Quantification of Pigments in Fermented Manzanilla and Hojiblanca Olives

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The color change observed in the fruits of the table olive during the process of lactic fermentation is confirmed as due exclusively to the structural modification of pigments, caused by the action of the enzyme chlorophyllase and the progressive acidity of the medium. The qualitative and quantitative knowledge of the pigments present in the fermented fruit has made the perfecting of a rapid spectrophotometric method for the evaluation of the principal fractions of pigment possible. From the absorption spectrum of the crude extract, the different fractions are calculated at the following wavelengths: total pigments at 406 nm, carotenoids at 476 nm, and chlorophyll derivatives at 668 nm. There is a good correlation between pigment content and fruit color.

INTRODUCTION

Recent innovations in the traditional process of elaboration of green table olives, to minimize the volume of wastewater, have affected the physicochemical and organoleptic characteristics (color, smell, and flavor) of the finished product (Fernández-Díez et al., 1985; Garrido-Fernández et al., 1986), although this is still not fully studied.

For some years, special attention has been given to research into the components responsible for color, as color is an important attribute of quality. The qualitative and quantitative evolution of chlorophylls and carotenoids has been followed during the traditional fermentation process of the olive, with the aim of determining the variables that take part in pigment transformation and attempting to direct their action without giving up the necessary modifications of the process.

This research, carried out on the variety Hojiblanca, has shown that the lactic fermentation process causes the total transformation of chlorophylls *a* and *b*, initially present, according to two different and coexistent mechanisms, giving rise to a mixture of pheophytins and pheophorbides in the elaborated fruit (Mínguez-Mosquera et al., 1989).

In the present work, a similar study is carried out on the variety Manzanilla, confirming the qualitative sequence of degradation of chlorophylls and carotenoids. At the same time, the differences between cultivars and the majority pigment responsible for color in fermented olive are determined.

As these pigments are liposoluble, the high lipid content of the olive is a serious obstacle in isolating chlorophyll pigments and makes prior attainment of a fat-free pigment extract necessary (Mínguez-Mosquera and Garrido-Fernández, 1989). From this, using the technique of thin-layer chromatography, it has been possible to separate and later identify the components that give color to the elaborated fruit. At the same time, it permits quantification of each individual pigment and determination of which are the most significant and to what degree.

But, for routine periodic control, the method is slow and tedious. The only possible simplification is by direct evaluation of the pigments in the extracts obtained with the solvents usual in this field, omitting previous purifications and separations. Correct interpretation of the resulting absorption spectrum, on the basis of previously acquired knowledge, may solve the problem.

Consequently, a rapid spectrophotometric method is described for the evaluation of the principal fractions of pigment present in the elaborated fruit. The relationship between color as quality and fruit pigment content is checked by using samples supplied and classified by the industry.

MATERIALS AND METHODS

Raw Material Used. The study was made during 2 consecutive years with olives of the variety Manzanilla (*Olea europaea pomiformis*). The experiment was carried out in 60 kg capacity fermenters. The traditional process of preparation for this type of olive includes a treatment with 2% sodium hydroxide for 6 h, a water washing for 8 h, and a later conditioning of the fruit in brine (10% NaCl). The sugars, vitamins, and amino acids of the fruit pass to the brine by an osmotic process, converting it gradually into a suitable medium for microorganism growth, where the fruits undergo a total lactic fermentation. Table I shows the sequence of the more characteristic microorganism species of this fermentation and their metabolic products (Fernández-Díez et al., 1985).

The complete process of fermentation and curing lasts approximately 6 months, at the end of which period the fruit should have certain organoleptic characteristics.

To evaluate the pigment content in the elaborated fruit, the study was carried out with selected fruits of the varieties Manzanilla and Hojiblanca, prepared as green table olives in Spanish or Sevillian style. They were provided by two firms of the Seville table olive industry and classified by quality by their respective experts. Each firm sent, for variety and quality, four glass containers of 5-kg capacity, each labeled with a subjective qualification (excellent, good, acceptable, or rejectable).

Preparation of Extract Free from Fatty Material. Samples were made from a triturate homogenized from 100 destoned fruit, about 500 g, weighing exactly between 5- and 15-g duplicates for each analysis according to the days fermentation. The pigment extraction was made with *N,N*-dimethylformamide. The filtrates were next treated with hexane in a decantation funnel to extract and separate the characteristic fatty olive matter from the previous solution. The hexane phase in its turn carried over the carotene fraction, while that corresponding to *N,N*-dimethylformamide retained chlorophylls, chlorophyll derivatives, and the rest of the carotenoids. De-

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Table I. More Relevant Species of Microorganisms Identified in Green Table Olive Fermentation Brines and Their Metabolic Products

species	frequency, %	isolation interval, days	metabolic products
Gram-negative bacteria		5 and 15	carbon dioxide,
Enterobacter cloacae	32		hydrogen,
Citrobacter freundii	12		acetic acid,
Enterobacter aerogenes	10		and ethanol
Flavobacterium diffusum	10		
Lactic cocci		7 and 55	lactic acid and
Streptococcus lactis	3		acetic acid
Pediococcus urinaeequi	36		
Leuconostoc paramesenteroids	61		
Lactobacilli		7 and 60	lactic acid
Lactobacillus plantarum	90		
Lactobacillus delbrueckii	10		

tails of the extraction process are given in a previous work (Mínguez-Mosquera and Garrido-Fernández, 1989).

Pigment Extraction by the Traditional Technique. Pigment extraction with acetone was carried out by a standard procedure according to the method of Smith and Benitez (1955), adding successive portions of acetone to 5–10 g of homogenized destoned fruit.

The extract was shaken after each addition and filtered on anhydrous Na_2SO_4. The solid residue was collected, and the operation was repeated until the filtrates were colorless. The extracts were combined and transferred to a rotavapor flask and evaporated at a temperature below 30 °C at reduced pressure. The fatty residue was dissolved in 5 mL of cychlohexane.

Pigment Separation. The pigment separation was carried out by thin-layer chromatography on silica gel 60 GF_{254} with the mixture light petroleum ether/acetone/diethylamine (10:4:1). For the separation of pheophorbides, the same developer was used, but pyridine was substituted for diethylamine.

Pigment Identification. The absorption spectra, as well as absorption properties and the color shown by these substances in TLC under white and UV light, served as a basis for identification of chlorophylls and derivatives (Smith and Benitez, 1955).

For carotenoids, the adsorption properties in thin layer, before and after saponification, and 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 the bibliography (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 ethyl 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 $E = E_0C$; the results were obtained as milligrams per kilogram of destoned fruit.

The values for the extinction coefficient E_0 in acetone were calculated from those given in the bibliography in ethyl ether for chlorophylls and pheophytins (Smith and Benitez, 1955) and in ethanol for carotenoids (Davies, 1976). The chlorophyllides and pheophorbides were eluted from the plate with the mixture acetone/pyridine (1:1), and the values of E_0 were calculated from that of E_0 for chlorophylls and pheophytins in acetone (Mínguez-Mosquera et al., 1989).

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

RESULTS AND DISCUSSION

Evolution of Pigments during the Fermentation. The chlorophyllic and carotenoid components were suitably characterized as the fermentation process went on. The progress of the qualitative and quantitative evolution of these pigments during the lactic fermentation process and later curing of the fruit in acid brine is shown in Tables II and III. All are in function of the changes in pH and time in days.

Table II. Qualitative and Quantitative Evolution of Chlorophylls and Derivatives in Manzanilla Variety Fruits during the Lactic Fermentation Process (Milligrams per Kilogram)[a,b]

pH	time, days	Chl *a*	Chl *b*	Chd *a*	Chd *b*	Phy *a*	Phy *b*	Pho *a*	Pho *b*
fresh		43.45	9.73						
6.12	5	39.48	6.01	3.94	3.85				
6.09	7	26.30	7.10	7.30	4.60				
5.87	13	26.41	6.10	7.10	4.20	5.29			
5.33	16	27.34	7.21	6.19	3.19	9.91		1.54	
5.12	22	14.41	6.65	6.98	2.74	12.83	1.02	7.88	1.05
4.56	28	11.82	4.22	5.38	1.75	16.70	2.78	6.62	1.40
4.29	34	7.10	3.86	3.30	1.00	18.36	3.50	6.83	2.80
4.11	45	4.09	2.90	1.48	0.70	19.63	5.73	6.63	2.96
4.06	66	2.74	2.59			24.43	4.80	8.09	3.45
4.03	72	3.23	3.20			24.55	4.77	7.63	2.28
4.03	104		1.23			24.68	5.74	6.61	2.56
4.02	114		1.72			25.36	5.62	6.08	2.47
3.96	139		1.38			26.61	5.39	6.73	2.06
3.90	210					26.50	6.65	6.70	2.20

[a] Destoned fresh fruit basis; average of duplicate analysis of two samples. [b] Key: Chl *a* = chlorophyll *a*; Chl *b* = chlorophyll *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*.

Table III. Qualitative and Quantitative Evolution of Carotenoids in Manzanilla Variety Fruits during the Lactic Fermentation Process (Milligrams per Kilogram)[a,b]

pH	time, days	β-carotene	lutein	viola-xanthin	neo-xanthin	neo-chrome
fresh		2.36	4.30	2.23	1.61	
6.12	5	2.92	4.93	1.24	1.03	
6.09	13	2.69	4.27	1.11	1.20	
5.33	16	3.34	6.41[a]	1.44	1.23	
5.12	22	2.18	6.12[a]	0.80	0.94	
4.56	28	3.04	5.80[a]	0.92	1.06	
4.29	34	2.34	6.08[a]	0.83	1.33	
4.11	45	2.59	5.16[a]		0.87	0.74
4.06	66	2.41	5.26[a]		0.40	1.24
4.03	72	2.84	5.19[a]			1.63
4.03	104	1.95	5.66[a]			1.06
4.02	114	2.58	5.74[a]			1.13
3.96	139	3.11	5.61[a]			1.18
3.90	210	2.60	5.90[a]			1.50

[a] Total lutein plus auroxanthin. [b] Destoned fresh fruit basis.

The qualitative sequence of results coincides with that found for the variety Hojiblanca (Mínguez-Mosquera et al., 1989). From this it is deduced that the lactic fermentation process causes total transformation of the chlorophylls initially present in the fresh fruit, independent of variety, by two mechanisms. One is of enzymatic origin, as the presence of chlorophyllides in the first days of fermentation indicates that hydrolysis of phytol is due to the action of chlorophyllase. The other is caused by the acid pH of the medium, as the rest of the chlorophyllic derivatives are a direct consequence of the replacement of the magnesium ion by hydrogen.

As in the case of the Hojiblanca variety, during the fermentation process, the carotenoid fraction has been affected only in those components that as a result of their molecular structure are sensitive to acid medium. It is confirmed that the total balance of pigmentary matter remains practically unaltered during the fermentation process.

Table IV. Pigment Content and Its Distribution (Percentages) of Lactic Acid Fermented Hojiblanca and Manzanilla Varieties and Rate between Fractions[a]

	Hojiblanca		Manzanilla	
pigment	mg/kg	%	mg/kg	%
pheophytin *a*	35.70	39.49	26.50	50.91
pheophytin *b*	8.70	9.62	6.65	12.78
pheophorbide *a*	23.80	26.33	6.70	12.87
pheophorbide *b*	7.20	7.96	2.20	4.23
β-carotene	3.52	3.89	2.60	5.00
lutein	9.38	10.38	5.90	11.34
neochrome	2.10	2.32	1.50	2.88
chlorophyllic fraction	75.40	83.41	42.05	80.79
carotenoid fraction	15.00	16.59	10.00	19.21
ratio pheophytins/pheophorbides	1.43		3.72	
ratio fractions chlorophyllic/carotenoid	5.43		4.20	

[a] Destoned fresh fruit basis.

Comparison between Cultivars. On comparison of varieties, percentage differences have been found in the final composition of pigments (Table IV). In the variety Manzanilla, the percentage of pheophytins is significantly higher than that of pheophorbides, the ratio between them being 3.7. In Hojiblanca, on the contrary, the percentage of pheophorbides is much higher, and so the ratio between these fractions is considerably lower at 1.4.

It is necessary to point out that in the fresh fruit the percentage composition of pigments is about the same for the varieties under study, although Hojiblanca is richer in pigments than Manzanilla (Mínguez-Mosquera et al., 1989). It seems logical to think that as the fruits of both varieties are processed similarly, the final percentage composition of pigments should be maintained in both cases. This is in fact the usual case for the carotenoid fraction between the proper limits for natural products.

To date, the exact causes of these differences in the chlorophyllic pigment composition in the final product are not known. It could be that the tissues of each variety differ in their chlorophyllase activity, which would directly affect the final proportion of pheophorbides. On the other hand, it is observed that the Manzanilla variety reaches acid pH values before Hojiblanca. The shorter time that the fruit is at a high pH, favorable to enzymatic action, could have an effect on the structural transformation of the chlorophyll.

The results for individual pigment composition indicate that the Hojiblanca variety is richer in pigmentation than Manzanilla and that in both the chlorophyll fraction is greater than the carotenoid. In general, the group of chlorophyll derivatives makes up more than 80% of the total pigmentation, pheophytin *a* being the majority pigment. The rest is distributed, in order of contribution, between pheophorbide *a*, pheophytin *b*, and pheophorbide *b*. As the compounds of series *a* are dominant and have similar absorption spectra, the group of chlorophyll derivatives can be quantified globally as pheophytin *a* by using its corresponding value of E_0. The contribution of the yellow pigmentation to the color of the fruit is around 20%, lutein being outstanding. This is assumed to be around 50% of the carotenoid contribution and permits evaluation of the group at the maximum absorption wavelength of this compound. These considerations lead to evaluation of the pigment content by fractions and permit the possible simplification of their measurements.

Rapid Method of Pigment Evaluation in the Fermented Olive. To evaluate the new method, a homogeneous triturate of olives was used as starting point. Equal quantities of sample were weighed, and the two extraction systems (acetone and *N,N*-dimethylformamide) were applied in parallel. After the filtrates were concentrated, the final residues were dissolved in the same volume of solvents and the respective absorption spectra obtained.

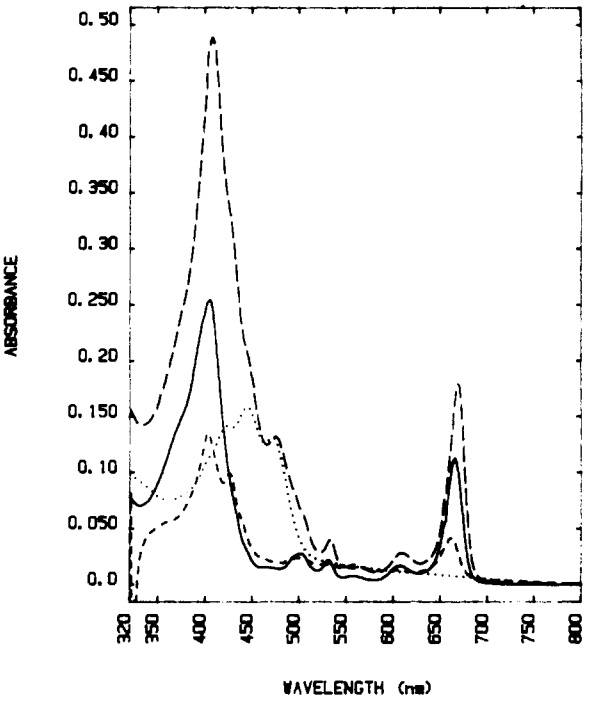

Figure 1. Absorption spectra from solution total pigments (– –), pheophytins (—), pheophorbides (- - -), and carotenoid (···) fractions.

Two aliquots from the *N,N*-dimethylformamide extraction were chomatographed separately by TLC. In one of them, the carotenoid fraction and chlorophyll were scraped from the plate independently and eluted in ethanol and ethyl ether, respectively. In the other, both were scraped together and eluted with ethyl ether. The respective absorption spectra of all of them were obtained and the characteristics studied in detail. That of one sample is given as an example in Figure 1.

It can clearly be seen that the shape corresponding to the total pigment is similar to that of pheophytins, as would be expected from the fact that pheophytin *a* is the major pigment component in the fermented olive.

The maximum observed at 476 nm is associated with the presence of yellow pigments, since in this zone of the spectrum the pheophytin and pheophorbide do not show absorption, making their evaluation possible in the total spectrum, following calculation of the corresponding coefficient of extinction at this wavelength. The carotenoid concentration is thus calculated at 446 nm as if it were all lutein (E_0 = 2550, in ethanol), and at the same time the extinction of this fraction is measured at 476 nm. When both values are substituted in the equation $E = E_0C$ the coefficient of extinction at 476 nm proves to be 2000.

Table V shows the results obtained by this procedure, comparing the values obtained with those found from the individual spectra of the different fractions (pheophytins, pheophorbides, and carotenoids). Fractions 1–3 come from scraping the indicated components from the chromatographic plate. They are eluted with the appropriate solvent in each case, and the concentrations are calculated, evaluating the different fractions such as lutein, pheophytin *a*, and pheophorbide *a*, which are the

Table V. Pigment Quantification from Absorption Spectra[a]

fraction	pigments in solution	solvent	λ_{Mx}, nm	E_0	concn, mg/kg Manzanilla	concn, mg/kg Hojiblanca
1	carotenoids	ethanol	446	2250	8.04	13.53
2	pheophytins	ethyl ether	406	1290	22.66	31.20
			664	613	20.25	39.63
3	pheophorbides	acetone/pyridine (1:1)	410	1237	10.63	24.12
			668	510	8.38	20.73
4	total pigments (fat-free)	cyclohexane	406	1290	45.61	72.23
			476	2000	8.22	13.40
			668	613	34.88	55.90
5	total pigments (with oil)	cyclohexane	406	1290	40.44	69.82
			476	2000	8.04	13.52
			668	613	33.29	52.60

[a] Destoned fresh fruit basis; average of 10 samples analyzed.

Table VI. Quantification of the Total Pigments, Carotenoids, and Chlorophyll Derivatives from Absorption Spectrum in Acetone Extract

fermenters	concn,[a] mg/kg total pigments (λ_{406})	concn,[a] mg/kg carotenoids (λ_{476})	concn,[a] mg/kg chlorophyll derivatives (λ_{668})
F_1	42.67	9.27	33.69
	35.04	8.67	27.49
	32.28	7.86	25.90
F_2	43.46	9.98	32.31
	36.11	7.77	27.40
	38.35	8.86	29.56
F_3	33.11	9.62	23.97
	37.33	11.78	31.44
	40.74	9.91	30.46
F_4	29.31	7.49	21.97
	36.94	9.34	26.84
	31.20	7.00	21.50

[a] Destoned fresh fruit basis.

majority components of each group, at the maximum absorption wavelengths. Fraction 4 corresponds to the fat-free extract of pigments, and fraction 5 comes from the crude extract (from acetone), which includes oil. The calculations in both cases are made from the respective absorption spectra.

The quantification of carotenoids at 476 nm in the complete extract (fraction 4) gives a similar value to that obtained in their own spectrum at 446 nm (fraction 1). The chlorophyll derivatives calculated at 668 nm coincide with the sum of pheophytins and pheophorbides (fractions 2 and 3). The concentration of pigments calculated at 406 nm is approximately equivalent to the sum of carotenoids and chlorophyll derivatives, already assigned to the former fractions due to the combination of absorbances of these compounds in the common zone of the spectrum. The two extraction systems applied in parallel (fractions 4 and 5) yield similar results.

Consequently, from the absorption spectrum of all the pigments in solution (oil included) the proposed method permits the evaluation of carotenoids at 476 nm and chlorophyll derivatives at 668 nm and the deduction of the total concentration of pigments at approximately 406 nm.

By use of this procedure, three replicated analyses of lactic acid fermented Manzanilla fruits corresponding to four different fermenters were carried out (Table VI). To verify that total pigment content calculated at 406 nm does not statistically differ from the value obtained by addition of the carotenoid fraction, estimated at 476 nm, and the chlorophyll derivatives, estimated at 668 nm, the data were reordered as shown in Table VII. Results from the corresponding analysis of variance point out that there is no statistical difference, according to the Duncan procedure (Ruiz-Maya, 1977).

Table VII. Total Pigment Analysis of Different Fermenters Using the Two Procedures (Milligrams per Kilogram)

procedure[a]	fermenter F_1	F_2	F_3	F_4
I[b]	42.67	43.46	33.11	29.31
	35.04	36.11	37.33	36.94
	32.28	38.35	40.74	31.20
II[c]	42.96	42.30	33.59	29.49
	36.16	35.17	43.22	36.18
	33.76	38.42	40.37	28.50

[a] Differences between procedures significant at $p < 0.005$. [b] Concentration of total pigments calculated at λ_{406}. [c] Concentration of total pigments calculated as addition of the concentrations at λ_{476} and λ_{668}.

Table VIII. Average Pigment Concentration in Olives (Manzanilla Variety) Previously Classified in Four Qualities by Their Surface Color

subjective classification	pigment concn,[a] mg/kg carotenoid fraction	chlorophyll fraction	total pigments
excellent	7.4	24.9	32.9
good	8.7	30.7	40.6
acceptable	10.2	37.8	47.8
rejectable	13.4	48.3	60.4

[a] Destoned fresh fruit basis.

Table IX. Individual Concentration (Milligrams per Kilogram) of Pigments in Olives Previously Classified by Their Surface Color

sample	subjective classification[a] excellent	good	acceptable	rejectable
	Carotenoid Fraction			
1	8.67	9.13	11.09	13.43
2	7.26	8.11	9.22	13.37
3	6.56	9.32	10.95	12.10
4	6.95	8.37	9.43	14.90
	Chlorophyll Fraction			
1	29.28	31.25	40.15	48.03
2	24.83	29.29	34.67	46.83
3	21.49	32.33	40.75	43.92
4	24.23	30.31	35.79	54.71
	Total Pigment			
1	38.06	41.78	51.40	61.29
2	33.06	39.46	44.38	59.15
3	29.26	43.53	51.82	54.65
4	31.59	37.79	43.64	66.67

[a] Differences between qualities significant at $p < 0.0005$ for the carotenoid and chlorophyllic fractions and total pigments.

Application of the Method. The need to evaluate the pigmentation of fermented olives rapidly and quantitatively is obvious for technicocommercial reasons. To establish the relationship between the color of the fruits and their pigment composition, the method described above is applied to samples of olives fermented in Spanish or Sevillian style, elaborated and supplied by the industry and classified by its experts in different grades of acceptability. Four samples for variety and quality were analyzed, and the mean values obtained are shown in Table VIII. The quantification of carotenoids and chlorophyll derivatives from the absorption spectrum of the crude extracts of

pigments is in total agreement with the subjective classification of the color of the fruits.

To study the differences found, the individual values are shown in Table IX. The analysis of variance corresponding to carotenoid and chlorophyll fractions as well as total content according to the Duncan procedure shows that the proposed method of evaluating color by pigment content may significantly differentiate the qualities by which olives can be classified subjectively.

Perhaps the most relevant and surprising result from the present study is that the subjective valorization of the color decreases as the pigment concentration increases. As the traditional process of lactic fermentation involves only the transformation of pigments, without their loss or destruction, it is clearly necessary to investigate where the problem of color lies by studying in detail how the modifications to the fermentation process act on these pigments.

Consequently, this method is considered of great interest for the possibilities given by the correlation found between the subjective classification of the fruits by their color and their concentration of chlorophyll derivatives and carotenoids. This makes possible the standardization of the color of table olives by their pigment content.

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Registry No. Chlorophyll *a*, 479-61-8; chlorophyll *b*, 519-62-0; chlorophyllide *a*, 14897-06-4; chlorophyllide *b*, 14428-12-7; pheophytin *a*, 603-17-8; pheophytin *b*, 3147-18-0; pheophorbide *a*, 15664-29-6; pheophorbide *b*, 20239-99-0; β-carotene, 7235-40-7; lutein, 127-40-2; violaxanthin, 126-29-4; neoxanthin, 14660-91-4; neochrome, 25548-02-1.