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High-performance liquid chromatographic study of alkaline treatment of chlorophyll

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Abstract

The degradation products of chlorophyll *a* and *b* obtained by alkaline treatment in the presence of atmospheric oxygen were studied. Reversed-phase ion-pair high-performance liquid chromatography, normal- and reversed-phase thin-layer chromatography and diode-array detection were used for the separation, isolation and preliminary identification of the following degradation products: Mg-rhodin g_7, Mg-chlorin e_6, MeO-lactone-chlorophyllide, Mg-purpurin, Mg-phytyl-rhodin g_7, Mg-phytyl-chlorin e_6, MeO-lactone-chlorophyll, Mg-phytyl-purpurin and some of their diasteroisomers. MeO-chlorophyll and OH-chlorophyll were tentatively identified. The alkaline treatment did not induce the specific de-esterification of phytol in any of the cases studied and oxidation of the isocyclic ring was simultaneously observed.

1. Introduction

Chlorophylls are the most important pigments in photosynthesis, but their interest with regard to food technology derives from their involvement in the green colour of fruit and vegetables. Chlorophylls are very stable in their natural environment inside the chloroplast, but whenever this organ becomes damaged the chlorophylls are very susceptible to undergo a wide range of chemical transformations as a consequence of the intervention of different factors such as high temperature, extreme pH values, enzymatic action, molecular oxygen and light. All these factors may combine during food processing, causing changes in the colour of the finished product [1,2]. The most appealing change is the separation under acidic conditions of the central atom of magnesium (pheophytinization) (Fig. 1), causing a drastic change in colour from bright green to olive brown [3,4]. Other possible changes are the formation of epimer at C-13[2] (chlorophyll *a'* and *b'*), decarbomethoxylation of C-13[2] leading to pyropheophytins [5,6], de-esterification of phytol at C-17[3] [7–11], oxidation of the isocyclic ring [12], insertion of divalent metals in the porphyrin ring (re-greening) [13,14] and oxidative opening of the porphyrin chromophore group causing decoloration [15,16].

The oxidation of the chlorophyll molecule by molecular triplet oxygen in alcoholic solutions causes the replacement of the atom of hydrogen of C-13[2] located in the isocyclic ring by oxygen or an oxygen-containing species. This reaction, named "allomerization", may occur by both

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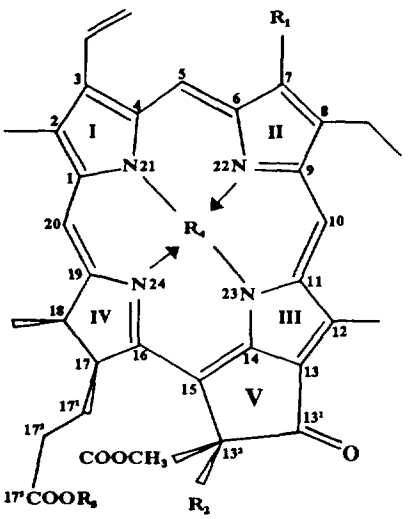

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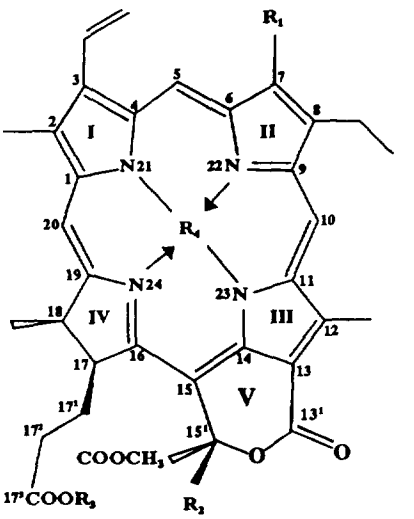

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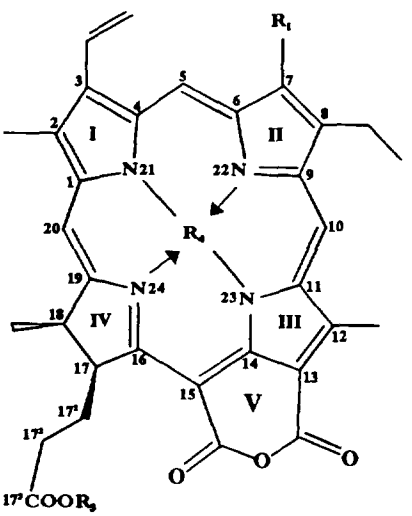

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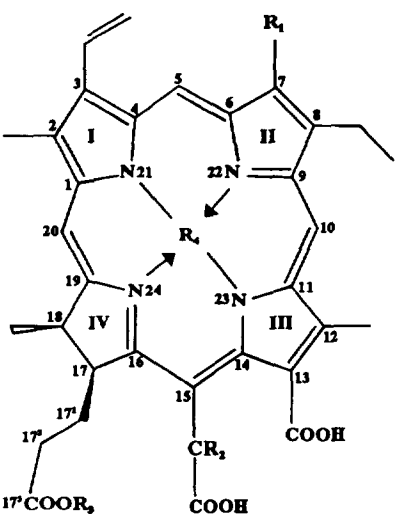

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No.	Trivial name	R_1	R_2	R_3	R_4	Abbreviation
1.1a	Chlorophyll *a*	CH_3	H	Phytyl	Mg	chl *a*
1.2a	Pheophytin *a*	CH_3	H	Phytyl	2H	phy *a*
1.3a	Chlorophyllide *a*	CH_3	H	H	Mg	chld *a*
1.4a	Pheophorbide *a*	CH_3	H	H	2H	pho *a*

Fig. 1. Structures, trivial names and numbering system for chlorophylls and derivatives. The replacement of the CH_3 group in R_1 by a CHO group forms chlorophyll *b* derivatives. The replacement of the CO_2CH_3 group at C^{13} by H forms pyro derivatives.

1.5a	13^2-Hydroxychlorophyll *a*	CH_3	OH	Phytyl	Mg	OH-chl *a*
1.6a	13^2-Methoxychlorophyll *a*	CH_3	OCH_3	Phytyl	Mg	MeO-chl *a*
2.1a	15^1-Methoxylactone chlorophyll *a*	CH_3	OCH_3	Phytyl	Mg	MeO-lactone-chl *a*
2.2a	15^1-Methoxylactone pheophytin *a*	CH_3	OCH_3	Phytyl	2H	MeO-lactone-phy *a*
2.3a	15^1-Methoxylactone chlorophyllide *a*	CH_3	OCH_3	H	Mg	MeO-lactone-chld *a*
2.4a	15^1-Methoxylactone pheophorbide *a*	CH_3	OCH_3	H	2H	MeO-lactone-pho *a*
3.1a	Mg-Phytyl-purpurin *a*	CH_3		Phytyl	Mg	Mg-phyt-purp *a*
3.2a	Mg-Purpurin *a*	CH_3		H	Mg	Mg-purp *a*
3.3a	Phytyl-purpurin *a*	CH_3		Phytyl	2H	phyt-purp *a*
3.4a	Purpurin *a*	CH_3		H	2H	purp *a*
4.1a	Mg-Phytyl-chlorin e_6	CH_3	2H	Phytyl	Mg	Mg-phyt-chlr
4.2a	Mg-Chlorin e_6	CH_3	2H	H	Mg	Mg-chlr
4.3a	Phytyl-chlorin e_6	CH_3	2H	Phytyl	2H	phyt-chlr
4.4a	Chlorin e_6	CH_3	2H	H	2H	chlr
4.5a	Mg-Purpurin *a* 7-dimethyl phytyl ester	CH_3	O	phytyl	Mg	Mg-phyt-purp 7
4.1b	Mg-Phytyl-rhodin g_7	CHO	2H	phytyl	Mg	Mg-phyt-rhd
4.2b	Mg-Rhodin g_7	CHO	2H	H	Mg	Mg-rhd
4.3b	Phytyl-rhodin g_7	CHO	2H	Phytyl	2H	phyt-rhd
4.4b	Rhodin g_7	CHO	2H	H	2H	rhd

Fig. 1 *(Continued)*

enzymatic and chemical pathways [17], forming MeO-lactone chl *a* (**2.1a**), OH-chl *a* (**1.5a**) and MeO-chl *a* (**1.6a**) [18–20] as major products. The acid hydrolysis of the phytol alcohol in the chlorophyll molecule is accompanied by the loss of Mg and produces pheophorbides (**1.4**). The excision of phytol without separation from Mg is a specific reaction catalysed by the endogenous enzyme chlorophyllase and which results in chlorophyllides (**1.3**). This de-esterification may also occur under mild alkaline conditions but the hydrolysis must be carried out in an inert atmosphere to avoid the possibility of other simultaneous oxidation reactions at C-13^2 [21].

Generally, the chlorophyllic derivatives have a very different chemical behaviour as a result of differences in their molecular structure, but their separation, isolation and identification entail important difficulties. Research in this field has advanced considerably in recent years owing to the high resolution of high-performance thin-layer chromatographic plates (HPTLC) and high-performance liquid chromatographic (HPLC) columns [22]. Schaber et al. [19] were the first to isolate by semi-preparative reversed-phase (RP) HPLC the products of the allomerization of chlorophyll *a* and to confirm the structure of the allomerized products 13^2-(*R*)- and 13^2-(*S*)-OH chl *a* (**1.5a**) and 15^2-(*R*)- and 15^2-(*S*)-MeO-lactone-chl *a* (**2.1a**) by NMR and ^{254}Cf plasma desorption mass spectrometry. Recently, Kuronen et al. [20] were successful in obtaining the analytical separation and preparative isolation by normal-phase (NP) HPLC of seven products of allomerization of chlorophyll *a* including, together with those mentioned above, 13^2-(*R*)- and 13^2-(*S*)-MeO-chl *a* (**1.6a**) and Mg-purpurin *a* 7-dimethyl-phytyl ester (**4.5a**). Recently in our laboratory we have investigated different conditions of chromatographic separation for most of the oxidized derivatives of the pheophorbides (**1.4**), including pyropheophorbides, chlorin e_6 (**4.4a**), rhodin g_7 (**4.4b**) and purpurin (**3.4**) [12].

The fruits of the olive tree processed for their direct consumption as table olives are submitted to an alkaline treatment to hydrolyse the bitter glucoside called oleuropeina and later, after several washes with water, they are placed in brine whereupon they experience natural lactic fermentation. This process causes the total degradation of the chlorophylls initially present in the fresh fruit to pheophytins and pheophorbides [23,24]. Recent innovations introduced in this system of processing (addition of culture initiators with recirculation, reusage of brine, etc.) seem to have modified the natural transformation of the chlorophylls and other minority

oxidative products have being detected but not identified [12,25]. We have therefore investigated the possible oxidation products of chlorophyll caused by alkaline treatment. We used normal- and reversed-phase thin-layer chromatography (NP and RP-TLC), ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) and diode-array detection (DAD) for the separation, isolation and preliminary identification of most of the oxidized derivatives of chlorophylls obtained under alkaline conditions and in the presence of atmospheric oxygen.

2. Experimental

2.1. Thin-layer chromatography

The TLC plates for normal-phase operation were prepared by spreading a layer of a mixture of silica gel GF_{254} (Merck, Darmstadt, Germany) and water (1:2, w/v) over 20×20 cm glass plates. A Unoplen thin-layer spreader (Shandon Southern, Runcorn, UK) was used and the thickness was adjusted to 0.7 mm. Reversed-phase TLC was performed using silica gel C_{18} plates (Nano SI F_{254} C_{18}-100; Sharlau, Barcelona, Spain) (10×10 cm, thickness 0.2 mm). As mobile phases we used light petroleum (b.p. 65–95°C)–acetone–diethylamine (10:4:1) for NP-TLC [25] and methanol–acetone–water (20:4:3) for RP-TLC [12]. Chromatography was performed in a normal saturation tank and for detection a Dessaga UV–Vis lamp provided white light and UV radiation of 254 and 366 nm.

2.2. Ion-pair reversed-phase high-performance liquid chromatography

HPLC was performed using a Waters Model 600E liquid chromatograph fitted with an injection valve (Rheodyne Model 7125) and a Waters Model 994 photodiode-array detector. Chromatograms were recorded on a register-integrator (Waters Model 5200). A stainless-steel column (25×0.4 cm I.D.), packed with 5-μm C_{18} Spherisorb ODS-2 (Teknokroma, Barcelona, Spain) was used. The column was protected by a cartridge (5×0.4 cm I.D.) packed with the same material. The pigments dissolved in acetone were centrifuged at 13 000 *g* (MSE Micro Centaur centrifuge) prior to injection into the chromatograph (20 μl) and were separated using an elution gradient (flow-rate 2 ml/min) with the mobile phases (A) water–ion pair reagent–methanol (1:1:8, v/v/v) and (B) acetone–methanol (1:1, v/v). The ion pair reagent was 0.05 *M* tetrabutylammonium acetate (Fluka, Buchs, Switzerland) and 1 *M* ammonium acetate (Fluka) in water. The gradient scheme has been described in detail in a previous paper [25].

2.3. Reagents

Acetone and methanol were of HPLC grade (Tecknokroma) and the remainder were of analytical-reagent grade. Water was deionized and filtered through a 0.45-μm nylon membrane (Supelco, Bellefonte, PA, USA).

2.4. Standards

Chlorophylls were isolated from fresh spinach leaves by pigment extraction with acetone [26] followed by TLC separation on silica gel GF_{254} (20×20 cm plates, thickness 0.7 mm) (Merck) using light petroleum (b.p. 65–95°C)–acetone–diethylamine (10:4:1) as eluent [25]. Chlorophyll C-13^2 epimers were prepared by treatment with chloroform according to Watanabe et al. [27]. Pheophytin *a* and *b* were prepared from the respective pure solutions of chlorophylls in diethyl ether by acidification with 2–3 drops of HCl (13%, v/v) [28]. Pheophorbides *a* and *b* were formed by enzymatic de-esterification of pheophytins; a protein precipitate of *Ailanthus altissima* (Mill.) leaves was extracted with sodium phosphate buffer (pH 7) containing 50 m*M* KCl and 0.24% Triton-X 100 and incubated with Tris buffer (pH 8.5) containing 0.24% Triton X-100 and the substrate dissolved in acetone in a 5:5:1 ratio [29]. The pheophorbides formed were transferred to diethyl ether by addition of water saturated with sodium chloride. The chlorophyll allomerization procedure was performed in the presence of methanol according to Schaber et al.

[19]. Solid chlorophyll *a* or chlorophyll *b* (1–2 μmol) was dissolved in 5 ml of methanol and exposed to atmospheric oxygen in darkness. The course of the reaction was followed by HPLC. Methyl esters of chlorine e_6 and rhodin g_7 were formed by treatment of pheophorbide *a* and pheophorbide *b*, respectively, with 0.5% KOH in methanol. Free chlorin e_6 and rhodin g_7 were obtained from their respective methyl esters in methanolic alkaline medium (30% KOH) at ambient temperature and under a nitrogen atmosphere. Purpurin *a* was obtained by alkaline oxidation of pheophorbide *a* with KOH (30%) in methanol in the presence of atmospheric oxygen, and purpurin *b* from pheophorbide *b* using the same procedure [30]. All standards were purified by NP- and RP-TLC [12,25].

2.5. Alkaline treatment of the chlorophyll in aqueous media

As oxidizing agents 0.5% aqueous solutions of sodium hydroxide and potassium hydroxide were employed. The chlorophyll (*a* or *b*), solid and chromatographically pure (1–2 μmol), was dissolved in 75 ml of acetone and mixed with 150 ml of the aqueous alkaline solution. This mixture was divided into three parts, each of which was placed in a decantation funnel. The three mixtures were exposed to atmospheric oxygen at room temperature for 10, 30 and 60 min respectively. On completion, 50 ml of diethyl ether and 200 ml of distilled water saturated with sodium chloride were added to induce separation into phases in each of the funnels. Although most of the products obtained are transferred to the organic phase, in the aqueous phase an important part of the coloration was retained. The respective ether phases were collected and washed three more times with water and another three times with 2% (w/v) sodium sulphate. The solvent was removed under reduced pressure at 30°C by means of a rotary evaporator and the residue was stored dry in a nitrogen atmosphere at −20°C until it was used (fraction I). The chlorophyll derivatives that were retained in the initial aqueous phase were recovered by incorporating a new portion of 50 ml of diethyl ether and adding 1 ml of 18% (v/v) HCl to force the transfer of pigments to the organic phase. The ether phases were washed repeatedly with water until the washings were neutral and finally they were passed through anhydrous sodium sulphate, the solvent was evaporated and the dry residue was stored in a nitrogen atmosphere at −20°C until required for use (fraction II).

2.6. Pigment identification

Pigments were identified by co-chromatography with authentic samples (TLC and HPLC) and from their spectral characteristics. The on-line UV–Vis spectra were recorded from 350 to 800 nm with the photodiode-array detector. The presence of phytol in the compounds was determined according to Bacon and Holden [31]: a solution of each pigment in 2.5% (w/v) methanolic KOH (3.5 ml) was incubated at 70°C for 30 min. After cooling in ice, 0.75 ml of light petroleum (b.p. 40–60°C) was added, and the phases were separated by addition of water (5 ml). The phytol was separated by TLC on silica gel GF_{254} with benzene–ethyl acetate (19:1, v/v) as solvent system and detected at 254 nm. The presence of magnesium in the compounds was checked by shaking an ethereal solution of the compound with 18% (v/v) HCl for 5 min [28]. A change in the UV–Vis spectrum indicates a positive test.

3. Results and discussion

3.1. Separation and isolation of the products of the alkaline treatment of chlorophylls

Ion-pair reversed-phase HPLC

As a result of the different alkaline treatments carried out, we obtained a total of ten degradation compounds of chlorophyll *a* and another nine derivatives of chlorophyll *b*. Fig. 2 shows the HPLC traces corresponding to the separation of the chlorophyll *a* derivatives which were obtained using the DAD technique at 430 nm and Fig. 3 those corresponding to the products obtained with chlorophyll *b* and detected at 460

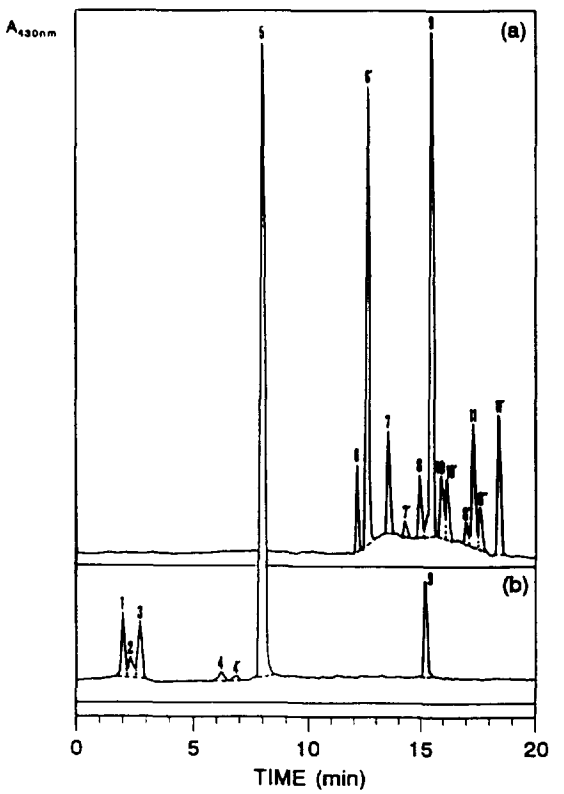

Fig. 2. HPLC separation of the alkaline treatment products from chlorophyll *a* using DAD at 430 nm. (a) Fraction I and (b) fraction II. Peaks: 1 = chlorin e_6; 2 = MeO-lactone chld *a*; 3 = Mg-purpurin *a*; 4 = MeO-lactone-pho *a*; 4′ = MeO-lactone-pho *a* isomer; 5 = purpurin *a*; 6 = Mg-phytyl-chlorin e_6; 6′ = Mg-phytyl-chlorin e_6 isomer, 7 = MeO-chl *a*; 7′ = MeO-chl *a*′; 8 = MeO-lactone-chl *a*; 8′ and 8″ = MeO-lactone-chl *a* isomers; 9 = Mg-phytyl-purpurin *a*; 10 = chl *a*; 10 = chl *a*′; 11 = OH-chl *a*; 11′ = OH-chl *a*′.

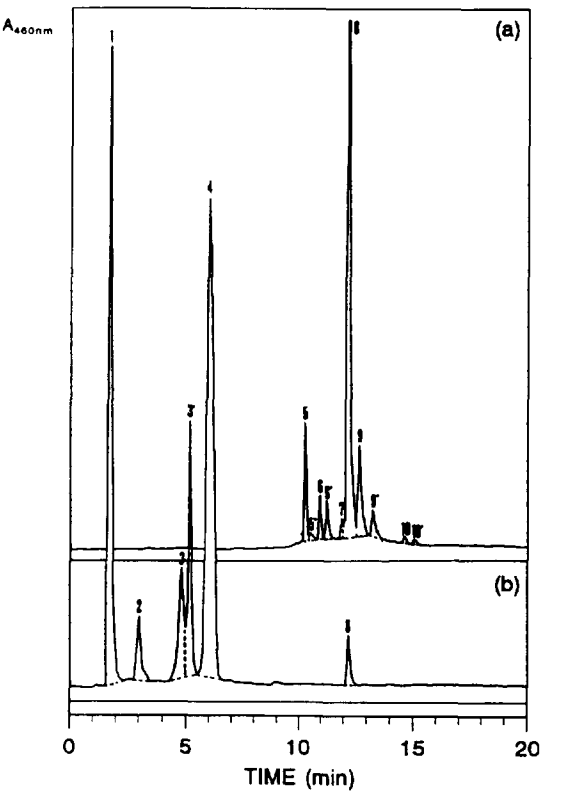

Fig. 3. HPLC separation of the alkaline treatment products from chlorophyll *b* using DAD at 460 nm. (a) Fraction I and (b) fraction II. Peaks: 1 = rhodin g_7; 2 = Mg-purpurin *b*; 3 = MeO-lactone-pho *b*; 3′ = MeO-lactone-pho *b* isomer; 4 = purpurin *b*; 5 = Mg-phytyl-rhodin g_7; 5′ = Mg-phytyl-rhodin g_7 isomer; 6 = MeO-lactone-chl *b*; 6′ = MeO-lactone-chl *b* isomer; 7 = MeO-chl *b*?; 8 = Mg-phytyl-purpurin *b*; 9 = chl *b*; 9′ = chl *b*′; 10 = OH-chl *b*?; 10′ = OH-chl *b*′?.

nm. Their chromatographic and spectroscopic characteristics are given in Tables 1 and 2, respectively. These tables include retention factors, electronic absorption maxima measured by DAD and the relationship between the absorbance of the Soret band and that of each absorption maximum that complete the UV–Vis spectral characteristics of these compounds. As the data show, the location of the absorption maxima of most of the compounds in each series derived from the chlorophylls is very different to that shown by the corresponding chlorophyll. Only two of the compounds in each series (peaks 7 and 11 for chlorophyll *a* and peaks 7 and 10 for chlorophyll *b*) have electronic absorption maxima located at exactly the same wavelengths as the chlorophyll from which they are derived. All components retained in fraction I, both in series *a* (Fig. 2a) and in series *b* (Fig. 3a), are resolved after 10–20 min of chromatographic analysis. Prior studies carried out by our group in which chlorophylls and derivatives were separated using the same gradient of chromatographic solvents as in this work guide their preliminary identification towards structures of polarity similar to those of the chlorophylls [25]. On the other hand, most of the peaks that constitute

Table 1
Chromatographic and spectroscopic properties of the alkaline treatment products from chlorophyll *a*

Peak No.	Pigment[a]	k_c	Spectral data in the IR-RP-HPLC eluent[b]											
			Soret	I		II		III		IV		V		VI	
				M	R	M	R	M	R	M	R	M	R	M	R
1	Chlorin e_6	0.27	400					500	12.25	(530)	61.25	606	35.00	660	2.92
2	MeO-Lactone-chld *a*	0.45	420					(524)	30.75	(570)	12.57	(614)	8.79	656	1.77
3	Mg-Purpurin *a*	0.72	428	(378)	2.30					584	8.79	646	4.64	686	1.91
4	MeO-Lactone-pho *a*	3.11	402					500	10.52	532	12.74	612	18.62	668	3.36
		3.49													
5	Purpurin *a*	4.21	410	360	2.52			506	21.55	546	5.39	(638)	19.75	698	2.66
6	Mg-Phytol-chlorin e_6	6.62	416					(524)	30.80	(600)	12.35			646	2.60
		6.91													
7	MeO-chl *a*	7.50	432	394	1.76	420	1.20	534	21.73	590	11.30	616	5.38	666	1.06
		7.98													
8	MeO-Lactone-chl *a*	8.40	420					(524)	30.75	(570)	12.57	(614)	8.79	656	1.77
		9.69													
		10.08													
9	Mg-Phytol-purpurin *a*	8.70	428	(378)	2.30					584	8.79	646	4.64	686	1.91
10	chl *a*	9.18	432	(394)	1.49	(420)	1.10	(534)	25.10	(590)	8.37	616	4.48	666	1.27
10′	chl *a*′	9.57	432	(394)	1.56	(420)	1.10	(534)	20.92	(590)	8.10	616	4.48	666	1.14
11	OH-chl *a*	9.89	432	(394)	1.88	(420)	1.15	(534)	24.80	(576)	11.27	616	6.20	666	1.20
		10.60													

Retention factor $k_c = (t_R - t_M)/t_M$, where t_R = retention time of the pigment peak and t_M = retention time of an unretained component; M = position maximum (nm) and R = ratio of absorbance at Soret band to absorbance at wavelength indicated.

[a] Abbreviations as in Fig. 1.

[b] The values in parentheses indicate inflection points in the absorption spectrum.

Table 2
Chromatographic and spectroscopic properties of the alkaline treatment products from chlorophyll *b*

Peak No	Pigment[a]	k_c	Spectral data in the IR-RP-HPLC eluent[b]											
			Soret	I		II		III		IV		V		VI	
				M	R	M	R	M	R	M	R	M	R	M	R
1	Rhodin g_7	0.09	426	350	7.00	(408)	2.85	(530)	15.31	(570)	14.41	(596)	17.50	648	6.45
2	Mg-Purpurin *b*	1.09	454	350	4.02	(430)	4.42					620	7.03	656	4.64
3	MeO-Lactone-pho *b*	2.39 2.69	432	366	5.44	(414)	1.78	520	16.60	556	20.75	600	31.13	650	7.78
4	Purpurin *b*	3.29	436			(420)	2.02	(518)	23.60	(560)	21.50	(616)	18.15	674	5.02
5	Mg-Phytol-rhodin g_7	6.09 6.35	450	350	4.28					578	9.79			624	7.83
6	MeO-Lactone-chl *b*	6.54 6.77	454							586	10.50			634	4.42
7	MeO-chl *b*	7.21	462	350	3.51					(570)	15.38	600	8.79	650	2.86
8	Mg-Phytol-purpurin *b*	7.36	454	350	4.02	(430)	4.42					620	7.03	656	4.64
9	chl *b*	7.88	466	350	3.15					(570)	16.80	600	8.69	650	2.80
9′	chl *b′*	8.24	466	350	3.32					(570)	15.75	600	8.40	650	2.83
10	OH-chl *b*	9.08 9.41	462	350	3.67					(570)	14.53	600	7.97	650	2.94

[a,b] See Table 1.

fraction II (Figs. 2b and 3b) were resolved in less than 9 min; this serves to indicate that these derivatives are significantly more polar than their corresponding chlorophylls.

Fundamentally, the effect of the treatment of chlorophyll with aqueous KOH or NaOH is the same although an inferior percentage transformation is obtained after treatment with KOH for the same concentration and time of reaction. As the time of contact between the aqueous alkaline solution and the organic solution of the chlorophyll increases, a higher percentage transformation of chlorophyll is obtained. When the time of reaction is just 10 min we obtained a mixture of products in which ca. 20% is unreacted chlorophyll. If the time of contact is increased to 30 or 60 min, the alkaline treatment is observed to be more effective (10% or 2% of undegraded chlorophyll maintained, respectively).

Normal-phase and reversed-phase TLC

In order to obtain a sufficient amount of each compound to allow the performance of additional chemical analyses, for the presence of both Mg and phytol, we proceeded with the separation of all the mixtures of the products of the alkaline treatment by TLC. Fig. 4 shows schematically the representation of the thin-layer chromatograms in both normal- and reversed-phase modes obtained with the different fractions of the products of degradation of chlorophyll *a* and chlorophyll *b* separated under the chromatographic conditions specified under Experimental. Table 3 details the chromatographic characteristics of the different bands isolated, including the R_F values and the colour on the TLC plate under daylight and UV radiation of 366 nm. Table 3 also shows the correspondence between the compounds developed by TLC and their location in the HPLC traces. The NP-TLC of the two fractions I showed five well defined bands in each. All of them have colours similar to or identical with the corresponding chlorophyll pattern, blue-green for series *a* and yellow-green for series *b*. Only band E has a darker coloration in both cases. It is important to note

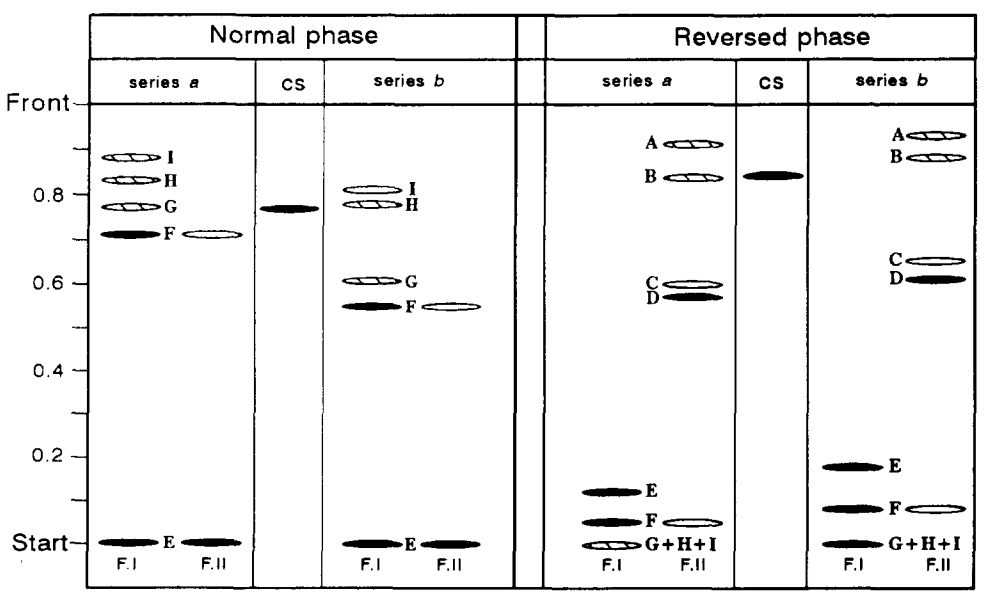

Fig. 4. TLC separation of alkaline treatment products from chlorophyll *a* and *b* and UV–Vis detection (daylight and 366 nm). NP-TLC was performed on silica gel GF_{254} plates (20 × 20 cm) eluted with light petroleum (b.p. 65–95°C)–acetone–diethylamine (10:4:1) and RP-TLC on silica gel C_{18} (10 × 10 cm) eluted with methanol–acetone–water (20:4:3). CS = chromatographic standard; chl *a* and chlorine e_6 were used as CS for NP and RP-TLC, respectively. Spots: black = major pigment; hatched = minor pigment; white = trace.

Table 3
Chromatographic characteristics of the alkaline treatment products from chlorophyll *a* and *b* isolated by TLC and location of peaks in the HPLC trace

Compound location			TLC characteristics			
TLC band		HPLC peak No.	R_f		Colour on plate	
Fraction I[a]	Fraction II		Reversed-phase	Normal-phase	Daylight	366-nm UV irradiation
Chl a derivatives						
	A	3	0.91	0.00	Blue-green	NF[b]
	B	1, 2	0.84	0.00	Grey-green	RF
	C	4, 4′	0.60	0.00	Grey	RF
	D	5	0.57	0.00	Grey-brown	NF
E		6, 6′	0.18	0.00	Brown-blue-green	IPF
F	F	9	0.05	0.71	Blue-green	NF
G, H, I[c]		7, 7′, 8, 8′, 8″, 10, 10′, 11, 11′	0.00	0.77, 0.83, 0.88	Blue-green	RF
Chl b derivatives						
	A	2	0.93	0.00	Yellow-green	NF
	B	1	0.88	0.00	Brown-green	RF
	C	3, 3′	0.65	0.00	Brown-green	RF
	D	4	0.61	0.00	Brown-green	NF
E		5, 5′	0.22	0.00	Brown-yellow-green	IPF
F	F	8	0.08	0.55	Yellow-green	NF
G, H, I[c]		6, 6′, 7, 9, 9′, 10, 10′	0.00	0.61, 0.78, 0.81	Yellow-green	RF

[a] Pigments fraction transferred to diethyl ether phase by addition of water saturated with NaCl (fraction I) or by addition of 18% (v/v) HCl (fraction II).
[b] NF = no fluorescence; RF = red fluorescence; IPF = intense pink fluorescence.
[c] Bands G, H and I were recovered together.

that the characteristic red fluorescence under 366-nm UV irradiation of chlorophyll and derivatives changes to a strong pink colour in band E while band F lacks this fluorescence. Band E is retained at the start of the chromatogram in NP-TLC and is very difficult to elute from the adsorbent. Under the conditions of RP-TLC the retention of this band was significantly lower and we recovered a larger amount of this compound, which permitted the performance of additional identification tests. The remaining pigments that constitute fraction I were accumulated without any problems from separations via NP-TLC. The fractions of pigments recovered in the organic phase by acidification (fraction II) remained at the start of the chromatogram in NP-TLC and only a light band was separated whose chromatographic features coincide with band F. RP-TLC allows the separation of, apart from this, another four bands. It is important to note that in this fraction of derivatives, as happened with fraction I, a major compound appears both in series *a* and in series *b* that does not show the characteristic red fluorescence under 366-nm UV irradiation (band D). All pigments that constitute this fraction were accumulated from separations via NP-TLC.

3.2. Identification of the products of the alkaline treatment in chlorophylls isolated by TLC

Fraction I

Band E from series *a* was shown by HPLC to consist of two isomers in the ratio 15:85 (Fig. 2a, peaks 6 and 6′) which were not separated by RP-TLC and show identical UV–Vis absorption spectra. This compound was submitted to a chemical treatment of de-esterification of the phytol alcohol group by incubation with 2.5% (w/v) methanolic KOH at 70°C. At the same time chlorophyll *a* standard was subjected to the same treatment. After de-esterification, the components recovered with light petroleum were separated by TLC over silica gel GF using benzene–ethyl acetate (19:1). In both instances a single component was separated that became visible with a blue-violet coloration under 254-nm UV irradiation and had an R_F value of 0.28. The treatment of this with dilute HCl to obtain the Mg-free derivative gave rise to a compound with an UV–Vis absorption spectrum identical (location of maxima and relationship between peaks) with that of chlorin e_6 standard. Nevertheless, the values of the retention factor (k_c) in HPLC and of R_F in RP-TLC were very different, which indicates that it is a compound with more apolar character than chlorin e_6 as a result of the presence in the molecule of the large monounsaturated chain of phytol alcohol. The set of all these chromatographic and spectroscopic characteristics together with the positive result of the test for the presence of the Mg ion and of phytol alcohol allows the preliminary identification of this pigments as Mg-phytyl-chlorin e_6 (**4.1a**). In series *b*, this band was shown to consist of two isomers with a ratio of 90:10 (Fig. 2b, peaks 5 and 5′) with identical UV–Vis absorption spectra. The form of these spectra resembles that of chlorophyll *b* but the absorption maxima in the red region is displaced 26 nm to the left and the absorption ratio of the Soret band to this band is 7.83, significantly higher than that for chlorophyll *b* (Table 2). The UV–Vis absorption spectra of the corresponding Mg-free derivative is identical with that of the rhodin g_7 standard but their chromatographic characteristics (k_c and R_F) indicate that it is a more apolar compound than rhodin g_7. The positive result of the test for the presence of phytol alcohol completed the preliminary identification of this pigment as Mg-phytyl-rhodin g_7 (**4.4b**).

The HPLC of band F from series *a* did not show isomers (Fig. 2a, peak 9). The UV–Vis absorption spectrum is very characteristic; although its form resembles that of chlorophyll *a* the absorption maximum in the red region shows a bathochromic displacement of 20 nm. This compound does not show red fluorescence under 366-nm UV irradiation and the Mg-free derivative obtained by acidification presents spectroscopic characteristics identical with those of standard purpurin *a*. However, its chromatographic properties indicate that it is a more apolar pigment. Finally, the positive result of the test for the presence of phytol allows the pre-

liminary identification of this compound as Mg-phytyl-purpuin *a* (**3.1a**). In series *b*, the UV–Vis absorption spectrum of the only peak that constitutes this band (peak 8, Fig. 2b) is characterized by the Soret band showing a hypsochromic displacement of 12 nm in relation to that of chlorophyll *b*, a shoulder appears at 430 nm and the Soret/maximum VI ratio (4.64) is higher than that for chlorophyll *b*. The spectroscopic characteristics of the Mg-free derivative are identical with those of the purpurin *b* standard but show values of k_c and R_F characteristic of a more apolar compound. Finally, the positive result of the test for presence of the phytol molecule allows the preliminary identification of this compound as Mg-phytyl-purpurin *b* (**3.1b**).

The small amounts of bands G, H and I did not allow their individual separation by NP-TLC and they were recovered from the adsorbent together. The HPLC of series *a* shows a mixture of four components spectroscopically different that were resolved into two or more isomers (Fig. 1a, peaks 7, 7′, 8, 8′, 8″, 10, 10′, 11 and 11′). The UV–Vis spectra of peak 8 and their isomers 8′ and 8″ are appreciably different from those of the rest of the components. Although its form is different to that chlorophyll *a*, in this case the point of inflection of the spectrum of the chlorophyll *a* to the left of the Soret band, located at the 394 and 420 mm, was not present. The remaining absorption maxima experience a hypsochromic displacement of ca. 10 nm and the ratio of the absorbence of the Soret band and the absorption maximum in the red region was very high (1.77). This value is very close to the 1.82 observed by Pennington et al. [17] for the allomerized derivative MeO-lactone-chl *a* (**2.1a**).

To confirm the identification of this compound, we performed a general process of allomerization of chlorophyll *a* in the presence of methanol and atmospheric oxygen, employing the technique described by Schaber et al. [19]. The course of the reaction was followed by HPLC. After 16 h (Fig. 5b), 20% of the initial chlorophyll *a* was transformed into three other compounds. According to the derivatives allomerized, mostly found by Schaber et al. [19] and

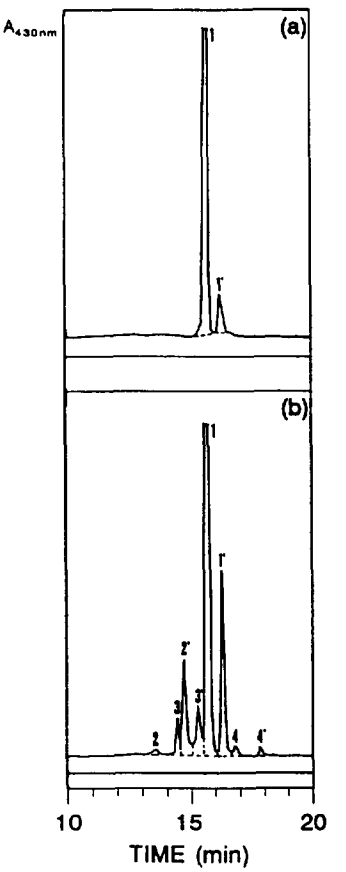

Fig. 5. HPLC separation of the allomerization products of chlorophyll *a* in presence of methanol and exposure to atmospheric oxygen. (a) Before exposure and (b) 16 h after exposure. Peaks: 1 = chl *a*; 1′ = chl *a*′; 2 = MeO-chl *a*; 2′ = MeO-chl *a*′; 3 = MeO-lactone-chl *a*; 3′ = MeO-lactone-chl *a* isomer; 4 = OH-chl *a*; 4′ = OH-chl *a* isomer.

Kuronen et al. [20], and in accordance with the absorption maxima and the relation of the peaks measured by Pennington et al. [17], compound 3 (peaks 3 and 3′) was identified as MeO-lactone-chl *a* (**2.1a**) and showed chromatographic and spectroscopic characteristics identical with those of the product of alkaline treatment of chlorophyll *a* numbered 8 in Table 1. The other two components of the set of derivatives formed in the allomerization reaction (Fig. 5b, peaks 2, 2′, 4, 4′) show UV–Vis absorption spectra identical

both in shape and in the location of the maxima with those of the chlorophyll *a* pattern. They show only slight differences in some of the ratios between the different peaks, especially between the Soret band and the maximum I that increases from 1.49 for chlorophyll *a* to 1.76 for compound 2 and 1.88 for compound 3 (10, 7 and 11 in Table 1). According to the allomerized derivatives found by Kuronen et al. [20] and their corresponding values of k_c it might be made up of a MeO-chl *a* (**1.6a**) and OH-chl *a* (**1.5a**), respectively. Also, the spectroscopic characteristics of these components are identical with those of the derivatives obtained in the alkaline treatment of chlorophyll *a* and numbered 7 and 11, but lacking other data the identification of 7 and 11 as MeO-chl *a* (**1.6a**) and OH-chl *a* (**1.5a**), respectively, can only be tentative. The identification of the peaks 10 and 10′ as chl *a* and chl *a*′, respectively, was confirmed by co-chromatography with the corresponding pure patterns.

As with the derivatives of series *a*, in series *b* bands G, H and I were recovered together and HLPC showed a mixture of four spectroscopically different components, three of them resolved into pairs of isomers (Fig. 2b, peaks 6, 6′, 7, 9, 9′, 10 and 10′). Although the shape of the UV–Vis spectrum of peak 6 was almost identical with that of chlorophyll *b*, the three absorption maxima showed a hypsochromic displacement of 12–14 nm. The ratio of absorbance between the Soret band and the absorption maximum in the red region reached a value of 4.42, significantly higher than that for chlorophyll *b* (2.80), and coincided with the value of 4.42 found by Hynninen and Ellfolk [32] for the allomerized derivative MeO-lactone-chl *b* (**2.1b**). We also performed a treatment of allomerization with chlorophyll *b* and the results of following the reaction by HPLC are shown in Fig. 6. Although we could not find recent references reporting the allomerization reaction of chlorophyll *b*, by similarity with the results found for chlorophyll *a* and according to the absorption maximum and relationship of the peaks recorded by Pennington et al. [17] and Hynninen and Ellfolk [32], we assume that peak 2 (Fig. 6b) is MeO-lactone-chl

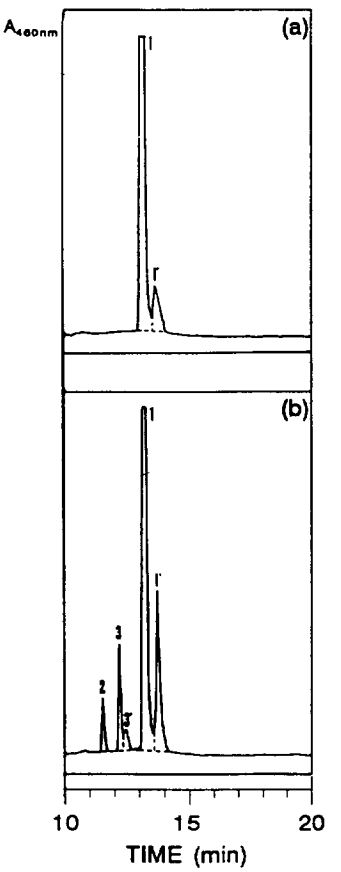

Fig. 6. HPLC separation of the allomerization products of chlorophyll *b* in presence of methanol and exposure to atmospheric oxygen. (a) Before exposure and (b) 16 h after exposure. Peaks: 1 = chl *b*; 1′ = chl *b*′; 2 = MeO-chl *b*; 3 = MeO-lactone-chl *b*; 3′ = OH-chl *b* isomer?.

b (**2.1b**); it shows chromatographic and spectrographic characteristics identical with those of the product of alkaline treatment of chlorophyll *b* numbered 6 in Table 2. The absorption spectrum of the other derivative resulting from the allomerization of chlorophyll *b* (Fig. 6b, peaks 3 and 3′) differs from that of chlorophyll *b* only in that the Soret band showed a small hypsochromic displacement of 4 nm and the ratio of absorbance of this band and that of the maximum IV is slightly larger (2.86). It could possibly be MeO-chl *b* (**1.6b**) or OH-chl *b* (**1.5b**). Lack of

further conclusive data leads to the identification of derivatives 7 and 10 obtained from the alkaline treatment of chlorophyll *b* as MeO-Chl *b* (**1.6b**) and OH-Chl *b* (**1.5b**), respectively, but only tentatively.

Fraction II

The addition of dilute HCl to this fraction of derivatives to reinforce their transfer from the aqueous to the organic phase required its identification from the corresponding magnesium-free derivatives. The UV–Vis absorption spectrum of band A from series *a* coincides exactly with that of Mg-phytyl-purpurin *a* (**3.1a**); nevertheless, under the conditions of HPLC it was resolved in less than 3 min (peak 3, Fig. 2b), which indicates that this is a compound of high polarity. The negative result of the phytol test and the positive result of the Mg test confirmed its preliminary identification as Mg-purpurin *a* (**3.3a**). In series *b* the UV–Vis absorption spectrum was identical with that of Mg-phytyl-purpurin *b* and the negative result of the test for phytol confirmed its preliminary identification as Mg-purpurin *b* (**3.2b**) (Fig. 3b, peak 2). The HPLC of band B from series *a* showed that it is formed by two peaks with a ratio of 4:1 (Fig. 2b, 1 and 2). The set of chromatographic and spectrographic properties of peak 1 is identical with that of chlorin e_6 standard (**4.4a**) and its definitive identification was confirmed by co-chromatography with the corresponding pure standard. The UV–Vis absorption spectrum of the other component coincides with that of MeO-lactone-chl *a* (**2.1a**) and its high polarity and chromatographic characteristics indicate that it may be the corresponding de-esterified derivative MeO-lactone-chld *a* (**2.3a**). In series *b*, band B is made up of a single peak (Fig. 3b, peak 1) whose chromatographic and spectrographic properties are identical with those of rhodin g_7 (**4.4b**). Its definitive identification was confirmed by co-chromatography with the corresponding pure standard. The small amounts of band C from series *a* did not allow sufficient accumulation to perform additional identification tests. The spectroscopic characteristics of the two isomers that comprise it (Fig. 2b, peaks 4 and 4′) are identical with those of the Mg-free derivative of MeO-lactone-chl *a* (**2.2a**) obtained by acid treatment of the corresponding parent. Nevertheless, the zone of the chromatogram in which this peak appears corresponds to that of more polar compounds, chlorophyll dephytolate derivatives, and therefore the compound could be an Mg- and phytol-free derivative of the MeO-lactone chl *a* called MeO-lactone-pho *a* (**2.4a**). In series *b* this band was shown by HPLC to consist of two isomers (Fig. 3b, peaks 3 and 3′) and the form of the UV–Vis absorption spectrum resembles that of pheophytin *b* (**1.2b**) [12], although the maximum shows a small hypsochromic displacement of 4–6 nm. The Mg-free derivative of MeO-lactone-chl *b* (**2.2b**) shows the same spectroscopic characteristics as this compound but a considerably smaller capacity factor; this indicates that it has a more polar character and may possibly be MeO-lactone-pho *b* (**2.4b**), that is, the corresponding phytol-free derivative. The absence of red fluorescence under 366-nm UV irradiation for band D together with the remaining chromatographic and spectroscopic properties of the single peak that comprises this band in series *a* (peak 5, Fig. 2b) and series *b* (Fig. 3b, peak 4) lead to their identification as purpurin *a* (**3.4a**) and purpurin *b* (**3.4b**) respectively. The definitive identification was confirmed by co-chromatography with the corresponding pure standard.

4. Conclusions

The alkaline treatment of chlorophylls under mild conditions and in the presence of atmospheric oxygen fosters the formation of compounds with a wide range of polarity. The study of the components of the apolar fraction indicated the presence of chlorophyllic derivatives obtained from both the solvolysis of the isocyclic ring generating Mg-phytyl-chlorin e_6 (**4.1a**) [or Mg-phytyl-rhodin g_7 (**4.1b**)] and the oxidative opening of the same forming Mg-phytyl-purpurin

(**3.1**) and MeO-lactone-chl (**2.1**). In the more polar fraction we have characterized degradation products in which a de-esterification at C-17[3] has occurred simultaneously with the oxidation of C-13[2] producing the corresponding phytol-free derivatives: Mg-chlorin e_6 (**4.2a**) [or Mg-rhodin g_7 (**4.2b**)], Mg-purpurin (**3.2**) and MeO-lactone-chld (**2.3**). The absence of chlorophyllides (**1.3**) and pheophorbides (**1.4**) in this group is important because this demonstrates that the specific formation of these dephytilate derivatives in a product is an evident sign that de-esterification has occurred by action of the chlorophyllase enzyme. This result reveals that in presence of atmospheric oxygen it is not possible to induce the specific de-esterification of the phytol by chemical treatment of the chlorophyll without promoting another series of oxidative reactions at C-13[2]. The change in colour observed in green table olives processed according to modifications introduced in the industrial processing system are probably directly related to the oxidation of chlorophylls caused by recirculation of the brines that introduces atmospheric oxygen under alkaline conditions. This situation would be in accord with the detection of oxidation products of chlorophylls in the final product of an industrial process that would have not been detected previously in fruits processed in the traditional manner in a pilot study in our laboratory [24]. The investigation carried out in this work has allowed the definitive preliminary identification of these derivatives (peaks 16 and 17 in Fig. 2 in Ref. [12]). The chromatographic and spectroscopic properties of these derivatives correspond to phytyl-rhodin g_7 and phytyl-chlorin e_6, respectively. This identification was confirmed by co-chromatography with the respective pattern.

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