Pigment Changes in Olives during Fermentation and Brine Storage

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The qualitative and quantitative variations taking place in the original chloroplastic pigment composition have been analyzed during lactic fermentation and subsequent brine conservation of the fruit. The study shows that chlorophyll degradation during the elaboration of table olives can take place by two different mechanisms, resulting in a mixture of pheophytins and pheophorbides in the final product. With respect to the carotenoid fraction, only β-carotene and lutein are resistant to the preparation process. The precursors phytofluene and ζ-carotene disappear, and violaxanthin, luteoxanthin, and neoxanthin give rise to their isomers auroxanthin and neochrome. The fact that the total balance of pigments remains constant with time indicates that the degradation taking place implies only transformation of the pigments and does not give rise to uncolored final products with pigmentation loss.

The changes undergone by vegetable chloroplastic pigments during ripening and storage, or as a consequence of the processes of freezing, scalding, dehydration, etc., which they are subjected to for the best conservation of their alimentary and organoleptic properties, have a considerable effect on the color of the final product (Clydesdale and Francis, 1982).

Walker (1964), on examining samples of frozen beans, observed first the conversion of the chlorophylls to pheophytins and then the degradation of both, which, as he put forward, is due to a peroxidation process of the fat. Wang et al. (1971) studied the effect of storage atmosphere on the degradation of chlorophyll in asparagus, and Jones et al. (1962, 1963) found the rapid formation of chlorophyllides and pheophorbides, as well as pheophytins, at 180 °F on testing the different treatments in commercial blanching processes.

There is general agreement that the main cause of green vegetable discoloration during these stages is due to the conversion of chlorophylls to pheophytins by the influence of pH. During this reaction hydrogen ions can transform the chlorophylls to their corresponding pheophytins by substitution of the magnesium ion in the porphyrin ring.

On the other hand, the enzyme chlorophyllase has some exceptional characteristics in vivo, so that unlike the majority of enzymes it acts at ambient temperature only in the presence of high concentrations of organic solvents and in aqueous medium acts only at temperatures between 65 and 75 °C. This enzyme hydrolyzes the phytyl ester group of chlorophylls and pheophytins, giving rise to chlorophyllides and pheophorbides, respectively.

In the case of table olives, the processed fruit does not always achieve the highly valuable yellowish green color. Since color is an important quality attribute, it is interesting to know what happens to the responsible pigments during processing.

The traditional elaboration system of olives in Spanish or Seville style consists, schematically, of an initial treatment of the fruits with dilute sodium hydroxide solution, the main aim being the elimination of the bitter glucoside oleuropein. Next there is a water washing to eliminate the excess of alkali remaining on the fruits, and finally they are put into brine, bringing about the lactic fermentation process to give the fruit its special and valued organoleptic characteristics.

The present work studies how the different steps of the preparation process affect the initial pigment composition of the fruit, what type of transformation has taken place, and what the origin of these changes is. At the same time, the possible degradation mechanism is suggested.

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EXPERIMENTAL PART

Raw Material Used. The study was carried out on olives of Hojiblanca variety, *Olea europaea arolensis*. The fruits were picked from the tree in the ripening stage corresponding to the color yellowish green. For the experiment, two 750-kg-capacity fermenters were controlled. Once filled with olives, 2% NaOH solution was added, a treatment that lasted 6 h. Then, the fruits were subjected to a water washing for 12 h. At the end of this period, the water was removed and 12% NaCl solution was added. At this point, the pH of the medium could be greater than 10 due to the alkali content still held by fruit. Under normal conditions, the pH reaches a value of approximately 6.5 in 2–3 days. The periodic control of pigments was started at this moment, with the object of following their evolution, in function of the parallelism between pH changes and the fermentation process.

The brine is transformed little by little into a suitable culture medium for microorganisms, and in 10–12 days the pH reaches a value of 6. During this first fermentation phase, the Gram-negative bacilli are the most characteristic microorganisms, their metabolism giving rise to carbon dioxide, hydrogen, acetic acid, lactic acid, ethyl alcohol, etc., as final products.

When, as a consequence of the fall in pH, strong development of Lactobacilli begins, the second phase of fermentation starts, leading to a considerable increase in acidity, reaching a pH of up to 4.5. *Lactobacillus plantarum* is the most representative microbe present, together with *Lactobacillus delbrueckii*. Both belong to the homofermentative lactic bacteria and produce practically only lactic acid as a final product of glucose fermentation.

During the third phase of fermentation the pH reaches 4.0 or less, acid formation ceasing as a result of the depletion of fermentable matter.

Last, at the end of this period the fruit still needs a time in its brine, until curing is achieved, before its packing and commercialization.

EXTRACT PREPARATION AND PIGMENT SEPARATION

The analyses were at all times made in duplicate. Sample (10 g) was taken from one homogenized from 15–20 destoned fruits. The pigment extraction was made with *N,N*-dimethylformamide. The filtrates were next treated with hexane in a separatory funnel in order to extract and separate the characteristic fatty matter from the previous solution. Hexane in turn carried over the carotene fraction,

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Table I. Absorption Maxima and Extinction Coefficients

pigment	λ_{max}	E_0	λ_{max}	E_0
	Acetone			
chlorophyll *a*	428	840	662	670
chlorophyll *b*	454	1450	646	518
pheophytin *a*	406	1290	666	540
pheophytin *b*	430	2060	654	395
lutein	446	2340		
β-carotene	450	2620		
violaxanthin	440	2340		
neoxanthin	438	2050		
neochrome	420	2270		
	Acetone/Pyridine (1:1)			
chlorophyllide *a*	438	813	668	648
chlorophyllide *b*	468	1471	654	504
pheophorbide *a*	410	1237	668	510
pheophorbide *b*	436	2075	660	400

while the residual to *N,N*-dimethylformamide retained chlorophylls, chlorophyll derivatives, and the rest of the carotenoids. Details on the extraction process are referred to in a previous work (Minguez and Garrido, 1989).

The pigment separation was carried out by thin-layer chromatography on silica gel 60 GF_{254} with light petroleum ether/acetone/diethylamine (10:4:1). For the separation of pheophorbides, the same developer was used, but substituting for diethylamine pyridine. The separation of pheophorbides and chlorophyllides was carried out on Kieselgur plates covered with peanut oil, using methanol/acetone/water (20:4:3) as developer. The bluish green and yellowish green chlorophyllides pass to the chromatogram front, and the gray and yellow-brown pheophorbides are retained with R_f 0.6 (Jones et al., 1972). For lutein purification silica gel 60 GF_{254} plates and the mixture benzene/ethanol (22:1) were used.

PIGMENT IDENTIFICATION

Chlorophylls and Derivatives. The absorption spectra, as well as the color shown by these substances in TLC under white and UV light, served as a basis for their identification (Smith and Benitez, 1955). Purified solutions of pheophytins were prepared according to the procedure described by Jones et al. (1968), of pheophorbides according to Hynninen (1973), and of chlorophyllides following the method proposed by Jones et al. (1972).

Carotenoids. The adsorption properties of these pigments in thin-layer, before and after saponification, absorption spectra in the visible and absorption bands in IR were taken into account. For the confirmation of functional groups, distinct physicochemical reactions specified in Davies (1976) were assayed.

Quantification. Once the chromatographic development of a known quantity of pigment extract was finished, the corresponding substance was scraped from the plate, eluted with acetone or diethyl ether, and made up to a determinate volume. Next the respective absorption spectrum was obtained, and the extinction value E_0, at the maximum absorption wavelength, was substituted in the equation $E = E_0C$. When the corresponding operations were carried out, the results were obtained in milligrams per kilogram of destoned fruit.

The values calculated for the extinction coefficient E_0 in acetone, from those given in the references in ethyl ether for chlorophylls (Smith and Benitez, 1955) and pheophorbides (Jones et al., 1962) and in ethanol for carotenoids (Davies, 1976), are given in Table I. The chlorophyllides were eluted from the plate with acetone/pyridine (1:1), and the value of E_0 was calculated from that of E_0 for chlorophylls in acetone.

Apparatus used: Buchi Rotavapor, Model R 110; DESAGA UV/vis lamp, provided with white light and ultraviolet $UV_{254,366}$; Hewlett-Packard UV/vis spectrophotometer, Model 8450, provided with Hewlett-Packard recorder, Model 7225A; Perkin-Elmer 782 IR spectrophotometer, with computer, Model 3600.

RESULTS AND DISCUSSION

Figure 1 shows the characteristics of the chromatograms obtained by spotting the plate in parallel with samples of the extracts purified of fatty matter, corresponding to fresh and processed fruit. The changes caused in the pigment composition by the fermentation process are obvious. Both the color of the substances on the plate and their R_f values have undergone considerable modifications. The only bands remaining in both developments are those numbered 1 and 6.

The component identification study (Table II) demonstrates that chlorophylls *a* and *b* present in the fresh fruit (Minguez and Garrido, 1989) disappear completely in the processed, giving rise to their corresponding derivatives, pheophytins *a* and *b* and pheophorbides *a* and *b* (Figure 2). Of the carotenoid fraction, only β-carotene and lutein remain, so that the compounds having 5,6-epoxide groups in their molecule, such as violaxanthin and neoxanthin, have passed to their corresponding isomers, auroxanthin and neochrome, both with 5,8-furanoid groups (Figure 3).

Consequently, we go on to consider when such modifications begin and how they evolve with time. Table III

Table II. Characteristics Used To Identify Table Olive Pigments, Separated on Silica Gel with Light Petroleum Ether (40–60 °C)/Acetone/Diethylamine (10:4:1)

band no.[a]	spectral abs: λ_{max}, nm: light petroleum ether	spectral abs: λ_{max}, nm: chloroform	IR: –OH	IR: ester C=O	epoxide test (HCl treatment): hypsochromic shift (EtOH), nm	color on TLC	carotenoid
			I. Hydrocarbons				
1	(426), 444, 470	(434), 458, 482	–	–	0	yellow	β-carotene
			II. Xanthophylls				
4	418, 442, 470	(430), 452, 482	+	–	0	brown and green	lutein
4′	380, 400, 422	388, 410, 436	+	–	0	blue	auroxanthin
5	399, 418, 446	402, 426, 454	+	–	0	blue-green	neochrome

band no.	spectral abs: λ_{max}, nm: acetone	spectral abs: λ_{max}, nm: diethyl ether	color white light	chlorophyll deriv
2	406, 470, 501, 534, 558, 608, 666	406, 468, 504, 532, 558, 608, 666	gray	pheophytin *a*
3	412, 432, 522, 558, 598, 654	412, 430, 522, 556, 598, 654	brown	pheophytin *b*
6	410, 468, 506, 536, 610, 668		gray	pheophorbide *a*
6′	410, 428, 526, 606, 656		yellow-green	pheophorbide *b*

[a] Bands 4 and 4′ were separated on silica gel 60 GF_{254} with benzene/ethanol (22:1). Bands 6 and 6′ were separated on Kieselgur impregnated with peanut oil in light petroleum ether (14%, v/v). Solvent: methanol/acetone/water (20:4:3).

Table III. Pigment Content in Fresh and Brined Olives during the Fermentation and Curing Processes (mg/kg)[a,b]

pH	time, days	chlorophylls and derivatives								carotenoids				
		chl *a*	chl *b*	chd *a*	chd *b*	phy *a*	phy *b*	pho *a*	pho *b*	1	2	3	4	5
fresh		72.58	19.65							3.41	7.27	2.50	2.22	
6.50	3	69.10	16.80	2.90	2.50					3.42	7.10	2.40	2.10	
6.43	5	60.30	16.32	10.57	3.04					3.45	8.30	2.18	1.64	
6.39	12	43.93	13.31	11.73	6.12	12.60		3.70		3.70	6.97	2.16	1.43	
5.61	15	41.75	12.90	7.85	6.22	12.11		10.31		4.06	9.10[c]	1.43	1.57	
5.10	22	26.10	10.90	7.65	5.38	12.42		11.20	2.83	3.64	8.37[c]	1.06	1.72	
4.44	27	22.14	6.69	5.07	2.44	16.85	2.64	12.16	5.30	3.76	7.49[c]	1.05	1.16	1.10
4.29	40	15.18	7.29	4.59	1.41	23.81	4.01	13.92	6.41	3.45	7.38[c]	0.71	0.99	1.20
4.11	49	11.40	5.37		2.50	21.93	3.78	20.40	5.43	3.74	7.51[c]	0.68	0.60	1.57
4.08	56	9.38	5.11		1.50	24.87	3.40	23.43	7.60	4.54	9.41[c]		1.02	1.13
4.03	84	6.39	4.78			29.38	5.43	20.67	7.89	3.29	8.59[c]		0.61	1.30
4.03	91	2.83	4.87			35.24	4.31	18.16	7.82	3.65	8.66[c]			2.33
4.03	104	2.81	4.45			32.50	4.85	21.40	7.50	4.14	8.25[c]			2.04
3.96	125		3.00			35.74	5.84	22.40	8.33	3.78	8.83[c]			2.10
3.50	210					35.70	8.70	23.80	7.20	3.52	9.38[c]			2.10

[a] Destoned olive basis, average of duplicate analysis of two samples. [b] Key: chl *a* = chlorophyll *a*; chl *b* = chlorophylll *b*; chd *a* = chlorophyllide *a*; chd *b* = chlorophyllide *b*; phy *a* = pheophytin *a*; phy *b* = pheophytin *b*; pho *a* = pheophorbide *a*; pho *b* = pheophorbide *b*; 1 = β-carotene; 2 = lutein; 3 = violaxanthin; 4 = neoxanthin; 5 = neochrome. [c] Total lutein + auroxanthin.

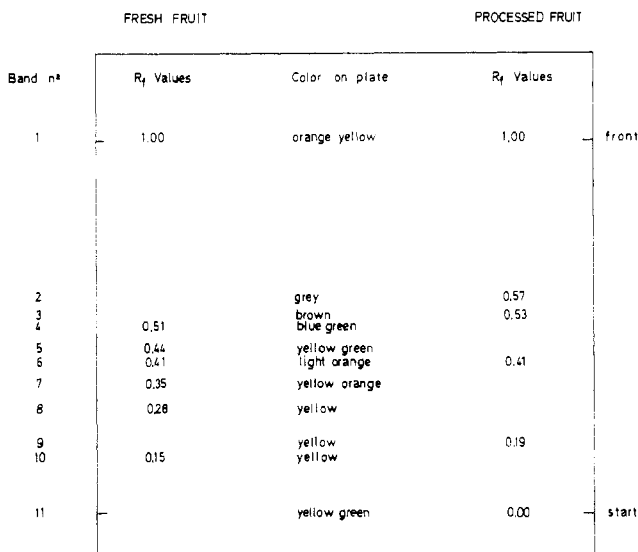

Figure 1. Scheme of the characteristic thin-layer chromatogram on silica gel GF_{254} of pigments from fresh and processed fruits (light petroleum ether/acetone/diethylamine (10:4:1)).

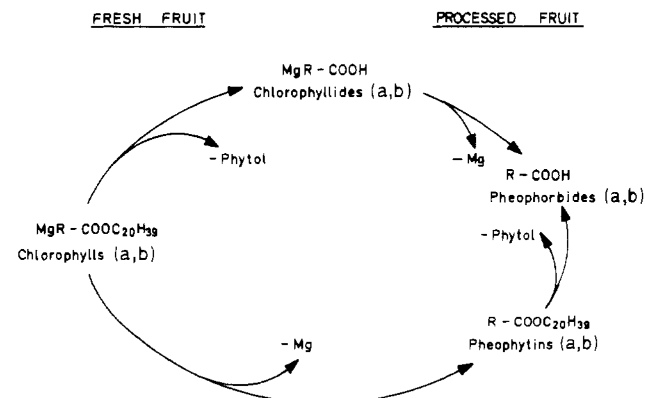

Figure 2. Structural changes of the chlorophylls and their derivatives identified in fresh and processed fruit.

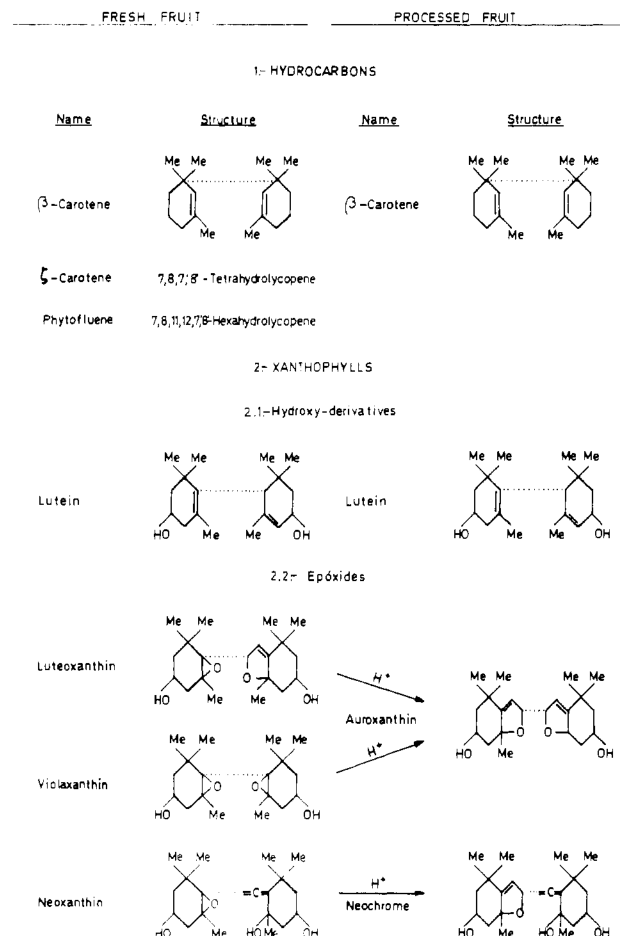

Figure 3. Structural changes of the carotenoid pigments identified in fresh and processed fruit.

shows the individual concentrations of chlorophylls, chlorophyll derivatives, and principal carotenoids during the fermentation process and later period of brine conservation. Figure 4 presents the pigment change that took place during the fermentation process and brine storage.

The alkaline treatment practically did not alter the initial pigment composition. During the first 15 days of brine pickling, the first fermentation phase, chlorophyll *a* was transformed to its corresponding Mg-free derivative pheophytin *a* and a mixture of chlorophyllide *a* and pheophorbide *a*. Also, along this period, chlorophyll *b*, being more resistant to the acid medium than chlorophyll *a*, is only partly converted to chlorophyllide *b*.

At the end of the second period, after 30 days, the pH of the medium falls to 4.5, and chlorophyll *a* undergoes an important transformation. The chlorophyll *b* content has fallen greatly, giving rise to pheophytin *b* and a mixture of chlorophyllide *b* and pheophorbide *b*. During the following month, the detection of chlorophyll *a* and *b* con-

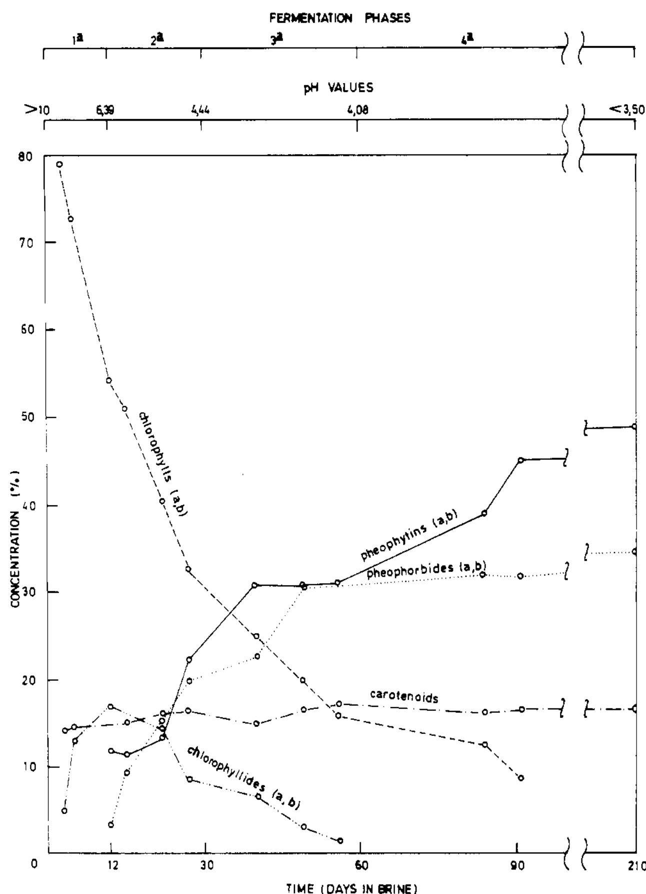

Figure 4. Evolution of pigments during fermentation and brine curing, expressed as the percentage of the total pigment recovered.

tinues, and the chlorophyllides have given rise to their corresponding pheophorbides, whose concentration rises in this stage, as does that of the pheophytins.

After 7 months, the fruit is now totally prepared for its commercialization. Neither chlorophylls nor chlorophyllides appear, and the levels of pheophytins and pheophorbides remain practically constant during this time.

Thus, the outstanding facts can be said to be that the alkaline treatment as such does not affect in principal the chlorophyllic fraction, although the high pH could favor the action of chlorophyllase later to produce chlorophyllides. During the first fermentation phase, chlorophyll *a* rapidly gives rise to pheophytin, chlorophyllide, and pheophorbide. The transformation to pheophytin is due to the effect of the slight acidity of the medium. The conversion to chlorophyllide and pheophorbide in the first days of brine pickling seems to indicate that this has taken place by means of an enzymatic, and not chemical, hydrolysis, caused by the action of chlorophyllase, present in almost all green tissues. The action of this enzyme is helped by a medium with optimum pH, in which the development of the microbial flora progressively leads to the formation of distinct organic components that can activate its action.

The pheophorbide level remains constant once the chlorophylls and chlorophyllides disappear. As, on the other hand, the pheophytin content remains invariable, it is improbable that the pheophorbides are formed from pheophytins by acid hydrolysis, since in this case their concentration would have increased with time, to the detriment of the pheophytins.

During the elaboration process, the carotenoid fraction has been affected only in those components that because of their molecular structure are sensitive to acid medium, and their concentration does not vary significantly with time.

The total balance of pigment material can be considered fixed during the fermentation process. The color changes that the fruits undergo during processing are due exclusively to pigment transformations, catalyzed by the acid pH of the medium and the action of chlorophyllase, which demonstrates the absence of other types of oxidative reactions that degrade them to uncolored products, such as the contact with oxidized lipids or caused by any lipoxidase.

Registry No. Chl *a*, 479-61-8; chl *b*, 519-62-0; chd *a*, 14897-06-4; chd *b*, 14428-12-7; phy *a*, 603-17-8; phy *b*, 3147-18-0; pho *a*, 15664-29-6; pho *b*, 20239-99-0; violaxanthin, 126-29-4; neoxanthin, 14660-91-4; ζ-carotene, 72746-33-9; phytofluene, 27664-65-9; luteoxanthin, 1912-50-1.

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