De-esterification of Chlorophylls in Olives by Activation of Chlorophyllase

M. Isabel Mínguez-Mosquera,* Beatriz Gandul-Rojas, and Lourdes Gallardo-Guerrero

Instituto de la Grasa y sus Derivados, CSIC, Avenida Padre García Tejero 4, 41012 Sevilla, Spain

The physicochemical conditions favoring the action of chlorophyllase in olive fruit itself have been investigated. Different temperatures and aqueous incubation media that contained acetone were tested. The highest levels of chlorophyllase activation in whole fruits (without extraction of the enzyme) were achieved by heating the fruits at 50 °C in a buffer (pH 8.5)–acetone (1:1) mixture. A study of the effect of pH on chlorophyllase activation in the fruit itself showed a maximum of activity at pH 7.5 and another fictitious maximum at pH 4.5. This second maximum may have been caused by a change in the composition of the substrate mixture as a function of the pH. The study highlights the activation of chlorophyllase in the olive in the processes used for obtaining virgin olive oil and green table olives.

INTRODUCTION

The olive fruit, *Olea europaea* (L.), is a green drupe that begins to form after flowering in May or June and ripens toward the end of autumn. During ripening it darkens to purple-black, at the same time as the oil content increases (Vázquez-Roncero, 1963). These fruits are used for the extraction of virgin olive oil or are consumed directly as table olives. There are, on the market, various commercial preparations of table olives that differ according to the stage of ripeness in which the fruit are harvested, the color of the final product, and the system used to eliminate the bitter glucoside oleuropein (Fernández-Díez et al., 1985).

The preparation of green table olives, Spanish or Sevillian style, includes an alkaline hydrolysis of the oleuropein from the fruit by means of an initial treatment with NaOH, followed by brining during which they undergo a normal lactic fermentation. During this process the fruits change color. This color change is due exclusively to a structural change in the pigments—both chlorophylls and carotenoids—initially present in the fresh fruit. The molecules of chlorophylls *a* and *b* are totally transformed, giving rise to a mixture of pheophytins *a* and *b* and pheophorbides *a* and *b* in the final product. In the carotenoid fraction, β-carotene and lutein remain unaltered, while the rest of the components with 5,6-epoxide groups in their molecule are transformed to the corresponding 5,8-furanoid derivatives (Mínguez-Mosquera et al., 1989). The detection of chlorophyllides in olives during the first days of processing suggests that they may be formed by the enzymatic action of chlorophyllase which hydrolyzes the phytol ester of chlorophyll molecules, giving rise to chlorophyllides. Subsequently, the acidity generated by the lactic fermentation would transform these into pheophorbides.

Virgin olive oil is obtained from ripe olives by mechanical extraction. Its color varies between green-yellow and golden yellow, depending on the degree of ripeness of the fruits. The qualitative and quantitative determination of chlorophyllic pigments present in ripe olives and their corresponding oils shows that, besides being transformed into pheophytins, these pigments are lost or destroyed during processing (Mínguez-Mosquera et al.,1990). The possibility exists that chlorophyllase is activated and acts in the stages prior to extraction. Enzyme action could be favored by the presence of hot water in contact with the tissues disintegrated during milling of the fruits. In these conditions the enzyme and substrate would come into contact at an optimum temperature for chlorophyllase activity (Terpstra and Lambers, 1983b). The action of this enzyme would cause the formation of a great amount of dephytilated chlorophyll derivatives which can be dissolved and eliminated in the water added and the vegetable water of the fruits. This would explain the loss of the chlorophyll fraction during the extraction process.

To demonstrate the possible involvement of chlorophyllase during the processing of olive fruits (preparation of olive oil and green table olives), the present work studies the physicochemical conditions (temperature, incubation medium, and pH) favoring the activation of chlorophyllase in the fruit itself.

EXPERIMENTAL PROCEDURES

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.

Raw Material and Reagents. The study was carried out on olives, *O. europaea* (L.), of the variety Picual (*rostrata*). 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.

Direct activation of the enzyme in fresh fruit was performed by immersion of the fruits in different aqueous media described in the bibliography (Jones et al., 1962; Levadoux et al., 1987; McFeeters et al., 1971; Terpstra and Lambers, 1983): H_2O, chlorophyllase buffer, pH 8.5 (20 mM Tris-HCl + 0.5 M NaCl + 10 mM $MgCl_2$ and mixtures of chlorophyllase buffer and acetone in the proportions 3:1 and 1:1. The temperatures used ranged from 20 (room temperature) to 70 °C (considered as the optimum for chlorophyllase activity). The reaction time ranged between 10 min and 85 h depending on the temperature used. Chlorophylls *a* and *b* were used as controls dissolved in the 3:1 mixture of chlorophyllase buffer and acetone.

Extraction of fruit pigments was performed with *N,N*-dimethylformamide according to the method of Mínguez-Mosquera and Garrido-Fernández (1989), with some modifications to prevent the loss of dephytilated derivatives in the wash waters. The technique is based on a selective separation of components between *N,N*-dimethylformamide and hexane. This system yields a solution of pigments free from the fatty matter which is characteristic of these fruits and which interferes with the later separation and quantification of pigments. The pitted and sliced olives (3 g) were treated repeatedly with *N,N*-dimethylformamide (50 mL) until complete extraction of color was achieved. The fatty matter was extracted with hexane (50 mL × 3) in a decanting funnel. The fat-free pigments were

0021-8561/93/1441-2254$04.00/0

Table I. Effect of Temperature, Time, and Incubation Medium on Chlorophyllase Activity

		activity (% product formed)					
		water			chlasa buffer[a]/cetone (3:1)		
temp (°C)	time	Chl a[b]	Chl b	ratio Chl a/Chl b	Chl a	Chl b	ratio Chl a/Chl b
70	15 min	2.19	1.72	1.27	4.91	4.02	1.22
	45 min	3.16	2.16	1.46	5.07	4.09	1.24
50	15 min	0.00	0.00		3.41	2.60	1.31
	45 min	2.40	1.95	1.23	8.78	5.93	1.49
30	24 h	2.99	2.05	1.46	28.45	23.77	1.20
20	3 h	0.00	0.00		0.00	0.00	
	24 h	0.00	0.00		5.58	3.40	1.64
	48 h	0.00	0.00		13.56	8.65	1.57
	74 h	0.00	0.00		24.65	16.90	1.46

[a] 20 mM Tris-HCl buffer + 0.5 M NaCl + 10 mM Cl_2Mg. [b] Substrate.

transferred to the 1:1 mixture of hexane and ethyl ether (150 mL), and 10% NaCl solution (400 mL) was added at 0 °C to force the transfer of de-esterified chlorophylls to the organic phase. Finally, the solution of pigments was evaporated and the dry residue collected in acetone. This extract was centrifuged at 13000*g* for 10 min, and the product of the enzymatic reaction was quantified by HPLC.

Separation and quantification of pigments was carried out by HPLC according to the method described by Mínguez-Mosquera et al. (1991), using a reversed-phase C_{18} column and an elution gradient with the solvents A [H_2O–ion-pair reagent–methanol (1:1:8 v/v/v)] and B [methanol–acetone (1:1 v/v)]. The ion-pair reagent was a solution of tetrabutylammonium (0.05 M) and ammonium acetate (1 M) in H_2O. Pigments were detected by absorbance at 430 nm before quantification.

Measurement of Activity. The amount of pigments measured was expressed either as a percentage of the product formed with regard to the initial amount of substrate or in units of enzymatic activity per kilogram of fruit. A unit of activity, katal (kat), is defined as the amount of enzyme needed to hydrolyze 1 mol of substrate/s.

RESULTS AND DISCUSSION

Effect of Temperature. The presence of chlorophyllase in different fruits has been demonstrated by direct activation of the enzyme by scalding (Jones et al., 1962, 1963; Clydesdale and Francis, 1968). According to Jones et al. (1962), thermal activation of this enzyme is greatest at 180 °F, with deactivation at higher temperatures (212 °F). The presence of acetone in the incubation medium enhances the enzymatic reaction, as chlorophyllase is an intrinsic membrane enzyme, and organic solvents at concentrations below 70% (Holden, 1961; Levadoux et al., 1987; Schoch and Brown, 1987) or detergents (Klein and Vishiac, 1961; McFeeters et al., 1971) enhance its extraction.

The experiments described were performed with these aforementioned considerations in mind. The incubation conditions and results are shown in Tables I and II. In all cases, the major reaction product was pheophorbide, with a much lower proportion of chlorophyllide. As a result, the measurement of enzymatic activity was verified on the total de-esterified product formed—chlorophyllides and pheophorbides—in the reaction. The control sample showed no de-esterification of the substrate, although epimerization and pheophytinization reactions did occur. Pheophorbide formation, even in aqueous medium, may be due to the effect of temperature and the long time needed for the incubation, as heat treatment of vegetables

Table II. Effect of Temperature, Time, and Incubation Medium on Chlorophyllase Activity

		activity (nkat/kg of fruit)					
		water			chlasa buffer[a]/acetone (3:1)		
temp (°C)	time	Chl a[b]	Chl b	ratio Chl a/Chl b	Chl a	Chl b	ratio Chl a/Chl b
70	15 min	6.20	1.78	3.48	13.91	4.15	3.35
	45 min	2.99	0.74	4.04	4.79	1.41	3.39
50	15 min	0.00	0.00		9.67	2.69	3.59
	45 min	2.27	0.71	3.20	8.26	2.04	4.05
30	24 h	0.09	0.02	4.50	0.84	0.26	3.23
20	3 h	0.00	0.00		0.00	0.00	
	24 h	0.00	0.00		0.16	0.04	4.00
	48 h	0.00	0.00		0.21	0.05	4.20
	74 h	0.00	0.00		0.24	0.06	4.00

[a] 20 mM Tris-HCl buffer + 0.5 M NaCl + 10 mM Cl_2Mg. [b] Substrate.

liberates acids in the fruit tissues, causing Mg^{2+} in the chlorophyll molecule to be exchanged for H^+ (Lin et al., 1970).

In aqueous medium most transformation of chlorophylls *a* and *b* took place at 70 °C after 45 min—a very long time compared with the few minutes needed for the activation of chlorophyllase at the same temperature in other vegetables. Jones et al. (1963) obtained 50% of chlorophyllide *a* in cucumbers after only 4 min of scalding at 180 °F, and Clydesdale and Francis (1968) found 13.4% of dephytilated chlorophyll *a* in spinach puree after 10 min of scalding at 155 °F. The low percentage of de-esterification in olives (3% for chlorophyll *a* and 2% for chlorophyll *b*) in conditions similar to those above indicates either that these fruits have low chlorophyllase activity or that the different characteristics of skin and texture modify the conditions of enzyme activation. At 50 °C chlorophyll hydrolysis was detected after 45 min. This temperature is similar to that used during olive oil extraction. At 30 °C the fruits needed 24 h of incubation for the action of chlorophyllase to yield levels of de-esterified chlorophylls similar to those obtained at 50 °C. At 20 °C no reaction product was detected even after 74 h.

The levels of pheophorbides found were considerably higher in the fruits incubated in the 3:1 mixture of chlorophyllase buffer and acetone at all temperatures. The highest value was at 30 °C after 24 h, with hydrolysis of 28.45% of the initial content of chlorophyll *a* and 23.77% of chlorophyll *b*, some 10-fold the levels found in aqueous medium. This activation may have been favored by adjusting the pH to 8.5—the optimum pH for the reaction catalyzed by chlorophyllase (Holden, 1961). Thus, Clydesdale and Francis (1968) increased the percentage of chlorophyll hydrolysis 4-fold in spinach puree by adjusting the pH with $MgCO_3$ during scalding.

When these results are expressed in units of enzymatic activity (Table II), the highest levels of activity were found at 70 °C after 15 min in the 3:1 buffer–acetone mixture, followed by those at 50 °C after 15 min. For both temperatures chlorophyllase activity decreased with increased incubation time, indicating that there is some inactivation or denaturation of the enzyme. In contrast, in the fruits kept at 20 °C enzymatic activity did not decrease with incubation time, but although an appreciable amount of product was formed, when values are expressed as units of activity, they are reduced to the lowest levels due to the long incubation time needed.

It is therefore deduced that for short times the optimum temperature for chlorophyllase activation in olives is 70

Table III. Effect of Acetone Concentration on Chlorophyllase Activity

incubation medium	activity[a,b] 30 °C Chl a	30 °C Chl b	50 °C Chl a	50 °C Chl b	70 °C Chl a	70 °C Chl b
water	0.00	0.00	0.00	0.00	0.14	0.04
buffer chlasa[c]	0.00	0.00	0.00	0.00	0.23	0.06
buffer/acetone						
3:1	0.00	0.00	0.11	0.00	0.43	0.12
1:1	0.00	0.00	0.46	0.12	0.39	0.10

[a] μmol/kg of fruit. [b] Incubation time 30 min. [c] 20 mM Tris-HCl buffer + 0.5 M NaCl + 10 mM Cl_2Mg.

°C and that the use of the 3:1 buffer–acetone incubation medium enhances the enzymatic action at all temperatures. It is, therefore, evident that the chlorophyllase enzyme, present in olive fruits, may be activated during extraction of virgin olive oil; the high temperatures reached during the milling period, together with the cell destruction, facilitate contact between chlorophyllase and its substrates (both present in the fruit) and thus provide optimum conditions for the enzymatic reaction.

As the fruits have been used directly as both substrate and source of enzyme, the concentration of neither can be fixed for an adequate study of enzyme–substrate behavior. The concentration of chlorophylls and enzyme depends on the stage of ripeness of the fruits. In addition, as stated above, the substrate composition depends on the incubation medium. Under these conditions, the study of substrate specificity is approximate and can only be carried out by indirect measurement.

The ratio between the levels of activity found for chlorophylls *a* and *b* in the enzymatic reaction shows, in all cases, that enzymatic activity is some 4-fold higher for chlorophyll *a*. This is because initially the ratio between the substrates chlorophylls *a* and *b* in the fruits is also of that order. However, considering the ratio between the percentages of product formed, it is observed that this falls to values around 1.20. This result does not allow the conclusion that the enzymatic activity is preferential for the *a* series to be drawn, since similar concentrations of substrate are not being compared. As the ratio of substrate to product formed falls from the initial 3:1 to 1:1, it seems there is a greater affinity of enzyme for the *b* series. Thus, preferential degradation of chlorophyll *b* has also been observed by Shimokawa (1979).

Effect of Acetone. Water, chlorophyllase buffer, and two levels of the chlorophyllase buffer–acetone mixture were assayed as incubation media with the same treatment time. The results are shown in Table III. In water and buffer, chlorophyllase was activated only at 70 °C. At 50 °C, in the media containing acetone, pheophorbide formation was favored by increasing the concentration of acetone. These same levels of de-esterification were found by incubation at 70 °C using 25% acetone and decreased slightly with increasing concentrations of acetone. Levadoux et al. (1987) studied the optimum conditions of pH and temperature for enzymatic action in mixtures of buffer–surfactant, buffer–acetone, and buffer–acetone–oil. They found that the instability of the enzyme at high temperatures is a function of the concentration of acetone in the incubation medium. These authors found that with acetone concentrations greater than 30% in the medium the enzyme was inactivated at temperatures higher than 50 °C, whereas with an acetone concentration of 6% chlorophyllase activity was unaffected, even at 60 °C.

As suggested in the previous section, enzyme activation seems to depend more on the concentration of acetone in the incubation medium than on increases of temperature. The best conditions for chlorophyllase activation in the fruits are heating at 50 °C with the 1:1 chlorophyllase buffer–acetone mixture.

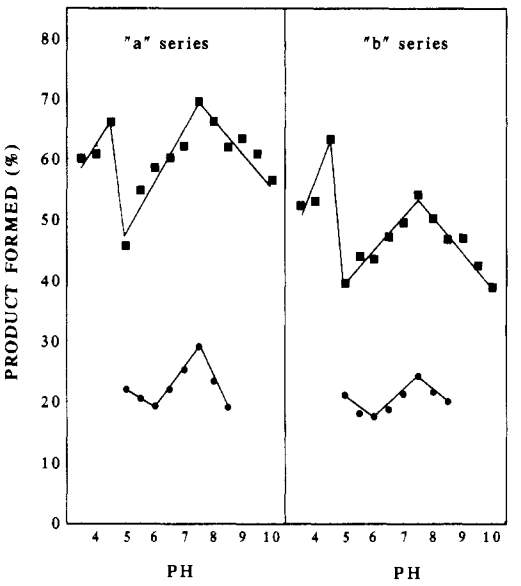

Figure 1. Effect of pH on chlorophyllase activity in olive fruits: (●) first assay; (■) second assay. Incubation was at 30 °C during 85 h in the medium buffer (0.01 M acetate + 0.05 M phosphate + 0.01 M borate containing 0.5 M NaCl and 10 mM Cl_2Mg)/acetone (1:1).

Effect of pH. Before this is considered, it must be remembered that, during the processing of green table olives, the lactic fermentation of olives is preceded by alkaline treatment, followed by washing of the fruits with water and immersion of them in brine. The initial pH of the fermentation medium is 8–9 units. Gradually by osmosis, the brine is converted into a suitable culture medium for microorganisms, resulting in the formation of organic compounds, liberation of acids, and a progressive decrease in the pH. This takes place at room temperature, which in September–October in Seville is close to 30 °C (Fernández-Díez et al., 1985). In the industrial process, chlorophyllide detection is performed 3 days after the fruits are placed in brine. Determination of pigments prior to this time is impractical due to the high pH of the fruits. Three days after the start of brining, the pH reaches 7 units. For these reasons, in the pH study the time and temperature conditions selected were similar to those used in the preparation of table olives, i.e., 30 °C for 85 h.

Figure 1 shows the percentage of product formed (chlorophyllides + pheophorbides) as a function of pH. The incubation medium used was acetone–buffer (0.01 M acetate + 0.05 M phosphate + 0.01 M borate containing 0.5 M NaCl and 10 mM Cl_2Mg) (1:1). The first assay curve shows that the optimum pH is 7.5 units for both *a* and *b* series. This agrees perfectly with the chlorophyllide formation detected, at the same pH value, during the actual processing of green table olives. At pH 6 the amount of product formed was reduced by some 10%, and at pH values lower than 5 units another maximum of activity is suggested. A second experiment, with a wider range of pH and without $MgCl_2$ in the incubation medium, confirmed the existence of another maximum of activity at pH 4.5.

Two hypotheses can be put forward to explain this tendency: the possible existence of two isoenzymes or the modification of the substrate by variation of the pH, which would allow two different enzymatic reactions. Given the

Table IV. Ratio of Percent Pheophytins to Percent Chlorophylls Found after the Enzymatic Reaction at Different Values of pH

	first assay		second assay	
pH	*a* series	*b* series	*a* series	*b* series
3.5			36.5	2.32
4.0			33.6	3.92
4.5			24.9	3.78
5.0	1.42	0.14	22.5	2.03
5.5	1.07	0.12	34.6	1.85
6.0	0.98	0.12	25.3	1.55
6.5	0.75	0.10	17.1	1.35
7.0	0.53	0.08	16.5	1.35
7.5	0.76	0.12	4.28	0.65
8.0	0.59	0.09	3.26	0.60
8.5	0.51	0.09	3.75	0.48
9.0			3.09	0.43
9.5			3.67	0.41
10.0			2.68	0.38

complexity of the substrate used in this study, it is more logical to consider factors connected with this rather than the enzyme.

As the fruits are used as a source of both enzyme and substrate, the latter in principle comprises a mixture of chlorophylls *a* and *b*, in the ratio naturally found in the olive—around 3.5–4.0 units (Mínguez-Mosquera and Garrido-Fernández, 1989). The instability of chlorophylls means that they are altered even under the best conditions. Table IV shows the ratio of pheophytin to chlorophyll found after the reaction at the different values of pH. In the first assay, at pH 8.5 this ratio for the *a* series is 0.51, while at pH 5 it reaches 1.42. For the *b* series, chlorophyll always predominates and the ratio varies from 0.09 to 0.14. This is in accord with the greater tendency of chlorophyll *a* for the pheophytinization reaction as described in the bibliography (LaJollo et al., 1971; Schwartz and Von Elbe, 1983). In the second assay, the ratio of pheophytin to chlorophyll is affected by the absence of $MgCl_2$ in the incubation medium and pheophytin *a* always predominates, although it decreases considerably from pH 3.5 to pH 10.0. For the *b* series, pheophytin predominates only up to pH 7. In any case, in the range of pH studied, pheophytin or chlorophyll predominated in the mixture of substrates as a function of the presence of Mg ions and of the pH value.

It may be that the enzyme acts indiscriminately on any tetrapyrrole phytilated derivative, each having its own rate constant or an optimum pH for activity depending on the substrate. If this were so, the most logical explanation for there being two maxima of activity would be the superimposition of two curves of activity against pH: one for pheophytin, with a maximum at pH 4.5, and the other for chlorophyll, with a maximum at pH 7.5. The optimum for pheophytins would be in accord with the maximum of activity found by McFeeters et al. (1971) for chlorophyllase of *Ailanthus altissima*. These authors state, furthermore, that the values of the kinetic rate constants of the enzyme (K_m) are almost 4 times greater for chlorophyll *a* than for pheophytin *a* (103 and 27 μM, respectively), indicating a greater affinity of chlorophyllase for pheophytin *a*.

In our opinion, the only explanation for the second optimum of activity at acid pH is that in this zone the dominant chlorophyllic pigments in the fruit are pheophytins. Thus, this maximum may be interpreted as a distortion of the graph, as it does not really show the activity of the enzyme against a single substrate but against two, for which, moreover, it has different affinities. However, during the actual procedure of processing green table olives, no new formation of de-esterified compounds at acid pH has been detected, despite the fact that when such conditions pertain, the mixture of substrates in the fruit is largely composed of pheophytins (Mínguez-Mosquera et al., 1989). However, it must be noted that in this case the condition of the fruits is not totally comparable, since in the actual system of processing green table olives there is a NaOH treatment which is not included in the model system. This hydrolyzes virtually all of the soluble matter of low molecular weight existing in the olive. It is feasible that in the fresh fruit a modulator (activator) of chlorophyllase exists, which is eliminated by the alkaline treatment, leading to a deactivation of the enzyme. On the other had, the time for which the fruits are in contact with the NaOH solution—approximately 6 h (Mínguez-Mosquera et al., 1989)—causes a gradual increase in the pH inside the olives. In the outer zones of the fruit pH values greater than 10 may be reached, which may lead to a certain degree of instability of the enzyme. In line with this suggestion, McFeeters et al. (1971) has indicated that the purified enzyme from *A. altissima* has only 66% of its original activity after incubation for 1 h at pH 11.1. Although these results are not completely comparable—in one case the enzyme is purified and in the present study the enzyme is determined *in vivo* , they emphasize the importance of such studies in the elucidation of the actual effect of alkaline treatment on the activity of chlorophyllase in olives.

The variation in the concentration of each substrate with pH does not allow simple comparison of activity from the height of the maxima, as although the concentration of chlorophyllic compounds in the reaction medium is constant, that of each derivative is not. Thus, the curve may be distorted by the uncertainty, in the case of pheophytin, of a real saturation of the enzyme by the substrate.

ACKNOWLEDGMENT

We express our sincere gratitude to CICYT for supporting this research project, ALI 91-1166-C03-02.

LITERATURE CITED

Clydesdale, F. M.; Francis, F. J. Chlorophyll changes in thermally processed spinach as influenced by enzyme conversion and pH adjustment. *Food Technol.* **1968**, *22*, 793–796.

Fernández-Díez, M. J., et al. *Biotecnología de la aceituna de mesa;* Consejo Superior de Investigaciones Científicas: Madrid, 1985.

Holden, M. The breakdown of chlorophyll by chlorophyllase. *Biochem. J.* **1961**, *78*, 359–365.

Jones, I. D.; White, R. C.; Gibbs, E. Some pigment changes in cucumbers during brining and brine storage. *Food Technol.* **1962**, *March*, 96–102.

Jones, I. D.; White, R. C.; Gibbs, E. Influence of blanching or brining treatments on the formation of chlorophyllides, pheophytins, and pheophorbides in green plant tissue. *J. Food Sci.* **1963**, *28*, 437–439.

Klein, A. O.; Vishiac, W. Activity and partial purification of chlorophyllase in aqueous systems. *J. Biol. Chem.* **1961**, *236*, 2544–2545.

LaJollo, F. M.; Lanfer Marquez U. M. Chlorophyll degradation in a spinach system at low and intermediate water activities. *J. Food Sci.* **1982**, *47*, 1995–2003.

Levadoux, W. L.; Kalmokoff, M. L., Pickard, M. D.; Grootwassink, J. W. D. Pigment removal from canola oil using chlorophyllase. *J. Am. Oil Chem. Soc.* **1987**, *64*, 139–144.

Lin, Y. D.; Clydesdale, F. M.; Francis, F. J. Organic acid profiles of thermally processed spinach puree. *J. Food Sci.* **1970**, *35*, 641–644.

McFeeters, R. F.; Chichester, C. O.; Whitaker, J. R. Purification and properties of chlorophyllase from Ailanthus altissima. *Plant. Physiol.* **1971,** *47,* 609–618.

Mínguez-Mosquera, M. I.; Garrido-Fernández, J. Chlorophyll and carotenoid presence in olive fruit (Olea europaea). *J. Agric Food Chem.* **1989,** *37,* 1–7.

Mínguez-Mosquera, M. I.; Garrido-Fernández, J.; Gandul-Rojas, B. Pigment changes in olives during fermentation and brine storage. *J. Agric. Food Chem.* **1989,** *37,* 8–11.

Mínguez-Mosquera, M. I.; Gandul-Rojas, B.; Montaño-Asquerino, A.; Garrido-Fernández, J. Determination of chlorophylls and carotenoids by high-performance liquid chromatography during olive lactic fermentation. *J. Chromatogr.* **1991,** *585,* 259–266.

Schoch, S.; Brown, J. The action of chlorophyllase on chlorophyll-protein complexes. *J. Plant. Physiol.* **1987,** *126,* 483–494.

Schwartz, S. J.; von Elbe, J. H. Kinetics of chlorophyll degradation to pyropheophytin in vegetables. *J. Food Sci.* **1983,** *48,* 1303–1306.

Shimokawa, K. Preferential Degradation of Chlorophyll *b* in Ethylene-Treated Fruits of Satsuma Mandarin. *Sci. Hortic.* **1979,** *11,* 253–256.

Terpstra, W.; Lambers, J. W. J. Interactions between chlorophyllase, chlorophyll a, plants lipids and Mg^{2+}. *Biochim. Biophys. Acta* **1983a,** *746,* 23–31.

Terpstra, W.; Lambers, J. W. J. Incorporation of Chlorophyll and Chlorophyllase into Artificial Membranes. *Photobiochem. Photobiophys.* **1983b,** *6,* 93–100.

Vazquez-Roncero, A. Olive Chemistry I. Organic Compounds. *Grasas Aceites* **1963,** *14,* 262–278.

Received for review April 8, 1993. Accepted September 28, 1993.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts,* November 1, 1993.