Effect of Storage on the Original Pigment Profile of Spanish Virgin Olive Oil

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ABSTRACT: The present study was carried out on 12 virgin olive oils to determine whether one year's storage under mild conditions of 15°C and darkness affected the initial pigment composition of recently extracted virgin olive oil. Although the total pigment content remained constant, the individual contribution of each pigment changed. The acid compounds liberated from the fruits during the oil extraction process promote the beginning of chlorophyll pheophytinization and the isomerization of the 5,6-epoxide groups of the minor xanthophylls. During the first 3 mon of storage, there was a generalized increase in pheophytinization that was different for each oil ($P < 0.01$, Duncan test) but was not correlated with the free acidity measured in them. At the same time, isomerized xanthophylls and allomerized pheophytins increased slightly. Following this stage, pyropheophytin *a* (a pigment not present in the initial oils), was detected; its concentration increased during storage. There were no significant differences in the final percentages of pyropheophytin *a* among the 12 oils, and the concentration of this new compound represented around 3% of the chlorophyll fraction. The pheophytin *a*/pyropheophytin a ratio always exceeded 20. All these small pigment transformations were signs that the oil had been stored. The content and class of pigments present in virgin olive oil are authentic indicators of its history prior to marketing.

Paper no. J10821 in *JAOCS 82*, 33–39 (January 2005).

KEY WORDS: Carotenoids, chlorophyll derivatives, chlorophyll transformation, oil storage, olive oil, olive variety.

The study of chlorophyll and carotenoid pigments in virgin olive oil has traditionally been of interest because of their contribution to the color (1). Other important aspects of these compounds are their influence on the oxidative stability (2,3). It is now known that the chlorophyll and carotenoid composition also can be used to characterize the authenticity of Spanish virgin olive oil.

A study carried out with 50 single-variety Spanish virgin olive oils established the intrinsic chlorophyll and carotenoid pigment profile of virgin olive oil and yielded two indices of authenticity and quality for this product (4). The authors of the work concluded that the chlorophylls/carotenoids ratio should be around 1, and that of minor carotenoids/lutein around 0.5, with limited variability. These parameters are independent of the olive variety from which the oil is extracted and have been verified in 70 single-variety oils of nine varieties of Spanish origin (4–6). Additionally, the percentage of lutein, the percentage of violaxanthin, and the total pigment content can be used as classificatory variables to distinguish between single-variety virgin olive oils.

It has been demonstrated that authenticity parameters of an oil, defined by the ratios mentioned above, are still stable after 12 mon of storage at 15°C in darkness—the usual industrial conditions for oil that is not marketed immediately following its production. The variables distinguishing between oil varieties also remain stable during the same period of storage (5).

The chlorophyll and carotenoid composition of the oils will depend on various factors, such as the degree of ripeness and the variety of the fruits employed, which will affect the content, percentage, and exclusivity of the pigments. Some olive varieties, such as Hojiblanca and Picual, have a high content in chlorophylls and carotenoids, whereas others, such as Arbequina and Blanqueta, have low pigmentation. This enables a statistically significant differentiation of oils by total pigment content (chlorophylls and carotenoids) into two groups: high and low pigmentation (4).

The chlorophyll and carotenoid profile of virgin olive oil is determined by the pigments initially present in the fruits and the derivatives formed during fruit milling and paste beating. Intermediate products of chlorophyll catabolism and associated derivatives are also detected at trace level. These include oxidation metabolites (13^2-OH-chlorophylls and 15^1 OH-lactone chlorophylls), intermediates of the degradation pathway initiated by chlorophyllase (chlorophyllides), and the corresponding Mg-free derivatives (8). The presence in the oil of carotenoids not described for this product, or chlorophyll derivatives at degradation levels other than those associated with the extraction process, shows whether the virgin olive oil is adulterated or has been subjected to an incorrect extraction process. Thus, the pigment profile of virgin olive oil can be used as an index of authenticity and quality (4).

However, it is not known whether, during one year of oil conservation, the chlorophyll or carotenoid molecules undergo any specific structural change altering the characteristic pigment profile of recently extracted virgin olive oil. The present study focuses on this aspect. As the pigment modifications associated with oil extraction are largely mediated by the release

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of acid compounds, the free acidity of the olive oil has also been measured, to determine whether this parameter has any influence on changes produced in the pigment molecules during storage.

MATERIALS AND METHODS

Raw materials. The study was carried out using 12 mono-variety virgin olive oils (that is, each oil was extracted from fruits of a single variety) of five different olive cultivars from the main producing areas of Spain. Recently extracted oils were requested from industry throughout the entire harvesting season to obtain the greatest possible variability in oil color. The samples provided were as follows: cv. Arbequina (A-1, A-2, and A-3) by the Cooperativa La Paz (Estepa, Seville); cv. Blanqueta (B-1) by the Cooperatives Oleicoles Valencianes (Muro, Alicante); cv. Cornicabra (C-1, C-2, and C-3) by Aceites Toledo S.A. (Los Yébenes, Toledo); cv. Hojiblanca (H-1, H-2, and H-3) by Olivarera Sor Angela de la Cruz (Estepa, Seville); and cv. Picual (P-1 and P-2) 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.

Extraction of pigments. Pigment extraction was performed with *N,N*-dimethylformamide (DMF) according to Mínguez-Mosquera *et al.* (9). 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 xanthophylls. This system yielded a concentrated pigment solution free of oil. All analyses were performed under green light.

Analysis of pigments. This was 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 Hewlett-Packard ChemStation (Rev. A.05.04). A stainless steel column (25 × 0.46 cm i.d.), packed with 5 µm C18 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* before 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 has been described in detail in a previous work (10). Detection was performed simultaneously at 410, 430, 450, and 666 nm. Details about the pigment identification and quantification by use of an external standard have been described in previous papers (6,11,12).

Acidity determination. Free acidity, given as the percentage of oleic acid, was determined by titration with ethanolic KOH of an oil solution in ethanol/ether (1:1) according to the European Official Method of Analysis (EC1991 Regulation 2568/91) (13).

Statistical analysis of data. All analyses were performed in triplicate. Significant differences were tested by ANOVA (Duncan test, setting *P* at 0.01). Linear regression tests were applied to obtain kinetic parameters. Calculations were made by using the program STATISTICA for Windows v.99 (StatSoft, Inc., Tulsa, OK;1984–1999).

RESULTS AND DISCUSSION

Acidity of the oils. Free acidity data in the initial oils and after 3, 6, 9, and 12 mon of storage are included in Table 1. The initial values were considerably lower than the limit of 1% oleic acid established by European Union legislation (13) for the extra virgin category. These values were maintained without significant differences during the first 3 mon of storage, although there was a slight increase from the sixth month on. After 12 mon, the free acidity increase was significant ($P < 0.01$) for some oils (A-1, B-1, C-3, H-1, H-2, P-1, and P-2), but the values did not in any case exceed the acidity limit established for the extra virgin category.

Initial pigment composition of virgin olive oils. As expected, the pigment profile was inherent to a virgin olive oil and was similar for all of them, independent of the degree of ripeness and variety of the fruit used in oil extraction. The chlorophyll fraction included mainly chlorophylls *a* and *b* and pheophytins *a* and *b*. Allomerized derivatives such as 13^2-OH-chlorophyll *a*, 15^1-OH-lactone chlorophyll *a*, 13^2-OH-pheophytin *a*, and 15^1-OH-lactone pheophytin *a* were also present in much lower quantities. The carotenoid fraction comprised lutein, β-carotene, and the minor xanthophylls neoxanthin, violaxanthin, antheraxanthin,

TABLE 1
Changes with Respect to Time in Free Acidity (% oleic acid) of the Oils During Storage[a]

Time	Virgin olive oil sample											
(mon)	A-1	A-2	A-3	B-1	C-1	C-2	C-3	H-1	H-2	H-3	P-1	P-2
0	0.18	0.20	0.18	0.24	0.21	0.23	0.23	0.12	0.13	0.12	0.20	0.16
3	0.18	0.19	0.18	0.23	0.23	0.23	0.23	0.13	0.13	0.13	0.20	0.15
6	0.19	0.19	0.19	0.26	0.24[b]	0.25	0.24	0.12	0.16[b]	0.13	0.21	0.15
9	0.22[b]	0.19	0.18	0.27[b]	0.23	0.23	0.28[b]	0.16[b]	0.15[b]	0.13	0.25[b]	0.16
12	0.22[b]	0.19	0.19	0.27[b]	0.23	0.25	0.32[b]	0.18[b]	0.19[b]	0.13	0.26[b]	0.19[b]

[a]Data are means of triplicate analyses (CV < 6% in all cases).
[b]Increased values with respect to the initial value are significant ($P < 0.01$).

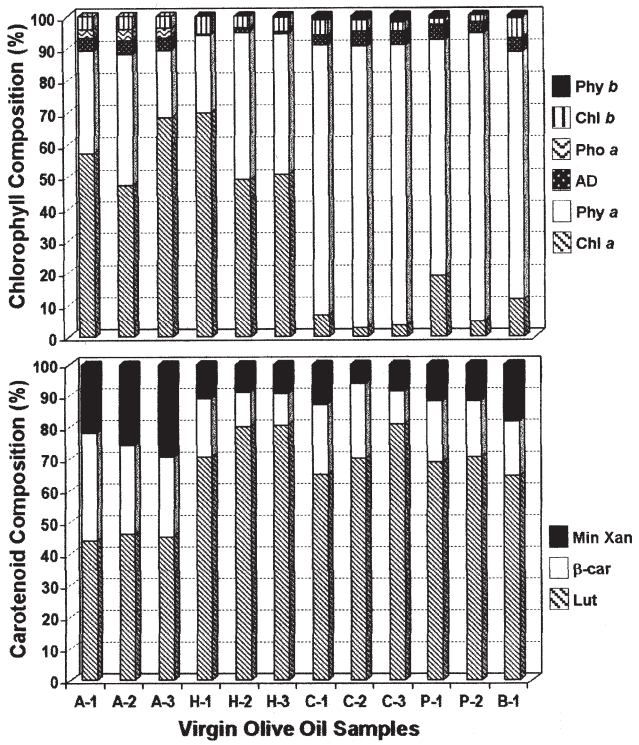

FIG. 1. Percentage composition of the chlorophyll and carotenoid pigments in recently extracted virgin olive oil. Abbreviations: Chl *a*, chlorophyll *a*; Phy *a*, pheophytin *a*; AD, allomerized derivatives; Pho *a*, pheophorbide *a*; Chl *b*, chlorophyll *b*; Phy *b*, pheophytin *b*; Lut, lutein; β-car, β-carotene; Min Xan, minor xanthophylls (CV < 10% in all cases).

β-cryptoxanthin, and the isomers 5,8-furanoid luteoxanthin and mutatoxanthin.

Although the pigment profiles were similar, the initial quantitative compositions of the various oils showed notable differences depending on variety and degree of ripeness of the fruit. The oils from olive fruit varieties with high pigmentation, such as Hojiblanca and Picual, showed greater pigment content than oils from olive fruit varieties with low pigmentation, such as Cornicabra, Arbequina, and Blanqueta.

Figure 1 shows the percentage composition of chlorophylls and carotenoids in the initial olive oils. The most important differences were found in the chlorophyll fraction. Oils from Hojiblanca and Arbequina varieties, which were visibly greener in color, retained a significantly higher percentage of chlorophyll *a* ($P < 0.005$), whereas those of Cornicabra, Picual, and Blanqueta, which were more golden, had a higher percentage of pheophytins. The proportion of allomerized derivatives was similar among the oils studied here except for the Hojiblanca variety, which was significantly lower ($P < 0.005$).

With respect to the carotenoid fraction, oils of the Arbequina variety had lower lutein percentages (around 45%), as was previously established (4). In the other varieties, lutein varied between 64 and 81% as a function of the degree of fruit ripening. β-Carotene and the minor xanthophylls were present at levels between 10 and 30%, with the oils of the Arbequina variety having a higher percentage of these pigments. On the other hand, the proportion of xanthophylls in the minor xanthophylls fraction having 5,8-furanoid groups was significantly higher ($P < 0.01$) in oils from Cornicabra and Blanqueta varieties (between 25 and 40%) than in oils from Hojiblanca and Arbequina varieties (between 12 and 20%). Therefore, the oils with lower proportions of isomerized xanthophylls were the same ones that had lower pheophytin percentages. These facts were a direct consequence of the different proportion of fruit pigments that were transformed during the oil extraction process.

Although pheophytinization and xanthophyll isomerization reactions are mediated by an acid medium, a patent correspondence with the initial free acidity values of the oils was not found in this work. In oils from Arbequina and Hojiblanca varieties, with free acidity values ≤0.2, the percentages of pheophytinization (≤50%) and xanthophyll isomerization (≤20%) were estimated to be lower than in oils from Blanqueta and Cornicabra varieties (acidity values ≥0.2) when the pheophytin percentage was 70% and the isomerized xanthophylls percentage was >20%. However, the percentages of pheophytins and isomerized xanthophylls showed significant ($P < 0.01$) differences between oil samples of the same variety and having the same free acidity value (for example A-1 vs. A-3 or H-1 vs. H-3) that could not be explained by this parameter.

An activity catalyzing the pheophytinization reaction and designated as "Mg-dechelatase" has been reported in plants (14). More recently, it has been found that this activity is due not to an enzyme but to a low-molecular-mass, heat-stable substance designated as "Mg-dequelating substance," which is functionally very similar to an enzyme (15). The proportion of pheophytins and isomerized xanthophylls in the oils probably is determined by the degree of cellular disintegration that is produced during the fruit milling and beating time of the paste, with disintegration favoring contact between pigments and any acid compounds and/or Mg-dequelating substances from the olive fruit that could be transferred to the oil.

Thus, the pigment profile of all the oils studied here was characteristic of virgin olive oil. Although the oils studied here had qualitatively similar pigment compositions, they differed initially in the proportion of each compound.

Changes in pigment composition of virgin olive oils during storage. As an example, Figure 2 shows typical HPLC chromatograms of pigment extracts from virgin olive oils at the beginning (Fig. 2A) and the end (Fig. 2B) of the experimental period. After 12 mon of storage, there were no losses of total pigment content, although there were some transformations of those initially present. The most important transformation was the conversion of chlorophylls (peaks 7 and 11) into pheophytins (peaks 13 and 16), which was complete in most of the oils. In much smaller proportions, the content of 13^2-OH-pheophytin *a* (peak 14) and 15^1-OH-lactone pheophytin *a* (peak 12) was increased, probably by allomerization reactions of pheophytin *a* and/or release of magnesium from allomerized chlorophylls. A new, unanticipated compound, pyropheophytin *a* (peak 17), was detected in the HPLC at the 12-mon analysis. This pigment formed by decarboxylation of pheophytin *a*. The

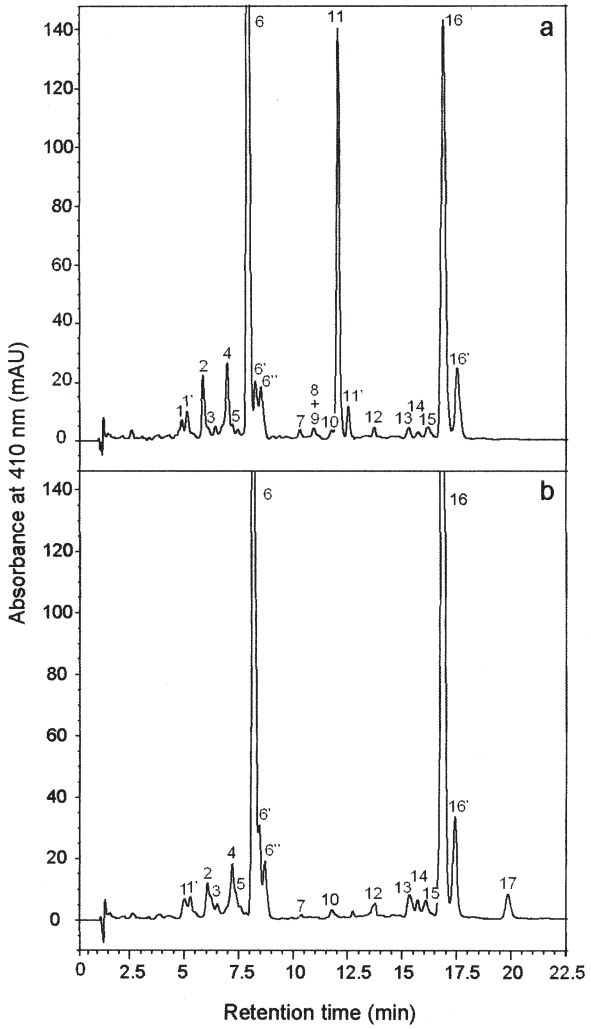

FIG. 2. HPLC separation of the pigments from Hojiblanca variety virgin olive oil at initial time (A) and after 12 mon of storage at 15°C in darkness (B). Detection by absorbance at 410 nm. Peaks: 1 and 1′ = neoxanthin and isomer; 2 = violaxanthin; 3 = luteoxanthin; 4 = antheraxanthin; 5 = mutatoxanthin; 6, 6′, and 6″ = lutein and isomers; 7 = chlorophyll *b*; 8 = 13^2-OH-chlorophyll *a*; 9 = 15^1-OH-lactone-chlorophyll *a*; 10 = β-cryptoxanthin; 11 and 11′ = chlorophyll *a* and *a*′; 12 = 15^1-OH-lactone pheophytin *a*; 13 = pheophytin *b*, 14 = 13^2-OH-pheophytin *a*; 15 = β-carotene; 16 and 16′ = pheophytin *a* and *a*′, 17 = pyropheophytin *a*.

carotenoid fraction of the oils remained practically unchanged with storage time, although a certain degree of isomerization of the 5,6-epoxide groups of the minor xanthophylls neoxanthin, antheraxanthin, and violaxanthin was detected in a few cases.

Figures 3–5 show the qualitative and quantitative transformation of the chlorophyll *a* derivatives (*a* series) in the oils of the Arbequina (Fig. 3), Hojiblanca (Fig. 4), and Cornicabra, Picual, and Blanqueta (Fig. 5) varieties. A detailed study of the formation of each pigment (pheophytins, the allomerized derivatives 13^2-OH-pheophytin *a* and 15^1-OH-lactone pheophytin *a*, and pyropheophytin a) now follows.

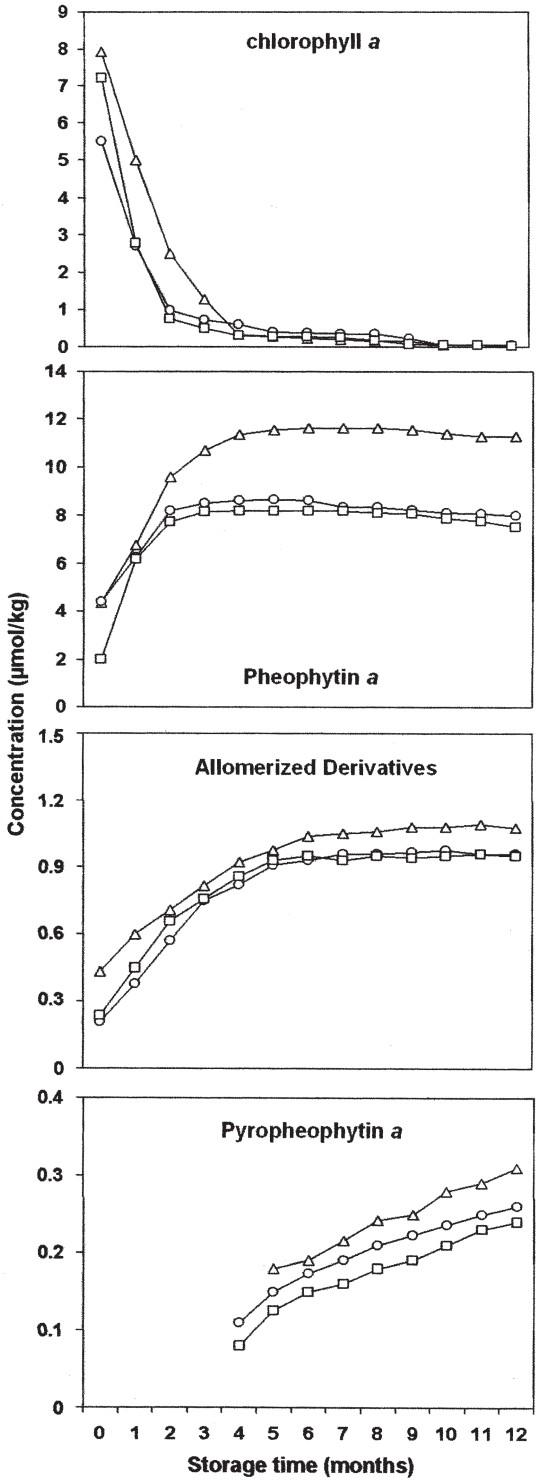

FIG. 3. Changes in concentration of chlorophyll *a*, pheophytin *a*, allomerized derivatives, and pyropheophytin *a* in oils of the Arbequina variety during storage: (△), sample 1; (○), sample 2; (□), sample 3. (CV < 10% in all cases).

The pheophytinization reaction, initiated in the oils during the extraction process, advanced with time under the established storage conditions, but differently and indiscriminantly

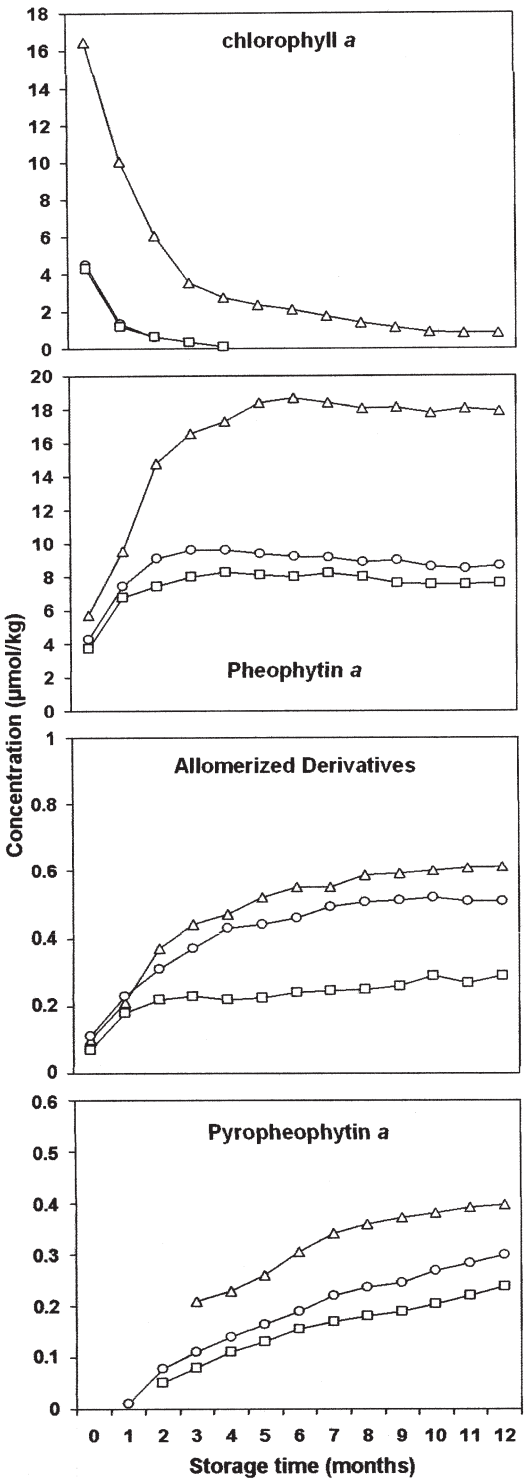

FIG. 4. Changes in concentration of chlorophyll *a*, pheophytin *a*, allomerized derivatives, and pyropheophytin *a* in oils of the Hojiblanca variety during storage. For symbols see Figure 3. (CV < 10% in all cases.)

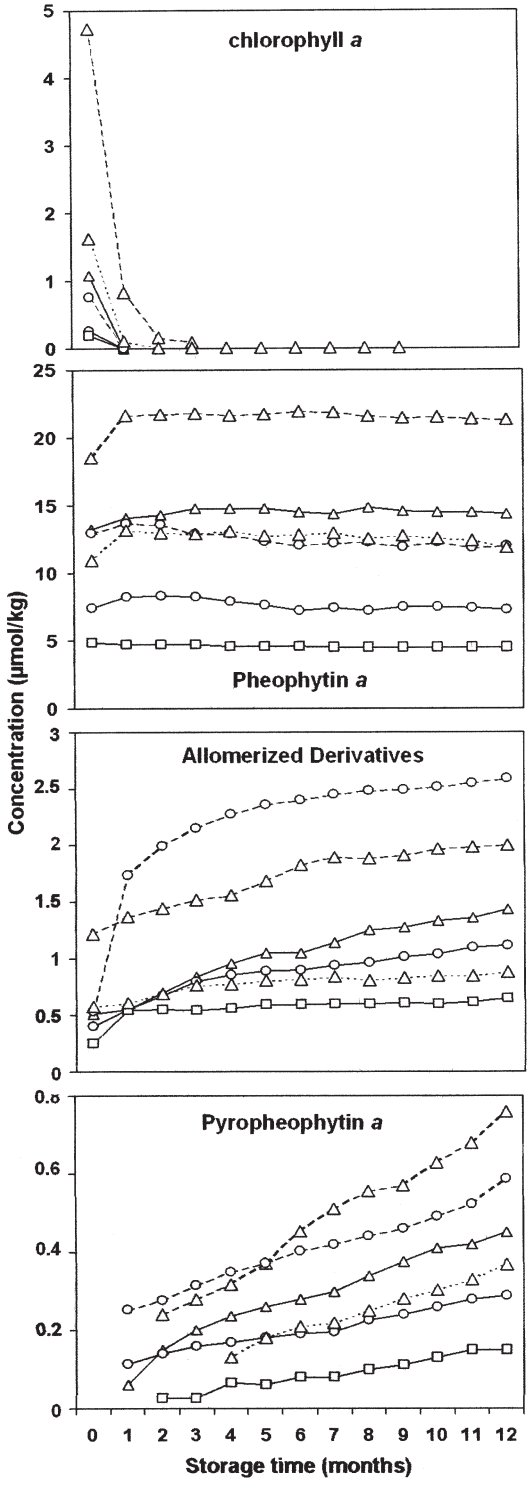

FIG. 5. Changes in concentration of chlorophyll *a*, pheophytin *a*, allomerized derivatives, and pyropheophytin *a* in oils of the Cornicabra (—), Picual (– –), and Blanqueta (····) varieties during storage. For symbols see Figure 3. (CV < 10% in all cases.)

in each oil. Although the initial concentration of chlorophyll *a* in each oil decreased with time, following first-order kinetics, the apparent rate constants (k) distinguished the oils of Arbequina significantly ($P < 0.01$) from the other varieties, except for the Hojiblanca sample 1 (H-1), which behaved similarly. In the Arbequina oils, k was between 0.37 and 0.48 mon^{-1} depending on the sample, and in no case did chlorophyll *a* disappear totally by the end of storage (Fig. 3). However, in the

Hojiblanca oils, except for sample H-1, the rate constants for the disappearance of chlorophyll *a* were much higher—between 0.92 and 1.37 mon^{-1} with around 70% of the chlorophyll *a* being transformed in the first month of storage; before the fifth month it had completely disappeared (Fig. 4). In H-1 oil, *k* was 0.24 mon^{-1}, closer to that for the Arbequina variety, and chlorophyll *a* was also detected up to 12 mon of storage. The different behavior of this oil with respect to the others of the same variety might result from the lower transformation (during the oil extraction process) of chlorophyll *a* into pheophytin *a*—around 25%—against the 44–46% that was transformed in the other Hojiblanca oils.

The results for the oils of the Cornicabra, Picual, and Blanqueta varieties were similar to those for Hojiblanca in that the transformation of chlorophyll *a* into pheophytin *a* was complete in the first months of storage (Fig. 5).

The pheophytinization of chlorophyll *b* (6–7% of the total chlorophylls) was complete only in Cornicabra. This behavior was in accord with kinetic studies on pheophytinization in which it was concluded that the reaction rate is always higher for chlorophyll *a* than for *b* (16,17). Nevertheless, and as in the case of chlorophyll *a*, the degree of transformation into pheophytin *b* varied among the oils of any one variety (data not shown). Again as seen for chlorophyll *a*, the lower the proportion of pheophytin *b* in the initial oils, the lower was the chlorophyll *b* transformation into pheophytin *b* during storage. At 12 mon, pheophytin *b* ranged from 12% (with respect to the chlorophyll *b* derivative series) in sample H-1 to 60% in H-3. Intervariety patterns again paralleled the transformation of chlorophyll *a.* In oils from Arbequina there was a lower transformation of chlorophyll *b* into pheophytin *b* (between 11 and 24%, with respect to the *b* series) than in oils from Hojiblanca and other varieties. The behavior of oil H-1 was again similar to that of Arbequina.

As has been previously commented on, the chlorophyll transformation into pheophytins occurred mainly during the first 3 mon of storage. However, the free acidity of the oils did not change significantly during the same period (Table 1). Again, this result demonstrated that the pheophytinization reactions that occurred during the storage time were not interrelated with the free acidity measured in the oils. We suggest that substances with acid character and/or Mg-dequelating properties can be released during fruit milling and paste beating and can remain occluded in the oil, thus stimulating reaction progress during oil storage. The content of those substances may vary as a function of the fruit variety and degree of ripeness.

With regard to the formation of 13^2-OH-pheophytin *a* and 15^1-OH-lactone pheophytin *a* (allomerized derivatives), all the oil samples used in the experiment showed considerable parallelism in the patterns of formation of these compounds. The initial concentration of allomerized derivatives of pheophytin *a* increased slightly in all cases during the first months —the period of storage coinciding with the greatest formation of pheophytin *a*. The formation of allomerized derivatives occurs when chlorophyll compounds are oxidized by triplet molecular oxygen, implying a reaction mechanism *via* free radicals (18). In this case, the oxidation reaction could be attributable to the availability of oxygen, present in the headspace of the sample containers, as suggested by Psomiadou and Tsimidou (19).

In general, the oils of the Hojiblanca variety, which were those that initially had fewer allomerized derivatives, also had a lesser formation of these compounds with storage time, whereas there presence in the oils of Arbequina, Cornicabra, and Picual was markedly greater.

Finally, it may be deduced that the storage conditions of the oils favored the formation of pyropheophytin *a*, a compound not present in the original oil. Although its formation was minimal (and it was not possible to make any kinetic study), the concentration increased with storage time. It was found that the higher the concentration of pheophytin *a*, the greater was the formation of the pyroderivative. Differences in the percentages of pyropheophytin *a* formed in the oils considered here were not significant after 12 mon and represented around 3% of the total chlorophyll pigments. The ratio of pheophytin *a* (the precursor pigment) to pyropheophytin *a* decreased, reaching a value between 30 and 20. Thus, ratios below 20 might indicate that the oil has been subjected to other, less proper storage conditions.

Among oils of different varieties there were some differences regarding the time when pyropheophytin *a* appeared. In the Hojiblanca, Cornicabra, and Picual oils, this compound was formed before the third month of storage, whereas in those of Arbequina and Blanqueta, it appeared later, at the fourth, or even—in the case of oil A-1—the fifth month of storage.

The formation of pyropheophytin in a foodstuff is generally attributed to heat treatments, with the extent of formation depending on the intensity of the treatment (16,20). For virgin olive oils stored for 10 mon at ambient temperature (25–35°C) in darkness, Gandul-Rojas *et al*. (21) detected the formation of pyropheophytin *a* in greater amounts (between 7 and 13% of the total chlorophyll pigments) than the 3% in the present experiment, in which the temperature was lower (15°C). Thus, the content and proportion of pyropheophytin *a* present in a virgin olive oil can indicate the storage conditions of the oil.

ACKNOWLEDGMENT

We express our sincere gratitude to the Comisión Interministerial de Ciencia y Tecnología (CYCIT-Spanish Government, AGL 2000-0699).

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[Received March 8, 2004; accepted December 7, 2004]