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Preparation of Cu(II) complexes of oxidized chlorophylls and their determination by thin-layer and high-performance liquid chromatography

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Abstract

Different allomerization products of pheophytins *a* and *b* have been obtained by alkaline treatment in the presence of atmospheric oxygen and isolated by normal phase thin-layer chromatography (NP-TLC) and semi-preparative high-performance liquid chromatography (HPLC). $3^1,3^2$-Didehydro-15^1-hydroxy-15^1-hydroxy-rhodochlorin-15-acetic acid δ-lactone-15^2-methyl-17^3-phytyl ester (*a* and *b*) and $3^1,3^2$-Didehydro-rhodochlorin-15-glyoxylic acid-17^3-phytyl ester (*a* and *b*) have been identified as principal products. Identification was based on UV-Vis and mass spectra, retention times and R_F values. The molecular mass of allomerized pheophytins was confirmed by positive-ion fast-atom bombardment mass spectrometry and positive/negative ion electrospray mass spectrometry. The corresponding Cu(II) complexes of these compounds were prepared by chelation reaction with $CuCl_2$ and separated by reversed-phase ion-pair high-performance liquid chromatography (RP-IP-HPLC) using a gradient of water and ion suppressor–methanol–acetone. The chromatographic characteristics in NP-TLC and RP-IP-HPLC of the Cu(II) complexes are reported. The corresponding UV-Vis spectra obtained with an on-line diode-array detector have been included.

Keywords: Complexation; Food analysis; Chlorophylls; Copper complexes; Pheophytins

1. Introduction

The application of modern technology to the processing of fruits and vegetables leads to various types of modification in the constituents of these foods. In the case of the chlorophyllic pigments, the most common, and most closely studied, transformation takes place during cooking, heat processing, freeze preservation or storage of the vegetables, and is due to the conversion of chlorophylls *a* and *b* into their respective pheophytins. This conversion causes a dramatic change in colour from bright green to olive green [1]. Mild heat treatments originate epimers at C-13^2 in the isocyclic ring, giving rise to the isomers known as chlorophylls *a'* and *b'*. Prolonged heat treatments induce the decarbomethoxylation of C-13^2 forming the pyro-derivatives [2,3]. Some metals, such as Cu and Zn, have a high binding affinity for the porphyrin ring, even higher than that of the native complex for Mg, and the binding of these to pheophytin and pheophorbide causes a re-greening [4–9]. The chlorophyll molecule may also

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be modified by the division of phytol. This can occur chemically by non-specific acid hydrolysis, in which case pheophorbides and other compounds are formed [10], or enzymatically through the action of chlorophyllase giving rise to chlorophyllides [11–13]. In addition, oxidation of the isocyclic ring (allomerization) may take place by both enzymatic and chemical pathways [14–16]. When this occurs in acidic or slightly alkaline media, the isocyclic ring is opened. The compounds resulting from this transformation are the so-called chlorins e_6 and rhodins g_7, originating from the *a* and *b* series, respectively [17].

In the case of olive processing to produce green table olives, studies on the olive varieties Manzanilla and Hojiblanca have demonstrated that certain transformations of the chloroplast pigments present in the fresh fruit are both desirable and necessary. During the fermentation of olives, chlorophylls *a* and *b* are totally degraded to pheophytins and pheophorbides by two different and coexisting mechanisms: one of these is enzymatic — chlorophyllase is active in the alkaline pH of the initial treatment of the fruits with NaOH solution — and the other results from the acid pH of the fermentation medium. The kinetic parameters of these degradation reactions have been elucidated, including the involvement of chlorophyllase [18]. The pigment transformation brought about by the lactic fermentation gives the olives their highly valued and characteristic golden colour. However, Gordal variety is at present posing some problems due to the occasional appearance of green stains on the surface of the processed fruits during their preservation in brine. The results from a study of pigments carried out during the processing of this variety were unexpected — treatment of the fruits with NaOH did not induce chlorophyllase activity but originated oxidized chlorophyllic derivatives that were preliminarily identified as phytol-chlorin and phytol-rhodin [19]. Recent work has shown that in the fruits affected by green staining, stable complexes are formed of pheophytin *a* and pyropheophytin *a* with Cu [20]. In addition, other chlorophyllic derivatives of bluish colour are present. These have not yet been identified, but are assumed to be stable complexes of Cu with the oxidized chlorophylls. The re-greening of vegetables is generally associated with the formation of metallocomplexes of chlorophyll derivatives, i.e. metallochlorophylls [4–7], that result from contamination by or additions of metal salts [8,9]. In this case however, the formation of metallochlorophylls seems due not to Cu(II) ions from an external source but to those contained in the fruit itself [20].

Although allomerized chlorophylls can also undergo chelation reactions with metal ions, such complexes have not been described previously. Until now, only the preparation and determination of metal complexes of pheophytins, pheophorbides and the corresponding pyroderivatives have been described. Jones et al. [21] described a reversed-phase thin-layer method for the detection of the zinc and the copper complexes in processed foods. More recently, the determination by HPLC of metallochlorophylls with different transition elements such as Cu [22], Zn [23,24], Mn [25], Fe [26], Ni [27] and Co [28] has been reported, and the formation kinetics of complexes of pheophytins, pyropheophytins and different pheophorbides with Zn [29] have been studied in model systems. In foodstuffs, the effects of Zn(II) ion concentration, pH, and solute on the rate of formation of metal complexes during the heat processing of vegetable purées have been studied [30–32]. The presence of metallochlorophylls in foodstuffs may be a symptom of alterations or adulterations [33,34], and accurate methods for their determination are necessary.

The aim of the present work is to describe the preparation and determination of Cu(II) complexes of oxidized chlorophyll in model systems to examine their chromatographic and spectrophotometric characteristics so as to enable their identification and determination in foodstuffs.

2. Experimental

2.1. Thin-layer chromatography

TLC plates in normal phase were prepared by spreading a layer of Silicagel GF_{254} mixture (Merck, Darmstadt, Germany)–water (1:2, w/v) on glass plates of 20 × 20 cm. A commercial spreader of thin layers was used (Shadon Southern, Mod. Unoplen, USA) with the thickness adjusted to 0.7 mm. TLC in reversed-phase was performed using commercial plates of Silicagel C_{18} (Nano SI F_{254} C_{18}-100,

Sharlau, Barcelona, Spain) (10 × 10 cm, thickness 0.2 mm). Qualitative NP-TLC was performed using commercial plates of Silicagel (Polychrom SI F_{254}, Sharlau) (4 × 8 cm, thickness 0.25 mm). The following were used as mobile phases: light petroleum (65–95°C)–acetone–diethyl amine (10:4:1) and light petroleum (65–95°C)–acetone–pyridine (10:4:1) and (10:4:2.5) for the NP-TLC [35], and methanol–acetone–water (20:4:3) for RP-TLC [36,37]. Chromatography was performed in a normal saturation tank and a Dessaga UV-Vis lamp provided white light and UV radiation of 366 nm for detection. Oxidation compounds were eluted from the plate with acetone–pyridine (1:1) and transferred to diethyl ether by addition of water saturated with sodium chloride.

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

HPLC analysis 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 for analytical separations. 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 Model Micro Centaur) prior to injecting them onto the chromatographic system (20 μl). Separation was performed using an elution gradient (flow-rate 2 ml/min) with the mobile phases (A) water–ion pair reagent–methanol (1:1:8, v/v) and (B) acetone–methanol (1:1, v/v). The ion pair reagent was 0.05 *M* tetrabutylammonium acetate (Fluka, Chemie AG Switzerland) and 1 *M* ammonium acetate (Fluka) in water. The gradient scheme has been described in detail in a previous work [35]. A semi-preparative column (25 × 1 cm I.D.) was used to isolate the different chlorophyll derivatives in pure form. Separation was performed using the same elution gradient at flow-rate 7.5 ml/min. Each fraction from semi-preparative HPLC was transferred to diethyl ether by addition of water saturated with sodium chloride. The solvent was removed under reduced pressure at 30°C on a rotary evaporator and the residue was stored dry in nitrogen atmosphere at −20°C until used.

2.3. Reagents

Acetone and methanol were of HPLC-grade (Teknokroma) and the remainder were analytical 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 [38] followed by TLC separation on silica gel GF_{254} (20 × 20 cm plates, thickness 0.7 mm) (Merck) using light petroleum (65–95°C)–acetone–diethyl amine (10:4:1) as eluent [35]. Chlorophyll C-13^2 epimers were prepared by treatment with chloroform according to Watanabe et al. [39]. Allomerized chlorophylls were obtained following a procedure similar to that reported by Schaber et al. [15]. Solid chlorophyll *a* (**1.1***a*) or chlorophyll *b* (**1.1***b*) (1–2 μmol) was dissolved in 5 ml of methanol and exposed to atmospheric oxygen for 16 h in darkness. All Mg-free derivatives were prepared from the corresponding chlorophyll parent dissolved in diethyl ether by acidification with two to three drops of HCl (13%, v/v) [40]. All standards were purified by NP and RP-TLC [35,36].

2.5. Oxidation of pheophytins by alkaline treatment in aqueous media

Oxidized chlorophylls were obtained by alkaline treatment in aqueous media. Solid and chromatographically pure pheophytin (*a* or *b*) (**1.2**) (0.1–0.2 μmol) was dissolved in 75 ml acetone and mixed with 50 ml of 0.5% sodium hydroxide. This mixture was placed in a decanting funnel and exposed to atmospheric oxygen at room temperature for 5–10 min. Then 50 ml of diethyl ether and 200 ml of distilled water saturated with sodium chloride were added to effect the separation into phases. The ether phase was washed three times with water and a further 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 used.

2.6. Preparation of copper(II) complexes of oxidized pheophytins

The copper complexes were prepared following a procedure similar to that reported by Jones et al. [41]. An excess of copper(II) ions as chloride was used in all reactions. The reaction system contained the pigment under study (≈10 μM) and copper(II) ions (1 M) in 4:1 (v/v) acetone–water. Some crystals of ascorbic acid (0.5%, w/v) were added to the reaction mixture to minimize oxidative changes. Reactions were carried out in 1.5-ml Eppendorf cones for 24–48 h at 25°C in darkness. Once the reaction was finished the metal chelates were transferred to diethyl ether and the organic layer washed with water and 2% (w/v) sodium sulphate. The diethyl ether was evaporated under nitrogen and the pigments dissolved in acetone.

2.7. Pigment identification

Identification of the allomerization products was based on colour and R_F values in TLC, retention times in HPLC, and UV-Vis and mass spectra. The on-line UV-Vis spectra were recorded from 350 to 800 nm with the photodiode-array detector. The molecular mass of the oxidized chlorophyllic species was obtained from the mass spectrum. Two types of mass spectrometer were used: a VG AUTOSPEC fitted with a fast-atom bombardment (FAB) ionization source and trisector geometry, and a VG PLATFORM fitted with atmospheric pressure electrospray ionization source and quadrupole analyzer. An aliquot of the sample dissolved in acetone (≈10 μg/ml) was added to a FAB matrix of thioglycerol to obtain the positive-ion FAB mass spectrum and another aliquot of sample was added to a matrix of methanol–water–trifluoroacetic acid to obtain the positive-ion electrospray mass spectrum. The trifluoroacetic acid was substituted by ammonia to obtain the negative-ion electrospray mass spectrum.

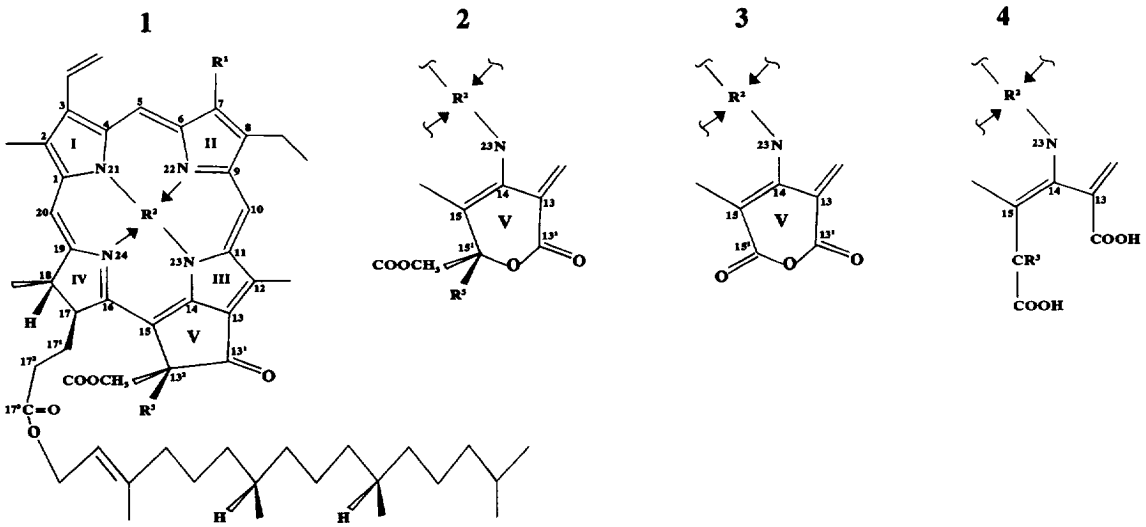
3. Results and discussion

3.1. Separation and isolation of allomerized pheophytins

Fig. 1 shows the structures, numbering system, trivial and semi-systematic name for pheophytins (phys) and their allomerization products. The present work employed a process of alkaline oxidation similar to that described in a previous work [37], but in this case starting from pure solutions of phys *a* and *b* (**1.2**).

The chromatographic characteristics of the different compounds are detailed in Table 1, including R_F values in three different solvent mixtures, and their colours on the TLC plate in daylight and under $UV_{366\ nm}$ radiation. During the development of the chromatogram performed with the petroleum ether–acetone–diethyl amine (10:4:1) mixture, phy *a* and *a'* (**1.2***a*) advanced with R_F values of 0.67 and 0.63 respectively, and phy *b* and *b'*(**1.2***b*) with R_F values of 0.64 and 0.59 respectively, while the major degradation products were retained at the base of the chromatogram (R_F=0.00). Attempts to separate these latter compounds by reversed-phase TLC were unsuccessful — better results were obtained by introducing pyridine into the solvent mixture used in the development of the normal-phase TLC, thus considerably increasing the polarity of the eluent. The chromatograms developed with petroleum ether (65–95°C)–acetone–pyridine (10:4:1) and (10:4:2.5) revealed, in addition to the pheophytins, two other well-separated bands. Elution of these compounds from the adsorbent was achieved using an acetone–pyridine (1:1) mixture, which allowed them to be accumulated from successive chromatographic runs.

Fig. 2 shows the analytical HPLC chromatograms corresponding to the separation of the oxidation products of phys *a* and *b* (**1.2**). These were obtained using the diode array detection (DAD) technique at 400 nm and 430 nm, respectively. Different times of contact between the phys and the alkalizing agents were tested and the best reaction time was found to be 10 min for phy *a* and 5 min for phy *b*. As can be seen in the chromatograms, these phy concentration/reaction time ratios were insufficient to produce complete transformation of the pheophytins; nevertheless, they achieved the desired aim of producing



No.	Semisystematic name	R_1	R_2	R_3	Abbreviation	Trivial name
1.1*a*	Chlorophyll *a*	CH_3	Mg	H	Chl *a*	Chlorophyll *a*
1.2*a*	Pheophytin *a*	CH_3	2H	H	Phy *a*	Pheophytin *a*
1.3*a*	Cu-Pheophytin *a*	CH_3	Cu	H	Cu-Phy *a*	Cu-Pheophytin *a*
2.1*a*	3^1, 3^2-Didehydro-15^1-hydroxy-15^1-hidroxy-rhodochlorin-15-acetic acid δ-lactone-15^2methyl-17^3-phytyl ester	CH_3	2H	OH	OH-Lact-phy *a*	15^1-OH-Lactone-phy *a*
2.2*a*	The Copper complex of 3^1, 3^2-didehydro-15^1-hydroxy-15^1-hidroxy-rhodochlorin-15-acetic acid δ-lactone -15^2methyl-17^3-phytyl ester	CH_3	Cu	OH	Cu-OH-Lact-phy *a*	Cu-15^1-OH-Lactone-phy *a*
2.3*a*	3^1, 3^2-Didehydro-15^1-hydroxy-15^1-methoxy-rhodochlorin-15-acetic acid δ-lactone-15^2methyl-17^3-phytyl ester	CH_3	2H	OCH_3	MeO-Lact-phy *a*	15^1-MeO-Lactone-phy *a*
3.1*a*	Purpurin 18 *a* phytyl ester	CH_3	2H		Phyt-purp 18 *a*	Purpurin 18 *a* phytyl ester
4.1*a*	3^1, 3^2-Didehydro-rhodochlorin-15 glyoxylic acid-17^3-phytyl ester	CH_3	2H	O	Glyox-phy *a*	Purpurin 7 *a* phytyl ester
4.2*a*	The Copper complex of 3^1, 3^2-didehydro-rhodochlorin-15 glyoxylic acid-17^3-phytyl ester	CH_3	Cu	O	Cu-Glyox-phy *a*	Cu-Purpurin 7 *a* phytyl ester
4.3*a*	Chlorine e_6 phytyl ester	CH_3	2H	2H	Phyt-chlr	Chlorine e_6 phytyl ester
4.3*b*	Rhodine g_7 phytyl ester	CH_3	2H	2H	Phyt-Rhd	Rhodine g_7 phytyl ester

Fig. 1. Structures, trivial names and numbering system for chlorophylls and derivatives. The replacement of the CH_3 group in R_1 by the CHO group forms chlorophyll *b* derivatives.

only the oxidation of C-13^2 in the phy molecule. Longer treatment times originate a greater number of undesirable oxidation products.

As the amount of oxidized phys obtained in each treatment is relatively small, two consecutive chromatographic runs are required to obtain enough of the various allomerized products for their identification. Successive NP-TLC runs with the mixture

Table 1
Chromatographic characteristics of the alkaline treatment products from pheophytin *a* and *b* isolated by TLC

Compound	Chromatographic characteristics in TLC				
	R_F value			Colour on plate	
	Eluent[a]			Daylight	UV_{366} radiation
	A	B	C		
Phy a derivatives					
Pheophytin *a*	0.67	0.69	0.91	Grey	RF[b]
Pheophytin *a'*	0.63	0.69	0.91	Grey	RF
OH-lactone-phy *a*[c]	0.00	0.56	0.63	Grey	RF
Glyox-phy *a*	0.00	0.32	0.56	Grey	RF
Phy b derivatives					
Pheophytin *b*	0.64	0.67	0.87	Brown	RF
Pheophytin *b'*	0.59	0.67	0.87	Brown	RF
OH-lactone-phy *b*	0.00	0.54	0.59	Brown	RF
Glyox-phy *b*	0.00	0.22	0.41	Brown	RF

[a]Eluents: A, light petroleum (65–95°C)–acetone–diethyl amine (10:4:1); B and C, light petroleum (65–95°C)–acetone–pyridine; B=(10:4:1) and C=(10:4:2.5).
[b]RF, red fluorescence.
[c]Abbreviations are as in Fig. 1.

petroleum ether–acetone–diethyl amine (10:4:1) accumulate all the oxidized derivatives retained at the base of the chromatogram. Each of these is then separated as the pure form by semi-preparative HPLC. Table 2 shows the chromatographic and spectroscopic characteristics of the different allomerized pheophytins that were isolated. Both the theoretical molecular mass and those given by the various mass spectrometry techniques used with the allomerized derivatives of pheophytin *a* are shown in Table 3. In a previous work these derivatives were obtained by acid treatment of the corresponding allomerized chlorophylls, and were preliminarily identified as 15^1-MeO-lactone phy *a* (**2.3***a*) and chlorine e_6 phytyl ester (**4.3***a*) [37]. However, the mass spectral data do not concur exactly with this identification. The molecular mass of compound 1 fitted 15^1-OH-lactone phy *a* (**2.2***a*)(OH-lact-phy *a*) exactly; that of compound 2 fitted purpurin 7 *a* phytyl ester (**4.2***a*) (Glyox-phy *a*). These results agree with those of Kuronen et al. [16]. Those authors found some 52% of 15^1-MeO-lactone (**2.3***a*) in the allomerization reaction of chlorophyll *a* in methanol. In our case the major derivative also had a lactone structure, but with the C-15^1 substitute being OH instead of MeO as a consequence of the aqueous reaction medium. For the same reason the second product of our oxidation reaction would have the same structure as purpurin 7-dimethyl phytyl ester reported by the latter authors, but lacking the two methyl esters on C-13^1 and C-15^2, respectively. These same results have been assumed for the identification of the allomerized derivatives of pheophytin *b*. A third allomerization product was also obtained (10%) tentatively identified as purpurin 18 phytyl ester (**3.1**).

3.2. Preparation of copper(II) complexes

The metallocomplexes of the different oxidized chlorophylls were obtained by mixing a solution of the corresponding pigment with an excess of Cu(II) ions. Once a sufficient amount of each compound of interest had been accumulated, the pigments were dissolved in acetone for the chelation reaction. Significant amounts of Cu-15^1-OH-lactone phy *a* (**2.2***a*) (Cu-OH-lact-phy *a*) and Cu-purpurin 7 *a* phytyl ester (**4.2***a*) (Cu-Glyox-phy *a*) were obtained after a reaction time of 24 h. However, the reaction times had to be prolonged to 48 h in the case of the pheophytin *b* derivatives. This lower reactivity of pheophytin *b* has also been observed during the

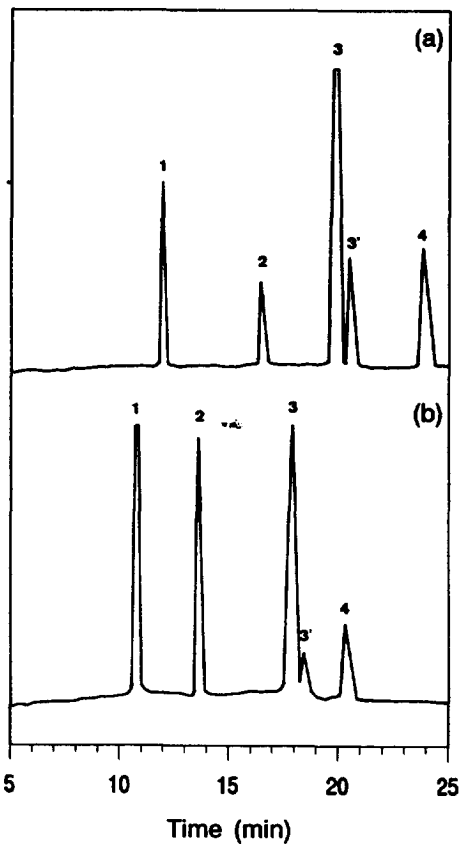


Fig. 2. HPLC separation of the alkaline treatment products from pheophytins using DAD at 400 nm for *a* series (a) and 430 nm for *b* series (b). Peaks: 1, Glyox-phy (**4.1**); 2, OH-lact-phy (**2.1**); 3, pheophytin (**1.2**); 3', pheophytin C-13^2 epimer; 4, phyt-purp 18 (**3.1**).

preparation of other metallochlorophyll complexes [22,23,28]; it is a consequence of the lower nucleophile capacity of pheophytin *b* to the chelation reaction with metal ions. Table 4 shows the TLC characteristics of the metallocomplexes of Cu-oxidized pheophytins prepared, R_F values, and colour in daylight and under $UV_{366\ nm}$ radiation. As can be seen, all of the metallocomplexes prepared lost the strawberry-coloured fluorescence under $UV_{366\ nm}$ radiation that is characteristic of chlorophyllic compounds. This effect is common with the complexes of Cu and chlorophyllic compounds [42], and is a feature that facilitates their identification. Their colour under white light is very similar to that of the corresponding chlorophyllic compounds: blue–green for the *a* series and yellow–green for the *b* series, with the exception of Cu-Glyox-phy *a* (**4.3***a*) which acquires a bluish colour completely different from that of chl *a*. In the case of Cu-Glyox-phy *b* (**4.3***b*), this effect was not observed.

Fig. 3 shows the HPLC chromatograms corresponding to the separation of the Cu-complexes of the oxidized pheophytins using DAD at 410 nm for *a* series (Fig. 3a) and 460 nm for *b* series (Fig. 3b). Table 5 shows the HPLC retention factors, electronic absorption maxima measured by DAD, and the ratio between the absorbance of the Soret band and that of each absorption maximum that completes the UV-Vis spectral characteristics of the Cu complexes. In general, insertion of the copper ion in the phy molecule considerably modifies the spectroscopic characteristics. The Soret band and the VI maxima are displaced, the former in a bathochromic direction and the latter in a hypsochromic direction. At the same time the ratio between these two parameters diminishes. The pattern of the absorption spectrum of Cu-OH-lact-phy *a* (**2.2***a*) is essentially the same as that of Cu-phy *a* (**1.3***a*) although the maxima are localized 10 nm to the left and the ratio between the Soret band and the VI maximum is somewhat larger (1.75). In the case of Cu-OH-lact-phy *b* (**2.3***b*) the displacement is also 10 nm and the ratio 3.52. However, the absorption spectrum characteristic of Cu-Glyox phy *a* (**4.2***a*) is completely different and no description of it has so far been found in the literature (Fig. 4a). The maximum shown by Cu-phy *a* (**1.3***a*) at 400 nm is converted into a point of inflection and there is a spectacular hypsochromic displacement of the VI maximum of almost 22 nm. The Soret/VI max ratio is also considerably lower. This UV-Vis spectrum is similar to that of a pigment present in green table olives with green staining; however, it has not been possible to identify this compound, since, although its spectroscopic characteristics are identical to those of Cu-Glyox phy *a* (**4.2***a*), it has higher R_F values and greater retention times (peak no. 12 in Ref. [20]), indicating a lower polarity. It may be a derivative of Cu-Glyox phy *a* (**4.2***a*) that has undergone esterification on C15^2 and C13^1. Further studies are needed for its definitive identification. A minor compound with spectroscopic characteristics almost identical to those of Cu-Glyox

Table 2
Chromatographic and spectroscopic properties of the allomerized pheophytins.

Pigment	k_c	Spectral data in the HPLC eluent[a]												
		Soret	I		II		III		IV		V		VI	
			M	R	M	R	M	R	M	R	M	R	M	R
Glyox-phy *b*	6.45	426	350	7.00	(408)	2.85	(530)	15.31	(570)	14.41	(596)	17.50	651	6.45
Glyox-phy *a*	7.67	399					500	12.25	(532)	61.25	607	35.00	662	2.92
OH-lact-phy *b*	8.35	427			(416)	1.89	522	15.38	(560)	17.57	(600)	20.50	651	4.47
OH-lact-phy *a*	10.89	399					499	10.97	531	18.00	614	22.91	670	2.57
Pheophytin *b*	11.23	435			412	2.27	524	13.62	(558)	20.42	598	16.33	654	3.83
Pheophytin *b'*	11.60	435			412	2.27	524	13.62	(558)	21.80	595	17.63	654	4.87
Pheophytin *a*	13.34	409	(400)	1.07	(376)	1.31	505	8.78	537	9.62	609	10.63	666	1.85
Pheophytin *a'*	13.84	409	(400)	1.07	(376)	1.31	505	9.18	537	10.10	609	11.22	666	2.35
Phyt-purp *b* 18	12.88	435			(420)	2.02	522	23.60	657	21.50	621	18.15	674	5.02
Phyt-purp *a* 18	16.28	407	(360)	2.52	(420)	1.10	507	21.55	545	5.39	(648)	19.75	699	2.66

Retention factor $k_c=(t_R-t_M)/t_M$ where t_R is the retention time of the pigment peak and t_M is the retention time of an unretained component. [a]M, position maximum (nm) and R, quotient of absorbance at Soret band divided by absorbance at wavelength indicated. The values in parentheses indicate inflection points in the absorption spectrum.

Table 3
Molecular mass by mass spectrometry techniques for allomerized pheophytin *a*

Compound	Molecular mass			
	Calculated	Observed		
		FAB	+Electrospray	−Electrospray
Pheophytin *a*	870.2	870	870.2	–
OH-lact-phy *a*	902.2	902	902.1	–
Glyox-phy *a*	888.2	888	888.2	888.1

Table 4
Chromatographic characteristics of the Cu complexes of oxidized pheophytin *a* and *b* isolated by TLC

Compound	Chromatographic characteristics in TLC				
	R_F value			Colour on plate	
	Eluent[a]			Daylight	UV_{366} radiation
	A	B	C		
Phy a derivatives					
Cu-Pheophytin *a*	0.61	0.69	0.91	Blue–green	NF[b]
Cu-Pheophytin *a'*	0.56	0.69	0.91	Blue–green	NF
Cu-OH-lactone-phy *a*	0.05	0.56	0.60	Blue–green	NF
Cu-Glyox-phy *a*	0.00	0.22	0.48	Blue	NF
Phy b derivatives					
Cu-Pheophytin *b*	0.55	0.67	0.87	Yellow–green	NF
Cu-Pheophytin *b'*	0.52	0.67	0.87	Yellow–green	NF
Cu-OH-lactone-phy *b*	0.07	0.47	0.54	Yellow–green	NF
Cu-Glyox-phy *b*	0.00	0.19	0.46	Yellow–green	NF

[a]As in Table 1.
[b]NF, no fluorescence.

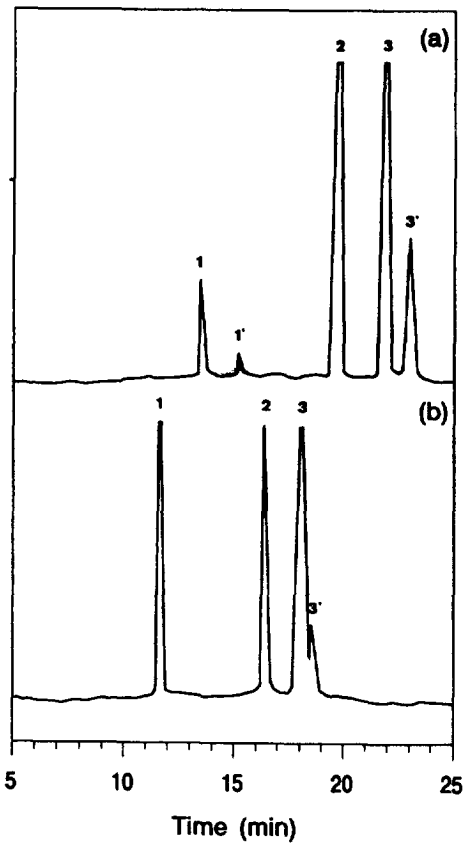


Fig. 3. HPLC separation of the Cu complexes of the oxidized chlorophylls using DAD at 410 nm for *a* series (a) and 460 nm for *b* series (b). Peaks: 1, Cu-Glyox-phy (**4.2**); 1′, Cu-Glyox-phy derivative?; 2, Cu-OH-lact-phy (**2.2**); 3, Cu-phy (**1.3**); 3′, Cu-phy C-13[2] epimer.

phy *a* (**4.2***a*) has been detected (Fig. 2a, peak 1′) but its maxima are localized some 6 nm to the right. Although this compound has not been identified, it could be an oxidized derivative formed during the chelation reaction with a very similar molecular structure.

The form of the Cu-Glyox phy *b* (**4.2***b*) spectrum (Fig. 4b) is more similar to that of Cu-phy *b* (**1.3***b*), although the VI maximum also undergoes a significant displacement of 16 nm and the Soret/VI max ratio increases to 5.93. In the *b* series no degradation products formed during the chelation reaction were detected.

4. Conclusion

A combination of the chromatographic techniques of NP-TLC and semi-preparative HPLC has enabled the major allomerization products of pheophytin *a* and *b* to be separated prior to preparation of the corresponding metallocomplexes with Cu. The chromatographic and spectroscopic characteristics of Cu-15[1]-OH-lactone pheophytin (*a* and *b*) (**4.3**) and Cu-purpurin 7 (*a* and *b*) phytyl ester (**4.2**) have been determined, and an HPLC method for their separation and determination described. This is a first step for their possible detection in processed fruits and vegetables.

Table 5
Chromatographic and spectroscopic properties of the Cu complexes of allomerized pheophytins

Pigment	k_c	Spectral data in the HPLC eluent[a]											
		Soret	I		II		III		IV		V		VI	
			M	R	M	R	M	R	M	R	M	R	M	R
Cu-Glyox-phy *b*	6.95	429							566	9.58			615	5.93
Cu-Glyox-phy *a*	8.64	406	(398)	1.18			502	22.73			(598)	12.50	630	2.55
Cu-Glyox-phy *a* der.[b]	9.86	412	(398)	1.25			502	22.73			(598)	10.42	635	2.34
Cu-OH-lact-phy *b*	10.10	432							574	8.68			622	3.52
Cu-OH-lact-phy *a*	12.96	411	392	1.20			500	16.50	(540)	17.71	599	7.75	645	1.75
Cu-Pheophytin *b*	11.17	443					540	15.53			590	6.47	633	2.38
Cu-Pheophytin *b′*	11.46	443					540	17.36			590	7.36	633	2.89
Cu-Pheophytin *a*	14.46	424	(384)	1.65	400	1.02	506	20.11	548	12.07	606	3.29	654	1.00
Cu-Pheophytin *a′*	15.28	424	(384)	1.17	400	1.84	506	14.43	548	8.42	606	4.21	654	1.00

[a]As in Table 2.
[b]der.=derivative.

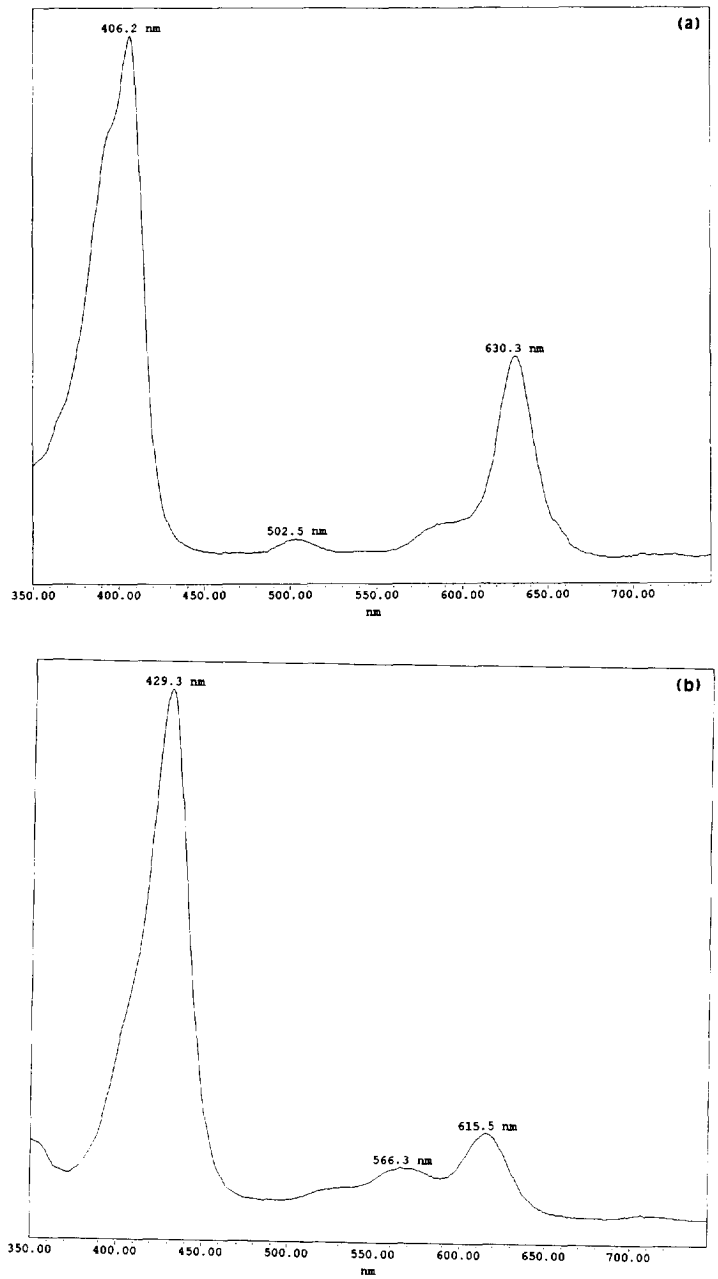


Fig. 4. UV-Vis spectra of the (a) Cu-Glyox-phy *a* (**4.2***a*) and (b) Cu-Glyox-phy *b* (**4.2***b*) recorded with the diode-array detector.

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