Mechanism and Kinetics of the Degradation of Chlorophylls during the Processing of Green Table Olives

M. Isabel Mínguez-Mosquera,* Beatriz Gandul-Rojas, and Julio Mínguez-Mosquera

Unidad Estructural de Biotecnología de los Alimentos, Instituto de la Grasa y sus Derivados (CSIC), Avenida Padre García Tejero 4, 41012 Sevilla, Spain, and Departamento de Matemáticas de IES, Avenida Blas Infante s/n, 41011 Sevilla, Spain

During the process of olive fermentation chlorophylls are transformed into pheophytins and pheophorbides. Chlorophyllides were detected as intermediate products. Research into the origin of these structural modifications has shown that the chlorophyllides are formed from the chlorophylls by the action of chlorophyllase, while it is the acid environment developed during fermentation that facilitates the interchange of magnesium ions for those of hydrogen in all of the porphyrin compounds present. The kinetic study carried out has shown that the degradation of chlorophylls to pheophytins and of chlorophyllides to pheophorbides fits first-order kinetics with respect to the pigment concentration. The degradation mechanism proposed is as follows: first stage (time = t_{chlase}), [chlorophylls] → [chlorophyllides] (k_1); second stage (time > t_{chlase}), [chlorophyllides] → [pheophorbides] (k_2) and [chlorophylls] → [pheophytins] (k_3). While the enzyme chlorophyllase is active (time = t_{chlase}), a proportion of the chlorophylls give rise to chlorophyllides. The remaining chlorophylls have not at this stage been affected by the action of the enzyme, and these constitute substrate available for the subsequent pheophytinization reaction. This begins once the active phase of the chlorophyllase enzyme has finished and takes place in parallel with the formation of pheophorbides. The latter compounds arise only from the chlorphyllides formed as a result of chlorophyllase action.

INTRODUCTION

The changes that occur both in the color of fruits and vegetables and in the pigments responsible for the color during the different stages of processing have been the subject of numerous studies, most of which have been summarized in recent reviews (Simpson, 1985; Schwartz and Lorenzo, 1990). The most common change is associated with the conversion of chlorophylls to pheophytins, which causes a dramatic color change from brilliant green to olive brown (Buckle and Edwards, 1969; Gold and Weckel, 1959). This reaction is intensified by prolonged thermal treatments and depends on the quantity of acids freed from the plant tissues during processing and storage (Lin et al., 1970). Mild thermal treatments, such as scalding, induce the formation of epimers in C_{10} producing chlorophylls *a'* and *b'*. Further heat treatments lead to the formation of large quantities of these isomers. During prolonged thermal treatments, such as canning, almost all of the chlorophylls are converted to pheophytins (Robertson, 1985) and to epimers of pheophytins. Furthermore, pyropheophytins can be identified as the most common chlorophyll derivatives present (Schwartz et al., 1981).

Another important pigment alteration in plants is that caused by the excision of phytol from the chlorophyll and pheophytin molecules through the action of the enzyme chlorophyllase (Holden, 1961), giving rise to the formation of chlorophyllides and pheophorbides, respectively. Wang et al. (1971) studied the effect of storage conditions on this transformation in asparagus, and Jones et al. (1962, 1963), investigating the different commercial scalding treatments, observed the rapid formation of chlorophyllides and pheophorbides at 82 °C. The degradation of chlorophylls to chlorophyllides by chlorophyllase during the fermentation process has also been described in cucumbers (Jones et al., 1963; White et al., 1963), peppers (Ruskov and Malchev, 1971), and tea leaves (Blanc, 1973). During the fermentation of wild rice the chlorophylls give rise to pheophytins and pyropheophytins (Schwartz and von Elbe, 1983).

The higher susceptibility to pheophytinization of chlorophyll *a* compared to that of chlorophyll *b* has been well documented in the literature (Buckle and Edwards, 1970a; Schwartz et al., 1981). Kinetic studies on pheophytin formation from chlorophylls have shown that chlorophyll *a* reacts with acid from 2 to 7 times more rapidly than chlorophyll *b* (Mackinney and Joslyn, 1941; Lajollo et al., 1971; Schwartz and Lorenzo, 1990).

Gold and Weckel (1959), after examining the conversion of chlorophylls to pheophytins in heat-processed peas, concluded that the degradation seemed to obey first-order kinetics. Robertson and Swinburne (1981) also observed apparent first-order kinetics in the loss of chlorophylls during the canning of kiwi fruits. Cho (1966) studied the conversion of chlorophylls *a* and *b* into pheophytins *a* and *b* in water/acetone buffer systems and described the reaction rate as being of second order with respect to the concentration of hydrogen ions and of first order with respect to chlorophyll concentration. Studying the kinetics of the degradation of pheophytins in canned spinach puree, Schwartz and von Elbe (1983) found that the formation rate of pyropheophytins from pheophytins is of pseudo-first order and that the rate of degradation of pheophytin *b* is 25–40% faster than that of pheophytin *a*. Canjura et al. (1991) studied the thermal degradation of chlorophylls and chlorophyllides in spinach puree and concluded that the degradation of both compounds follows first-order kinetics. Furthermore, the same authors found that chlorophyllides are less stable than chlorophylls, which suggests that any method aimed at maximizing the

* Address correspondence to this author at the Instituto de la Grasa y sus Derivados (CSIC), Avenida Padre García Tejero 4, 41012 Sevilla, Spain.

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Table 1. Qualitative and Quantitative Changes in Chlorophylls during Table Olive Processing [Process Type Long (PL1)] as a Function of pH

			pigment concn (μmol/kg)											
	pH		*a* series[a]						*b* series[a]					
time (days)	fruit	brine	Chl[b]	Phy[b]	Chl + Phy	Chld[b]	Pho[b]	Chld + Pho	Chl	Phy	Chl + Phy	Chld	Pho	Chld + Pho
0	6.08		49.97		49.97				12.37		12.37			
4	8.44	8.28	40.73		40.73	7.04		7.04	7.62		7.62	4.52		4.52
6	7.28	6.93	33.09	8.47	41.56	4.70	2.70	7.40	7.29	0.37	7.66	3.86	0.85	4.71
8	6.67	5.49	26.18	15.44	41.62	3.18	3.90	7.08	6.61	0.72	7.33	3.28	1.43	4.71
10	5.17	5.49	25.01	17.20	42.21	2.60	4.95	7.55	6.58	1.06	7.64	2.58	2.12	4.70
14	5.40	4.56	15.12	26.52	41.64	1.40	5.61	7.01	5.95	1.43	7.38	2.11	2.35	4.46
19	5.41	4.76	13.91	27.63	41.54	0.70	6.10	6.80	4.86	2.43	7.29	1.41	3.10	4.51
20	5.08	4.65	12.59	29.01	41.60	0.28	6.80	7.08	4.83	2.48	7.31	1.05	3.40	4.45
26	4.83	4.57	11.76	29.53	41.29		7.02	7.02		3.79	2.74	6.53	4.14	5.10
30	4.69	4.60	6.16	35.40	41.56		7.05	7.05	3.99	3.65	7.64	0.35	4.36	4.71
33	4.55	4.52	5.78	35.32	41.10		7.23	7.23	3.66	3.17	6.83		4.61	4.61
40	4.61	4.42	3.69	36.99	40.68		7.16	7.16	3.14	4.42	7.56		4.60	4.60
50	4.53	4.46	1.52	38.50	40.02		7.18	7.18	2.42	5.20	7.62		4.66	4.66
54	4.44	4.35	1.54	38.24	39.78		7.18	7.18	2.24	5.70	7.97		4.70	4.70
60	4.43	4.29	0.90	39.10	40.00		7.15	7.15	1.88	6.18	8.06		4.70	4.70
70	4.41	4.32	0.37	40.53	40.90		7.20	7.20	1.47	6.30	7.77		4.71	4.71
89	4.33	4.21	0.23	40.82	41.05		7.12	7.12	0.95	6.30	7.25		4.78	4.78
104	4.45	4.25		40.77	40.77		7.14	7.14	0.61	6.59	7.20		4.58	4.58
117	4.37	4.26		40.69	40.69		7.15	7.15		6.57	6.57		4.90	4.90
161	4.21	4.10		40.85	40.85		6.94	6.94		6.41	6.41		4.80	4.80
203	4.34	4.19		40.81	40.81		6.97	6.97		6.68	6.68		4.51	4.51
287	4.27	4.08		40.76	40.76		7.17	7.17		6.59	6.59		4.67	4.67

[a] *a* series: chlorophyll *a* and derivatives. *b* series: chlorophyll *b* and derivatives. [b] Chl, chlorophyll; Chld, chlorophyllide; Phy, pheophytin; Pho, pheophorbide.

chlorophyllide content will not be effective in improving the stability of the green color.

Since the color of foodstuffs is an important quality attribute, a great deal of attention has been paid in the past few years to the chloroplast pigments present in olive fruits (Mínguez-Mosquera and Garrido-Fernández, 1989) and to the transformations undergone during the processing of the fruits for table olives (Mínguez-Mosquera et al., 1989, 1991). The involvement of chlorophyllase in this process has been demonstrated in a model system and the physicochemical conditions favoring the action of chlorophyllase in olive fruit itself have been investigated (Mínguez-Mosquera et al., 1993).

The aim of the present study was to elucidate the origin of the modifications that occur in the chlorophyll fraction during processing and to establish the mechanism and kinetics of its degradation.

MATERIALS AND METHODS

Apparatus. A Büchi Rotavapor, Model R 110, a Waters 600 E multisolvent delivery system, a Waters 994 programmable photodiode array detector, and a Waters 5200 printer-plotter were used.

Reagents. All reagents were of analytical grade except those used for HPLC, which were of chromatographic grade. The water used was deionized and filtered through a nylon membrane of 0.45 μm.

Standards. Chlorophylls *a* and *b* were isolated from fresh spinach leaves by pigment extraction with acetone (Holden, 1976) followed by TLC separation on silica gel GF_{254} with development using petroleum ether (65–95 °C)/acetone/diethylamine (10:4:1) (Mínguez-Mosquera and Garrido-Fernández, 1989). Alkaline derivatives of chlorophylls *a* and *b* were obtained by adding to pure solutions two or three drops of 0.5% KOH in methanol and leaving them to react for 10 min (Hynninen, 1973).

Raw Material Used. The olives used in this study, *Olea europaea* (L.), of the variety Manzanilla (*pomiformis*), were picked in the stage of maturity prior to ripening. The color of the fruits varied between light green and yellowish green. To process the fruits as table olives, fermenters with a capacity of 60 kg were employed. In general terms, the process consists of treating the fruits initially with NaOH solution, washing them with water, and afterward, brining them in a NaCl solution. The sugars, vitamins, and amino acids pass by osmosis from the fruit into the brine, converting it into a medium ideal for microbial culture. In this medium the fruits undergo a natural lactic acid fermentation. The whole process of fermentation and curing takes approximately 6 months, by which time the fruits should have acquired certain determined organoleptic characteristics (Fernández-Díez et al., 1985).

The study was performed for 2 consecutive years and the pigments were measured every 2–3 days during the first 2 weeks of fermentation and, subsequently, every 8 days. Analyses were performed in duplicate on 10-g samples of pulp prepared from 15–20 triturated and homogenized pitted fruits. Two fermenters were prepared each year. The first year the study was carried out in a "long type" process (2.22% NaOH for 7 h 15 min, washing for 6 h, and brining in a 10.6% NaCl solution) (PL1 and PL2 codes) and the second one in a "short type" process (2.38% NaOH for 5 h 10 min, washing for 7 h, and brining in a 10.6% NaCl solution) (PS1 and PS2 codes).

Measurement of pH in the Fruit. pH was measured by triturating three to five stoned fruits in a minimum volume of distilled water which was added to facilitate homogenization. It should be mentioned that during the first days of the process, the measure of the internal pH of the fruit is affected by errors due to NaOH in the external zones of the fruit.

Extraction, Separation, and Quantification of Pigments. Analysis of chlorophyll from olive fruits requires an extraction technique that results in a solution of pigments free from fatty material. The presence of lipids in the final extract, which occurs in the normal extraction processes, interferes in the subsequent stages of identification and quantification. The method used here was that developed by Mínguez-Mosquera and Garrido-Fernández (1985, 1989) which consists of a selective separation between the hexane and *N,N*-dimethylformamide solvent phases. Pigments were separated and quantified using reversed phase HPLC, following the methodology described by Mínguez-Mosquera et al. (1991).

RESULTS AND DISCUSSION

Transformation of Chlorophylls during the Processing of Table Olives. In Tables 1–4, the initial concentrations of chlorophylls *a* and *b* in the fresh fruit are shown for each fermenter, as well as the qualitative and quantitative changes in these pigments during processing, as a function of the pH of the brine and of the fruit itself. The concentrations of chlorophylls and

Table 2. Qualitative and Quantitative Changes in Chlorophylls during Olive Processing [Process Type Long (PL2)] as a Function of pH

			pigment concn (μmol/kg)											
	pH		*a* series[a]						*b* series[a]					
time (days)	fruit	brine	Chl[b]	Phy[b]	Chl + Phy	Chld[b]	Pho[b]	Chld + Pho	Chl	Phy	Chl + Phy	Chld	Pho	Chld + Pho
0	6.08		49.97		49.97				12.37		12.37			
4	7.41	6.71	41.21		41.21	8.08		8.08	7.02		7.02	5.32		5.32
6	7.30	6.58	36.70	4.60	41.30	5.70	2.80	8.50	6.69	0.34	7.03	4.32	0.96	5.28
8	6.69	5.69	32.01	7.90	39.91	5.65	4.37	10.02	6.28	0.76	7.04	4.04	1.10	5.14
10	5.27	4.50	28.88	12.51	41.39	2.50	5.90	8.40	6.05	0.98	7.03	2.92	2.40	5.32
14	5.47	4.58	22.09	17.37	39.46	1.69	6.90	8.59	5.21	1.63	6.84	2.50	2.90	5.40
19	5.39	4.79	18.96	20.21	39.17	0.70	7.31	8.01	4.27	2.57	6.84	1.97	3.12	5.09
20	4.90	4.69	14.15	25.07	39.22	0.30	7.58	7.88	4.19	2.60	6.79	1.03	3.80	4.83
26	4.81	4.61	12.90	26.86	39.76		8.90	8.90	4.11	2.64	6.75	0.70	4.34	5.04
30	4.71	4.53	8.70	30.70	39.40		8.50	8.50	3.67	3.36	7.03	0.39	4.93	5.32
33	4.69	4.58	7.92	32.24	40.16		8.86	8.86	3.04	3.87	6.91		4.96	4.96
40	4.64	4.50	5.40	34.58	40.02		8.28	8.28	2.86	4.33	7.19		4.84	4.84
50	4.52	4.48	2.61	38.78	41.39		8.50	8.50	2.22	4.80	7.02		5.27	5.27
54	4.47	4.46	2.01	38.24	40.25		8.42	8.42	2.17	4.77	6.94		5.03	5.03
60	4.45	4.39	1.43	39.96	41.39		8.56	8.56	1.73	5.30	7.03		5.30	5.30
70	4.40	4.28	0.78	40.20	40.98		8.50	8.50	1.35	5.60	6.95		5.32	5.32
89	4.38	4.26	0.20	40.10	40.30		8.41	8.41	0.95	5.97	6.92		4.91	4.91
104	4.40	4.28		40.06	40.06		8.21	8.21	0.62	6.34	6.96		4.94	4.94
117	4.36	4.24		40.64	40.64		8.56	8.56		6.64	6.64		5.30	5.30
161	4.22	4.10		40.06	40.06		9.04	9.04		6.79	6.79		5.05	5.05
203	4.25	4.16		40.33	40.33		8.45	8.45		6.68	6.68		4.87	4.87
287	4.06	3.82		40.59	40.59		8.80	8.80		6.57	6.57		5.28	5.28

[a] Same as in Table 1. [b] Same as in Table 1.

Table 3. Qualitative and Quantitative Changes in Chlorophylls during Table Olive Processing [Process Type Short (PS1)] as a Function of pH

			pigment concn (μmol/kg)											
	pH		*a* series[a]						*b* series[a]					
time (days)	fruit	brine	Chl[b]	Phy[b]	Chl + Phy	Chld[b]	Pho[b]	Chld + Pho	Chl	Phy	Chl + Phy	Chld	Pho	Chld + Pho
0	6.11		53.76		53.76				12.13		12.13			
3	7.27	6.79	40.34		40.34	11.42		11.42	7.52		7.52	4.44		4.44
4	6.69	6.21	32.73	9.66	42.39	11.35	0.97	12.82	7.50		7.50	4.54		4.54
6	6.12	5.54	30.64	11.90	42.54	7.60	3.45	11.05	7.10		7.10	5.07		5.07
8	5.60	5.06	25.75	16.64	42.39	5.10	6.25	11.35	6.03	0.94	6.97	3.82	1.46	5.28
10	5.31	5.02	21.22	20.29	41.51	3.37	8.04	11.41	5.98	1.92	7.90	3.19	1.94	5.13
12	5.17	5.00	14.10	27.78	41.88	1.20	9.07	10.27	4.84	2.12	6.96	2.66	2.29	4.95
19	4.94	4.78	11.08	30.34	41.42	0.60	9.65	10.25	4.33	2.45	6.78	1.34	3.47	4.81
26	4.84	4.69	7.53	34.43	41.96		11.22	11.22	3.98	3.56	7.54	0.50	4.84	5.34
30	4.78	4.65	6.87	35.52	42.39		11.29	11.29	3.26	4.15	7.41		5.28	5.28
33	4.77	4.64	5.15	36.81	41.96		11.01	11.01	2.47	4.18	6.65		5.09	5.09
40	4.75	4.57	3.77	38.60	42.37		11.30	11.30	2.26	4.90	7.16		5.50	5.50
47	4.71	4.56	2.87	39.60	42.47		11.27	11.27	1.72	5.72	7.44		5.04	5.04
50	4.69	4.54	2.07	40.10	42.17		11.29	11.29	1.52	5.40	6.92		5.60	5.60
60	4.64	4.52	1.10	41.03	42.13		11.18	11.18	1.21	5.73	6.94		5.68	5.68
70	4.60	4.49		41.30	41.92		11.20	11.20	0.88	5.96	6.84		5.70	5.70
82	4.58	4.50		41.76	42.16		11.61	11.61	0.70	6.53	7.23		5.13	5.13
96	4.55	4.51		42.18	42.18		11.61	11.61	0.58	6.57	7.15		5.05	5.05
110	4.59	4.51		42.89	42.89		11.34	11.55	0.16	6.42	6.58		5.19	5.19
157	4.53	4.24		42.53	42.53		11.55	11.55		6.53	6.53		5.20	5.20
196	4.26	4.31		42.00	42.00		10.80	10.80		6.48	6.48		5.13	5.13

[a] Same as in Table 1. [b] Same as in Table 1.

chlorophyllides fall progressively, while those of the pheophytins and pheophorbides gradually increase. The results confirm that both the alkaline treatment and the subsequent lactic acid fermentation inherent in the traditional process for producing table olives cause the total transformation of the chlorophylls present in the fresh fruit. Initially, the formation of chlorophyllides was detected in all fruits. Despite washing, the initial treatment of the fruits with NaOH caused an alkaline pH during the first few days in brine, as well as provoking the formation of large quantities of CO_2 and acetic acid (Fernández-Díez et al., 1985). The optimum pH for chlorophyllase activity lies between 7.5 and 8.5 (Holden, 1961; Mínguez-Mosquera et al., 1993; Terpstra and Lambers, 1983), and this enzyme is only active at room temperature in the presence of organic compounds (Levadoux et al., 1987; Schoch and Brown, 1987) or detergents (McFeeters et al., 1971; Johnson-Flanagan and Thiagarajah, 1990). These conditions, in the present study, favored chlorophyllase activity, provoking the enzymatic hydrolysis of phytol in the chlorophyll molecule with the consequent formation of chlorophyllides. Subsequently, the acidity generated in the fermentation medium favored the formation of chlorophyll derivatives free from magnesium, giving rise in the final product to a mixture of pheophytins and pheophorbides. It should be noted that, in general terms, this transformation fits the same pattern in all cases. The presence of pheophorbides and pheophytins is due to the structural transformation of the chlorophyll and chlorophyllide molecules caused by the acidity of the medium, which favors the exchange of magnesium ions for protons.

Origin of Chlorophyllide Formation. Although in the literature it is postulated that *in vitro* the acid or

Table 4. Qualitative and Quantitative Changes in Chlorophylls during Table Olive Processing [Process Type Short (PS2)] as a Function of pH

			pigment concn (μmol/kg)											
	pH		*a* series[a]						*b* series[a]					
time (days)	fruit	brine	Chl[b]	Phy[b]	Chl + Phy	Chld[b]	Pho[b]	Chld + Pho	Chl	Phy	Chl + Phy	Chld	Pho	Chld + Pho
0	6.11		53.76		53.76				12.13		12.13			
3	7.65	6.96	39.61		39.61	12.46		12.46	6.92		6.92	4.82		4.82
4	7.02	6.25	31.45	9.82	41.27	12.48		12.48	6.65		6.65	5.47		5.47
6	6.18	5.33	30.05	13.48	43.53	8.65	4.17	12.82	6.49	0.50	6.99	5.41	0.57	5.98
8	5.70	5.16	24.74	16.53	41.27	5.60	6.87	12.47	5.78	0.86	6.64	4.39	1.08	5.47
10	5.13	4.97	21.53	20.25	41.78	3.82	8.28	12.10	5.69	1.28	6.97	3.14	1.59	4.73
12	5.09	4.95	14.10	28.50	42.60	2.04	10.40	12.44	4.25	2.72	6.97	2.64	2.54	5.18
19	4.90	4.76	12.61	30.61	43.22	0.56	11.65	12.21	4.10	2.87	6.97	1.99	3.83	5.82
26	4.83	4.65	8.16	34.05	42.21		12.03	12.03	3.43	3.58	7.01	0.93	4.66	5.59
30	4.80	4.66	6.60	34.66	41.26		12.40	12.40	2.67	3.97	6.64		4.72	4.72
33	4.71	4.60	5.82	37.40	43.22		12.17	12.17	2.46	4.23	6.69		4.86	4.86
40	4.69	4.58	3.62	37.64	41.26		12.41	12.41	2.18	4.76	6.94		4.71	4.71
47	4.66	4.53	3.50	38.71	42.21		12.38	12.38	1.71	5.38	5.55		4.76	4.76
50	4.60	4.51	1.99	39.28	41.27		11.89	11.89	1.50	5.32	6.82		5.03	5.03
60	4.59	4.51	1.09	40.18	41.27		12.50	12.50	1.23	5.71	6.94		5.22	5.22
70	4.55	4.49	0.59	40.67	41.26		12.36	12.36	0.88	5.99	6.87		5.30	5.30
82	4.56	4.47	0.30	42.01	42.31		12.43	12.43	0.63	6.32	6.95		4.93	4.93
96	4.54	4.46		42.18	42.18		12.13	12.13	0.43	6.41	6.84		4.93	4.93
110	4.58	4.46		42.60	42.60		12.58	12.58	0.18	6.38	6.56		4.92	4.92
157	4.48	4.20		42.52	42.52		11.98	11.98		6.44	6.44		5.04	5.04
196	4.34	4.25		42.81	42.81		12.44	12.44		6.49	6.49		5.05	5.05

[a] Same as in Table 1. [b] Same as in Table 1.

alkaline hydrolysis of chlorophylls gives rise to chlorophyllides in one step (Jackson, 1976), the present authors could find no evidence to support this hypothesis. This theory was therefore tested experimentally. Acid de-esterification is improbable, given that the pH in the medium at the time when these compounds were detected, at around 7, does not favor this type of reaction. Alkaline hydrolysis, on the other hand, could theoretically cause the formation of chlorophyllides. For this reason, the authors proceeded to verify the hydrolysis of the chlorophyll phytols *in vitro*. The compounds formed, however, did not include chlorophyllides. Thus, neither acid nor alkaline treatments are favorable for the formation of chlorophyllides. Even if a process could be developed that promoted, solely and exclusively, the de-esterification of chlorophyll, without promoting other types of reactions, this would still be nonspecific, since it would affect equally the phytol group in C_9 and the acetate group in C_{10}, giving rise, at best, to a mixture of compounds. This mode of action does not reflect what happened in the fruit in the present study. Here, after alkali treatment, one derivative only was detected. Due to the specificity of the reaction, it must have been formed by enzyme action.

During the first days of the processing, the pH in the fruit is still very alkaline and altered chlorophylls appeared in the pigment extract. However, in subsequent samples the latter compounds were not detected, which demonstrates that these derivatives are not actually present in the fruit but rather that they are altered chlorophylls arising as a result of the pigment extraction process in highly alkaline conditions. Then, pigment analysis could not start until the fruit had reached a pH near neutral.

Origin of Pheophorbide Formation. To check whether the formation of pheophorbides occurs exclusively via chlorophyllides or whether, on the contrary, there is a step from pheophytins to pheophorbides, a balance was performed on the material during the whole fermentation process, grouping on one side those components with phytol (chlorophylls and pheophytins) and on the other those without phytol (chlorophyllides and pheophorbides) (Tables 1–4). The permanency or otherwise of these fractions would enable clarification of whether formation of pheophorbides follows only one of the two most probable pathways or whether the two are used indifferently.

Whether one considers the table of initial chlorophyllide contents or that of the cumulative sum of the molar concentrations of pheophorbides and chlorophyllides, the final value of compounds with the phytol group removed can be seen to have remained constant during the whole process. The gradual fall in the concentration of chlorophyllides together with the corresponding increase in pheophorbides cannot be interpreted as representing the result of enzymatic activity, because the final product of the reaction (total of chlorophyllids plus pheophorbides) remained stable from the first measurement. On the other hand, since the balance of chlorophylls plus pheophytins also remained constant during the same time period, a pheophytin to pheophorbide step cannot even be considered to be probable.

Chlorophyllase was only active during the period prior to the start of the fermentation process, and its cumulative activity yielded around 15 μmol/kg of reaction product, this remaining constant throughout fermentation. Subsequently, the acidity of the medium removed chlorophyllides from the initial equation, while the formation of pheophorbides, the final product, progressively increased. Consequently, the phytol- and magnesium-free derivatives, the pheophorbides, were produced solely and exclusively from the chlorophyllides initially present, and these, due to the progressive acidification of the medium, exchanged Mg^{2+} ions for protons, excluding the possibility of chlorophyllase acting on the pheophytins.

A recent study on the effect of pH on chlorophyllase activation in the fruit itself (Mínguez-Mosquera et al., 1993) indicated that this enzyme has a maximum activity at pH 7.5 and another fictitious maximum at pH 4.5. However, during the processing of table olives, the formation of chlorophyllides occurs prior to the start of fermentation and no chlorophyllase activity was detected at acid pH. The explanation for this may be that, as a consequence of treating the fruits with NaOH, the permeability of the skin is enhanced, provoking an intense process of osmosis that eliminates virtually all of the low molecular weight soluble material from the olive. In this case equilibrium between the fruit and the medium is never reached, and the substances eliminated from the fruit are used by the microbial flora, therefore making the presence of an internal modulator, which activates or inhibits the

activity of the enzyme, impossible to be detect. This situation contrasts with that during enzymatic activation in the fruit.

Influence of pH. Except for the enzymatic conversion of the chlorophylls to chlorophyllides by chlorophyllase, the structural transformation occurring in the rest of the pigments during the fermentation process is catalyzed in every case by the presence of acids.

The degradation of chlorophylls and chlorophyllides to pheophytins and pheophorbides, respectively, which occurs in both series of pigments, is induced by the acidity produced by the lactic acid fermentation that occurs naturally and spontaneously in the fruits. The kinetic equation that describes the degradation of the pigments is $-dc/dt = k[H^+]^n[\text{pigment}]^n$. Although physically the reaction occurs inside the chloroplasts, the medium in which it takes place is the fermenter, since it is diffusion across the membranes by osmosis that leads to fermentation. The intracellular pH is thus altered by the pH of the brine.

Considering the change in pH that takes place in the fermentation brine, it might at first seem that when the interior of the fruit reaches a pH of 8, the concentration of the chlorophyll in the fruit is greater than that of hydrogen ions, in which case, if pheophytinization occurs, its kinetics would be of second order. However, taking into account the fermenter as a whole, the concentration of hydrogen ions in the fermentation medium is not at all affected by this reaction, which thus takes place without this reagent being limiting. As a result, the concentration of H^+ ions in the fermentation medium, compared with the existing chlorophylls is, from the start of the process, in excess and can thus be considered to be constant. In this case the kinetic reaction can be expressed as $-dc/dt = k'[\text{pigment}]^n$, where $k' = k[H^+]^n$, which has been called a pseudo-order reaction (González-Ureña, 1991; Hill, 1977).

Mechanism and Kinetics of Chlorophyll Degradation. Once the cause of the structural modifications in the chlorophyll molecule was determined, attention was switched to establishing the stages that, simultaneously or consecutively, take part in the overall degradation of these pigments. In elucidating the number of basic steps in the degradation of chlorophylls, we considered only those stages in which a structural change could be recognized.

The initial chlorophyll content of the fresh fruit is degraded by two perfectly distinguishable pathways: that which through the action of chlorophyllase gives rise to derivatives without phytol and that which leads exclusively to pheophytins, once chlorophyllide formation has occurred. The fact that the total balance of material remains constant is proof enough that there is no other type of reaction that affects the pigments, such as oxidative reactions, which would yield colorless products. By way of example, Figure 1 shows, on a semilogarithmic scale, the percentage retention of chlorophylls *a* and *b* as a function of time for the fermenter PL2. The data fit a straight-line equation and this conforms to a pseudo-first-order kinetic model with respect to chlorophyll concentration. The semilogarithmic representation of the percentage retention of chlorophyllides *a* and *b* against time (Figure 2) shows that the degradation of chlorophyllides to pheophorbides also fits first-order kinetics. According to the above, three basic reactions of degradation occur: (1) chlorophylls to chlorophyllides, (2) chlorophyllides to pheophorbides, and (3) chlorophylls to pheophytins.

In the first and second stages the final product could be considered to arise as a result of a consecutive reaction

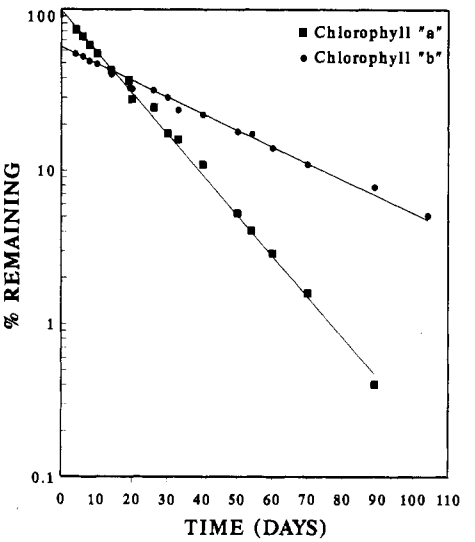
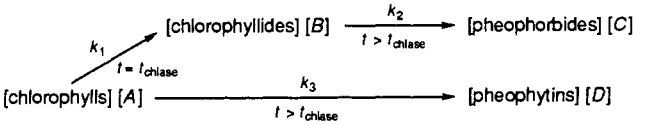


Figure 1. First-order degradation rate of chlorophylls *a* and *b* in table olive processing (fermenter code PL2) as a function of time.

in which the chlorophyllides act as the intermediate product. Pheophytin formation would take place in a parallel reaction. As a first step, and coinciding with the alkaline phase, while the enzyme chlorophyllase (t_{chlase}) is active, a proportion of the chlorophylls give rise to chlorophyllides. The remaining chlorophylls have not at this stage been affected by the action of the enzyme, and these constitute substrate available for the subsequent pheophytinization reaction which begins once the active phase of the chlorophyllase enzyme has finished and takes place in parallel with the formation of pheophorbides which arise only from the chlorophyllides formed as a result of chlorophyllase action. The kinetic scheme would be



The corresponding kinetic equations are

first stage ($t = t_{chlase}$)

(a) $-dA/dt = k_1A$; (b) $dB/dt = k_1A$; (c) $dC/dt = 0$; (d) $dD/dt = 0$

second stage ($t > t_{chlase}$)

(a) $-dA/dt = k_3A$; (b) $-dB/dt = k_2B$; (c) $dC/dt = k_2B$; (d) $dD/dt = k_3A$

The kinetic equations integrated for each of the compounds are all exponential-type equations ($y = \exp(kt + b)$) and are shown in Table 5. The apparent first-order rate constants and the correlation coefficients were calculated from the linear regression analysis obtained with a statistical software and are shown in Table 6. The results showed that the mechanism and kinetics of the degradation of chlorophylls during the processing of green table olives were the same in all cases, independent of the alkaline treatment used in the fruit processing. In keeping with these results, the graphs which mathematically fit the kinetic model for degradation of chlorophylls *a* and *b* during the processing of table olives are shown in Figures 3 and 4.

The rate constant for the formation of pheophorbide *a* (k_{2a}) is of the order of 3 times greater than the kinetic

Table 5. Equations Integrated Corresponding to the First-Order Kinetics of Chlorophyll Degradation during the Fermentation Process of Green Table Olives

pigment degradation	pigment formation
First Step: Time = t_{chlase}	
chlorophyll: $A = A_0 \exp(-k_1 t)$	chlorophyllide: $B = A_0[1 - \exp(-k_1 t)]$
Second Step: Time > t_{chlase}	
chlorophyllide: $B_1 = A_0[1 - \exp(-k_1 t_{chlase})] \exp(-k_2 t)$	pheophorbide: $C = A_0[1 - \exp(-k_1 t_{chlase})][1 - \exp(-k_2 t)]$
chlorophyll: $A_1 = A_0 \exp(-k_1 t_{chlase}) \exp(-k_3 t)$	pheophytin: $D = A_0[1 - \exp(-k_1 t_{chlase})][1 - \exp(-k_3 t)]$

Table 6. Apparent Rate Constants and Correlation Coefficients for the Chlorophyll Degradation during the Fermentation Process of Green Table Olives

fermentater code	chlorophyllide	pheophorbide		pheophytin	
	k_1[a]	k_2	r^2[b]	k_3	r^2
	Chlorophyll Derivatives (*a* Series)				
PL1	0.048	0.1765	95.92	0.0635	98.74
PL2	0.047	0.1829	96.31	0.0614	99.69
PS1	0.067	0.2017	95.23	0.0575	99.22
PS2	0.075	0.2034	99.15	0.0589	99.22
	Chlorophyll Derivatives (*b* Series)				
PL1	0.12	0.0878	95.31	0.0246	99.64
PL2	0.14	0.0966	96.15	0.0237	99.44
PS1	0.16	0.0979	95.66	0.0318	97.66
PS2	0.15	0.0756	93.30	0.0314	99.05

[a] k, apparent rate constant (days^{-1}). [b] r^2, correlation coefficient multiple × 100; $p < 0.001$.

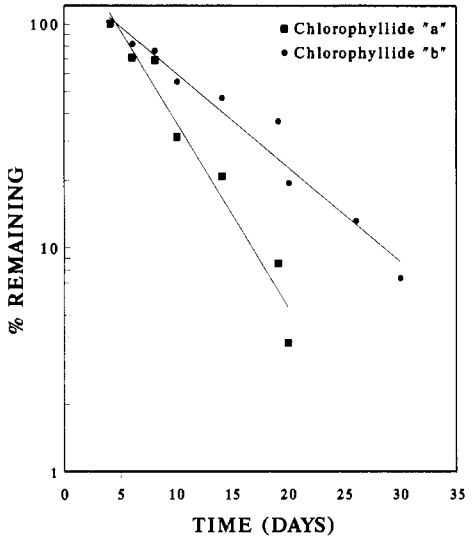


Figure 2. First-order degradation rate of chlorophyllides *a* and *b* in table olive processing (fermenter code PL2) as a function of time.

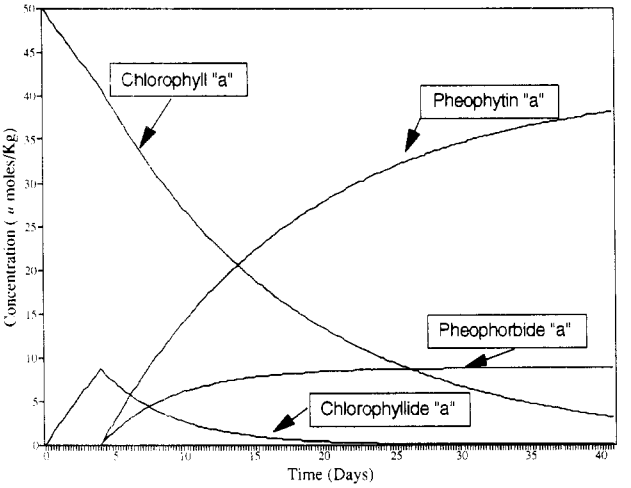


Figure 3. Kinetic model for degradation of chlorophyll *a* in table olive processing.

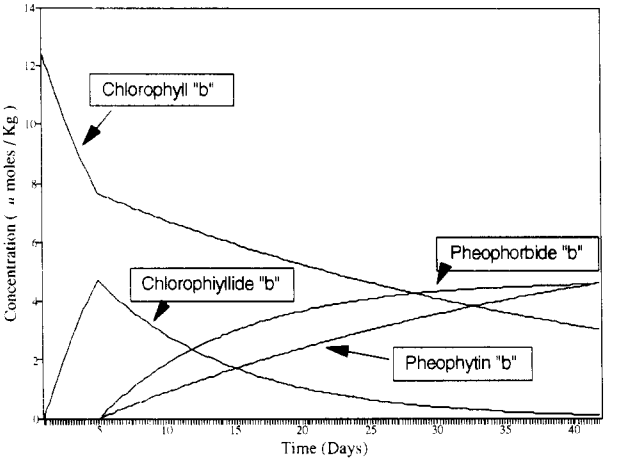


Figure 4. Kinetic model for degradation of chlorophyll *b* in table olive processing.

constant for the formation of chlorophyllide (k_{1a}). This means that if the formation of pheophorbides is considered as a single two-stage reaction, the slow stage depends on the enzyme activity. According to these results, the kinetic constants of series *a* fit what is found in the majority of the consecutive reactions in which the product of the primary reaction is the limiting factor, i.e., $k_{2a} \gg k_{1a}$ (González-Ureña, 1991), although in this case the reaction is consecutive but not simultaneous. The fact that the kinetic constant for formation of pheophorbide *a* (k_{2a}) is 3.3 times greater than that for pheophytinization (k_{3a}) is quite significant. This arises since the lack of a phytol group in these derivatives (pheophorbides) eliminates the steric interference of this alcohol group and allows the Mg^{2+} ion to be more readily replaced by the H^+ ion in the porphyrin ring (Ogura et al., 1987).

With respect to series *b* the fact that the rate constant of formation for pheophorbide *b* (k_{2b}) is lower than that for chlorophyllide *b* is noteworthy. The higher affinity of the enzyme for the substrate makes the slow stage of the overall reaction the one not controlled by the enzyme but by the H^+ concentration. The constants k_{2b} and k_{3b}, involved in the rate of formation of pheophorbide *b* and pheophytin *b*, respectively, show the same effect as in series *a*, with a greater tendency toward pheophorbide formation.

From a comparison of the reactions that occur in series *a* and in series *b*, it can be seen that k_{1b}, the rate constant of the de-esterification reaction for chlorophyll *b*, is 3 times greater than k_{1a}, the kinetic constant for chlorophyll *a* de-esterification, agreeing with the observations of other authors with respect to the higher affinity of chlorophyllase for the substrate chlorophyll *b* (Shimokawa et al., 1978). The following reaction, the conversion of chlorophyllides to pheophorbides, controlled by H^+ ions, demonstrates the greater resistance of series *b* to the loss of Mg^{2+}, resulting in a k_{2b} inferior to k_{2a} (0.1 and 0.2, respectively). The kinetic constant in the pheophytinization reaction (k_3) is of the order of 2 or 3 times greater for series *a* than for *b*, as found in kinetic studies performed by other authors which show that the rate of pheophytinization is of the order of 2.5–10 times greater for series *a* (Lajollo et al., 1971; Buckle and Edwards, 1970b; Schwartz et al., 1981).

In summary, the results found for the different kinetic constants are

chlorophyll *a*	chlorophyll *b*	comparison between the two
$k_{2a} > k_{1a}$	$k_{2b} < k_{1b}$	$k_{1a} < k_{1b}$
$k_{2a} > k_{3a}$	$k_{2b} > k_{3b}$	$k_{2a} > k_{2b}$
		$k_{3a} > k_{3b}$

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