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Short Communication

Characterization and separation of oxidized derivatives of pheophorbide *a* and *b* by thin-layer and high-performance liquid chromatography

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ABSTRACT

The separation and purification of the main oxidized derivatives of pheophorbide *a* and *b* by reversed-phase TLC afforded their characterization by physical and spectroscopic properties. Their subsequent separation and identification by reversed-phase HPLC was achieved using a system of gradient elution. Under these conditions the separation of rhodin g_7, chlorin e_6, pheophorbide *b*, pheophorbide *a*, purpurin *b*, pyropheophorbide *b*, pyropheophorbide *a* and purpurin *a* was achieved in 15 min.

INTRODUCTION

The process of lactic fermentation during the preparation of table olives involves the degradation of the chlorophylls to their corresponding pheophytins and pheophorbides in the final product [1]. However, it has been observed that during the subsequent conservation of these fruits in brine, another series of reactions takes place that may involve the oxidation of chlorophyllic compounds.

In numerous studies [2,3] HPLC has been used to follow the colour transformation from bright green to olive green in processed vegatables. This change is due mainly to the degradation of chlorophylls to pheophytins and pheophorbides. Nevertheless, there may be a reaction involving C-10 decarbomethoxylation or the opening of the isocyclic ring, giving rise to compounds with a higher degree of oxidation, whose control, by either TLC or HPLC, has not been sufficiently studied.

Hynninen [4] described the preparation and purification of some oxidized derivatives of chlorophylls (chlorines, rhodines and purpurins) by different alkaline treatments. These compounds were separated by TLC on cellulose plates with *n*-heptane–pyridine (7:3) as eluent [5].

In this work we investigated different conditions for the separation of pheophorbide derivatives by in normal- and reversed-phase TLC and ion-pair HPLC.

EXPERIMENTAL

Sample preparation

The study was carried out on olives, *Olea europaea* (L.), of Hojiblanca variety (*arolensis*). The

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fruits were processed using the traditional method of Spanish-style fermentation in brine [1]. The fat-free extract pigment was prepared by extraction with N,N-dimethylformamide. Details of the extraction process have been published previously [3,6].

Standards

Chlorophyll *a* and *b* were supplied by Sigma (St. Louis, MO, USA) (chlorophyll *a*, No. C-6144, and chlorophyll *b*, No. C-3878). Chlorophyllides *a* and *b* were produced by enzymatic deesterification of chlorophylls: a protein precipitate of *Ailanthus altissima* (Mill.) leaves was incubated with acetone–Tris–HCl buffer (pH 8) (1:1) [7]. Pheophorbide *a* and *b* were obtained from their respective chlorophyllides by acidification with 0.01 *M* HCl [8]. Pyropheophorbide *a* and *b* were prepared by heating the corresponding pheophorbides in pyridine under reflux at 100°C for 24 h [2].

The remaining standards were prepared according to the method described by Hynninen [4], which is summarized as follows. Methyl esters of chlorin e_6 and rhodin g_7 were formed by saponification of pheophorbide *a* and pheophorbide *b*, respectively, with 0.5% KOH in methanol. Free chlorine e_6 and rhodin g_7 were obtained from their respective methyl esters by saponification with KOH (30%) at ambient temperature and under a nitrogen atmosphere. Purpurin *a* was obtained by alkaline oxidation of pheophorbide *a* with 30% KOH in methanol in the presence of atmospheric oxygen, and purpurin *b* from pheophorbide *b* using the same procedure.

Separation of pigments by TLC

Reversed-phase TLC was carried out on commercial aluminium plates of 20 × 20 cm covered with a 0.1-mm layer of cellulose (CE F_{254}; Sharlau, Barcelona, Spain), Kieselguhr plates 20 × 20 cm with a 0.25-mm layer of fluorescent indicator (Macherey–Nagel, Düren, Germany) of impregnated with 14% (v/v) of maize oil in light petroleum (b.p. 65–110°C) by vertical immersion up to 18 cm (spotting was done on the non-impregnated area) and plates of 10 × 10 cm covered with a 0.2-mm layer of silica gel C_{18} (Nano SI F_{254} C_{18}-100; Sharlau). The mobile phases used were *n*-heptane–pyridine (7:3), methanol–acetone–water (20:4:6) and methanol–acetone–water (20:4:3), respectively. The chromatography was performed in a normal saturated tank with detection using a Desaga UV-Vis lamp providing with white light and UV radiation of 254 and 366 nm.

Separation of pigments by HPLC

HPLC analysis was performed using a Waters Model 600 E chromatograph fitted with a Waters Model 994 photodiode-array detector and a Waters Model 5200 register–integrator.

The pigment extract (20 μl) was filtered through a 0.45-μm nylon membrane and injected into the liquid chromatograph. Separation was performed on a 25 x 0.4 cm I.D. C_{18} Spherisorb ODS-2 analytical column of 5-μm particle size (Supelco, Bellefonte, PA, USA). The column was protected by a cartridge (5 × 0.4 cm I.D.) packed with the same material. Pigments were separated using a linear gradient system at a flow-rate of 2 ml/min. The eluents used were (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 and 1 *M* ammonium acetate in water. Elution was from 100% A linearity to 100% B in 15 min, returning to the initial conditions in 5 min. Multiple detection was performed at 400 and 430 nm. Pigments were identified by comparing the retention times with those of authentic standards and from their spectral characteristics.

Reagents

All reagents were of analytical-reagent grade, except acetone and methanol, which were of HPLC grade. Water was deionized and filtered through a 0.45-μm nylon membrane (Supelco).

RESULTS AND DISCUSSION

Separation of pigments by TLC

Different systems of sorbents and mobile phases were examined. The separation on cellulose plates using *n*-heptane–pyridine (7:3) as mobile phase [5] did not afford the expected results. The pigments advanced to the front of the chromatogram, except chlorin and rhodin, which remained at the base. Development in the reversed-phase mode with Kieselguhr plates impregnated with maize oil, and methanol–acetone–water (20:4:6) as mobile phase [9],

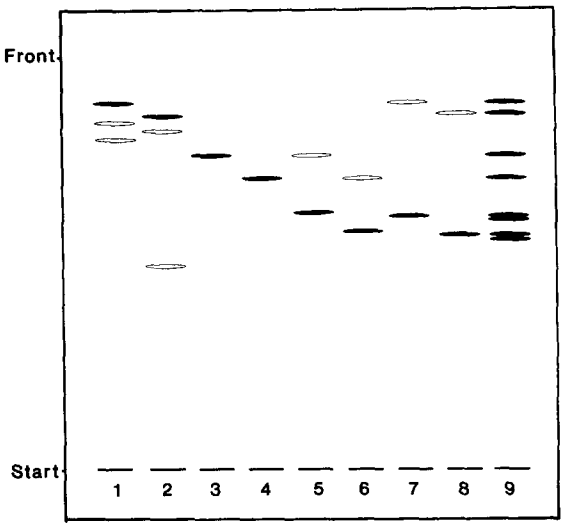

Fig. 1. Thin-layer chromatogram on silica gel C_{18} eluted with methanol–acetone–water (20:4:3): 1 = rhodin g_7; 2 = chlorin e_6; 3 = pheophorbide *b*; 4 = pheophorbide *a*; 5 = pyropheophorbide *b*; 6 = pyropheophorbide *a*; 7 = purpurin *b*; 8 = purpurin *a*; 9 = mixture of all purified standard pigments. Black spots, main pigment; white spots, traces of precursors.

gave similar results, with the pigments moving to the front of the elution. Using this mobile phase, decreasing the amount of water to give a ratio of 20:4:3, and cellulose plates as support, the pigments were well separated but poor resolution was obtained because the tails of the bands quite overlapped. To reduce this effect, the cellulose support was changed for commercial plates of silica gel C_{18} (HPTLC). This modification gave optimum results. A chromatogram was obtained in which the bands were sharp and well defined, permitting complete separation and purificaton of all the pigments under study. All the compounds were separated from the standard mixture of pheophorbide *a* and *b*, chlorin,

TABLE I

RESULTS OF TLC ON SILICA GEL C_{18} OF PURE STANDARD PIGMENTS

Solvent system: methanol–acetone–water (20:4:3).

Pigment	R_F value	Colour on plate	
		White light	UV radiation
Rhodin g_7	0.88	Brown-green	Red fluorescence
Chlorin e_6	0.84	Green-grey	Red fluorescence
Pheophorbide *b*	0.75	Brown-green	Red fluorescence
Pheophorbide *a*	0.69	Grey	Red fluorescence
Pyropheophorbide *b*	0.62	Brown-green	Red fluorescence
Purpurin *b*	0.61	Lemon-green	No fluorescence
Pyropheophorbide *a*	0.58	Grey	Red fluorescence
Purpurin *a*	0.56	Brown	No fluorescence

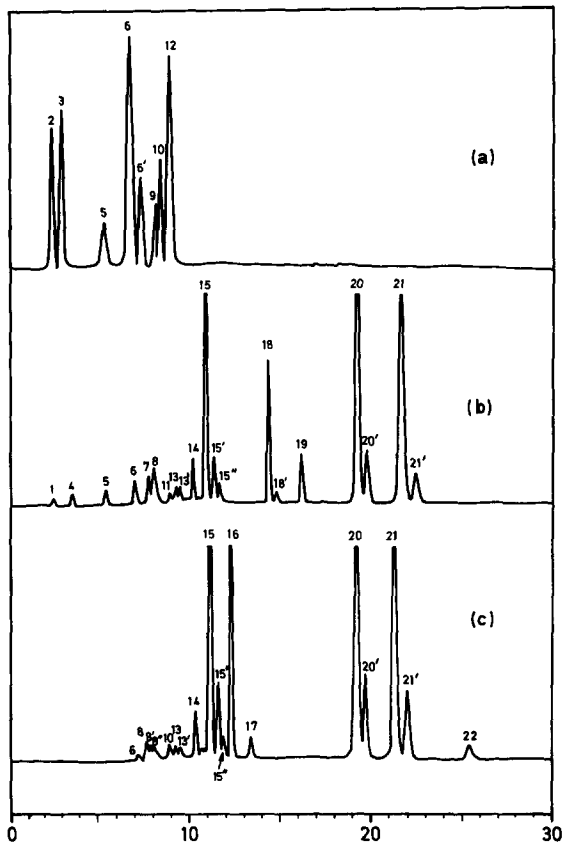

Fig. 2. HPLC: (a) standards obtained from the oxidation of pheophorbides; (b) pigments present in fermenting olives; (c) pigments present in fruits conserved in brine. Peaks: 1 = chlorophyllide *b*; 2 = rhodin g_7; 3 = chlorin e_6; 4 = chlorophyllide *a*; 5 = pheophorbide *b*; 6 = pheophorbide *a*; 6′ = pheophorbide *a*′ and purpurin *b*′ mixture; 7 = neoxanthin; 8 = neocrome; 8′ and 8″ = neocrome isomers; 9 = pyropheophorbide *b*; 10 = pyropheophorbide *a*; 11 = luteoxanthin; 12 = purpurin *a*; 13 = auroxanthin; 13′ = auroxanthin isomer; 14 = mutatoxanthin; 15 = lutein; 15′ and 15″ = lutein isomers; 16 and 17 = unknown pigments; 18 = chlorophyll *b*; 18′ = chlorophyll *b*′; 19 = chlorophyll *a*; 20 = pheophytin *b*; 20′ = pheophytin *b*′; 21 = pheophytin *a*; 21′ = pheophytin *a*′; 22 = pyropheophytin *a*. Gradient system described in ref. 3.

rhodin and purpurin *a* and *b*, including pheophorbides from their corresponding pyro derivatives. Only the mixture of purpurins and pyropheophorbides offered any resistance to separation, but as this situation does not occur very frequently, it is not a real problem. Therefore, there conditions were chosen to achieve individually purified pigments and to obtain standards that were used later in HPLC.

Fig. 1 shows the chromatogram of the standards obtained from the oxidation of pheophorbides. Small amounts of pheophorbides which had not undergone decarbomethoxylation were separated from the pyro derivatives. At the same time, purpurin *a* and purpurin *b* were accompanied by remains of chlorin and rhodin, respectively, and these in turn by traces of these esterified compounds. Once the pure standards had been obtained, a mixture of them was developed and successfully separated. Table I shows the chromatographic characteristics found for each pure component studied.

Separation of pigments by HPLC

Fig. 2a, b and c show the chromatograms for the separation, applying the gradient system described by Mínguez-Mosquera *et al.* [3], of the mixture of all purified standard pigments, of the pigments present in fermenting olives and of pigments present in fruits conserved in brine, respectively. In the last instance it can be seen that peaks 16 and 17 corresponding to the new pigments, possibly formed during the conservation in brine of table olives, do not correspond to any of the studied chlorophyll derivatives. At first they were identified incorrectly as isomers of pheophorbide *b* and *a*, respectively [3]. However, it was later shown that the localization of the maxima and the peak ratios in the absorption spectra of these compounds do not coincide exactly with those of pheophorbide *a* and *b*, but are identical with those of chlorin and rhodin. This result and the fact that the unknown pigments were eluted later than chlorin and rhodin, which means that they must be compounds of lower polarity, suggested that they might be esters of rhodin and chlorin, respectively. They could not be identified and at present it is not known when they were formed.

A detailed study of the standard separation by HPLC showed that all the pigments were eluted during the first 10 min of the 30-min gradient and that peak 6′ corresponded to a mixture of purpurin *a* and pheophorbide *a*. To improve the performance and reduce the separation time, different new gradient systems were tested. The optimum results were achieved with that given under Experimental, with which the total elution time was only 15 min. Using these conditions, the mixture of all the standards was separated by HPLC as shown in Fig. 3. Table II shows the spectroscopic and chromato-

TABLE II

SEPARATION OF STANDARD PIGMENTS BY HPLC

Capacity 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.

Peak No.	k'_c	Pigment	Spectral data in the eluent									
			Maxima (nm)[b]									Peak ratio[a]
			I	II	III	IV	V	VI	VII	VIII	IX	
1	0.76	Rhodin g_7	–	(408)	426	–	–	(530)	(570)	596	648	6.54
2	1.61	Chlorin e_6	–	–	400	–	500	532	–	602	660	3.86
3	4.13	Pheophorbide *b*	374	418	438	–	–	532	–	600	654	4.25
4	5.15	Pheophorbide *a*	(380)	(400)	410	–	504	536	–	608	666	2.48
5	5.36	Purpurin *b*	–	(420)	436	–	–	522	560	622	674	5.10
6	5.57	Pheophorbide *a*	(380)	(400)	410	–	504	536	–	608	666	2.48
7	6.08	Pyropheophorbide *b*	374	418	438	–	–	532	–	600	654	4.25
8	6.25	Pyropheophorbide *a*	(380)	(400)	410	–	504	536	–	608	666	2.48
9	6.60	Purpurin *a*	358	–	410	474	503	544	–	638	698	2.74

[a] Soret band (peak III) absorbance divided by the maximum absorbance in the red region (peak IX).

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

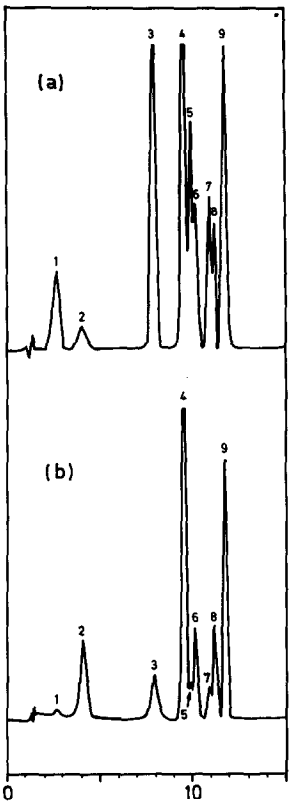

Fig. 3. HPLC of standard pigments mixture (20 μl). Detection by absorbance at (a) 430 and (b) 400 nm. Peaks: 1 = rhodin g_7; 2 = chlorin e_6; 3 = pheophorbide *b*; 4 = pheophorbide *a*; 5 = purpurin *b*; 6 = pheophorbide *a* isomer; 7 = pyropheophorbide *b*; 8 = pyropheophorbide *a*; 9 = purpurin *a*. Linear gradient system: 100% water–ion-pair reagent–methanol (1:1:8, v/v/v) to 100% acetone–methanol (1:1, v/v) in 15 min.

graphic characteristics of the pigments separated, including the capacity factors, absorption maxima in the eluent and peak ratios.

CONCLUSIONS

The study carried out on highly oxidized derivatives of pheophorbides permitted the development of a rapid HPLC method for the separation of these compounds. The method is of interest for easily detecting oxidation reactions that are undesirable during green table olive fermentation and for stricter control of this process. Equally, this method could be applied to detect these anomalous compounds in other feed products with chlorophylls as constituents and which have been processed, *e.g.*, canned vegetables, pickles and virgin olive oil.

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