Pigment Parameters Determining Spanish Virgin Olive Oil Authenticity: Stability During Storage

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ABSTRACT: The chlorophyll and carotenoid pigment composition in 12 mono-variety virgin olive oils was examined every 30 d during 1 yr of storage at 15°C in darkness. The oil authenticity parameters, as defined by the ratio of chlorophylls/carotenoids and the ratio of minor carotenoids/lutein, remained stable throughout storage, irrespective of the variety and degree of ripeness of the source fruit. The percent of violaxanthin, percent of lutein, and total pigment content, the classifying variables chosen as the best possible discriminators among olive varieties, also remained stable during storage. The prediction model for olive variety, which was based on a discriminant multivariate analysis of the observed values of these variables, gave a correct classification in 99.6% of the oils analyzed. The discriminant criterion established remained valid after 1 yr of oil storage. The detection of chlorophyll derivatives other than those associated with the physical extraction process was seen as a quality index, as small, analytically detectable transformations in the structure of pigments were indicative of oil storage.

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KEY WORDS: Authenticity, carotenoid, chlorophyll, multivariate statistical analysis, oil storage, olive oil, olive variety, stability of pigment parameters.

The crop from the olive tree alternates between high and low production from year to year, a condition known as "veceria." As a result, when the olive crop is plentiful, large amounts of oil are produced, part of which is stored in reserve for its commercialization the following year. The conditions under which the virgin olive oil must be stored before marketing can affect its chemical composition, and thereby its flavor and stability.

The literature includes studies aimed at understanding how parameters related to the oxidative stability of packaged virgin olive oil behave during its shelf life before reaching the consumer. Thus, Pagliarini *et al.* (1) have proposed models to predict the stability of extra virgin olive oil under various commercial conditions, and Procida and Cichelli (2) have studied changes in the antioxidant fraction of extra virgin olive oils stored in different types of packaging.

The photosensitizing capacity of the chlorophyll pigments has stimulated research aimed at knowing the effect of such compounds on the oxidative stability of oils under different storage conditions. Thus, Gutiérrez-Rosales *et al.* (3) studied the effect of adding chlorophylls on the stability of virgin olive oil during 3 mon of storage at 36°C in both light and darkness. The authors found that for oils stored in darkness, the concentration of the chlorophyll fraction remained constant throughout storage, whereas for those stored in light, the chlorophylls disappeared after 7 d. In no case did they observe a prooxidant effect associated with the photosensitizing nature of these compounds. However, Khan and Shahidi (4), who studied virgin olive oils kept at 60°C in light and in darkness, concluded that the chlorophylls do play a part in photo-oxidation. Psomiadou and Tsimidou (5,6) studied the stability of virgin olive oils to autoxidation and photo-oxidation by monitoring the presence of different minor components, among which were chlorophylls. They found an antioxidant role for such compounds that favored the stability of oils stored in darkness, whereas in the light the effect of the compounds could be prooxidant, depending on their concentration. The effect of the oxidative stability of a virgin olive oil on its initial organoleptic characteristics also has been established (7), since these qualities can be lost, above all when the oil is stored in the light (8).

One of the organoleptic characteristics most seriously affected during oil conservation is color, a quality attribute conferring commercial value. The color of a virgin olive oil is due to the solubilization of the lipophilic chlorophyll and carotenoid pigments present in the source fruit. Recently, Gandul-Rojas *et al.* (9) established the profile of intrinsic chlorophyll and carotenoid pigments of virgin olive oil in a study carried out with 50 mono-variety virgin olive oils, and proposed its use as an index of authenticity. The presence in virgin olive oil of carotenoid or chlorophyll pigments not described for this product can be used to determine whether its color is adulterated. Those authors also established that in all virgin olive oils, irrespective of the olive variety, the ratio of total chlorophylls/total carotenoids is around 1.14 ± 0.04 (SE). Similarly, the ratio of minor carotenoids/lutein is around 0.47 ± 0.03, except in the particular case of oils from cv. Arbequina olives, which have a value greater than 1, and in which this parameter—besides authenticating the oil as "virgin olive"—distinguishes the olive variety. In addition to these two parameters of authenticity, the percentage of violaxanthin (%V), the percentage of lutein (%L), and the total pigment content (TPC) were ideal classificatory variables because they distinguished between mono-variety virgin olive oils, enabling a model to be developed to predict the olive variety of the oil.

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The aim of the present study was to monitor possible variations in the established profile of chlorophyll and carotenoid pigments inherent in recently extracted virgin olive oil, and in the ratios between the different pigment fractions, when the oil must be stored in seasons of surplus production.

MATERIALS AND METHODS

Raw materials. The study was carried out using 12 mono-variety virgin olive oils (that is, each extracted from the fruit of a single variety) of five different olive cultivars from the main producing areas of Spain. Recently extracted oils were requested from industries throughout the entire harvesting season to obtain the greatest possible variability in oil color. The olive oil extraction process consisted of the following operations: preparation of a paste by milling and beating the fruit to form an oily continuous phase, solid–liquid separation by centrifugal decantation, and separation of the vegetable water by centrifugation (10). Oil samples were taken in the olive mill at the end of this process, at the outlet from the centrifuge. The samples provided were as follows: cv. Arbequina (A1, A2, A3, and A4) by the Cooperativa La Paz (Estepa, Seville); cv. Blanqueta (B1) by the Cooperatives Oleicoles Valencianes (Muro, Alicante); cv. Cornicabra (C1 and C2) by Aceites Toledo S.A. (Los Yébenes, Toledo); cv. Hojiblanca (H1, H2, and H3) by Olivarera Sor Angela de la Cruz (Estepa, Seville); and cv. Picual (P1 and P2) by Finca Venta del Llano (Menjibar, Jaén). An immediate analysis (initial, or time zero) was performed on the extracted oils. Next, the oils were distributed into amber glass jars of 65-mL capacity, with 3% (vol/vol) headspace. The jars were closed so as to be airtight and stored in a refrigerated chamber at 15°C in darkness. The samples were analyzed monthly for 1 yr. All analyses were performed in triplicate.

Extraction of pigments. Pigment extraction was performed with *N,N*-dimethylformamide (DMF) according to Mínguez-Mosquera *et al.* (11). The technique is based on the selective separation of components between DMF and hexane. The hexane phase carried over lipids and the carotene fraction, whereas the DMF phase retained chlorophylls and xantophylls. This system yielded a solution of pigments free from oil, which interferes with subsequent separation and quantification of pigments. All analyses were performed under green light.

Analysis of pigments. Analyses were carried out by HPLC using a liquid chromatograph fitted with an automatic injector and diode array detector. Data were collected and processed with an LC HP ChemStation (Rev. A.05.04). A stainless-steel column (25 × 0.46 cm i.d.), packed with 5 µm C_{18} Spherisorb ODS-2 (Teknokroma, Barcelona, Spain) was used. The column was protected with a precolumn (1 × 0.4 cm i.d.) packed with the same material. The solution of pigments in acetone was centrifuged at 13,000 × *g* prior to injection into the chromatograph (20 µL). Separation was performed using an elution gradient (flow rate 2 mL min^{-1}) with two mobile phases: (A) water/ion pair reagent/methanol (1:1:8, by vol), and (B) acetone/methanol (1:1, vol/vol). The ion pair reagent was 0.05 M tetrabutylammonium acetate (Fluka Chemie AG, Buchs, Switzerland) and 1 M ammonium acetate (Fluka) in water. The gradient scheme was described in detail in a previous work (12). Detection was simultaneously performed at 410, 430, 450, and 666 nm. Details on pigment identification and quantification by use of an external standard have been described in previous papers (13–15).

Statistical analysis of data. Data were interpreted by ANOVA, linear regression, and multivariate statistical analyses using the program SPSS for Windows v. 8.0.1S (1989–1998, SPSS Inc., Chicago, IL). Discriminant analysis was used to classify the oils, according to their mono-variety origin, into mutually exclusive groups based on the values observed for the set of independent variables, %V, %L, and TPC. The discriminant rule obtained thereby enabled a virgin olive oil of unknown origin to be assigned to a particular variety.

RESULTS AND DISCUSSION

Chlorophyll and carotenoid composition of the recently extracted oils. All the oils used in the present study showed the same initial qualitative composition in chlorophyll and carotenoid pigments, irrespective of the olive variety and the time of picking, in accordance with the profile determined in a previous work for virgin olive oil (9). Thus, the oils contained chlorophylls *a* and *b*, pheophytins *a* and *b*, and (in some samples) traces of oxidized chlorophyll derivatives, such as 13-OH-pheophytin *a* and 15^1-OH-lactone pheophytin *a*. The carotenoid fraction included lutein, β-carotene, β-cryptoxanthin, violaxanthin, neoxanthin, antheraxanthin, and the isomers 5,8-furanoid luteoxanthin, auroxanthin, and mutatoxanthin. Additionally, esterified xanthophylls and dephytylated chlorophyll derivatives were detected in the oils of cv. Arbequina; these compounds are present exclusively in this olive variety (15).

To confirm the parameters of authenticity established in the earlier work (9), as defined by the ratio of chlorophylls/carotenoids and the ratio of minor carotenoids/lutein, correlations were calculated between each pair of variables. The correlation found between chlorophylls and carotenoids was a straight line of slope B ± SE = 1.18 ± 0.07 ($R = 0.98$, $n = 12$). The linear regression model passing through the origin showed the best correlation coefficient, with a significance lower than 0.01%. Thus, the slope directly expressed the value of this parameter or ratio. This model was able to fit the 12 oils of the overall study, independent of the variety and degree of ripeness of the source olive, and was not statistically different (Duncan's test, $P < 0.05$) from that established earlier (B ± SE = 1.14 ± 0.04) (9). Analysis of the residual values showed that 2 of the 12 oils used in the study had a SD with an absolute value greater than 1× the mean SD of the residuals. One of them, H1, with a positive residual value, was an oil obtained from barely ripe olives (beginning of harvest); the other, H3, with a negative residual value, was an oil from very ripe olives (the end of picking).

Regarding the correlation between the minor carotenoids and lutein, the process was similar, yielding the straight line B ± SE = 0.48 ± 0.06 ($R = 0.91$, $n = 12$). In this case, all the residual values greater than 1× the mean SD of the residuals were for oils of cv. Arbequina. This made it possible to analyze correlations

that were specific to these cases (B ± SE = 1.20 ± 0.02; $R = 1$, $n = 4$). Besides authenticating oils as virgin olive oils, the ratio between minor carotenoids and lutein enabled oils from cv. Arbequina olives to be differentiated from the others. Of the remaining oils, H3, obtained from very ripe olives, again had a residual value below 1× the mean SD of the residuals.

Table 1 shows the initial values of the two parameters of authenticity mentioned above. The experiment was carried out with oils from fruit having different degrees of ripeness; thus there was some variability in these parameters, although always within the previously established limits (9). All the samples conformed in that, for each olive variety, the value of the chlorophyll/carotenoid ratio and the minor carotenoid/lutein ratio fell as the degree of ripeness of the source olives increased.

Table 1 also includes the values for the %L, %V, and TPC —parameters established as indices of authenticity for mono-variety oils (9). In the four samples of cv. Arbequina oils, %L was around 45%, whereas in the other varieties, it varied between 65 and 80% (depending on whether the oil was from the beginning, middle, or end of the harvest season). In general, the percentage increased with the ripeness of the source fruit. On the other hand, %V differentiated, not only for cv. Arbequina oils (with a mean value of 12.8%), but also for cv. Blanqueta oil (with a value of 9%) and for Hojiblanca oils extracted from less-ripe fruit (with values between 4.9 and 5.9%). For the other varieties, Cornicabra and Picual, and for the sample of Hojiblanca oil from the end of the harvest (H3), the percentage of violaxanthin was between 3 and 4%.

Applying the discriminant multivariate analysis technique, Gandul-Rojas *et al.* (9) obtained a classification of mono-variety oils from the values observed for these parameters (%V, %L, and TPC), enabling a model to be established that would predict the olive variety. Figure 1 shows the scores for the first and third discriminant functions obtained by applying the model to the 12 oils studied here, and for the oils used to construct the model. A correct assignment was achieved in all cases.

These results confirmed that each of the oils used in the present study complied with the parameters of authenticity regarding the pigments demanded of a virgin olive oil, and with those others inherent in each olive variety.

TABLE 1
Parameters of Authenticity of the Virgin Olive Oils Studied in the Present Work[a]

		Virgin olive oil		Mono-variety virgin olive oil		
Sample	Variety	Chls/Cars	Minor Cars/L	V (%)	L (%)	TPC (mg/kg)
A-1	Arbequina	1.50	1.18	12.55	45.89	21.30
A-2	Arbequina	1.44	1.26	12.43	44.17	20.03
A-3	Arbequina	1.15	1.17	12.73	46.16	17.09
A-4	Arbequina	1.08	1.19	13.47	45.68	16.51
B-1	Blanqueta	1.09	0.55	9.01	64.45	23.85
C-1	Cornicabra	1.33	0.54	3.00	65.09	24.18
C-2	Cornicabra	0.91	0.43	3.35	70.09	15.47
H-1	Hojiblanca	1.37	0.53	4.90	65.56	45.68
H-2	Hojiblanca	1.23	0.42	5.89	70.48	37.93
H-3	Hojiblanca	0.59	0.25	2.96	80.39	21.97
P-1	Picual	1.36	0.45	4.04	69.01	38.49
P-2	Picual	1.04	0.41	3.63	70.76	26.23

[a]The oil samples of each variety are numbered in decreasing order of TPC. Data are means of triplicate analyses (CV < 5% in all cases). Chls, total chlorophylls; Cars, total carotenoids; Minor Cars, minor carotenoids; L, lutein; V, violaxanthin; TPC, total pigment content.

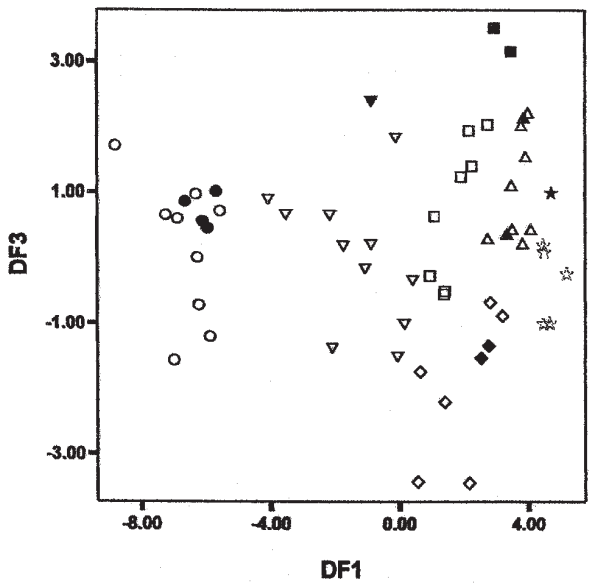

FIG. 1. Discriminant analysis of pigment parameters [percent violaxanthin (%V), percent lutein (%L), and total pigment content (TPC)] in recently extracted olive oils. Scatter plot of the discriminant function (DF) scores for each observation in the olive oil variety data set of six groups: Arbequina (○), Blanqueta (▽), Cornicabra (◇), Hojiblanca (□), Picual (△), and end-of-picking oils (☆). Open symbols are data from Reference 9, and closed symbols are data obtained in the present work.

Changes in pigments during oil storage. All the oils were subjected to the same storage conditions, and their pigment composition was analyzed monthly. Qualitatively, the most significant change detected was a generalized progression of the chlorophyll pheophytinization reaction, initiated during the oil extraction process as a consequence of the released acidity. The rate of pheophytinization differed, probably because of the FFA content inherent in each oil. This observation is currently under further study. At the same time, there was a very slight rise in the hydroxylation on C-13[2] of pheophytin *a* and a small formation of pyropheophytin *a*, a pigment absent from the recently extracted oil.

In the carotenoid fraction, which is more stable than that of chlorophylls, the progressive isomerization of the 5,6-epoxide groups of the minor xanthophylls antheraxanthin, violaxanthin, luteoxanthin, and neoxanthin was detected in only some oils.

Changes in the indices of oxidative stability are currently under study to discover whether transformation of the chlorophyll molecule during oil storage affects oil quality to any extent.

The qualitative pigment profile, established earlier as a parameter of virgin olive oil authenticity, was also seen as an index of quality, as small, analytically detectable transformations in the structure of pigments were indicative of oil storage.

Stability of the parameters of authenticity. Similar to the earlier study, the linear regression model passing through the origin showed the best correlation coefficient between chlorophylls and carotenoids on one hand—B ± SE = 1.16 ± 0.02 ($R = 0.98$, $n = 156$)—and minor carotenoids and lutein on the other—B ± SE = 0.47 ± 0.02 ($R = 0.91$, $n = 156$)—in the set of 156 samples

analyzed (12 oils × 13 mon). In the first case, analysis of residual values differentiated the 13 oils (those of the initial sample H1) in the model for the beginning of harvest and another 13 (those of the initial sample H3) fitting the correlation for the end of picking. In the case of the correlation between minor carotenoids and lutein, oils of the cv. Arbequina were again differentiated.

ANOVA for the calculated values of the ratio of total chlorophylls/total carotenoids and the ratio of minor carotenoids/lutein for each oil during the storage period demonstrated that no significant differences between the values were obtained for each of these virgin olive oil authenticity indices (Duncan's test, <0.05), confirming their constancy during 1 yr of storage. Thus, the parameters of authenticity established for virgin olive oil remained stable in the oil stored at 15°C in the darkness, irrespective of the oil variety and whether it came from fruit at different degrees of ripeness.

The classifying parameters %V, %L, and TPC were selected as indices of variety authenticity as the best possible discriminators between the groups (varieties). ANOVA of the values obtained for each parameter during the storage time demonstrated no statistically significant changes (Duncan's test, <0.05), confirming their constancy during 1 yr of storage.

Therefore, applying the model to predict the olive variety from the set of 156 oils analyzed yielded a correct classification in 99.4% of cases. Figure 2 displays the values obtained for the corresponding first and third discriminant functions. The first discriminant function, which showed the best correlation with the variable %V and explained 85% of the variance, enabled separation of the cvs. Arbequina, Blanqueta, Picual, and the end-of-harvest group. The third discriminant function, which explained 100% of the accumulated variance, was correlated mainly with the variable TPC and distinguished between the cvs. Cornicabra and Hojiblanca.

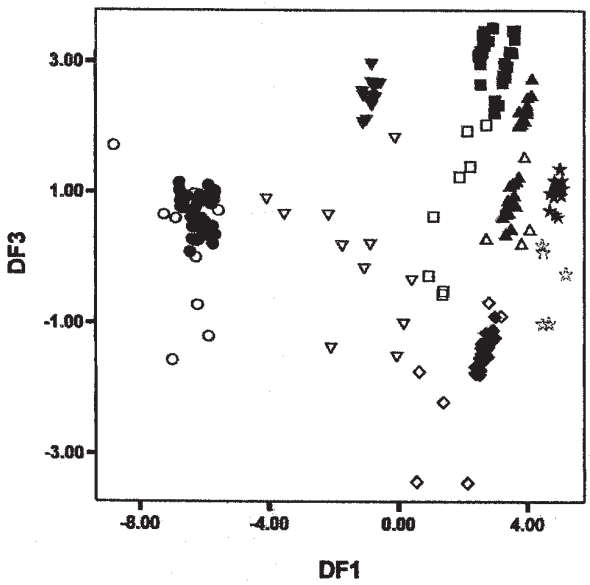

FIG. 2. Discriminant analysis of pigment parameters (%V, %L, and TPC) in recently extracted olive oils and throughout the 12 mon of storage. Scatter plot of the DF scores for each observation in the olive oil variety data set of six groups. Symbols and abbreviations are as shown in Figure 1.

These results demonstrated that the parameters of authenticity of mono-variety virgin olive oils remained statistically unaltered during 1 yr of storage; therefore, the model used to predict the olive variety based on these parameters remained valid throughout this period.

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