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High-performance liquid chromatographic screening of chlorophyll derivatives produced during fruit storage

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Abstract

Reversed-phase high-performance liquid chromatography with photodiode array and fluorescence detection was applied to the systematic screening of chlorophylls and derivative pigments. The chromatographic procedure proposed made it possible to successfully separate and identify eight chlorophyll derivatives (the *a* and *b* forms of chlorophyll, chlorophyllide, pheophytin and pheophorbide) by using a linear gradient of methanol, acetone and ammonium acetate. The method has been routinely applied to study chlorophyll degradation during the postharvest storage of cherimoya (*Annona cherimola*, Mill.) fruits. The brilliant green colour even at maturity, and its high chlorophyllase and Mg-dechelating activities, indicate that this plant material might be suitable for investigating the as yet not well known chlorophyll breakdown processes.

Keywords: Fruits; Food analysis; *Annona cherimola*; Chlorophylls

1. Introduction

High-performance liquid chromatography (HPLC) is the most widely applied technique in the analysis of plant pigments. Most of the methods proposed employ conventional reversed-phase (RP) HPLC [1–7] but when broad polarity compounds are simultaneously analysed this technique requires highly polar solvents and complex elution programs. This results in lengthy chromatograms, which are too long for routine application, and a large band width in the tailing peaks.

Photodiode array detection (PAD) systems are powerful tools for chromatographic analysis since they enable immediate identification of peaks from their absorbance spectrum and provide purity criteria. The use of fluorescence detection in HPLC not only complements conventional UV–Vis absorption methods but in many instances achieves greater sensitivity and specificity, making it the best detection method in the analysis of chlorophyll and its derivative pigments [8–10], although some degradation products which come after pheophorbide formation are non- or weakly fluorescent.

The changes in colour of ripening and senescent fruits are visible results of chlorophyll degradation. This loss of chlorophyll during postharvest and senescence of some fruits, as *Annona*, causes a shift in colour from brilliant green to olive brown, a change which is associated with inferior quality. Chlorophylls, the pigments responsible for the characteristic green colour of fruit and vegetables,

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may degrade to undesirable grey–brown compounds such as pheophorbide and pheophytin. This degradation processes can be initiated by external factors such as temperature changes, increased levels of ethylene, light, oxygen, water stress, or some other factor or combinations thereof [11–14], and are mediated by acids and/or enzymes. In studying pigmentation during fruit growth, ripeness and the postharvest or senescence process, an accurate knowledge of the pigments involved is of fundamental interest.

The actual chlorophyll breakdown mechanism remains largely unknown, with chlorophyllase (EC 3.1.1.14) being the only enzyme which has so far been demonstrated to be involved in this degradative process. This enzyme catalyzes the removal of the phytol chain from the porphyrin ring to form chlorophyllide [14–16]. Several authors have also suggested the existence of an Mg-releasing enzyme (Mg-dechelatase) which would catalyze the removal of the Mg^{2+} ion from the tetrapyrrolic ring [17,18].

For the HPLC analysis of acidic pigments, such as chlorophyllides and pheophorbides ($2<pK_a<4$) [19], ion-suppression or ion-pairing techniques must be used to achieve optimal resolution. Acetic acid and tetrabutylammonium acetate have been used as suppression ion and ion-pairing reagents, respectively, in the analysis of chlorophyll derivatives [1,5,19], although neither of them provided totally satisfactory results because of the broad polarity spectrum of these compounds. Ammonium acetate has been used as buffer in the RP-HPLC analysis of porphyrins; it provides excellent results in the separation of the high polarity chlorophyll derivatives because of its ability to speed up the proton equilibrium in the chromatographic process [3,6,20,21].

This paper presents an RP-HPLC method for systematically analysing chlorophylls and their breakdown products during the postharvest storage of *Annona* fruits. These pigments arise from the action of the chlorophyllase and an Mg-dechelating process, and the method is applied to the quantitation of both reactions. *Annona* fruits, with their climateric behaviour, cannot be stored at low temperatures because they suffer chilling injury; this circumstance, together with the action of chlorophyllase and the existence of Mg-dechelating activity and enzymatic browning processes, produce important green colour losses which rapidly spoil the external appearance and the commercial value of these fruits.

2. Experimental

2.1. Samples

Cherimoya fruits (*Annona cherimola*, Mill.) cv. ''Fino de Jeta'' were grown in Motril, on the Mediterranean coast of Granada (Spain). Mature fruit samples were harvested and taken immediately to the laboratory, where they were cut into small pieces and frozen in liquid N_2 and stored at -70°C.

2.2. Pigment standards

The reference samples of chlorophyll *a* and *b* were obtained from plant extracts by semipreparative HPLC and rechromatographed in an analytical column. The purity of the isolated pigments was examined by further chromatography and the pigments were fractionated again if necessary. The authenticity of these standards was confirmed by comparison of their absorption spectra in diethyl ether with those of the literature [5,19]. Chlorophyllide *a* and *b* were prepared by enzymatic de-esterification of the respective chlorophyll. Pheophytins were obtained by controlled acidification with oxalic acid of the respective chlorophyll solutions [22]. Pheophorbides were prepared by enzymatic de-esterification with chlorophyllase of the respective pheophytins. The identification of all the isolated pigments was confirmed from their spectral maxima.

2.3. Chromatography

The HPLC equipment consisted of a modular Shimadzu (Kyoto, Japan) liquid chromatographic system equipped with two LC-6A pumps operated from a Shimadzu SCL-6A controller. A Rheodyne (Cotati, CA, USA) Model 7125 injector with a sample loop of 20 or 100 μl (depending on the column) was used. A Shimadzu fluorescence detector RF-10A and a Shimadzu SPD-M6A photodiode UV–Vis detector were used on line. The fluoresence detector was operated at 440 nm (excitation) and 660

nm (emission). Spectral data from the PAD system were recorded between 400 and 670 nm.

Analytical separations were performed on a 5 μm Spherisorb ODS-2 column (25 cm×0.4 cm I.D.) (Phase Separations, Norwalk, CT, USA). Semipreparative separations were carried out on a 5 μm Spherisorb ODS-2 column (25 cm×1 cm I.D.). Pigments were eluted using a linear gradient in 15 min from 100% solvent A (80% methanol in 1 *M* ammonium acetate) to 100% solvent B (80% methanol in acetone). Solvent B was maintained until the pigments were completely eluted. The flow-rate was 1 ml/min with the analytical column and 4 ml/min with the semipreparative column. Isolated pigments were identified according to their retention times and visible absorption characteristics compared with those in the literature.

2.4. Enzymatic extracts

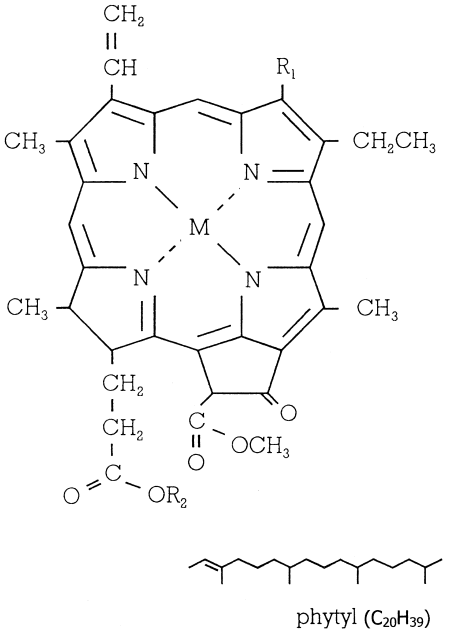
Samples of fruit peel (2 g) were homogenised with 100 ml of 400 m*M* sucrose in 50 m*M* Tris–HCl at pH 8.0 for 120 s in a Polytron homogeniser at high speed on ice bath. The extract was filtered through four layers of gauze and the filtrate was then centrifuged for 10 min at 7000 *g* in a Sorvall RC-5 centrifugue (DuPont, Willmington, DE, USA) at 4°C. The pellet, which contained most of the chloroplast material, was suspended in 10 ml of 100 m*M* phosphate buffer (pH 6.5) containing a 0.2% of Triton X-100. In an aliquot of these extracts were determined the chlorophyllase and Mg-dechelating activities according to Refs. [23] and [24], respectively. Both activities were evaluated in a thermostatically controlled bath at 30°C. Protein–dye binding [25] was used for protein determination.

2.5. Chemicals

In general, analytical-reagent grade chemicals from Panreac (Barcelona, Spain) were used. For HPLC analyses HPLC-grade solvents from Romil (Loughborough, UK) were used. Eluents for HPLC were filtered through 0.45-μm membrane filters (Millipore, Bedford, MA, USA) and degassed prior to use.

3. Results and discussion

Chlorophyll derivatives include a broad spectrum of green–grey–brown pigments of very different polarities. Fig. 1 shows the structures and names of the different compounds considered. Pheophytins and chlorophyllides are Mg-free (replaced by 2 H) and phytyl-free derivatives, respectively, of the parent chlorophylls, whereas the pheophorbides are free of both Mg and phytol. Consequently, chlorophyllides and pheoporbides are considered as acidic



PIGMENT	M	R_1	R_2
Chlorophyll a	Mg	CH_3	$C_{20}H_{39}$
Chlorophyll b	Mg	CHO	$C_{20}H_{39}$
Chlorophyllide a	Mg	CH_3	H
Chlorophyllide b	Mg	CHO	H
Pheophytin a	2 H	CH_3	$C_{20}H_{39}$
Pheophytin b	2 H	CHO	$C_{20}H_{39}$
Pheophorbide a	2 H	CH_3	H
Pheophorbide b	2 H	CHO	H

Fig. 1. Structures of the chlorophyll derivative pigments analysed. (The C7 position of the chlorophyll ring could be either one vinyl or one ethyl group. According to the spectral data presented in Table 1 we think it is an ethyl group.)

pigments, while chlorophylls and pheophytins present low polarity.

We attempted to optimise the RP-HPLC separation with PAD and fluorescence detection in a single stage suitable for the analysis of the above mentioned photosynthetic pigments. These pigments, particularly the chlorophyllides, show a broad polarity spectrum, which causes some problems since they are dissociated even at neutral pH and can interact hydrophobically with the stationary phase of reversed-phase C_{18}-bonded columns. Under these conditions polar compounds were quickly eluted using methanol–water or acetonitrile–water as eluents. However, low polar or non-polar compounds show intense interactions with the residual silanol groups of reversed-phase packings, which results in severe tailings [1]. To resolve this problem and to obtain good resolution, methods such as ion-suppression in acidic mobile phase or ion-pairing buffered at neutral pH, are used [3,5,19].

In our experiment the use of 0.5–1.0 *M* ammonium acetate in the mobile phase permitted good column selectivity, efficiency and resolution. A high concentration of ammonium acetate was essential to speed up proton equilibrium in the chromatographic process, particularly when ionogenic compounds (chlorophyllides and pheophorbides) are analysed.

Fig. 2 shows the absorbance and fluorescence chromatograms of an extract of chlorophyll pigments developed in the above mentioned conditions, where eight chlorophyll derivatives can be identified. Although good resolution can be appreciated visually, the chromatographic parameters *k* (retention factor), R_s (resolution) and α (separation factor) were calculated (Table 1) to confirm this valuation. The values of α and R_s reveal, respectively, the absence of overlapping peaks and complete resolution between adjacent bands [26].

The combination of photodiode array and fluorescence detection proved adequate for chloropigment analysis. Fluorescence detection (excitation 440 nm, emission 660 nm) was particularly sensitive for those pigments which were only present in small amounts, such as pheophytin *b* (peak 7) or pheophorbide *b* (peak 3), whose signals, as detected by PAD, were very weak (Fig. 2). Table 2 shows the identities and spectral maxima for the chlorophyll derivative pigments analysed.

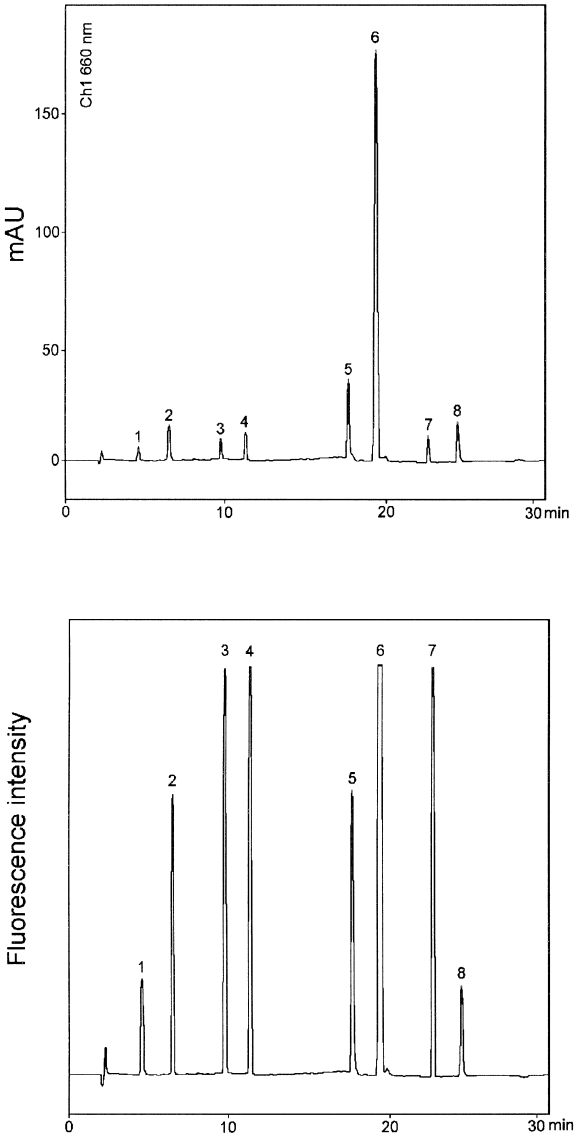


Fig. 2. Elution profile by RP-HPLC of the chlorophyll derivative pigments analysed. The pigments were detected spectrophotometrically at 660 nm and fluorimetrically using excitation and emission wavelengths at 440 and 660 nm, respectively. Peaks: 1=chlorophyllide *b*; 2=chlorophyllide *a*; 3=pheophorbide *b*; 4=pheophorbide *a*; 5=chlorophyll *b*; 6=chlorophyll *a*; 7=pheophytin *b*; 8=pheophytin *a*.

3.1. Analysis of chlorophyll derivatives produced during the storage of annona fruits

The analytical method presented was applied to

Table 1
Chromatographic parameters of the separation of chlorophylls and derivative pigments by RP-HPLC[a]

Peak No.	t_R	k	W_b	α	R_s	Purity
1	3.10	1.20	0.62	–	–	0.999
2	4.98	1.94	0.57	1.61	2.63	0.999
3	7.44	2.89	0.53	1.49	3.67	0.998
4	8.85	3.44	0.51	1.18	1.05	0.999
5	14.74	5.73	0.52	1.66	4.15	0.997
6	16.40	6.38	0.54	1.11	1.21	0.999
7	21.49	8.36	0.61	1.31	8.27	0.999
8	23.38	9.10	0.59	1.08	1.58	0.998

[a] Symbols: t_R=adjusted retention time (min); k=retention factor; W_b=peak width at base (min); α=separation factor; R_s=resolution.

the single-stage determination of the chlorophyll derivatives resulting from the chlorophyllase and Mg-dechelating activities in the peel of mature *Annona* fruits stored at 10°C in order to systematically appraise both processes. Chlorophyllase hydrolyses chlorophyll into chlorophyllide and phytol. This hydrolytic action can also be performed on pheophytin to produce the corresponding dephytylated pigment, pheophorbide [14–16,27]. In our experiment the substrates of the reaction were chlorophylls and pheophytins. It is important to stress that chlorophylls and pheophytins have the same spectral properties than chlorophyllides and pheophorbides, respectively. So, when spectrophotometric

Table 2
Identities and spectral maxima for chlorophyll derivative pigments analysed

Peak No.	Solvent	Absorbance maxima (nm)	Ref.	Pigment
1	Eluent	427, 616, 668	–[a]	Chlorophyllide *a*
	Diethyl ether	430, 662	–[b]	
	Diethyl ether	428, 662	[5]	
2	Eluent	468, 608, 653	–[a]	Chlorophyllide *b*
	Diethyl ether	595, 644	–[b]	
	Diethyl ether	458, 594, 643	[19]	
3	Eluent	407, 508, 666	–[a]	Pheophorbide *a*
	Diethyl ether	408, 505, 667	–[b]	
	Diethyl ether	408, 665	[19]	
4	Eluent	438, 532, 654	–[a]	Pheophorbide *b*
	Acetone	430, 525, 654	–[b]	
	Acetone	430, 523, 656	[30]	
5	Eluent	429, 616, 660	–[a]	Chlorophyll *a*
	Diethyl ether	430, 661	–[b]	
	Diethyl ether	430, 662	[31]	
6	Eluent	468, 575, 651	–[a]	Chlorophyll *b*
	Diethyl ether	430, 452, 642	–[b]	
	Diethyl ether	453, 643	[31]	
7	Eluent	407, 502, 666	–[a]	Pheophytin *a*
	Diethyl ether	410, 505, 667	–[b]	
	Diethyl ether	408, 667	[31]	
8	Eluent	525, 597, 652	–[a]	Pheophytin *b*
	Diethyl ether	526, 598, 654	–[b]	
	Diethyl ether	525, 599, 654	[19]	

[a] Data obtained from PAD.
[b] Data obtained after redissolution in the pure solvent.

methods are used to appraise this activity it is necessary to separate the product of the reaction from the remain substrate. The use of HPLC avoids this tedious process, diminishing the time of analysis and increasing the reliability of the result. The Mg-dechelating activity specifically uses as substrate chlorophyllides *a* and *b*, or bacteriochlorophyllides, but not chlorophylls or other Mg-protoporphyrin substances [24]. The quantitation of this activity was expressed as the appearance of pheophorbides. Fig. 3 shows the evolution of both activities in *Annona* fruits during storage.

Chlorophyll degradation measured in chloroplast fragments from the peel of mature *Annona* fruits was quite rapid, complete transformation of the chlorophyll being achieved in only 15 min. This confirms that in these fruits the chlorophyll breakdown processes are highly favoured as compared with other plant materials [15,23,28,29].

During the postharvest storage of *Annona* fruits the chlorophyllase activity was almost constant for the first 5–6 days (around 10 μM/min mg protein), this point would correspond to the maximum climateric. During this time the physiological characteristics of the fruit remained quite stable and no endogenous degradative processes due to overmaturation were observed. Between the sixth and eighth day of storage there was a slight decrease in chlorophyllase activity although this subsequently stabilised. Mg-dechelating activity increased sharply towards the sixth day and then diminished. This maximum formation of pheophorbides coincides with the maximum of chlorophyllase activity. These results suggest that chlorophyllides are the immedi-

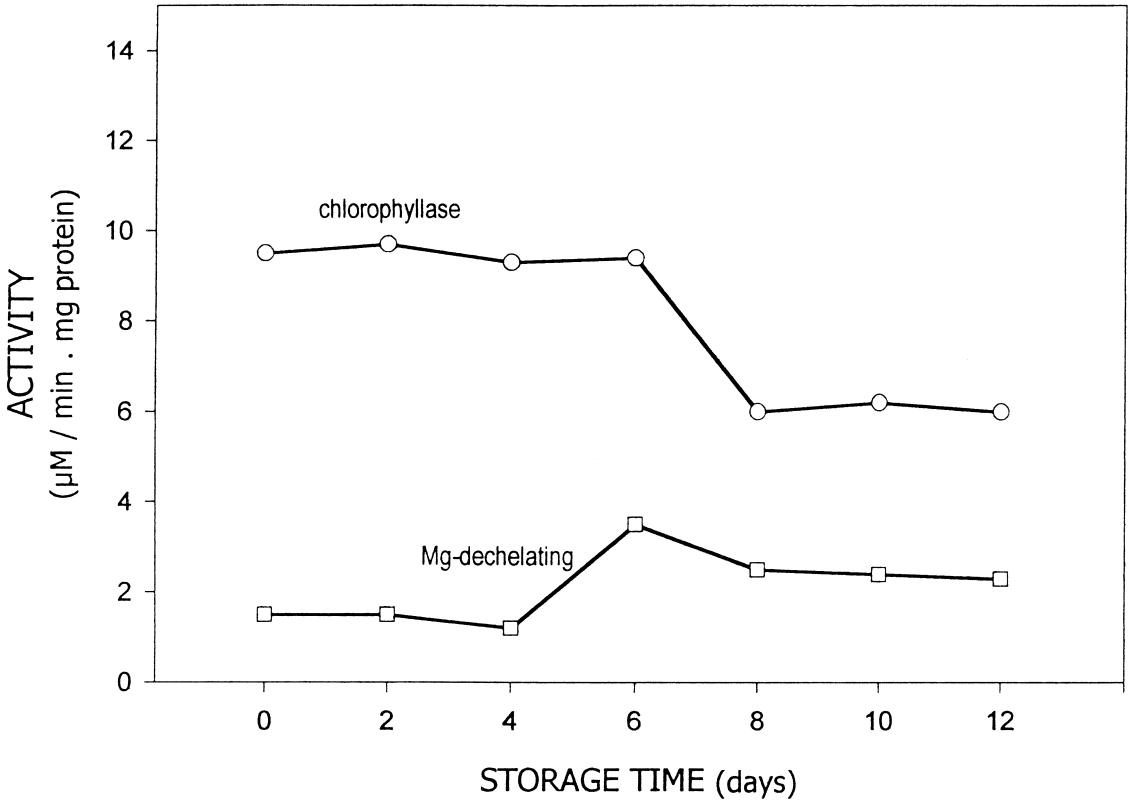


Fig. 3. Chlorophyllase and Mg-dechelating activities in peel extracts of *Annona* fruits stored at 10°C. Both activities were assayed by chloroplast incubation in 100 m*M* phosphate buffer (pH 6.5) containing 0.2% Triton X-100, 33% acetone and 25 μM exogenous substrate (chlorophylls in the case of chlorophyllase and chlorophyllides for the Mg-dechelating activity).

ate substrate of the dechelating activity. However, we observed no direct relationship between the disappearance of chlorophylls and the appearance of pheophorbides; the range of Mg-dechelating activity was around 25–30% of the chlorophyllase activity, which may be the consequence of other degradations suffered by the pigments (chlorophyllides and pheophorbides) in the incubation extracts.

We conclude that RP-HPLC using buffered mobile phase with ammonium acetate provides a valuable approach for the investigation of the complex degradative processes of chlorophyll. The proposed analytical method should prove very useful in the quantitation of two of the activities (chlorophyllase and Mg-dechelating) involved in these reactions. *Annona* fruits are a plant material very suitable for these investigations due to chlorophyll catabolism is highly favoured.

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