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Journal of Chromatography A, 994 (2003) 85–92
JOURNAL OF CHROMATOGRAPHY A
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Rapid separation of chlorophylls *a* and *b* and their demetallated and dephytylated derivatives using a monolithic silica C_{18} column and a pyridine-containing mobile phase

José L. Garrido[a,*], Francisco Rodríguez[b], Emilia Campaña[b], Manuel Zapata[b]

[a] *Instituto de Investigacións Mariñas de Vigo (CSIC), Eduardo Cabello 6, E-36208 Vigo, Spain*
[b] *Centro de Investigacións Mariñas, Consellería de Pesca, Xunta de Galicia, Apdo. 13, E-36620 Vilanova de Arousa, Spain*

Received 4 October 2002; received in revised form 12 February 2003; accepted 11 March 2003

Abstract

A monolithic C_{18}-bonded silica rod column (Merck Chromolith) was applied to the separation of mixtures of chlorophylls *a* and *b* and their derivatives originated by hydrolysis of the phytyl ester linkage (chlorophyllides), loss of the central Mg atom (pheophytins), or both processes (pheophorbides). Mobile phases containing two different ion-pair reagents, ammonium acetate and buffered pyridine, were tested. Both eluents achieved the resolution of the eight pigments in less than 5 min. The method based on the pyridine-containing mobile phase was applied to the separation of chlorophylls and their green coloured degradation products in senescing leaves of deciduous trees, green fruits and a marine microalga.
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Keywords: Monolithic columns; Chlorophylls; Chlorophyllides; Pheophytins; Pheophorbides

1. Introduction

The emergence of the autumnal colours in the leaves of deciduous trees and the changes in colour of ripening fruits are the most visible results of chlorophyll degradation. However, chlorophyll catabolism occurs not only as part of senescence processes but throughout the plant life, in germination, growth and reproductive development [1,2].

Biochemical information on the breakdown of chlorophyll has been very scarce until the past decade, but the processes are now understood [2–4]. In most cases, chlorophyll is first dephytylated to chlorophyllide by the action of chlorophyllase, and subsequently the central Mg atom is removed by Mg-dechelatase [2–4]. In certain species like *Ginkgo biloba*, the removal of Mg from chlorophyll to form pheophytin occurs in advance of dephytylation [5]. The product of these reactions, pheophorbide (Fig. 1), is the last green coloured intermediary catabolite of the pathway [2–4].

Whereas in senescing leaves chlorophyll breakdown appears to be a component of the retrieval of nitrogen from dying organs, in fleshy fruits its disappearance exposes carotenoids or anthocyanins, which render the fruit attractive for seed-dispersing

*Corresponding author. Tel.: +34-986-214-461; fax: +34-986-292-762.
E-mail address: garrido@iim.csic.es (J.L. Garrido).

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doi:10.1016/S0021-9673(03)00486-2

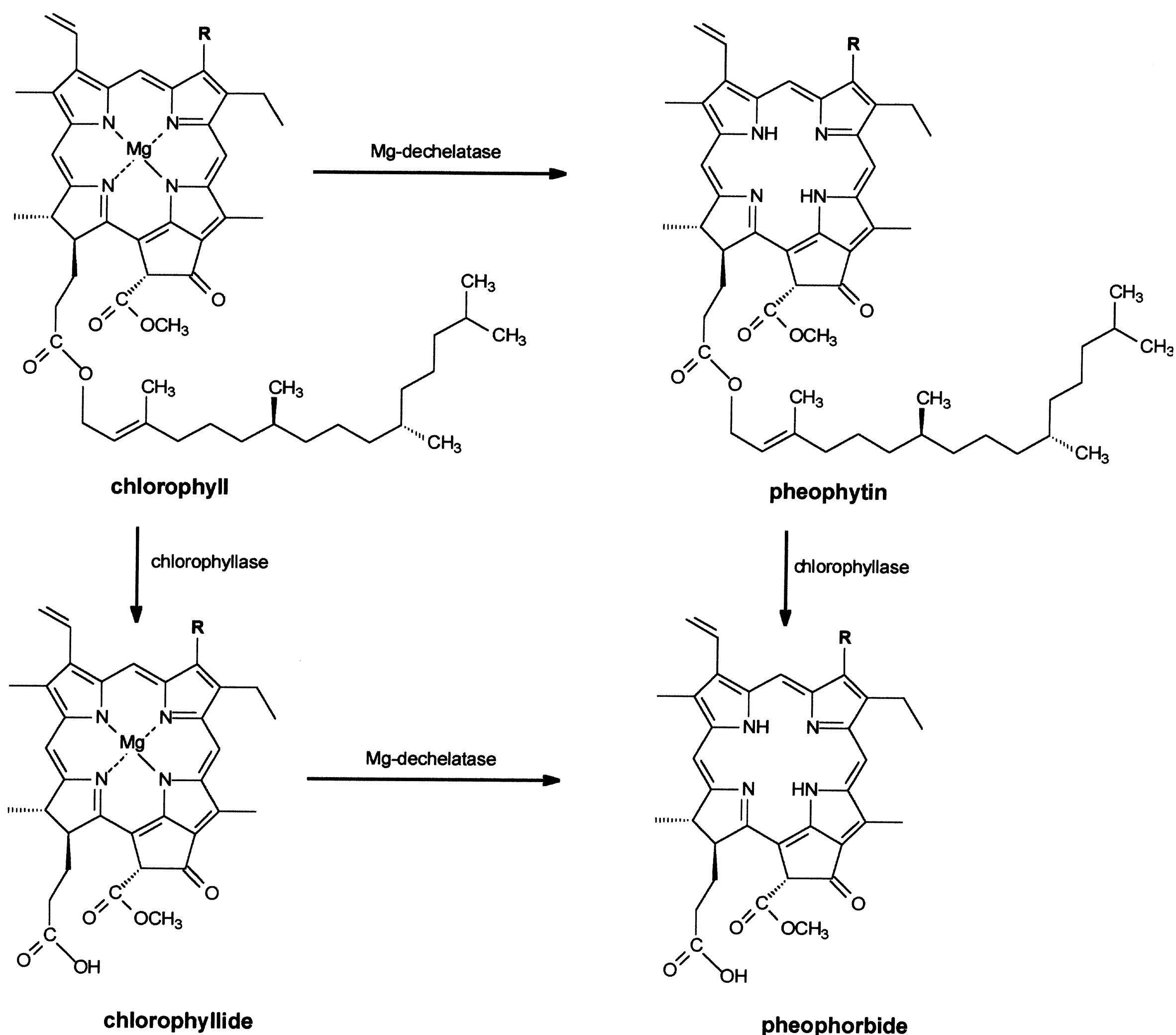

Fig. 1. Structures of chlorophylls and their dephytylated and demetallated derivatives. For chlorophyll *a* and derivatives, R=–CH_3; for chlorophyll *b* and derivatives, R=–CHO.

animals [1]. On the other hand, the loss of chlorophyll during post-harvest of green fruits and vegetables causes a shift in their colour. As consumers perceive the green colour of chlorophyll as an indicator of the freshness of food, many studies are being carried out to understand and to control the degradation of these molecules [6].

On a global scale, chlorophyll breakdown in aquatic systems is quantitatively and ecologically at least as significant as in terrestrial environments [2]. The accurate estimation of chlorophyll is essential in studies of aquatic ecology [7,8] and, as chlorophyll and chlorophyllide exhibit the same spectral properties, the production of these derivatives can seriously alter estimations made with spectrophotometric methods employed in oceanographic studies [8,9].

The global importance of chlorophyll degradation in terrestrial and aquatic environments, and its economic impact in food, point out the need for analytical methods to trace chlorophylls and their still coloured derivatives. Several high-performance liquid chromatography (HPLC) methods employing particle based reversed-phase (RP) columns have been developed for that purpose [6,10–16]. Their

analysis times typically last from 20 to 45 min, followed by the necessary re-equilibration.

Recently monolithic HPLC columns have been introduced [17–19]. A monolithic column consists of ''one piece of solid that possesses interconnected skeletons and interconnected flow paths (through-pores) through the skeletons'' [19]. Monolithic columns with small-sized skeletons and large through-pores are suitable for high speed separations, as they exhibit reduced flow resistance combined with reduced diffusion path length, compared to particle-packed columns [17–19]. A sol–gel procedure for the preparation of monolithic silica rods has been recently developed [20] and applied to the preparation of columns with high efficiencies and low backpressures [21] that show performances [22] and reproducibilities [23] similar to particle-based ones.

In this study we report the application of a monolithic C_{18}-bonded silica rod (Merck Chromolith) to the fast separation of chlorophylls *a* and *b* and their demetallated and dephytylated derivatives. Examples of the application of the method to the analysis of these compounds in senescent leaves, ripening fruits and a microalga are provided.

2. Experimental

2.1. Algal cultures

Dunaliella salina (originally from the Culture Collection of Marine Microalgae, Instituto de Ciencias Marinas de Andalucía, Cádiz, Spain) was grown on f/2-enriched seawater medium at 16±1 °C, under a 12:12 h light–dark cycle with an irradiance of 150 μmol photons m^{-2} s^{-1} during the light period.

2.2. Fruit samples

Fully ripe fruit samples of green apple (*Malus domestica* Borkh., cv. ''Granny Smith'') and cherimoya (*Annona cherimola* Mill., cv ''Fino de Jete'') were obtained from a local market.

2.3. Leaves

Senescing (yellow–green or fully yellow) leaves of *Castanea sativa* Mill. and *Ginkgo biloba* were collected in Campolongo Gardens (Pontevedra, Spain) in September 2002.

2.4. Sample preparation

Phytoplankton cells were harvested during the exponential phase of growth by centrifugation at 2000 *g*. The algal pellets were either (i) directly extracted with cold 90% acetone, or (ii) resuspended in 2 ml 50% aqueous acetone and incubated in the dark at room temperature to induce chlorophyllase activity [8]. After 30 min 8 ml 100% acetone was added to stop the reaction and to complete the pigment extraction. Samples of fruit peel (2 g) or senescing leaves (16 cm^2) were homogenised with 8 ml of 90% acetone and left for 16 h at −10 °C. All extracts were filtered through 25 mm, 0.2 μm GHP Acrodisc filters (Pall Gelman Laboratory, Ann Arbor, MI, USA) prior to injection.

To avoid peak distortion which affect early eluting pigments, 0.3 ml of Milli-Q water was added to each ml of acetone or methanol extracts, respectively, just immediately before injection [24,25]. All sample preparations were done under subdued light.

2.5. Pigment standards

The reference samples of chlorophylls *a* and *b* were obtained from algal extracts by semi-preparative HPLC and re-chromatographed in an analytical column. The purity of the isolated pigments was examined by further chromatography using two different chromatographic methods based on monomeric C_8 [16] and polymeric C_{18} [26] columns; and the pigments were fractionated again if necessary. The authenticity of these standards was confirmed by comparison of their absorption and fluorescence spectra in different solvents with those of the literature [27]. Chlorophyllides *a* and *b* were prepared by enzymatic de-esterification of the respective chlorophyll [8]. Pheophytins and pheophorbides were obtained by controlled acidification with 10% HCl of the respective chlorophyll and chlorophyllide solutions in diethyl ether [28]. The identification of all the isolated pigments was confirmed from their Vis (350–700 nm) and emission fluorescence (600–800 nm, excitation at the corresponding absorbance maximum) spectra, obtained with a Beckman DU-70

UV–Vis spectrophotometer (Fullerton, CA, USA) and a Perkin-Elmer LS 50 B luminescence spectrometer (Norwalk, CT, USA).

2.6. Chromatography

Aliquots of sample extracts were analysed using a Waters (Milford, MA, USA) Alliance HPLC system consisting of a 2690 separations module, a Waters 996 diode array detector (1.2 nm optical resolution) interfaced with a Waters 474 scanning fluorescence detector by means of a Sat/In analog interface. The column was thermostated at 25 °C by means of a Thermo Neslab RTE-200 (Portsmouth NH, USA) recirculating refrigerated water bath. A monolithic Chromolith Performance RP-18e (100×4.6 mm) (E. Merck, Darmstadt, Germany) column was used. Mobile phase consisted of (A) methanol, (B) 0.025 *M* aqueous pyridine solution (pH 5.0 with acetic acid) and (C) acetone. Solvent B was prepared in a fume cupboard by dissolving 1.98 g pyridine (J.T. Baker HPLC grade) to 1000 ml with Milli-Q water and adjusting the pH to 5.00 with acetic acid (J.T. Baker HPLC grade). A linear gradient from A–B (80:20, v/v) to A–C (80:20, v/v) was pumped during 3 min, followed by an isocratic hold at A–C (80:20, v/v) during a further 2 min. The initial solvent composition was recovered immediately after the end of the analysis by changing eluent B by eluent C at time 5 min (no reversed gradient was used). The flow-rate was 4 ml/min.

For semi-preparative isolation of the standards a polymeric Vydac 201 TP 510 (250×10 mm) column (Vydac—The Separation Group, Hesperia, CA, USA) was used as described by Zapata et al. [29].

For both analytical and semi-preparative HPLC solvents were HPLC grade from Riedel-de Häen (Seelze, Germany).

2.7. Pigment identification

Visible absorption spectra obtained by the diode array detector (wavelength range 350–750 nm) were used for routine pigment identification. After checking for peak homogeneity, spectral information was compared with a library of chlorophyll and carotenoid standard spectra from pigments prepared from reference phytoplankton cultures [30]. The scanning fluorescence detector was used as a selective detector for chlorophylls and derivatives. Excitation and emission wavelengths of 430 and 650 nm, respectively, were selected.

3. Results and discussion

The set of compounds constituted by the substrates and the products of chlorophyllase covers a wide range of chromatographic polarities, from the very non polar chlorophylls and pheophytins (with a C_{20} phytyl side chain) to the acidic (dephytylated) chlorophyllides and pheophorbides (Fig. 1). To achieve their separation in one single run, ion-pair chromatography combined with gradient elution has been the method of choice [10,11,16], as the acid-labile nature of chlorophylls precludes the application of ion-suppression methods. Most of these techniques employ ammonium acetate-buffered eluents, based on that initially developed by Zapata et al. [11] in a method that separated chlorophylls *a* and *b* and their derivatives (chlorophyllides and pheophorbides) together with carotenoids. Some years after, the same results were obtained when the method was applied to the analysis of chlorophylls and derivatives in cherimoya [15]. Recently this methodology has been adapted to the analysis of chlorophylls and their alteration products in processed green vegetables, and modified to adapt it to mass spectrometry (MS) operating conditions in HPLC–MS [6]. Later on, pyridine (as pyridinium acetate) was introduced as ion pair reagent for the analysis of acidic chlorophylls [31]. Pyridine shows several advantages in the analysis of both acidic and esterified chlorophylls: it is miscible with water and most organic solvents and shows adequate viscosity and boiling point values; although it absorbs strongly in the ultraviolet, it is transparent in the visible region of the spectrum, thus not interfering with pigment detection; and, being a tertiary amine, it does not react with acetone (frequently employed as an eluent in RP-HPLC separation of chlorophylls) to form hemiaminals and then imines (as ammonium ion, also commonly employed in pigment HPLC analysis, does) [26]. The major drawbacks to the use of this solvent are the noxious odour of concentrated pyridine and an increased health hazard relative to other mobile phase com-

ponents. However, with proper care and handling pyridine is basically a safe and effective chromatographic additive, with safety parameters similar to those of acetonitrile and available as a HPLC-grade solvent from many manufacturers [32]. Pyridine-containing mobile phases have been successfully employed in the HPLC analysis of phytoplankton pigments [16].

Both ammonium acetate and pyridinium acetate were tested for the analysis of chlorophylls and derivatives on reversed-phase monolithic columns. Fig. 2 shows the chromatographic traces obtained with no ion pair reagent (Fig. 2a), with ammonium acetate (Fig. 2b) or with pyridinium acetate (Fig. 2c) in the eluent. As could be expected, no retention was obtained for acidic chlorophylls when no ion pair reagent was added to the mobile phase (Fig. 2a), so the four compounds (chlorophyllides *a* and *b* and their demetallated derivatives, pheophorbides *a* and *b*) coeluted at the beginning of the chromatogram. However, the four phytyl esterified pigments (chlorophylls *a* and *b* and the demetallated pheophytins *a* and *b*) eluted well separated in less than 5 min. The resolution of the four non polar compounds is conserved when the mobile phase is changed to include ammonium acetate or pyridine (Fig. 2b and c). Both mobile phases achieve satisfactory separation of the eight compounds, with slightly higher resolutions of the acidic derivatives when using the pyridine-containing mobile phase (Fig. 2c). In consequence, the method with the last mobile phase (as described in the Experimental section) was chosen. A flow-rate of 4 ml/min was selected as it provided the smallest analysis time while keeping resolution values higher than 2 for all pigment pairs. The operation at high flow-rates (typically 2 to 10 ml/min) while keeping high column performance is a characteristic of monolithic silica columns. As they show very flat height equivalent to a theoretical plate (HETP) versus linear velocity curves, monolithic columns can be operated at high flow-rates with only very small decreases of efficiency [33]. In this way, the analysis is accelerated while the time needed to recover the initial conditions after a gradient is reduced [33]. Under the reported conditions the total analysis time, including re-equilibration, was 5.5 min, so the number of samples to be analysed relative to methods employing particle-based columns is by a factor of 4 higher, while the solvent consumption is slightly reduced due to the reduction in re-equilibration time.

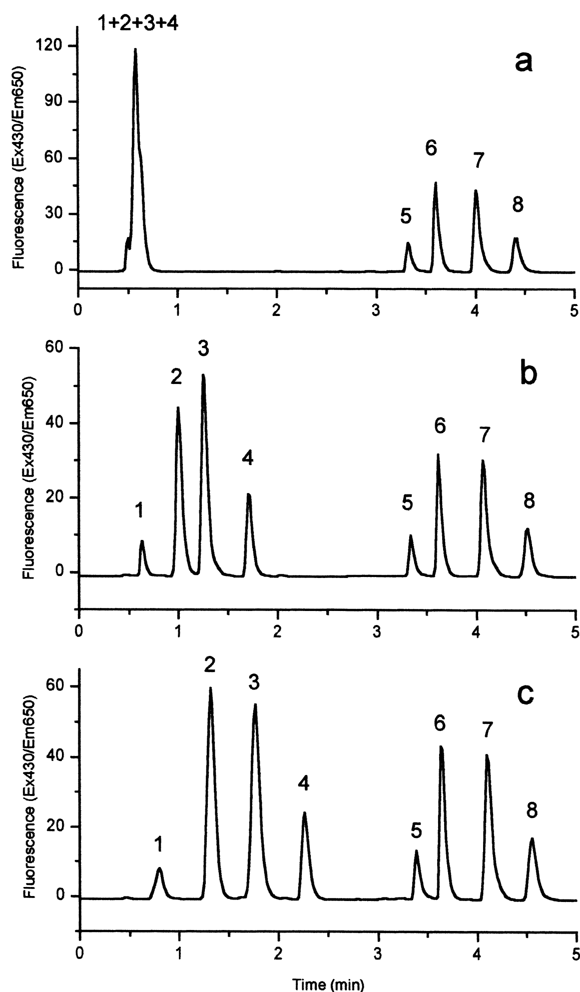

Fig. 2. Fluorescence chromatograms of standards of chlorophylls *a* and *b* and their derivatives, employing different aqueous solutions as solvent B in the mobile phase. (a) Solvent B = water; (b) solvent B = 1 *M* aqueous ammonium acetate; (c) solvent B = 0.025 *M* aqueous pyridine solution (pH 5.0 with acetic acid). Other chromatographic conditions as described in the Experimental section. 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*.

The chlorophyll composition during the senescence of the leaves of deciduous trees can be monitored with the method proposed in this work. Fig. 3a shows the chromatogram of a pigment extract

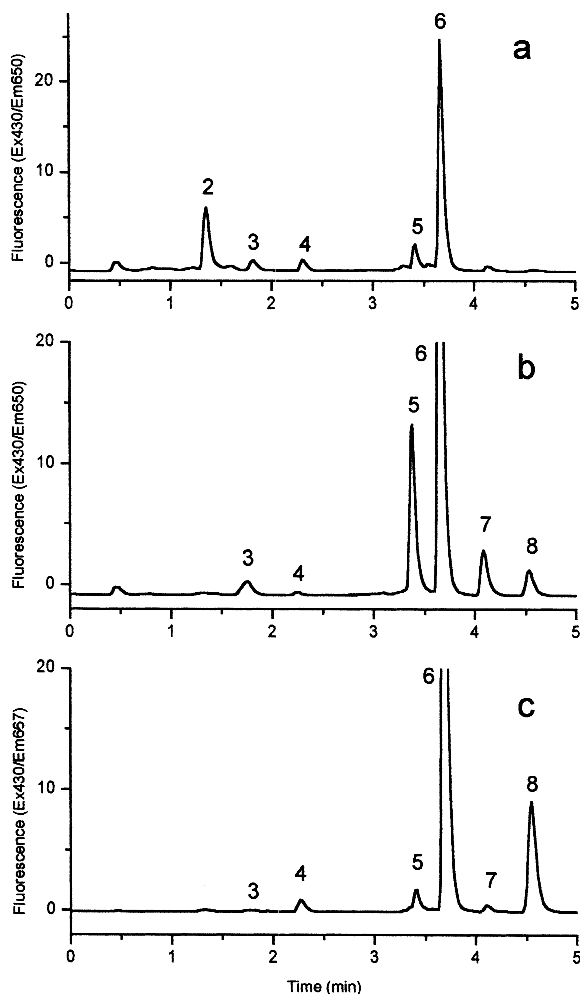

Fig. 3. Fluorescence chromatograms of pigment extracts from senescing leaves. (a) *Castanea sativa* (excitation at 430 nm, emission at 650 nm). (b) *Ginkgo biloba* (excitation at 430 nm, emission at 650 nm). (c) *Ginkgo biloba* (excitation at 430 nm, emission at 667 nm). Peak identification as in Fig. 2.

of senescing leaves of *Castanea sativa*, in which the absence of pheophytins and the presence of chlorophyllide are characteristic of a degradation pathway in which the de-esterification of the phytol side chain precedes the loss of Mg. As previously observed [5] a different chlorophyll composition is shown by senescing leaves of *Ginkgo biloba*, in which significant amounts of pheophytins *a* and *b* are found (Fig. 3b) together with smaller proportions of pheophorbides *a* and *b*, supporting a degradation pathway in which the Mg-dechelatase activity is previous to chlorophyllase activity. It has to be noted that whereas Tang et al. [5] only detected chlorophylls *a* and *b* and pheophytin *a* in senescing leaves of *Ginkgo biloba*, we found pheophorbide *a* together with chlorophyll *b*-related catabolites (pheophytin *b* and pheophorbide *b*). This difference could be explained by the sensitivity and the adequacy of the excitation and emission wavelengths in the fluorometer employed as a detector. When we recorded the same chromatogram at the wavelength pair used by Tang et al. [5], (excitation at 430 nm and emission at 667 nm), the peaks corresponding to chlorophyll *b*, pheophorbide *b* and pheophytin *b* decreased severely (Fig. 3c).

The method was also applied to the screening of the chlorophyll composition of two ‘‘green-ripe’’ fruits. It has been suggested [34] that green-ripe fruits retain significant photosynthetic activity, which helps them to offset the respiratory costs of fleshy fruit, providing them a certain eco-physiological (nutritional) advantage, whereas the bright colours of other fruits play an important role in the attraction of seed dispersing animals. Fig. 4 shows the fluorescence chromatograms of green apple and cherimoya peel extracts. Their chlorophyll patterns are quite different, with very scarce or absent dephytylated compounds in green apple (Fig. 4a) whereas cherimoya peel showed accumulation of chlorophyllides *a* and *b* and the presence of small quantities of pheophorbides *a* and *b* (Fig. 4b).

It seems, in consequence, that different biochemical strategies can underlie the colour of green-ripe fruits, with strong differences in the net content of the photosynthetically active chlorophylls *a* and *b*. As only limited information on the pigment composition of fruit peels is available [15,35], the method here proposed could be used for monitoring the proportions of different chlorophylls and their degradation products during the pre- and post-harvest ripening periods or during fruit senescence.

The method was also employed for monitoring induced chlorophyllase activity in the marine microalga *Dunaliella salina*. Fig. 5 shows the chromatograms, at 440 nm, of pigment extracts before (Fig. 5a) and after (Fig. 5b) 30 min incubation. The conversion of chlorophylls into chlorophyllides is almost complete, but no Mg-free derivative appears.

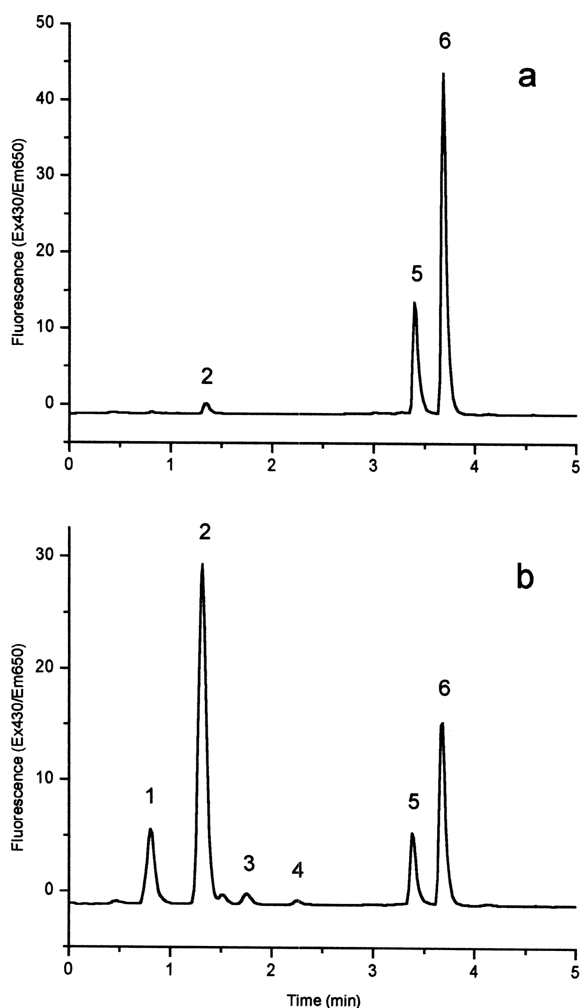

Fig. 4. Fluorescence chromatograms of pigment extracts from "green-ripe" fruits. (a) Green apple *Malus domestica*, cv. "Granny Smith". (b) Cherimoya (*Annona cherimola*, cv. "Fino de Jete"). Peak identification as in Fig. 2.

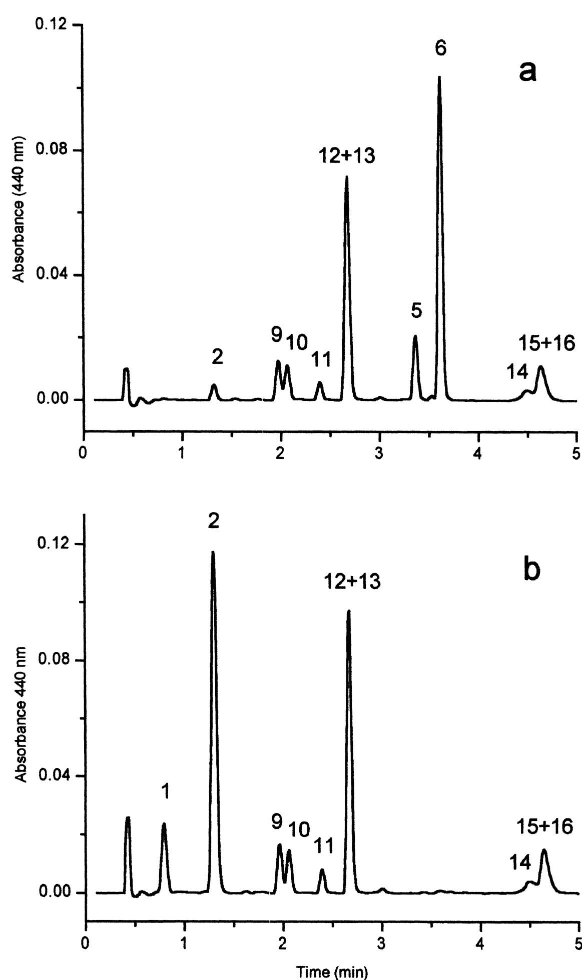

Fig. 5. Absorbance (440 nm) chromatograms of pigment extracts from the marine microalga *Dunaliella salina*. (a) Before incubation to promote chlorophyllase activity. (b) After incubation. Peaks: 1–8=as in Fig. 2; 9=neoxanthin; 10=violaxanthin; 11=antheraxanthin; 12=lutein; 13=zeaxanthin; 14=β,ψ-carotene; 15=β,ε-carotene; 16=β,β-carotene.

The method enables the simultaneous separation of chlorophylls and carotenoids of *D. salina*, with the coelution of two pairs of carotenoids: the isomeric xanthophylls lutein and zeaxanthin, and the isomeric carotenes β,ε-carotene and β,β-carotene.

The need for a rapid method for separating the remaining substrate and the reaction product of chlorophyllase has been pointed out [15,28,36]. The method here proposed can be employed for measuring chlorophyllase activity as it enables the separation of the four substrate–product pairs for this enzyme in very short analysis times. Thus, chlorophyll *b* and chlorophyllide *b* can be separated in less than 3.5 min, chlorophyll *a* and chlorophyllide *a* in less than 4 min, pheophytin *b* from pheophorbide *b* in less than 4.5 min and pheophytin *a* from pheophorbide *a* in less than 5 min. These analysis times contrast with the times (typically 10–15 min) needed for the separation of each of these pairs using particulated C_{18} columns, even using methods specially improved for such separations [28,36].

Acknowledgements

We thank VWR International Eurolab (Barcelona, Spain) for the generous gift of the Merck monolithic column used in this work. This work was supported by Xunta de Galicia through project PGIDT99MAR50102.

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