[20-22] The conclusion of previous studies is that because the olive tree is an living organism, the inherent chemical characteristics of the oils from a particular cultivar in the areas studied in this work are modulated by the climate (e.g. temperature, humidity), the availability of elements and nutrients (i.e. soil), the agricultural practices (e.g. irrigation, fertilization), the olive maturation at harvesting time and the extraction and storage technologies as the main external variables although the latter has little or no influence since almost all the VOOs are produced by centrifugation processes (mainly 2-phases). Changes in these variables cause the variability in the concentrations of the chemical compounds. The variability in the profile of the chemical compounds observed over the years is then decisive to determine if a geographical area (e.g. PDO, PGI) produces particular VOOs regardless of the above cited variables.

3.1. Studying a PDO neighboured by other PDOs with the same main cultivar and soils.

Concerning the three selected cases to be studied, the first one analyses the differences of oils from three neighbouring geographical areas with the same main cultivar (var. Hojiblanca) and similar climate and soils. The first two areas corresponded to the PDOs “Estepa” and “Antequera”, while the third area corresponded to the PDO “Lucena”, although in this PDO only the samples from cooperative societies in the vicinity of the two previous PDOs were selected.

Applying the Brown-Forsythe algorithm, nine chemical compounds of Table 2 showed partial ability (Table 3) to distinguish VOOs produced in PDO Estepa (P1) from its neighbouring orchards: PDO “Hojiblanca” (P2) and PDO “Lucena” (E1). As the major cultivar (Hojiblanca) is common to all these geographical zones, the differences among P1 and its neighbourhoods P2 and P6 are not in the concentrations of major but in the minor compounds, perhaps due to variations in olive ripeness when harvesting or agricultural practices. On the other hand, the differences in the minor composition of the oils may be modified by the presence of percentages of minor varieties (e.g., Arbequina, Picual, Manzanilla, Lechín, Gordal) in each one these three geographical areas.

Palmitic and palmitoleic acids were the only two fatty acids selected to distinguish oils from PDO “Estepa” from those of the surrounding areas. Fatty acids have been previously applied for the geographical identification of oils by country and the two selected acids have also shown ability to distinguish the oils produced in Andalusia from the oils produced in other Spanish Autonomous Communities.^[21] The difference in the olive ripeness when harvesting could explain the variations in the concentration values, rather than assuming climate or altitude effects. Thus, palmitic acid is the end-product in the biosynthesis of fatty acids and the concentration of this fatty acid evolves during maturity, reducing the concentration in the last ripeness stages.^[23] The olives are usually harvested at earlier stages of ripeness in PDO “Estepa” in comparison with PDOs “Antequera” and “Lucena”. Thus, an earlier harvest would have an

impact in sensory properties and phenolic composition. On the other hand, the ability of palmitic and palmitoleic acids to classify the geographical origin of olive oils is also due to their low variability between crop seasons.^[24]

The lower concentration of 24-methylene-cycloarthanol in P1 compared to P2 and E1 is even more remarkable, the latter two showing similar values (~100 mg/100 g). Although it is thought that 24-methylene-cycloarthanol is synthesized by a methylation reaction of cycloarthenol,^[25] the latter does not show as many differentiation properties as the former. 24-Methylene-cycloarthanol is an end product in sterol biosynthesis and the concentration of this compound is highly dependent on ripeness; in fact, accumulation has been observed in the last ripeness stages.^[26] Since the concentration of this compound increases during,^[27] the concentration values in P1 point out again that their olives are milled when they are less ripe than those from P2 and P6. This difference in concentration also agrees with the percentage of VOOs from var. Arbequina in P1, around 8%,^[7] since this cultivar is harvested less ripe than var. Hojiblanca and Picual. The presence of Arbequina cultivar in the blends of this PDO would associate these oils with green and bitter attributes due to an earlier harvest, which would also explain other observed differences in composition. In fact, the concentration of 24-methylene-cycloarthanol has been used in classifying models to distinguish oils from Arbequina varieties from other varieties due to the low concentration found in this cultivar.^[28]

The concentration of Δ^5 -avenasterol has been associated with the percentage of stone with respect to the pulp in milled olives.^[29] The oils from P6 show slightly lower values for this compound and this result agrees with the percentage of VOOs from var. Picual in this area. The lower concentration of aliphatic alcohols is characteristic of P1^[30] and it has been related to a particular management of the olive oil extraction process (hammer milling and malaxing conditions). The differences in the percentages of fatty acids are also due to the varietal effect,

since the percentage of VOOs from var. Hojiblanca is much higher (>90%) in P2 (Table 1). Finally, hydrocarbons are related not only to cultivars but also to the kinds of soil.^[17, 21]

None of the compounds selected by the Brown-Forsythe test was, however, able to discriminate oils from the three geographical origins (P1, P2 and P6) simultaneously. The non-supervised statistical procedure of Principal Component Analysis (PCA) was then applied with the nine selected compounds (Table 3) to discriminate VOOs from the three geographical zones simultaneously by means of two factors. The objective was to check the effect of the chemical differences on the distribution of the samples when a multivariate exploratory analysis was applied.

Figure 2 shows the PCA plot with the first two principal components of VOOs. There are three great groups surrounded by their ellipses of confidence at 85%. The ellipses show that it might be possible to distinguish VOOs produced inside P1 (PDO “Estepa”) from those produced in P2 (PDO “Antequera”) although the proximity of olive tree groves from both PDOs mean a problem of not easy solution in terms of proposing analytical parameters for authenticity control. The proximity between PDOs has its paradigm in the VOOs produced in the geographical area of Puente Genil, a village of the province of Córdoba. Thus, a part of the olive tree groves of this village belongs to PDO “Lucena” – those ones located in the right bank of Genil river - while other olive tree groves belong to PDO “Estepa” – those ones located in the Southwest area of Genil river that is named Miragenil. This explains how close some samples from P1 (PDO “Estepa”) are to two samples collected inside P6 (samples from olive mills of Puente Genil) as displayed in Figure 2.

On the other hand, the geographical area of the PDO “Antequera” (P2) includes olive groves of the provinces of Málaga and Córdoba (Palenciana). In fact, the geographical area of this PDO is so large that data show that there is not homogeneity in the chemical composition of the VOOs there produced as Figure 2 shows. Furthermore, this PDO limits with PDOs “Estepa”

and “Lucena (Figure 1) as well. This explains, for instance, that in the PCA plot (Figure 2), two VOOs from the olive tree groves of Iznájar (Córdoba) and Rute (Córdoba) are near the samples from PDO “Antequera”.

3.2. Studying PDOs neighboured among them and with olive tree groves including different soils and cultivars.

The Northern part of the province of Córdoba (Figure 1), in which concerns olive tree groves, is split into two neighbourhood areas, “Los Pedroches” (E1), whose VOOs do not have an associated PDO, and “Sierra de Córdoba”, whose olive tree groves belong, almost completely, to PDO “Montoro-Adamuz” (P3). These geographical areas have very similar climate and soils (mainly Regosols and Cambisols).^[31] The province of Córdoba also has other three PDOs (“Baena”, “Priego de Córdoba” and “Lucena”, coded as P4, P5, and P6 respectively), which are located not too far from the PDO “Montoro-Adamuz” (<100 km) but in the Southern part and separated by the countryside of Guadalquivir river. Between these three PDOs and the Guadalquivir river there are the “Campiñas” areas (formerly “Campiña alta” and “Campiña baja”), coded as E2. “Campiñas” is located in-between the areas of PDO “Montoro-Adamuz” (P3) and the PDOs “Baena” (P4) and “Lucena” (P6).

The geographical area of PDO “Lucena is separated from the other two PDOs in the province of Cordoba (“Baena”-P4 and “Priego de Córdoba”-P5) by 31568 ha of The National Park “Sierras Subbéticas”. On the contrary, there is not any relevant natural barrier between geographical areas of the PDOs “Baena” and “Priego”. Furthermore, the terrain, which has slight undulations, is composed of clay soils with loamy materials in PDO “Lucena” but soils of these three PDOs are, in general, mainly Cambisols associated to Leptosols and Regosols.^[31] Table 1 shows that the main cultivar is Picual in all the PDOs excepting PDO “Lucena” in which the main cultivar is Hojiblanca.

The chemical characterization of oils from the PDOs located in the province of Córdoba to protect them against oils from other PDOs and non-PDO olive groves is a challenge. In fact, none of the ten chemical compounds selected by the Brown-Forsythe test (Table 4) is able to discriminate oils from the four PDOs simultaneously. However, two of them (24-methylene-cycloarthanol and cycloeucalenol) are able to distinguish VOOs of PDO “Montoro-Adamuz” from the other PDOs with $p < 0.95$. The low concentration of Δ^5 -avenasterol in VOOs from PDO “Montoro-Adamuz” seems to be related to the percentage of var. Picual in VOOs while the low concentration of 24-methylene-cycloarthanol has already been explained by the early harvesting of the olives.^[26] Table 4 also shows that the VOO chemical compounds of two neighbouring PDOs, Baena (P4) and Priego (P5), are very similar. In fact, only the concentration of octacosane hydrocarbon shows a slight difference between the olive oils from these PDOs.

The non-supervised statistical procedure of Principal Component Analysis (PCA) was also run with the selected compounds (Table 4) in order to know to what extent these compounds are able to discriminate simultaneously VOOs produced in the geographical zones by means of two factors. Figure 3 shows the distribution of the samples from the PDOs “Baena” (P4), “Priego” (P5) and “Lucena” (P6), and two non-PDOs “Los Pedroches” (E1) and “Campiñas” (E3) in the PCA plot. The samples from PDO “Montoro-Adamuz” (P3) are surrounded by their confidence ellipse at 80%. Inside this ellipse there is a sample from “Campiñas” (E2), which is a VOO from a cooperative society of Villa del Río that is near Montoro village (~11 km), one of the main municipalities of this PDO. There are also two samples of “Los Pedroches” (E1) in the vicinity of the confidence ellipse. They belong to VOOs produced in Pozoblanco, which is at less than 30 km away from PDO “Montoro-Adamuz” geographical area.

Figure 3 also shows that samples from PDO “Lucena” (P6) seem to be different enough from the other two PDOs. On the contrary, Figure 3 displays that there is not clear difference between VOO samples from “Baena” and Priego”. If we had also applied Linear Discriminant Analysis (LDA), we would have got a 100% of correct classification for all the PDO and non-PDO VOO samples. However, applying this supervised statistical procedure in this case would have been like to catch lightning in a bottle because LDA, with such amount of variables (chemical compounds) - would arrive hyper-optimist conclusions that would not be validated when adding new data of the same cooperative societies in successive crops.

3.3. Studying PDOs neighboured among them and with olive tree groves including similar soils and cultivars

The province of Jaén, which produced more than 20% of worldwide VOOs, has three PDOs that are located in the mountain region of this province (Figure 1). All the three PDOs neighbour a non-PDO area called “La Loma” (J3 in Figure 1) that is made of plains also planted with olive trees of the same cultivar (Picual). The PDO “Sierra Mágina” (P7) also neighbours a mountainous area so called “Sierra Sur” (J1) that is planted of Picual olive trees while PDO “Sierra del Segura” (P8) is adjacent to another mountainous area so-called “El Condado” (J2), which major cultivar is Picual as well. All the samples from the non PDO areas were grouped under the code E3 (Table 1). An analysis of the soils shows that the three PDOs and the other two non-PDO areas, which are located in mountainous areas,^[32] have very similar soils, basically constituted by calcaric regosols and lithic leptosols at high altitudes (> 700m) while soils contain calcic cambisols with variable amounts of rendzic leptosols and calcaric phaeozems at medium altitudes (between 400m and 700m). The soil of “La Loma” is constituted mainly by vertisols and vertic cambisols that change to calcaric regosols in the hills.^[31]

The altitude of the olive tree groves is also important for the content of some chemical compounds and they were already decisive for characterizing VOOs of PDO “Sierra del Segura” (P8) from the rest of the province when applied a successful expert system model.^[16, 33] In that study, however, there were not two other PDOs located in mountain ranges (“Sierra Mágina”, P7, and “Sierra de Cazorla”, P9) as there are now, which makes the selection of chemical compounds with ability to distinguish PDOs rather difficult when applying Brown-Forsythe at $p < 0.05$.

Almost half of the selected chemical compounds characterizing these PDOs (Table 5) are hydrocarbons, which had already proved their usefulness in geographical characterization.^[30] One of them is α -copaene, which is a mono-unsaturated sesquiterpene, that has been identified in all the varieties^[17]. The selection of α -copaene as a traceability marker for geographical identification agrees with Zunin et al. (2005)^[34], who used this compound together with other terpenoid hydrocarbons to distinguish Italian and non-Italian oils. Furthermore, this compound has been used in the geographical identification. Phytol, a diterpene alcohol, has also been selected as a variable in multivariate models to classify VOOs according to their geographical origin.^[24,30] The selection of this compound, which is a breakdown product of chlorophyll, is probably partially due to the correlation between phytol and the degree of ripeness of the olives.^[35] Other selected compounds have been explained previously (e.g., 24-methylene-cycloarthanol, β -sistosterol) or are probably related with the percentage of minor cultivars (Royal and Manzanillo de Jaén) in VOOs (e.g., stearic and linoleic fatty acids).

Despite the Brown-Forsythe test shows that some compounds are able to distinguish VOOs from PDOs and non-PDO areas, Figure 4 shows that the multivariate procedure of PCA displays a separation of samples that is slightly worse than the two previous cases. One of the reasons is that all the PDOs are located in mountainous areas with similar climate, soils and

altitude. Besides, they neighbour one another, which add more problem to their very similar pedoclimate conditions and cultivars. However, PCA plot seems to point out differences in composition between P8 “Sierra del Segura” and the rest of olive tree groves.

4. CONCLUSIONS

The study of these three cases shows that each PDO has a different degree of difficulty to be distinguished from VOOs produced in its surrounding areas, including other PDOs or non-PDOs. Thus, in the two first cases (Figures 2 and 3) some distinctive chemical characteristics were found for some PDOs. However, the results also prove that it is difficult to establish discreet limits defining the PDO areas. In fact, some VOOs obtained from olive tree groves located near the borderlines were misclassified. At the same time, the third case (Figure 4) involves a major challenge in the classification of samples due to the predominance of a single cultivar (Picual) and the large extension of land that are covered by olive tree groves, which lacks of distinct pedoclimate variables.

The chemical compositions of major and minor compounds provided a support to certify the singularity of VOOs from PDOs versus others when applied an expert system based on a large database that requires of enormous amount of information and efforts but it is not easy when applying unsupervised statistical algorithms.^[33] Furthermore, the number of PDOs increases year by year, which can reach a point that the singularity of PDOs will not be supported by chemical or sensory variables but only by administrative controls. The current physical-chemical information should be used by both administration and PDO councils to supplement arguments for approving or denying new PDOs.

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FIGURE CAPTIONS

Figure 1. Geographical areas of virgin olive oil Protected Designations of Origin (PDOs) located in Andalusia. They are: “Estepa” (P1) in the province of Sevilla, “Antequera” (P2) in the province of Málaga, “Montoro-Adamuz” (P3), “Baena” (P4), “Priego” (P5) and “Lucena” (P6) in the province of Córdoba, “Sierra Mágina” (P7), “Sierra del Segura” (P8) and “Sierra de Cazorla” (P9) in the province of Jaén, “Poniente de Granada” (P10) and “Montes de Granada” (P11) in the province of Granada, and “Sierra de Cádiz” (P12) in the province of Cádiz. The non-PDOs geographical areas are: “Los Pedroches” (E1) and “Campiñas” (E2) in the province of Córdoba, and “Sierra Sur” (J1), “El Condado” (J2) and “La Loma” (J3) in the province of Jaén.

Figure 2. Principal Component Analysis (PCA) of virgin olive oils produced inside three Protected designations of Origin (PDOs): P1 (“Estepa”), P2 (“Antequera”) and P6 (“Lucena”) that are near the two previous PDOs.

Figure 3. Principal Component Analysis (PCA) of virgin olive oils (VOOs) produced inside four Protected Designations of Origin (PDOs) - P3 (“Montoro-Adamuz”), P4 (“Baena”), P5 (“Priego”) and P6 (“Lucena”) - and two non-PDOs - E1 (“Los Pedroches”) and E2 (“Campiñas”).

Figure 4. Principal Component Analysis (PCA) of virgin olive oils produced inside three Protected Designations of Origin (PDOs) - P7 (“Sierra Mágina”), P8 (“Sierra del Segura”) and P9 (“Sierra de Cazorla”) - and from olive tree groves of non-PDO geographical areas near the borderline of the PDOs (E3) that are located in the areas: “La Loma”, “El Condado” and “Sierra Sur”.

Table 1. Codes of the samples analyzed in each protected designation of origin (PDO) and of the samples from neighbouring areas (non-PDO) with information of main cultivars and number of samples.

Code	Sample description	Province	Samples	Main Cultivars
P1	PDO “Estepa”	Sevilla	12	Hojiblanca (>80%), Arbequina, Lechin
P2	PDO “Antequera”	Málaga	16	Hojiblanca (≈90%), Picual, Arbequina
P6	PDO “Lucena” ¹	Córdoba	12	Hojiblanca (>90%), Ocal, Chorro
P3	PDO “Montoro-Adamuz”	Córdoba	16	Picual, Nevadillo negro
E1	“Los Pedroches” area	Córdoba	12	Picual, Nevadillo
E2	“Campiñas” area	Córdoba	15	Picual, Hojiblanco
P4	PDO “Baena”	Córdoba	23	Picual, Picudo, Pajarero ,Chorro
P5	PDO “Priego”	Córdoba	14	Picual, Picudo, Hojiblanca
P6	PDO “Lucena”	Córdoba	17	Hojiblanca (>90%), Ocal, Chorro
P7	PDO “Sierra Mágina”	Jaén	20	Picual (>90%)
P8	PDO “Sierra del Segura”	Jaén	20	Picual (>80%), Royal, Verdala
P9	PDO “Cazorla”	Jaén	12	Picual(>90%), Royal
E3	Neighbouring non-PDO ²	Jaén	40	Picual (>90%)

Note: ¹, only VOOs from olive tree groves located near the provinces of Sevilla and Málaga; ², VOOs from olive tree groves of the areas “Sierra Sur” (J1), “El Condado” (J2) and “La Loma” (J3) that are near the PDOs (P7-P9) according to Figure 1.

Table 2. Chemical compounds quantified in the samples.

Fatty Acids	n-Alkanes	Terpenic Hydrocarbons	Linear Alcohols
Palmitic	C17	α -Copaene	Docosanol
Palmitoleic	C21	Eremophylene	Tetracosanol
Margaric	C23	Muurolene	Pentacosanol
Margaroleic	C24	α -Farnesene	Hexacosanol
Stearic	C25	Allofarnesene	Octacosanol
Oleic	C26		
Linoleic	C27	Terpenic Alcohols	Methyl-sterols
Linolenic	C28	Taraxerol	Gramisterol
Arachidic	C29	Dammaradienol	Cycloeucalenol
Gadoleic	C30	β -Amirine	24-Ethyllophenol
Behenic	C31	Butirospermol	Citrostadienol
	C32	Cycloarthenol	Obtusifoliol
Sesquiterpenes	C33	24-Methylene-cycloarthanol	
Tridecene	C35	Cyclobranol	Sterols
Copane		24-Methylene-lanosterol	Campesterol
Valencene			Stigmasterol
Muurolene		Diterpenic Alcohols	β -Sitosterol
Farnesene		Phytol	Δ^5 -Avenasterol
Allofarnesene		Erythrodiol	Δ^7 -Stigmasterol

Table 3. Values (mean $\pm$ standard deviation) of the chemical compounds characterizing VOOs produced in PDO “Estepa” (P1), PDO “Antequera” (P2) and samples from PDO “Lucena” (P6).

Chemical compound	P1	P2	P6 ³	P ⁴
Palmitic acid ¹	10.15 $\pm$ 0.24	8.36 $\pm$ 0.26	9.39 $\pm$ 0.35	<i>a,b</i>
Palmitoleic acid ¹	0.86 $\pm$ 0.07	0.54 $\pm$ 0.03	0.78 $\pm$ 0.06	<i>a,b</i>
Docosanol ²	1.58 $\pm$ 0.13	2.46 $\pm$ 0.24	2.57 $\pm$ 0.53	<i>a,c</i>
Tetracosanol ²	2.92 $\pm$ 0.96	5.20 $\pm$ 0.55	5.12 $\pm$ 0.94	<i>a,c</i>
Δ^5 -Avenasterol ²	12.17 $\pm$ 0.70	13.09 $\pm$ 1.15	10.11 $\pm$ 0.64	<i>b,c</i>
β -sitosterol ²	168.64 $\pm$ 5.98	162.37 $\pm$ 12.55	146.65 $\pm$ 5.97	<i>b</i>
24-Methylene-cycloarthanol ²	70.78 $\pm$ 6.06	98.81 $\pm$ 8.81	101.62 $\pm$ 10.32	<i>a,c</i>
Cycloarthenol ²	15.26 $\pm$ 1.35	19.31 $\pm$ 1.27	12.73 $\pm$ 5.32	<i>a,b</i>
Pentatriacontane (C35) ²	0.02 $\pm$ 0.00	0.03 $\pm$ 0.00	0.04 $\pm$ 0.01	<i>a,c</i>

Note: ¹, %; ², mg/100g; ³, samples from cooperative societies near the provinces of Sevilla and Málaga; ⁴, test for independent samples by groups ($p < 0.050$: *a*, between P1 and P2; *b*, between P1 and P6; *c*, between P2 and P6).

Table 4. Values (mean $\pm$ standard deviation) of the chemical compounds characterizing VOOs with PDOs (“Montoro-Adamuz”-P3-, “Baena” -P4-, “Priego de Córdoba”-P5- and “Lucena” -P6-) and VOOs from their neighbouring non-PDO olive grove (“Los Pedroches” -E1- and “Campiñas” -E2-) of the province of Córdoba.

Chemical compound ¹	P3 ²	P4 ²	P5 ²	P6 ²	E1 ²	E2 ²	P ³
Cicloeucaleenol	3.58 $\pm$ 0.50	5.56 $\pm$ 0.50	5.60 $\pm$ 0.40	8.04 $\pm$ 0.90	5.02 $\pm$ 0.53	4.84 $\pm$ 0.61	a,b,c,g,j,n
Phytol	6.24 $\pm$ 0.87	4.00 $\pm$ 0.29	4.07 $\pm$ 0.36	5.29 $\pm$ 0.53	6.45 $\pm$ 0.74	3.57 $\pm$ 0.29	a,g,n
Hexacosanol	9.06 $\pm$ 1.18	6.69 $\pm$ 0.53	6.48 $\pm$ 0.27	6.21 $\pm$ 0.60	12.29 $\pm$ 1.50	9.47 $\pm$ 0.67	a,b, k,m,n
Δ^5 -Avenasterol	7.28 $\pm$ 0.64	10.10 $\pm$ 0.42	9.34 $\pm$ 0.68	10.46 $\pm$ 0.53	8.12 $\pm$ 0.43	8.57 $\pm$ 0.52	a,b,c,j,k,n
Taraxerol	0.92 $\pm$ 0.05	0.70 $\pm$ 0.05	0.62 $\pm$ 0.04	0.82 $\pm$ 0.05	0.96 $\pm$ 0.07	0.66 $\pm$ 0.06	a,c,j,n
24-Methylene-cycloarthanol	67.87 $\pm$ 4.01	100.30 $\pm$ 4.65	101.93 $\pm$ 5.84	97.21 $\pm$ 6.99	81.89 $\pm$ 4.44	76.46 $\pm$ 5.49	a,b,c,d,f,k,m,n
Tridecene	0.38 $\pm$ 0.03	0.60 $\pm$ 0.03	0.58 $\pm$ 0.05	0.23 $\pm$ 0.04	0.35 $\pm$ 0.04	0.61 $\pm$ 0.03	a,b,c,j,n
Copaene	0.05 $\pm$ 0.00	0.11 $\pm$ 0.02	0.11 $\pm$ 0.02	0.20 $\pm$ 0.03	0.05 $\pm$ 0.01	0.04 $\pm$ 0.01	a,b,c,j,k,m,n
Tetracosane (C24)	0.10 $\pm$ 0.01	0.07 $\pm$ 0.00	0.07 $\pm$ 0.01	0.08 $\pm$ 0.01	0.10 $\pm$ 0.01	0.07 $\pm$ 0.00	a,c,j
Octacosane (C28)	0.10 $\pm$ 0.00	0.12 $\pm$ 0.01	0.08 $\pm$ 0.01	0.13 $\pm$ 0.02	0.11 $\pm$ 0.01	0.11 $\pm$ 0.01	b,h,j

Note: ¹, mg/100g; ², mean $\pm$ standard deviation; ³, Test for independent samples by groups (p >0.05): a, between P3 and P4; b, between P3 and P6; c, between P3 and P5; d, between P3 and E1; f, between P3 and E2; g, between P4 and P6; h, between P4 and P5; j, between P5 and P6; k, between P4 and E2; m, between P5 and E2; n, between P6 and E2.

Table 5. Values (mean $\pm$ standard deviation) of the chemical compounds characterizing VOOs produced in PDOs named “Sierra Mágina” (P7), “Sierra del Segura” (P8) and “Cazorla” (P9) and from their neighbouring orchards (E3).

Chemical Compound	P7	P8	P9	E3	p³
Stearic acid ¹	2.77 $\pm$ 0.16	2.30 $\pm$ 0.11	2.80 $\pm$ 0.19	2.76 $\pm$ 0.12	b,c,d,e,f
Linoleic acid ¹	4.31 $\pm$ 0.21	4.16 $\pm$ 0.13	4.88 $\pm$ 0.27	4.36 $\pm$ 0.15	a,b,e,f
Gramisterol ²	0.56 $\pm$ 0.04	0.64 $\pm$ 0.34	0.79 $\pm$ 0.07	0.70 $\pm$ 0.03	a,b,d,e
Campesterol ²	4.73 $\pm$ 0.17	4.08 $\pm$ 0.09	4.30 $\pm$ 0.09	4.68 $\pm$ 0.10	c,d,e
β -Sitosterol ²	122.16 $\pm$ 3.53	106.44 $\pm$ 2.15	112.68 $\pm$ 2.04	118.84 $\pm$ 2.28	c
Phytol ²	3.05 $\pm$ 0.19	3.34 $\pm$ 0.13	3.08 $\pm$ 0.13	2.96 $\pm$ 0.09	e
24-Methylene-cycloarthanol ²	93.75 $\pm$ 8.65	84.18 $\pm$ 3.79	106.74 $\pm$ 8.19	92.69 $\pm$ 4.78	b,e,f
α -Copaene ²	0.04 $\pm$ 0.00	0.06 $\pm$ 0.00	0.05 $\pm$ 0.01	0.03 $\pm$ 0.00	c,d,e,f
Valencene ²	0.04 $\pm$ 0.01	0.07 $\pm$ 0.01	0.05 $\pm$ 0.01	0.04 $\pm$ 0.01	c,e
Tridecene ²	0.42 $\pm$ 0.04	0.31 $\pm$ 0.04	0.44 $\pm$ 0.03	0.47 $\pm$ 0.03	b,d
Tricosane ²	0.27 $\pm$ 0.08	0.14 $\pm$ 0.01	0.15 $\pm$ 0.01	0.16 $\pm$ 0.01	a,c,d
Hentriacontane ²	0.40 $\pm$ 0.02	0.38 $\pm$ 0.03	0.49 $\pm$ 0.04	0.47 $\pm$ 0.02	a,b,d,e
Tritriacontane ²	0.20 $\pm$ 0.01	0.19 $\pm$ 0.01	0.26 $\pm$ 0.02	0.24 $\pm$ 0.02	a,b,d,e

Note:; E3, VOO samples from olive tree groves that are neighbouring PDOs but are located inside the non-PDO areas of “Sierra Sur” (J1), “El Condado” (J2) and “La Loma” (J3); ¹, values in %; ², mg/100g; ³, Test for distinguishing samples (p <0.05): a, for P7 vs. P9; b for P8 vs P9; c, for P7 vs. P8; d, for P7 vs. E3; e, for P8 vs. E3; f, for P9 vs. E3.