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Pigment Metabolism of 'Sikitita' Olive (*Olea europaea* L.): A New Cultivar Obtained by Cross-Breeding

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ABSTRACT: The new olive cultivar 'Sikitita' was obtained from a cross between the 'Picual' and 'Arbequina' varieties. 'Sikitita' was selected for its features, making it particularly suited to high-density olive hedgerow orchards. From the standpoint of chloroplast pigment metabolism, the fruits of the 'Picual' and 'Arbequina' varieties have significant differences. It is therefore extremely interesting to analyze the descendants of both cultivars. With regard to chlorophyll catabolism, 'Sikitita' has proven to be a cultivar with low pigmentation and low levels of chlorophyllase activity. This is contrary to the findings obtained to date, where varieties with low pigmentation are a consequence of high chlorophyllase activity ('Arbequina') and highly pigmented fruits are due to low chlorophyllase activity ('Picual'). 'Arbequina' was, until recently, the only cultivar described that had developed a carotenogenic process, despite its anthocyanic ripening. However, from its father ('Arbequina'), the 'Sikitita' cultivar has inherited the pool of enzymes necessary to esterify xanthophylls at the chromoplast level. This makes 'Sikitita' a very interesting cultivar, with potential chemotaxonomic differences (such as esterified xanthophylls in the olive oils), and demonstrates the interest in genetic improvement programs for olive cultivars with different organoleptic characteristics.

KEYWORDS: 'Sikitita', 'Picual', 'Arbequina', olive varieties, olive oil, fruits, ripening, chlorophylls, carotenoids, catabolism, chlorophyllase

INTRODUCTION

In the past few years, the spread of high-density hedgerow olive orchards has promoted the selection of new olive cultivars suitable for this new growing system. This new concept of olive orchard is based on the use of planting densities of around 2000 trees/ha. This means plants can form hedgerows 2−3 years after planting, which can be collected by straddle-harvesting machines. The lower harvesting cost using this machinery compared to standard olive orchards is due to the drastic reduction of both labor and time needed to harvest the crop. From the agronomic point of view, low vigor cultivars are needed for this system: to control long-term tree size, for the harvesting machines to pass over the hedgerow, and to ensure the illumination of the canopy cropping area.[1,2] In Spain, 'Sikitita' was registered in 2007 as a new olive cultivar after crossing the 'Picual' and 'Arbequina' varieties.[3] It is characterized by an early bearing, high oil content and yield efficiency. Its low vigor and compact and weeping growth habit make it particularly suitable for high-density hedgerow orchards.

From the standpoint of chloroplast pigments, the fruit of the two 'Sikitita' parents is very different. 'Picual' is considered a highly pigmented cultivar, while the 'Arbequina' cultivar has low pigmentation.[4] These differences in pigment levels reflect a different metabolism of chlorophylls and carotenoids. During the catabolism of chlorophylls, chlorophyllase is the first enzyme involved in the degradation path responsible for dephytilating the chlorophyll molecule to form chlorophyllide. Measurements made during fruit growth and ripening[5] show that the fruits of the 'Arbequina' cultivar are about 100 times more active than those of the 'Picual' cultivar.

Moreover, during the transition period from growth to the beginning of ripening, the 'Arbequina' cultivar fruit accumulates the catabolite 13^2-OH-chlorophyll *a* (following an accumulation and degradation curve), reaching up to 15% of the chlorophyll fraction. In contrast, in the fruits of the 'Picual' cultivar, this catabolite is never more than 1% and remains constant, regardless of the state of maturity of the fruit.

In fruit with anthocyanic ripening (such as olive, sweet cherry, red currant, or strawberry), when the ripening process begins, the photosynthetic activity decreases and the chloroplast pigments (chlorophylls and carotenoids) begin to break down, while the synthesis of anthocyanins start. In contrast, in carotenogenic fruits (pepper or tomato type), at the start of ripening, the chlorophyll and carotenoid degradation process begins but, concomitantly, the *de novo* synthesis of carotenoid pigments accumulating in chromoplasts starts. For a typical anthocyanin fruit, such as olives, the development of a carotenogenic process was demonstrated for the first time in the 'Arbequina' cultivar fruit, albeit expressed at a very low level compared to typical carotenogenic fruit.[6] This process involves the esterification of certain carotenoids, which is a reaction that takes place in these

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chromoplasts.[7] Thus, these esterified carotenoids are exclusively detected in the 'Arbequina' cultivar fruit.[6,8]

Because the 'Sikitita' cultivar fruit is the result of a cross between 'Picual' and 'Arbequina', it is of great interest, from the standpoint of pigment metabolism, to observe how the chloroplast pigments behave in this fruit, subject to the new rules. It is interesting to find out not only the traits inherited from each parent but also their actual inter-relationship in the new organism. From a practical standpoint, the pigment composition of a food, such as olive, is crucial for determining the color of an oil, with some markets preferring greener or browner colors. Nutritionally, at present, both the chlorophylls and carotenoids[9,10] are considered as functional ingredients, because of the demonstrated beneficial effects on the organism consuming them. This translates into added value for those foods containing them. In addition to this eminently necessary aspect when launching a new product on the market, such as a new cultivar of olive,[11,12] the objective of this work is focused more on the implications for crossing such different varieties, from the standpoint of pigment metabolism, and if these results allow us to obtain more general conclusions regarding the catabolism of chlorophylls and carotenoids in fruit.

MATERIALS AND METHODS

Chemicals. Tetrabutylammonium acetate and ammonium acetate were supplied by Fluka (Zwijndrecht, The Netherlands). High-performance liquid chromatography (HPLC)-reagent grade solvents were purchased at Teknokroma (Barcelona, Spain). Analysis-grade solvents were supplied by Panreac (Barcelona, Spain). The deionized water used was obtained from a Milli-Q 50 system (Millipore Corp., Milford, MA).

Chlorophyll (chl) *a* and *b* were purchased from Sigma. Chlorophyllide was formed by enzymatic de-esterification of chl.[5] The C-13 epimer of chl *a* was prepared by treatment with chloroform.[13] The 13^2-OH-chl *a* and *b* were obtained by selenium dioxide oxidation of chl *a* at reflux heating in pyridine solution.[14] β-Carotene, lutein, violaxanthin, neoxanthin, and anteraxanthin were obtained from a pigment extract of green olives saponified with 3.5 M KOH in methanol[15] and isolated by thin-layer chromatography (TLC). All standards were purified by normal-phase (NP)- and reversed-phase (RP)-TLC.[15]

Plant Material. The study was carried out with fruits of three olive (*Olea europaea* L.) cultivars that are used for oil production. The 'Picual' cultivar is typical of Andalucia (southern Spain). The 'Arbequina' cultivar, originally from Cataluña (northeast Spain), has been grown in Andalucia in the past few years. 'Sikitita' is a new cultivar obtained from a cross between 'Picual' and 'Arbequina'.[3] All of the olive fruits were collected in plants cultivated in the experimental farm of IFAPA, Centro Alameda del Obispo of Córdoba (Spain), during the harvesting season of 2009−2010 (from September to December). Fruit sampling began when the developing fruit was still green and finished when it was ripe (detected by the overproduction of anthocyanins) (Table 1). For each sampling date, 1 kg of fruit was collected from around the whole perimeter of the tree and 100 fruits were chosen at random to evaluate the most representative color. The sequence of color changes was green, light green, small reddish spots, turning color, purple, and black.[16] The light green stage is not the same for all of the varieties; for instance 'Arbequina' has a homogeneous yellowish color. The morphological characteristics of the fruits are not the same, enabling varieties to be distinguished by direct observation. The final average fruit and stone weights were 1.58 and 0.30 g, 2.53 and 0.42 g, and 4.53 and 0.62 g, for 'Arbequina', 'Sikitita', and 'Picual', respectively.

Olive oils were obtained for the above-mentioned samples but only in the ripening stages indicated in Table 1. For that, the ripening index was calculated[16] according to color changes of peel and pulp classified into eight groups or categories: green intense (0), yellow or yellowish green (1), green with reddish spots (2), reddish or light violet (3), black with white pulp (4), black with <50% purple flesh (5), black with ≥50% purple flesh (6), and black with 100% purple flesh (7). Olive oils were extracted by the Abencor System (Comercial Abengoa, S.A., Sevilla, Spain) in the Almazara Experimental of Instituto de la Grasa (CSIC, Sevilla). This unit consists of three basic elements: olive crusher, thermobeater to mix the paste, and centrifuge to eliminate the solid residue. Such a system reproduces the industrial process on a laboratory scale, following the same phases of grinding, malaxation (25 °C for 30 min), centrifugation, and decantation. The oil was separated from wastewater by decanting and later filtered prior to analysis.

Table 1. Apparent Color and Ripening Index of the Olive Fruits and Oils Analyzed

harvesting number	'Arbequina'	'Picual'	'Sikitita'
	Apparent Color of Olive Fruit Samples		
1	intense green	intense green	intense green
2	green	small reddish spots	green
3	yellow−greenish	turning color	small reddish spots
4	turning color	purple	turning color
5	black	black	black
	Ripening Index of Fruit Samples for Olive Oil Extraction[a]		
4	2.67	3.27	2.23
5	3.11	4.04	2.80
6	3.45	4.90	3.06

[a] Explained in the Materials and Methods.

Pigment Extraction. For fruit, samples were taken from a homogenized triturate, prepared from 100 destoned fruits (ca. 40 g) of the most representative size by accurately weighing from 4 to 15 g for each analysis depending upon the degree of ripeness of the fruits. Pigments were extracted with *N*,*N*-dimethylformamide (DMF) saturated with $MgCO_3$.[15] The solid residue was collected by vacuum filtration, and the extraction was repeated until filtrates were colorless. For olive oil, samples of 15 g of virgin olive oil were dissolved with DMF saturated with $MgCO_3$. The extracts combined in a funnel were repeatedly treated with hexane. In both materials, chlorophylls, chlorophyll derivatives, and xanthophylls were retained in the DMF phase. The hexane phase contained lipids and carotenes. The DMF phase was treated with 10% (w/v) NaCl solution at 0 °C, and the chlorophylls and xanthophylls transferred to 100 mL of a mixture of diethyl ether/hexane (1:1, v/v). The aqueous layer was washed with diethyl ether and finally discarded, eliminating polyphenols and other water-soluble compounds. The combined organic phases were filtrated through anhydrous Na_2SO_4 and evaporated to dryness under vacuum at a temperature below 30 °C. The dry residue was dissolved in 1.5 mL of acetone prior to HPLC. Analysis was immediate or followed storage at −20 °C not more 18 h. All analysis was performed under green light.

Analysis of Chlorophylls and Carotenoids by HPLC. The separation and quantification of pigment products were carried out by HPLC using a HP 1100 Hewlett-Packard liquid chromatograph fitted with a HP1100 automatic injector HPLC. A stainless-steel column (20 × 0.46 cm inner diameter), packed with 3 μm C_{18} Mediterranea Sea (Teknokroma, Barcelona, Spain), was used. The column was protected by a precolumn (1 × 0.4 cm inner diameter) packed with the same material. Separation was performed using an elution gradient[17] (flow rate

of 1.25 mL min^{-1}) with the mobile phases: water/ion pair reagent/methanol (1:1:8, v/v/v) and methanol/acetone (1:1, v/v). The ion pair reagent was 0.05 M tetrabutylammonium and 1 M ammonium acetate in water. The column was stored in methanol/water (1:1, v/v). Sequential detection was performed with a photodiode array detector at 410, 430, 450, and 666 nm. Data were collected and processed with a LC HP ChemStation (Rev.A.05.04). Pigments were identified by co-chromatography with authentic samples and from their spectral characteristics. The online ultraviolet−visible (UV−vis) spectra were recorded from 350 to 800 nm with the photodiode array detector.

Measurement of Chlorophyllase Activity (EC 3.1.1.14). The enzyme was extracted from an acetone powder.[5] The standard reaction mixture (1.1 mL) contained about 0.1 μmol of chl *a* in acetone, 100 mM Tris buffer (pH 8.5) containing 0.24% (w/v) Triton X-100, and solubilized enzyme in a 1:5:5 ratio. The HPLC method is an adaptation[5] to the new 3 μm column. The chromatographic gradient is from 25 to 43.7% B in 3 min and then to 100% B in 3 min, followed by an isocratic elution for 7 min. The results are expressed as units of enzymatic activity per kilogram of acetone powder. One unit of enzymatic activity, the katal (kat), is defined as the amount of enzyme that catalyzes the formation of 1 mol of product per second. For each acetone powder, two enzymatic extractions were made, and for each extraction, two incubations were made.

Statistical Analysis of Data. All pigment analysis were carried out in triplicate, and the enzymatic determinations were carried out in quadruplicate. Data were expressed as the mean ± standard deviation (SD). The SD was always <10%. The data were analyzed for differences between the means using one-way analysis of variance (ANOVA). Duncan's multiple-range test was used as a post-hoc comparison of statistical significance ($p < 0.05$). All statistical analyses were performed using Statistica for Windows (version 5.1, StatSoft, Inc., Tulsa, OK).

RESULTS AND DISCUSSION

Pigment Content and Chlorophyll Metabolism in Fruits. Figure 1a shows the chromatogram correspondent to a pigment extract of 'Sikitita' cultivar fruits at 430 nm. At the level of total chlorophylls and carotenoides, it can be seen that the 'Sikitita' cultivar has no significant differences ($p < 0.05$) with the 'Arbequina' cultivar during the ripening of the fruit (Table 2). However, it is statistically different ($p > 0.05$) to the 'Picual' cultivar fruit (except at the last stage of ripening for the carotenoid fraction). If we consider the first control analyzed (prior to the start of ripening), we see that the 'Picual' cultivar fruit has a higher pigment content than 'Arbequina' and 'Sikitita'. We have used this state[4] to define varieties of high and low pigmentation; therefore, 'Sikitita' can be defined as a low pigmentation cultivar, similar to 'Arbequina'. However, when the ripening period begins (samples 2−5), the 'Picual' cultivar fruit experiences a very rapid degradation process compared to the fruit of the other two varieties. It is shown that the rate of degradation of chlorophylls and carotenoids is higher in the 'Picual' cultivar fruit than in the 'Arbequina' cultivar fruit.[4] However, throughout ripening, the pigment composition of the 'Picual' fruit is superior to the 'Arbequina' fruit.[4] Nevertheless, in this study, the 'Picual' fruit ripened ahead of the 'Arbequina' and 'Sikitita' varieties, with the 'Picual' fruit reaching the first turning color in the third harvesting compared to the other two cultivars, which reached it in the fourth harvesting. In fact, if we compare these stages of ripening (harvesting 3 for 'Picual' and harvesting 4 for 'Arbequina' and 'Sikitita'), we still observe the highest pigment content in 'Picual'. The higher speed of fruit ripening for the 'Picual' cultivar in this work is possibly due to its reduced fruit load in the season evaluated.

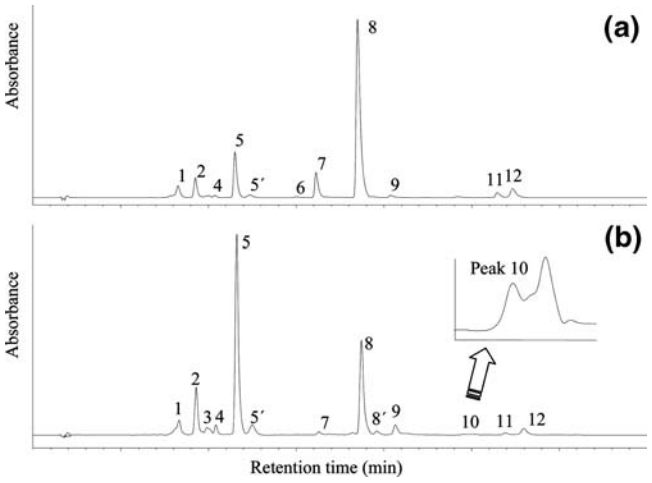

Figure 1. Chromatograms (at 430 nm) of (a) 'Sikitita' fruits and (b) olive oil. (Inset) Enlargement of peak 10. Peaks: 1, neoxanthin; 2, violaxanthin; 3, luteoxanthin; 4, anteraxanthin; 5, lutein; 5′, lutein isomer; 6, 13^2-OH-chl *a*; 7, chl *b*; 8, chl *a*; 8′, chl *a*′; 9, oxidized chl *a*; 10, esterified xanthophylls; 11, pheophytin a; and 12, β-carotene.

In relation to the oxidative metabolism of chlorophylls in the 'Picual' cultivar fruit, the percentage of allomerized chlorophyll (Table 2) does not exceed 1% and rapidly degrades. In contrast, an accumulation and subsequent degradation of 13^2-OH-chlorophyll *a* and 13^2-OH-chlorophyll *b* is seen in the 'Arbequina' variety fruit. The significance of oxidative metabolism within the general catabolism of chlorophylls is under investigation at this time, although some initial efforts have been made in search of the oxidative enzymes involved, such as peroxidase or chlorophyll oxidase in fruit.[18,19] Recently, the reaction mechanism of the peroxidase enzyme has been described[20] and identified 13^2-OH-chlorophyll *a* as an initial reaction product. The oxidative activity of chlorophylls in the 'Sikitita' fruit seems to have no relevance within the overall context of the catabolism of chlorophylls, as with the 'Picual' cultivar fruit and unlike the 'Arbequina' cultivar fruit.

In the fruit, the catabolic pathway of chlorophylls starts with the loss of the phytol chain (catalyzed by chlorophyllase), resulting in chlorophyllide, which later releases the central magnesium (through a metal-chelating substance), forming pheophorbide. From then on, a series of enzymatic reactions leads to the rupture of the macrocycle and, consequently, to loss of color. The sum of the initial products of the degradation route, chlorophyllide and pheophorbide, is known as dephytilated catabolites (Table 2). As expected,[5] the 'Picual' cultivar fruit does not accumulate dephytilated derivatives, while the 'Arbequina' cultivar progressively accumulates dephytilated catabolites with advancing ripening. No accumulation of dephytilates is detected in the 'Sikitita' cultivar fruit, except in the last stage of ripening.

As shown in Figure 2, the chlorophyllase activity of the 'Arbequina' cultivar fruit can be 100 times greater than the 'Picual' cultivar average activity.[5] On this occasion, higher levels of activity than those previously published were obtained, because the states analyzed in this study represent more advanced stages of maturity. Although the levels of chlorophyllase activity of the 'Sikitita' cultivar fruit are somewhat higher than those of 'Picual' (86.63 nkat/kg of acetone powder compared to 20.70 nkat/kg of acetone powder), they can be considered minimal compared to the levels determined in 'Arbequina'. In the three varieties, the

Table 2. Pigment Composition during Fruit Ripening in 'Picual', 'Arbequina', and 'Sikitita' Olive Varieties[a]

		harversting date				
		1	2	3	4	5
total chls[b]	'Picual'	445.18 ± 14.48 d	83.95 ± 12.45 d	83.95 ± 12.88 d	5.89 ± 1.35 d	2.38 ± 0.58 d
	'Arbequina'	313.78 ± 27.36 e	143.45 ± 6.48 e	108.66 ± 19.93 e	22.55 ± 8.93 e	6.38 ± 2.11 e
	'Sikitita'	324.04 ± 22.73 e	179.72 ± 26.57 e	120.37 ± 3.29 e	22.81 ± 3.33 e	7.83 ± 1.79 e
total carotenoids[b]	'Picual'	69.29 ± 0.90 f	15.67 ± 2.41 f	18.11 ± 1.58 f	3.69 ± 0.25 f	3.23 ± 0.27 f
	'Arbequina'	61.74 ± 12.35 g	29.47 ± 1.26 g	21.01 ± 3.01 g	6.80 ± 1.03 g	3.55 ± 0.05 f
	'Sikitita'	62.32 ± 5.02 g	30.55 ± 3.85 g	21.04 ± 1.02 g	7.23 ± 1.19 g	4.10 ± 0.67 f
oxidized chls[c] (%)	'Picual'	0.90 ± 0.10 h	0.41 ± 0.03 h	0.16 ± 0.01 h	0.00 ± 0.00 h	0.00 ± 0.00 h
	'Arbequina'	4.88 ± 0.45 i	6.02 ± 0.61 i	3.67 ± 0.31 i	2.70 ± 0.23 i	0.33 ± 0.04 i
	'Sikitita'	1.24 ± 0.01 h	0.36 ± 0.02 h	0.21 ± 0.02 h	0.53 ± 0.04 j	0.50 ± 0.03 j
dephytilated chls[c] (%)	'Picual'	0.00 ± 0.00 k	0.00 ± 0.00 k	0.00 ± 0.00 k	0.00 ± 0.00 k	0.00 ± 0.00 k
	'Arbequina'	0.26 ± 0.01 l	0.21 ± 0.04 l	0.12 ± 0.01 l	1.19 ± 0.14 l	5.65 ± 0.45 l
	'Sikitita'	0.00 ± 0.00 k	0.00 ± 0.00 k	0.00 ± 0.00 k	0.00 ± 0.00 k	0.29 ± 0.01 m

[a] Data were expressed as the mean ± SD. For each parameter (total chls, total carotenoides, oxidized chls, and dephytilated chls), different letters for the same harvesting date indicate significant differences ($p < 0.05$) between varieties. [b] Milligrams per kilogram of dry weight. [c] chls = chlorophylls.

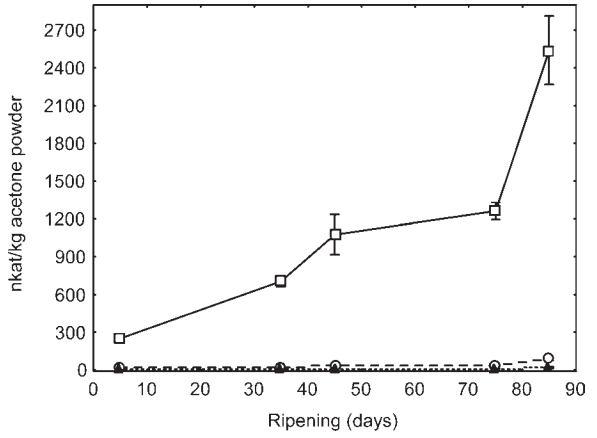

Figure 2. Chlorophyllase activity during the ripening of (□) 'Arbequina', (▲) 'Picual', and (○) 'Sikitita' olive fruit varieties during the ripening period. The ripening days are calculated on the basis of the harvesting dates (Table 1). Each symbol represents the mean ± SD.

chlorophyllase activity increases with advancing ripening, which is expected because it is a catabolic enzyme. However, there is no explanation in the last stage of ripening for such a high chlorophyllase activity for the low chlorophyll content present in the fruit. At this point, we must take into account the nonphysiological extraction conditions required for measuring enzyme activity. This may cause the *in vitro* determination to be a nonphysiological activation of the enzyme, which the enzyme does not reach *in vivo*. However, if we correlate the enzyme activity levels found *in vitro* (Figure 2) with the levels of dephytilated catabolites found *in vivo* (Table 2), there is a parallel relationship among the three varieties. Consequently, the measurement of chlorophyllase activity *in vitro* is a good indicator of enzyme activity in the fruit.

There is a surprisingly low chlorophyllase activity in the 'Sikitita' cultivar fruit, which can be considered low pigmentation (levels similar to 'Arbequina'; Table 2). Chlorophylls in their membranes are subjected to a continuous turnover,[21] and it is assumed that chlorophyllase is one of the enzymes involved in the degradation of chlorophyll in that turnover.[22] It has thus far been considered that a cultivar of highly pigmented olive (green 'Picual' fruit) has a low chlorophyllase activity, because of less pressure to be degraded, which leads to higher net levels of chlorophyll. Instead, a cultivar with low chlorophyll content ('Arbequina' in the green state) has a high chlorophyllase activity, which has a high chlorophyll degradation turnover and, consequently, low levels of pigmentation. The data for the 'Sikitita' cultivar fruit imply that it may be another chlorophyll-degrading enzyme (pheophorbide a oxygenase path) [23] that is responsible for the low chlorophyll content of a cultivar, without it necessarily having to be chlorophyllase, as seemed clear to date. The maternal inheritance of the 'Sikitita' chlorophyllase genes (from chlorophyllase values) is remarkable.

Carotenoid Metabolism in Fruits. The carotenoid biosynthetic pathway is divided into two pathways from the all-*trans* lycopene:[24] the path called β, β, because of the action of a β-cyclase resulting in two β,β rings forming β-carotene and from this, via several modifications, to anteraxanthin, violaxanthin, and neoxanthin xanthophylls, and the path called β, ε, because of the action of a ε-cyclase to form α-carotene, which hydroxylates to form lutein. The primary role of carotenoids in green fruit and leaves is its accessory role in light-harvesting centers and photoprotective pigments in pigment−protein complexes. Therefore, the carotenoid composition in the chloroplasts of higher plants is almost uniform. β-Carotene is found in the reaction centers and is usually between 25 and 30%. The light-harvesting antenna complexes contain the pool of xanthophylls. Among these, lutein (40−45%), violaxanthin (10−15%), and neoxanthin (10−15%) constitute the majority.[25] This distribution is approximately what is observed in the fruit of the three varieties tested (Table 3), as would be expected from a green fruit (state 1).

However, with advancing fruit ripening, different carotenoid profiles are observed[26] depending upon the carotenogenic processes or different degradation rates. In the 'Picual' cultivar fruit, a clear trend toward the dominance of the β, ε series (lutein) is observed during ripening, which reaches nearly 90% of the carotenoids in ripe fruit. This is because β-carotene generally degrades more rapidly than lutein.[26] Therefore, the carotenoid content in the β, β series is less in the 'Picual' cultivar fruit, especially at the xanthophyll level (violaxanthin, anteraxanthin, and neoxanthin), whose presence in ripe fruits is almost residual.

Table 3. Percentage Composition of Individual Carotenoids in Fruits of the 'Picual', 'Arbequina', and 'Sikitita' Cultivars during Ripening[a]

		harversting date				
		1	2	3	4	5
lutein	'Picual'	46.64 ± 0.97 b	50.14 ± 2.36 b	54.01 ± 2.17 b	62.09 ± 4.10 b	88.62 ± 4.04 b
	'Arbequina'	43.86 ± 0.38 c	38.80 ± 0.50 c	38.43 ± 0.17 c	44.57 ± 0.13 c	60.49 ± 2.92 c
	'Sikitita'	37.14 ± 0.24 d	39.52 ± 1.12 c	39.21 ± 1.19 c	63.60 ± 3.74 b	71.94 ± 1.46 d
β-carotene	'Picual'	17.19 ± 0.37 e	21.66 ± 4.04 e	25.08 ± 3.72 e	31.82 ± 4.63 e	6.46 ± 4.06 e
	'Arbequina'	20.13 ± 1.92 e	24.72 ± 0.35 e	28.13 ± 2.96 e	29.24 ± 4.50 e	18.41 ± 4.40 f
	'Sikitita'	24.34 ± 2.01 f	27.20 ± 2.48 e	27.89 ± 1.88 e	13.20 ± 2.17 f	11.64 ± 1.48 e
β-cryptoxanthin	'Picual'	0.00 ± 0.00 g	0.21 ± 0.07 g	0.39 ± 0.04 g	0.00 ± 0.00 g	0.00 ± 0.00 g
	'Arbequina'	0.00 ± 0.00 g	0.12 ± 0.01 h	0.51 ± 0.03 h	0.88 ± 0.13 h	0.70 ± 0.03 h
	'Sikitita'	0.00 ± 0.00 g	0.00 ± 0.00 i	0.07 ± 0.01 i	0.12 ± 0.02 i	0.57 ± 0.02 i
violaxanthin	'Picual'	17.27 ± 0.37 j	12.50 ± 0.62 j	7.26 ± 0.35 j	1.94 ± 0.18 j	1.27 ± 0.15 j
	'Arbequina'	18.49 ± 1.27 j	20.20 ± 2.20 k	16.35 ± 2.77 k	13.49 ± 2.57 k	9.29 ± 0.39 k
	'Sikitita'	22.13 ± 0.82 k	16.94 ± 0.73 k	17.33 ± 0.16 k	12.00 ± 0.62 k	7.11 ± 1.16 l
anteraxanthin	'Picual'	2.34 ± 0.04 m	2.58 ± 0.01 m	2.34 ± 0.39 m	0.86 ± 0.00 m	0.99 ± 0.08 m
	'Arbequina'	2.66 ± 0.05 m	3.31 ± 0.13 n	4.98 ± 0.21 n	3.93 ± 0.04 n	3.68 ± 0.19 n
	'Sikitita'	1.58 ± 0.17 n	1.55 ± 0.09 o	1.80 ± 0.10 m	2.37 ± 0.11 o	3.19 ± 0.57 n
neoxanthin	'Picual'	16.56 ± 0.29 p	12.91 ± 0.78 p	10.92 ± 0.86 p	3.29 ± 0.35 p	2.53 ± 0.25 p
	'Arbequina'	14.86 ± 0.23 q	12.85 ± 2.34 p	11.59 ± 0.11 p	7.89 ± 1.97 q	7.42 ± 0.48 q
	'Sikitita'	14.81 ± 0.80 q	14.81 ± 0.64 p	13.70 ± 0.66 q	8.71 ± 1.70 q	5.55 ± 0.39 r

[a] Data were expressed as the mean ± SD. For each parameter (lutein, β-carotene, β-cryptoxanthin, violaxanthin, anteraxanthin, and neoxanthin), different letters for the same harvesting date indicate significant differences ($p < 0.05$) between varieties.

The carotenoid composition of the 'Arbequina' cultivar fruit is a reflection of the emerging carotenogenesis[4] that they experience. The fruit of this cultivar has a higher content of xanthophylls in the β, β series (violaxanthin, anteraxanthin, and neoxanthin) at the end of maturation compared to the 'Picual' cultivar fruit, and it therefore has a lower lutein content.

In general, the 'Sikitita' cultivar fruit has a carotenoid profile quite similar to 'Arbequina'. Toward the end of ripening, the xanthophyll content of the β, β series (violaxanthin and neoxanthin) is significantly higher than 'Picual' ($p < 0.05$). At the end of ripening, the lutein content of 'Sikitita' is between the 'Picual' and 'Arbequina' varieties. These results could be indicative of a carotenogenic process, but no net carotenoid synthesis has been found in the 'Sikitita' fruit. However, there are two aspects that clearly differentiate 'Arbequina' and 'Sikitita'. First, 'Sikitita' has a lower lutein degradation rate in advanced stages of ripening, which translates into higher values (statistically different, $p < 0.05$) compared to 'Arbequina'. Second, 'Sikitita' has a very low initial anteraxanthin content (below 2%), which increases toward the end of ripening, eventually equaling that of 'Arbequina'. In conclusion, a carotenogenic process similar to that of 'Arbequina' can be detected in the 'Sikitita' cultivar fruits, although it also has certain maternal ('Picual') tendencies.

Pigment Profile in Olive Oils. The chloroplast pigment content of olive oil (Figure 1b) is a direct consequence of the metabolism of chlorophylls and carotenoids intrinsic to the fruit, as amended by the extraction conditions of virgin olive oil. Although color is currently not part of the quality indices of virgin olive oil, it remains essential in marketing. Therefore, adulteration of the color is a practice in the sector.[17] For the three varieties tested, the total pigment content is similar in the first sample analyzed (Table 4), although previous studies[27] show the pigment content of the 'Picual' cultivar to be higher than that of 'Arbequina'. As commented earlier, trees of the 'Picual' cultivar matured very quickly compared to the 'Arbequina' and 'Sikitita' cultivars, so that, in the advanced stages of maturity, the 'Picual' cultivar oil has very low pigmentation.

The balance between the two fractions of pigments decreases with maturation, because of the faster degradation of the chlorophyll fraction compared to the carotenoid.[5] This index has been proposed as an indicator of the authenticity of virgin olive oil,[27] between 1.5 (early season oil) and 0.5 (end of season). In general, the oils analyzed meet this standard (Table 4), except for 'Arbequina' and 'Sikitita' at the beginning of harvesting and at the end for the 'Picual' cultivar. It must be taken into account that the oils analyzed in this study are not commercial but the ripening stages have been chosen to study the widest possible ripening range.

At the level of carotenoids, 'Sikitita' cultivar oil differs from that of 'Picual' in terms of its low percentage of lutein (significantly different, $p < 0.05$) and high levels of violaxanthin and neoxanthin (significantly different, $p < 0.05$). This is a direct reflection of the metabolism of the fruit, which resembles the 'Arbequina' cultivar. However, it is striking that the 'Sikitita' cultivar oil has higher β-carotene content than the 'Arbequina' and 'Picual' varieties. These differences are not attributable to the metabolism of carotenoids in fruit during ripening because, as we have seen (Table 3), there were no significant differences. This result could be explained in terms of the different behavior during the extraction process. Contrary to what was originally thought, the pigments are largely occluded in the olive pomace (60% chlorophylls and 25% carotenoids).[28] It may be that there is less β-carotene occlusion during 'Sikitita' oil milling. Also, given that the destruction losses are also lower for this pigment because of the increased antioxidant capacity of its structure,[29] a greater proportion may be transferred to the oil. In any case, the fact that

Table 4. Main Pigments Indices of 'Picual', 'Arbequina', and 'Sikitita' Olive Oils[a]

		harvesting date		
		4	5	6
total pigments[b]	'Picual'	23.53 ± 0.39 c	6.62 ± 0.08 c	2.99 ± 0.16 c
	'Arbequina'	27.64 ± 1.79 d	12.46 ± 0.41 d	4.39 ± 0.22 c
	'Sikitita'	20.29 ± 1.81 e	13.73 ± 0.68 e	11.31 ± 0.96 d
ratio of chlorophylls/carotenoids	'Picual'	1.56 ± 0.06 f	0.55 ± 0.01 f	0.19 ± 0.03 f
	'Arbequina'	1.64 ± 0.32 f	0.83 ± 0.02 g	0.48 ± 0.07 g
	'Sikitita'	1.58 ± 0.05 f	0.87 ± 0.03 g	0.78 ± 0.05 h
lutein (%)	'Picual'	69.50 ± 2.82 i	80.11 ± 0.38 i	87.92 ± 0.23 i
	'Arbequina'	46.50 ± 0.83 j	41.86 ± 0.19 j	50.69 ± 1.16 j
	'Sikitita'	40.78 ± 0.49 k	54.31 ± 1.62 k	60.71 ± 0.04 k
β-carotene (%)	'Picual'	16.62 ± 0.49 l	11.38 ± 0.64 l	8.51 ± 0.13 l
	'Arbequina'	22.21 ± 1.23 m	17.17 ± 1.13 m	18.91 ± 1.12 m
	'Sikitita'	28.26 ± 0.97 n	21.52 ± 1.89 n	20.88 ± 1.08 m
violaxanthin (%)	'Picual'	5.26 ± 0.34 o	3.41 ± 0.12 o	1.17 ± 0.11 o
	'Arbequina'	11.49 ± 0.18 p	24.93 ± 0.50 p	15.84 ± 0.69 p
	'Sikitita'	18.49 ± 0.31 q	13.79 ± 0.27 q	11.00 ± 0.83 q
anteraxanthin (%)	'Picual'	3.76 ± 0.02 r	2.50 ± 0.14 r	1.24 ± 0.02 r
	'Arbequina'	10.29 ± 0.17 s	5.49 ± 0.10 s	7.32 ± 0.21 s
	'Sikitita'	3.26 ± 0.05 r	2.16 ± 0.05 t	2.15 ± 0.21 t
neoxanthin (%)	'Picual'	4.19 ± 0.15 u	1.68 ± 0.05 u	0.79 ± 0.09 u
	'Arbequina'	5.79 ± 0.04 v	5.18 ± 0.33 v	3.09 ± 0.32 v
	'Sikitita'	7.65 ± 0.09 w	6.18 ± 0.01 w	3.70 ± 0.05 w
β-cryptoxanthin (%)	'Picual'	0.66 ± 0.02 x	0.93 ± 0.05 x	0.37 ± 0.03 x
	'Arbequina'	2.00 ± 0.04 y	1.29 ± 0.00 y	1.20 ± 0.01 y
	'Sikitita'	0.59 ± 0.01 z	0.60 ± 0.01 z	0.52 ± 0.09 z
esterified xanthophylls (%)	'Picual'	0.00 ± 0.00 aa	0.00 ± 0.00 aa	0.00 ± 0.00 aa
	'Arbequina'	1.73 ± 0.03 ab	4.09 ± 0.02 ab	2.94 ± 0.24 ab
	'Sikitita'	0.97 ± 0.01 ac	1.44 ± 0.06 ac	1.05 ± 0.06 ac

[a] Data were expressed as the mean ± SD. For each parameter (total pigment content, ratio of chorophylls/carotenoides, lutein, β-carotene, violaxanthin anteraxanthin, neoxanthin, β-cryptoxanthin, and esterified xanthophylls), different letters for the same harvesting date indicate significant differences ($p < 0.05$) between varieties. [b] Milligrams per kilogram of dry weight.

the 'Sikitita' cultivar oil has a higher content of β-carotene, a pigment with a high antioxidant capacity and provitamin A value, is a factor to evaluate positively.

However, the most spectacular of the results is the presence of esterified xanthophylls in the 'Sikitita' cultivar oil. The esterification of carotenoids occurs only at the chromoplast level, i.e., chloroplasts forming biosynthesized organelles. This implies that a carotenogenic process occurs in the 'Sikitita' cultivar fruit, similar to the 'Arbequina' cultivar fruit, although at a lower level (judging by the percentage of carotenoid esters). Therefore, 'Arbequina' and 'Sikitita' are currently the only two varieties of olive that have developed a carotenogenic process. In 'Sikitita' fruits, esterified xanthophylls are not detected because they are present at low levels; however, the more lipophilic pigments become more concentrated during oil extraction, thus allowing their quantification. The carotenogenic process in the 'Sikitita' cultivar fruit explains the high neoxanthin and violaxanthin content in the fruit and oil because of the biosynthetic process and the consequent proportional decrease in lutein, as with the 'Arbequina' cultivar fruit.

The carotenoid esterification process has been described in many fruits and flowers; however, the molecular and enzymatic mechanisms underlying this reaction are unknown.[30] Esterification is a reaction that increases the lipophilicity of carotenoids, which facilitates its accumulation in the plastoglobules. It has been proposed that the generation and availability of free fatty acids determine the esterification and their accumulation.[31] Specifically, enzymes that generate fatty acids and esterified carotenoids, such as a type of lipase, could regulate the accumulation of carotenoids.[32] Despite the high fatty acid content of olive fruit (20−25% of fresh weight), carotenoids are present only in the free (non-esterified) form. Uniquely, the 'Arbequina' cultivar fruit and now also the 'Sikitita' cultivar fruit are the only ones with esterified carotenoids. It follows that something more than biosynthesized fatty acid enzymes are required to produce the esterification of carotenoids in a fruit. The 'Sikitita' cultivar fruit has inherited the paternal enzyme pool and lipophilic structures necessary to produce the phenomenon of carotenogenesis. The presence of carotenoid esters in the 'Sikitita' cultivar oil is a chemotaxonomic differentiating factor from other olive oil varieties.

In conclusion, the new 'Sikitita' cultivar has a very characteristic pigment composition, which is a mixture of the characteristics of its progenitors, in both the fruit and oil. This demonstrates the potential of breeding to produce cultivars of olives with different qualities to make them more attractive in the olive oil market.

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