Use of Chlorophyll and Carotenoid Pigment Composition to Determine Authenticity of Virgin Olive Oil

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ABSTRACT: The chlorophyll and carotenoid pigment profile of 50 mono-variety virgin olive oils was used to develop an index of authenticity for the product. The presence of carotenoids other than those described, or chlorophyll derivatives at another level of degradation, were found to be determing elements of this index for "virgin" olive oil quality. In addition, the ratio of chlorophyll/carotenoid should be around 1, and the ratio of minor carotenoids/lutein should be about 0.5, with a limited variability. These characteristics may be expected of virgin olive oil in general and are independent of variety. Finally, the percentage of lutein, violaxanthin, and total pigment content may be used to distinguish between mono-variety virgin olive oils.

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KEY WORDS: Authenticity, carotenoid, chlorophyll, multivariate statistical analysis, olive oil, olive variety, pigment.

Virgin olive oil, an excellent natural food, is obtained from olive fruit (*Olea europaea*, L.) by mechanical or physical procedures (such as milling, beating, centrifugation, and decantation). Its composition varies widely, depending on fruit variety, degree of fruit ripeness, environmental conditions, growing region, and techniques of processing and storage (1). These factors influence oil color, which is one of the basic quality characteristics of virgin olive oil. Olive oil color is due solely to the solubilization of pigments present in the original fruit. The product is best defined by a green-yellowish color, although the green hue may vary depending on the variety and the predominant state of ripeness of the mixture of fruits used (2,3).

Until relatively recently, this quality attribute was evaluated by visual comparison with standard solutions, using an adaptation of the methods developed for oilseeds (4–6). Currently, colorimeters are being introduced to automate this process (7). However, colorimetric methods are not well suited for olive oil because the instruments have been optically designed for oils without green or blue hues. As a result, color measurement is distorted and inaccurate in oils with green coloration (8).

The study of chlorophyll and carotenoid pigment composition in edible products derived from olives is relatively recent. Analysis of chlorophylls in a lipid matrix required the special adaptation of methods to isolate, identify, and quantify the chlorophyll and carotenoid pigments in the olive fruit (9,10). The use of high-performance liquid chromatography (HPLC) in the separation and quantification of these pigments in olive oil allows the simultaneous analysis of chlorophylls and carotenoids, and is applicable to any type of vegetable oil (11). Considerable advances have been made in understanding the metabolism of these compounds during the olive ripening process (12) and the effect of the different systems of industrial processing on their initial structure in fruits meant for use as table olives (10) or for oil extraction (2). At the same time, the apparent color (measured from the chromatic coordinates L, a, and b) has been correlated with the pigment composition in virgin olive oil (3).

Analysis of the content and type of pigments present in both fruits and the corresponding oil shows that the oil-obtaining process causes a structural transformation of pigments, due to the liberation of acids, and a considerable loss of pigmentation, mainly in the chlorophyll fraction. This loss may involve co-oxidative reactions of lipid peroxides and the enzyme lipoxygenase (2). The effect of the extraction conditions on the color and quality of oil has been studied by Papaseit (13). This work compared absorbance indices of carotenoid color and chlorophyll color in oils with different extraction systems at different temperatures. In addition, Ranalli (14) used chromatographic methods to identify variation in carotenoid composition in virgin olive oils extracted by different methods. Escolar *et al.* (15) proposed that the chromatic parameters defining the color of commercial virgin olive oil are within a narrow, fixed range, and might be related to the quality of virgin olive oil. Ranalli and Modesti (16) concluded that genetic factors (olive variety) also have a significant effect on the quality of extracted oil.

Hence, the pigments responsible for fruit color are used to verify the authenticity of the derived products. As an example, the anthocyanin pattern of each fruit authenticates the source of both commercial jam (from strawberry, blackberry, raspberry, blueberry, blackcurrant, and cherry) (17) and juices (including aronia, raspberry, blackberry, blackcurrant, cherry, and red grape) (18).

Earlier studies characterizing chlorophyll and carotenoid composition in mono-variety virgin olive oils from the main producing areas of Spain showed the existence of variety-

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conditioned characteristics affecting the content, proportion, and exclusiveness of pigments. Independent of pigment content, the ratio between the chlorophyll and carotenoid fractions remained constant, with a value close to unity (19). Therefore, the presence of a specific pigment profile could become a requirement of virgin olive oil, and the ratio between pigments could be used to guarantee the authenticity of the product. This would help prevent the marketing of fraudulent mixtures with vegetable oils from another source or type of processing.

In the present work, the pigment composition of a wide range of mono-variety oils was used to standardize new parameters and to develop criteria of quality assurance for the authenticity of virgin olive oil. The pigments responsible for its color, an attribute that at present is evaluated only organoleptically, are proposed as a new index of quality and authenticity of virgin olive oil.

MATERIALS AND METHODS

Raw materials.The study was carried out using 50 mono-variety virgin olive oils (each one made by processing fruits from a single variety) of six different olive varieties from the main producing areas of Spain. The oils requested from industry had recently been extracted to avoid any kind of contamination. Oil was extracted from fruits picked at the beginning, middle, and end of the harvest to obtain the greatest possible variability in oil color. These oils were classified as "extra virgin" or "common virgin" by sensorial characteristics and analytical indices (1). The process to extract olive oil 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 (1). Sampling was carried out in the olive mill at the end of this process, at the outlet from the centrifuge. The nine oils of the Arbequina variety were provided by Cooperativa La Paz (Estepa, Sevilla, Spain): one of them from the beginning, five from the middle, and three from the end of the harvesting period. The oils of the Blanqueta variety were provided by Cooperatives Oleicoles Valencianes (Muro, Alicante, Spain): five oils were from the beginning and six from the middle of the harvesting period. The samples of Cornicabra were provided by Aceites Toledo S.A. (Los Yébenes, Toledo, Spain): two samples were from the beginning, five from the middle, and two from the end of the harvesting period. The Hojiblanca variety oils were provided by Olivarera Sor Angela de la Cruz (Estepa, Sevilla, Spain): one sample was from the beginning, seven were from the middle, and three were from the end of the harvesting period. The Picual variety oils were provided by Finca Venta del Llano (Menjibar, Jaén, Spain): two samples were from the beginning and six samples from the middle of the harvesting period. The Lechín variety oils were provided by Finca Venta del Llano (Menjibar, Jaén, Spain): one sample was from the beginning and one from the middle of the harvesting period.

Extraction of pigments. Pigment extraction was performed with *N,N*-dimethylformamide (DMF) according to Mínguez-Mosquera *et al.* (2). The technique is based on the selective separation of components between DMF and hexane. The hexane phase carried over lipids and the carotene fraction while the DMF phase retained chlorophylls and xanthophylls. This system yielded a solution of pigments free from oil which interferes with subsequent separation and quantification of pigments. All analyses were performed in triplicate under green light.

Analysis of pigments. Details about the pigment identification have been described in previous papers (11,19,20). Separation and quantification were carried out by HPLC using an HP 1100 Hewlett-Packard (Godalming, Surrey, England) liquid chromatograph fitted with an HP 1100 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* (MSE Model micro centaur; Fluka, Chemic Ab, Buchs, Switzerland) prior to injection into the chromatograph (20 μL). Separation was performed using an elution gradient (flow rate 2 mL min^{-1}) with the mobile phases: (A) water/ion pair reagent/methanol (1:1:8, vol/vol/vol) and (B) acetone/methanol (1:1, vol/vol). The ion pair reagent was 0.05 M tetrabutylammonium acetate (Fluka, Chemie AG, Loughborough, England) and 1 M ammonium acetate (Fluka) in water. The gradient scheme has been described in detail by Mínguez-Mosquera *et al*. (11). Detection was simultaneously performed at 410, 430, 450, and 666 nm. External standard calibration was used for the quantitation of 25 pigments for sample. These data were interpreted by analysis of variance, linear regression, and multivariate statistical analysis using the program SPSS for Windows v. 8.0.1S (SPSS Inc., Chicago, IL, 1989–1998).

RESULTS AND DISCUSSION

Intrinsic pigment profile of extra virgin olive oil. The qualitative study of pigments in the 50 virgin olive oils demonstrated a common pattern that is not dependent on variety, ripeness of the fruit, or extraction technique. A typical chromatogram of pigment extract from virgin olive oil is shown in Figure 1. Apart from the pigments that conform to the general pattern, in varieties such as Arbequina, other unique pigments have been found. Their identification has been described previously (19).

The color of virgin olive oil is determined by the chlorophyll pigment fraction and carotenoids initially found in the fruits plus derivatives formed by acids released during milling and beating. During the extraction process, a proportion of the native chlorophylls is transformed into pheophytins when the central Mg^{2+} ion of the porphyrin ring is substituted by H^+. This reaction is visually very striking, because it directly affects the chromophore group of the chlorophyll, and the

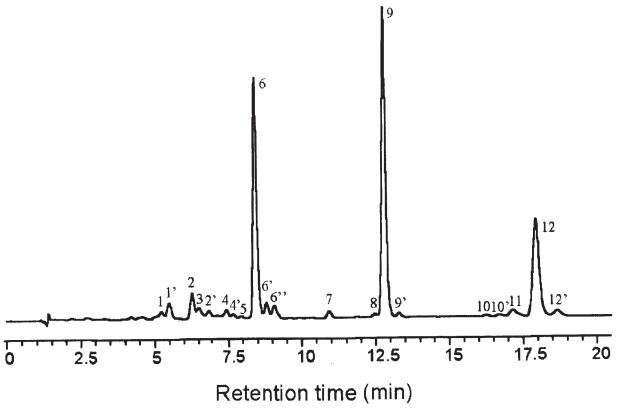

FIG. 1. High-performance liquid chromatogram of the pigments from Hojiblanca variety virgin olive oil (H3 code). Peaks measured at 410 nm: 1, neoxanthin; 1′, neoxanthin isomer; 2, violaxanthin; 2′, violaxanthin isomer; 3, luteoxanthin; 4, antheraxanthin; 4′, antheraxanthin isomer; 5, mutatoxanthin; 6, lutein; 6′ and 6″ lutein isomers; 7, chlorophyll *b*; 8, β-cryptoxanthin; 9, chlorophyll *a*; 9′, chlorophyll *a*′; 10, pheophytin *b*; 10′, pheophytin *b*′; 11, β-carotene; 12, pheophytin *a*; 12′, pheophytin *a*′.

color changes from bright green to olive brown. Only a part of the original chlorophyll content of the fruit remains intact in the oil. In the carotenoid fraction, the acidity of the medium causes isomerization of the 5,6-epoxide groups to 5,8-furanoids. As a consequence, luteoxanthin, auroxanthin, neochrome, and mutatoxanthin have been found in the oils, in addition to violaxanthin, neoxanthin, and antheraxanthin (2,11).

In some virgin olive oils, traces have been detected of easily formed chlorophyll derivatives oxidized on carbon-13 (OH-pheophytins and lactone-pheophytins), whose presence may be due both to the conditions inherent to the extraction process and to the possible activity of the enzyme chlorophyll oxidase (21). In oils from fruits with a high chlorophyllase activity, as is the case of the Arbequina variety, chlorophyllides and pheophorbides also have been found. These compounds are products of the enzymatic deesterification of the alcohol phytol in the molecules of chlorophyll and pheophytin, respectively (19). α-Carotene and esterified xanthophylls, typical of the exclusive carotenoid pattern of the fruits of this variety (22), also were present.

Consequently, virgin olive oil should contain only pigments from the fruit plus derivatives associated with the extraction process: specifically, pheophytins (*a* and *b*), 5,8-furanoid isomers of the original xanthophylls, traces of oxidized chlorophylls, and, in certain cases, deesterified chlorophyll derivatives. The presence of carotenoids other than those described, or of chlorophyll derivatives at another level of degradation, indicates that the oil is not of "virgin olive" quality.

Isochromic pigment fractions. With the intrinsic pigment profile of virgin olive oil established, it becomes important to know whether these pigments naturally maintain constant ratios that could be required as a guarantee of an oil's authenticity. The color intensity of the oil is determined by changes in the source fruits' pigment content during ripening. Although the pigment concentration in the fruit differs greatly with the variety, it decreases with ripening where chlorophylls disappear faster than carotenoids (9,12,22). Thus, color intensity and hue depend not only on the source fruit variety but also on the predominant ripeness state of the raw material. The olive has an intense green color that turns green-yellowish with the gradual decrease in chlorophylls and carotenoids. Then, anthocyanin compounds are synthesized and begin to cover the skin with reddish spots from the apex to the peduncle (a color stage denominated pintón), until the fruit becomes intensely violet (purple) and, finally, black. Coloring continues inward through the mesocarp toward the pit. At the start of picking, when the proportion of green fruits on the tree is still high, the oils obtained are greenish because their chlorophyll pigment fraction is clearly predominant. As harvesting proceeds, the proportion of green fruits decreases, while that of pintón, purple, and black fruit increases. In this progression, the oils become more golden, and even yellow at the end of the processing period with the decrease in the chlorophyll/carotenoid ratio.

Figure 2A shows pairs of values for the two isochromic fractions (total chlorophylls and total carotenoids) after

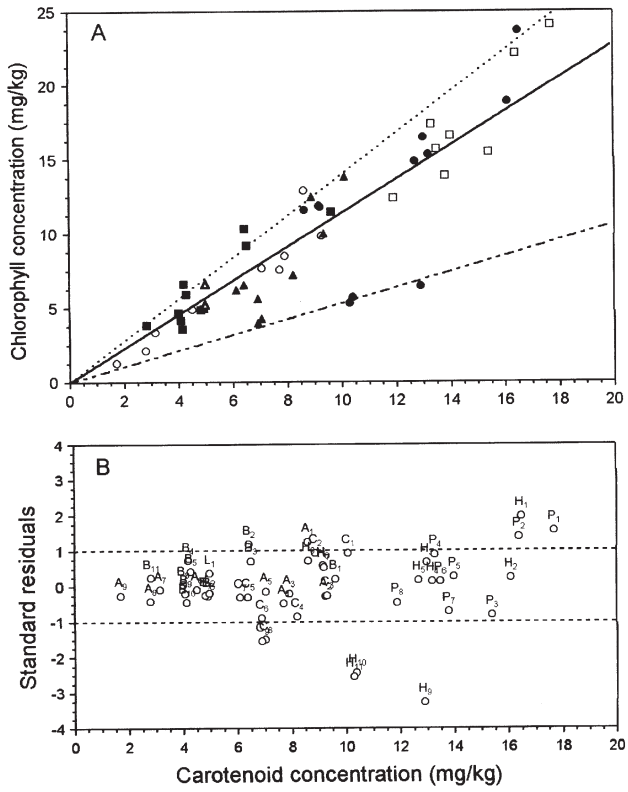

FIG. 2. Relationship between chlorophyll and carotenoid pigment fractions in virgin olive oils. Samples represented oils from Arbequina (○), Blanqueta (■), Cornicabra (▲), Hojiblanca (●), Picual (□), and Lechín (△) varieties; (A) linear correlations and (B) standard residuals vs. independent variable. Regression models are set to zero and each slope grouping, 1.14 (—), 1.40 (·····) and 0.53 (– · –), corresponds to the best oils samples. The oils of each variety were numbered in decreasing order of total pigment content and A (Arbequina), B (Blanqueta), C (Cornicabra), H (Hojiblanca), P (Picual), and L (Lechín).

grouping the data on pigment composition in the 50 oil samples. First, it can be observed that the distribution of the different oils is not wholly random. Oils from the Picual and Hojiblanca varieties contain the highest values of chlorophylls and carotenoids. The other varieties, Arbequina, Blanqueta, and Cornicabra, have a lower pigment content. Duncan's test confirmed that these two groups can be distinguished statistically by total pigment content (chlorophylls + carotenoids) ($P < 0.01$).

Joint analysis of the natural ratio between chlorophylls and carotenoids, independent of variety and production date, demonstrated a linear correlation between the variables; the regression model passing through the origin shows the best correlation coefficient, with a significance lower than 0.01% [slope (B) ± SE = 1.14 ± 0.04, $R = 0.975$, and $n = 50$]. Thus, the slope of the straight line directly expresses the ratio between chlorophylls and carotenoids.

As the total pigment content (chlorophylls + carotenoids) falls in a particular variety, the chlorophyll/carotenoid ratio also decreases. Thus, oils obtained at the beginning of picking, from fruits that are less ripe and therefore have more pigmentation, showed the highest values for the chlorophyll/carotenoid ratio, with all being greater than unity. In contrast, fruits from the final production stage were overripe and yielded oils with little color where the carotenoid fraction was predominant. The chlorophylls/carotenoids ratio in these cases was inverted, and lower than unity.

Analysis of the residual values (Fig. 2B) showed that of the 50 cases studied, 11 had a standard deviation (SD) with absolute value above 1 × mean SD of the residuals, making an alternative study necessary for this set of data. The oils of each variety are numbered in decreasing order of total pigment content. Those showing a positive residual value are generally those of the more pigmented oils of each variety: Arbequina (A1), Blanqueta (B2), Picual (P1 and P2), and Hojiblanca (H1). The relationship was linear slope (B) ± SE = 1.40 ± 0.03, $R = 0.999$, and $n = 5$. These oils, obtained at the beginning of the picking season, are the most fruity, aromatic, and bitter in flavor. They are not normally marketed directly, but are used to obtain *coupages* (oil mixtures) with balanced organoleptic characteristics similar to those of a more proper picking (1). The oils showing a negative residual value were those of less pigmentation, the varieties Cornicabra (C8 and C9) and Hojiblanca (H8, H9, and H10). In this case, a linear regression was obtained: slope (B) ± SE = 0.53 ± 0.02, $R = 0.998$, and $n = 5$. These oils, obtained from very ripe fruits at the end of the picking season, have faint, mild sensorial character and lower stability. They usually present slight alterations in their physicochemical parameters or sensorial characteristics and are not marketed directly as "virgin" quality, but are used in the blending of "olive oils" (refined olive oil + virgin olive oil in varying proportions) (1). In 80% of the other oils studied, the correlation found between the chlorophyll and carotenoid pigment fractions fits a linear model: slope (B) ± SE = 1.15 ± 0.02, $R = 0.993$, and $n = 40$.

Carotenoid fraction. In the search for a relationship between content and type of pigments of the different oils, attention was centered initially on lutein, the major pigment of the carotenoid fraction (2,19). Figure 3A plots, for each oil studied, the concentration of the minor carotenoids vs. lutein. Again, a linear correlation was found between these variables: slope (B) ± SE = 0.47 ± 0.03, $R = 0.926$, and $n = 50$. Analysis of the residual values (Fig. 3B) posed the same dilemma as in the section above, and, following the same criterion, an alternative study was carried out for those cases showing an SD (absolute value) greater than 1 × mean SD of the residuals. Again, these points are those of the less pigmented oils of the varieties Cornicabra (C8 and C9) and Hojiblanca (H8, H9, and H10), where the minor carotenoids/lutein ratio was smaller: slope (B) ± SE = 0.23 ± 0.05, $R = 0.999$, and $n = 5$. With increasing fruit ripeness, the rate of disappearance of lutein is less than the rest of the carotenoid fraction (2).

Oils with an SD of the residuals less than 1 × mean SD had a concentration of lutein lower than that of the minor carotenoids, and were all the Arbequina variety. Only three oils from this variety fit the general model; these were the least pigmented (A7, A8, and A9). This variety also showed the feature mentioned above: the percentage of lutein in-

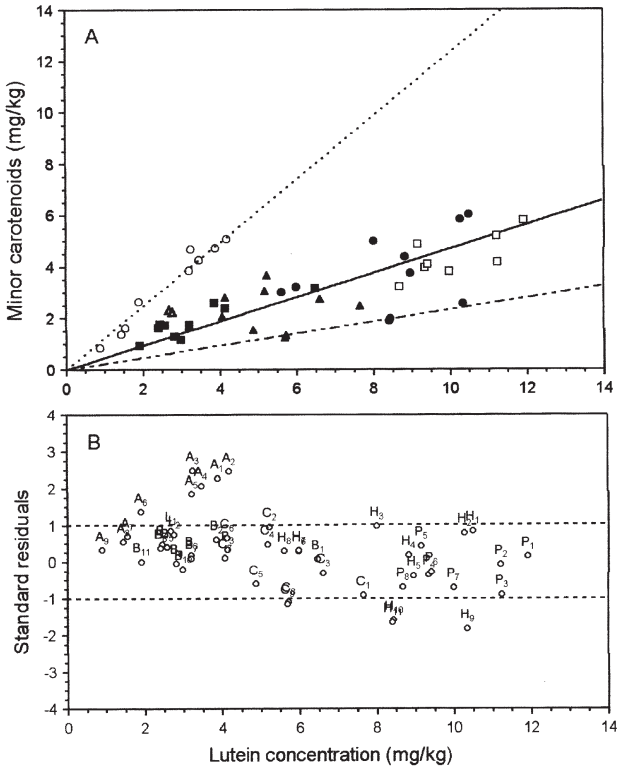

FIG. 3. Relationship between minor carotenoids and lutein in virgin olive oils. Samples represent oils from the varieties Arbequina, Blanqueta, Cornicabra, Hojiblanca, Picual, and Lechín; (A) linear correlations and (B) standard residuals vs. independent variable. Regression models are set to zero and each slope grouping, 0.47 (—), 1.23 (·····) and 0.23 (– · –), corresponds to the best oil samples. See Figure 2 for abbreviations.

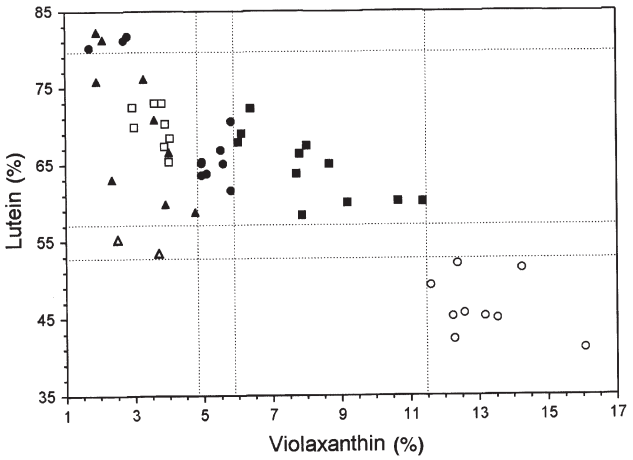

FIG. 4. Percentage of lutein vs. the sum of violaxanthin and its isomers in virgin olive oils. Samples represent oils from Arbequina, Blanqueta, Cornicabra, Hojiblanca, Picual, and Lechín varieties. See Figure 2 for abbreviations.

creased as the total pigment content decreased. As a consequence, a different linear model was used for all cases of this variety: slope (B) ± SE = 1.23±0.04, $R = 0.996$, and $n = 9$. Thus, the percentage of lutein may be a distinguishing characteristic in this variety.

Excluding the oils from the Arbequina variety and those from the end of picking, the minor carotenoids/lutein ratio fits a linear model: slope (B) ± SE = 0.48 ± 0.02, $R = 0.981$, and $n = 36$. This ratio may therefore be established as another index of authenticity for virgin olive oil in general.

In addition, the group of violaxanthin and its furanoid isomers (luteoxanthin + auroxanthin) marked certain differences between varieties. Figure 4 compares the percentage of lutein vs. that for the sum of violaxanthin and its isomers. The data show an inverse relationship between these two parameters. The percentage of lutein again mirrored the results already discussed. The Arbequina variety oils showed the lowest average percentage of lutein (46.3 ± 3.8), followed by the Lechín variety oils (54.4 ± 1.3). These values were statistically different one from the other and from the other varieties (Duncan's test, $P < 0.01$). They also formed a separate group of end-of-picking oils in which the percentage of lutein increased up to 81%. In the rest of the oils studied, the percentage of lutein showed values within the range 57 to 77%, with no significant differences between the Cornicabra, Hojiblanca, Picual, and Blanqueta varieties.

The percentage of violaxanthin and its isomers was highest in the Arbequina variety (13.1 ± 1.4), followed by Blanqueta (8.2 ± 1.7) and Hojiblanca (5.3 ± 0.4). These mean values were statistically different from each other and from those corresponding to the other varieties (Duncan's test, $P < 0.02$). The Picual, Cornicabra, and Lechín varieties were in a lower range (2 to 4%), which was common to the three. The percentage of this group of minor xanthophylls thereby enables differentiation of the virgin olive oils from the Arbequina, Blanqueta, and Hojiblanca varieties. The Lechín variety is distinguished by the percentage of lutein. Hence, it is possible to differentiate Picual by the total pigment content.

It can therefore be concluded that some parameters related to the carotenoid fraction should be required of all olive virgin oils, regardless of the variety and the state of ripeness of the source fruit. Other parameters can be used to distinguish mono-variety oils.

Statistical analysis of the results. A stepwise discriminant analysis was applied to these data for the purpose of establishing criteria for distinguishing virgin olive oils by the source olive variety. Three variables were selected for the groupings: % of violaxanthin, % of lutein, and total pigment content. The small number of available samples of the Lechín variety was excluded from the statistical analysis. With 48 cases, an adequate classification was obtained in 100% of the samples for olive variety and distinguishing six categories: Arbequina, Blanqueta, Cornicabra, Hojiblanca, Picual, and end-of-picking. The category end-of-picking included samples C8, C9, H8, H9, and H10, whose low ratio of chlorophyll/carotenoid and high percentage of lutein made them statistically different from the others, and they received a different mathematical treatment in all the cases.

The canonical discriminant functions explain, respectively, 83.9, 95.1, and 100% of the accumulated variance. This analysis selected the percentage of violaxanthin as the variable showing the best correlation with the first discriminant function (0.922) and presenting the greatest discriminating power, followed by percentage of lutein (0.736) and total pigments (0.715). Five samples (one of each variety) were used to test the validity of the functions. In all cases, the oils were correctly classified in their corresponding groups.

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