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Journal of Chromatography A, 692 (1995) 239–245

JOURNAL OF CHROMATOGRAPHY A

Separation of photosynthetic pigments and their precursors by reversed-phase high-performance liquid chromatography using a photodiode-array detector

Benoît Schoefs[a,*], Martine Bertrand[b], Yves Lemoine[a]

[a]*Plant Cytophysiology and Phycology Laboratory (SN2), University of Lille 1, 59655 Villeneuve d'Ascq, France*
[b]*National Institute for Techniques and Sciences of the Sea, Digue de Collignon, 50110 Tourlaville, France*

Abstract

A reversed-phase HPLC method is described for the separation of photosynthetic pigments, allowing the rapid and efficient separation of protochlorophyll(ide)s, chlorophyll(ide)s and *cis*- and *trans*-carotenoid isomers. The pigment contents of non-illuminated and illuminated bean leaves were determined and compared. Both sample types present the same carotenoid pattern. The main carotenoids are always *trans*-violaxanthin, *trans*-lutein and β-carotene. The tetrapyrrole pigment contents are very different. Non-illuminated leaves mainly contain protochlorophyllide and protochlorophyll esters whereas illuminated leaves contain mainly chlorophylls.

1. Introduction

Tswett, who discovered liquid chromatography, was also the first to analyse plant pigments [1]. Since these pioneer assays, the number of papers dealing with this technique has increased dramatically. The Association of Official Analytical Chemists [2] recommends the use of silica gel as the major adsorbent to separate carotenoids and chlorophylls (Chls) on a thin-layer chromatographic plate or by open-column chromatography. Both methods are lengthy and losses, degradations or modifications of the pigments could occur [3,4]. Moreover these techniques cannot separate compounds with very similar structures.

During the 1980s, the technique of HPLC was introduced for the separation and determination of plant pigments (for reviews, see [5–7]). The introduction of photodiode-array detection systems has increased the analytical power of HPLC because such detectors allow the simultaneous recording of chromatograms at various wavelengths during a single run and real-time identification and determination of the pigments. General advantages of such a detector have been reviewed [5,8]. The C_{18} reversed-phase column is particularly suitable for the separation of complex mixtures containing tetrapyrrole rings and hydrocarbon molecules [9–11]. Moreover, the bound phase is very inert and there is no risk of decomposition or structural modification of (proto)chlorophyll(ide)s and carotenoids when these molecules are in contact with the bound phase [12]. The utilization of a ternary system of non-aqueous solvents, rather than a solvent system containing water or buffer, avoids partial

* Corresponding author. Present address: Photobiology Laboratory, Department of Botany (B22), University of Liège, 4000 Liège, Belgium.

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SSDI 0021-9673(94)01066-8

precipitation or crystallization of the solutes on the solid phase, optimizes sample solubilization, gives a better peak shape and prolongs the column lifetime [12]. HPLC represents the best method for pigment separation owing to advantages such as speed, high resolution and sensitivity (for a review, see [5]). Several methods for the separation of either protochlorophyllides (Pchlide) or protochlorophylls (Pchl) [13,14], Chls [15–17], carotenoids [18–20], or both Chls and carotenoids [9,19–22] have been described. To our knowledge, the only method exhibiting a good separation of Pchl(ide)s, chlorophyllide (Chlide), Chls and carotenoids in the same run was that of Eskins and Harris [23]. However, this method does not achieve the separation of carotenoid isomers or of Pchlide esters.

Pigments of green plants are generally known as carotenoids and Chls. Carotenoids consist of carotenes and xanthophylls and can be found in vivo as *cis/trans*-(*Z*/*E*)-isomers (for reviews, see [5,24,25]). In addition to Chl *a* and *b*, it has been shown that pheophytin *a* and Chl *a* epimers (i.e., Chl *a'*) play an important role in photosynthesis [26,27] and not only occur as degradation products of Chl *a*. Chl precursors are particularly abundant in dark-grown plants [28–30]. As soon as such plants are illuminated, Pchlides are enzymatically transformed to Chlides [31], which are subsequently esterified in several steps to Chl *a* [32–34]. Pchlides and their esters have also been detected spectroscopically in greening and in green material [35,36].

The HPLC–photodiode-array detection system described in this paper allows in one step a simple, reproducible and rapid separation and identification of over twenty plant pigments including the different Pchlide esters and the *cis/trans*-carotenoid isomers. Such a resolution is not achieved by the method of Eskins and Harris [23].

2. Experimental

2.1. Plant material and culture conditions

Seeds of *Phaseolus vulgaris* cv. Red Kidney were cultivated in the dark at 298 K for 10 days as described by Schoefs et al. [37]. In order to obtain their greening, the seedlings were placed under fluorescent (Sylvania, Natural Super, 36 W) for different time periods between 0 and 16 h.

Wheat seeds (*Triticum aestivum* var. Castran) were grown for 6 days in the dark on a perlite–vermiculite (50:50) bed at 298 K.

Cucumber seeds (*Cucumber sativus* var. Marketer, Le Paysan, Avignon, France) were grown in the dark for 3 days on a perlite–vermiculite (50:50) bed at 298 K. The cotyledons were immersed in a 1.82 m*M* solution of δ-aminolevulinic acid (ALA) (Sigma, St. Louis, MO, USA) in order to increase the content of divinyl- (DV) Pchlide [38].

A green safe light was used while harvesting.

2.2. Preparation of authentic references

Monovinyl- (MV) Pchlide was prepared from etiolated wheat and bean leaves. The leaves were cut into small segments and immersed in diethyl ether for 24 h in the dark at 253 K. The extract was filtered through a Whatman (Maidstone, UK) ashless filter [39]. The filtrate essentially contains MV-Pchlide. DV-Pchlide was prepared from δ-ALA-fed cucumber cotyledons as described by Wellburn [38].

The carotenoids were separated on silica gel thin-layer plates (5513, Merck, Darmstadt, Germany) using the method described by Eichenberger and Grob [40]. The coloured bands were scraped off and the pigments were eluted with methanol. The pigments were characterized by their fluorescence properties using a Hitachi (Tokyo, Japan) F 1301 spectrofluorimeter and/or by absorption spectra recorded with a Uvikon 940 double-beam spectrophotometer (Kontron, Milan, Italy). The identification was done by comparison of the absorbance maxima obtained here with those in the literature [24]. The pigments were injected or dried under vacuum using a Heito Rotavapor. The pigments were stored in amber-coloured bottles with screw-caps at 243 K under nitrogen. Under these conditions, they remain stable for at least 1 month without any damage. Differentiation of *cis*- and *trans*-

carotenoid isomers can be done on the basis of the "*cis*-peak" in the near-UV region [24].

All the manipulations were performed under a green safe light at 277 K.

2.3. Preparation of samples

Dark-grown bean leaves were harvested using a weak green light in order to avoid any photo-transformation of pigments [5]. Just after the harvest, the leaves were hand-ground in methanol. The extracts were clarified by centrifugation at 50 000 *g* for 10 min at 277 K. The supernatant was filtered through a 0.45-μm PTFE filter membrane (Millipore, Bedford, MA, USA) and vacuum-dried using the Rotavapor and again solubilized in 0.5 ml of methanol. The sample was either dried again under nitrogen and stored in the dark at 253 K or immediately used for HPLC analysis.

2.4. Instrumentation and HPLC analysis

The chromatographic separation of pigments was carried out on an HPLC system that consists of one pump and its solvent-delivery system (SP 8700; Spectra-Physics, Darmstadt, Germany) and a Model 991-25 UV–Vis photodiode-array detector (Waters, Milford, MA, USA). The acquisition and treatment of data were performed using the Waters 991 software. The time and wavelength resolution were 1.1 ± 0.1 s and 2 nm, respectively. The pigment extract was injected with a Rheodyne (Cotati, CA, USA) Model 7125 sample valve equipped with a 100-μl loop. Up to 100 μl of methanolic extracts were injected. Separations were carried out with a Zorbax (DuPont, Willmington, DE, USA) reversed-phase column (particle size of the packing 4.65 nm; 25 cm × 4.6 mm I.D.).

The solvents used as the mobile phase were as suggested by Foyer et al. [10], but their proportions and the type of gradient were modified to obtain an adequate separation and detection of the different pigments (i.e., esters of Pchlide). The solvents were degassed prior use by bubbling helium (Quality U; Air Liquide, Paris, France) through them. Solvent A [acetonitrile–methanol (70:30, v/v)] was mixed with increasing proportions of solvent B (dichloromethane) during all the runs. The programme was as follows: solvent A was delivered isocratically from 0 to 7 min followed by a 2-min linear gradient to 20% solvent B. This solvent mixture was run isocratically until 30 min. The column was re-equilibrated between two sample analyses for a minimum of 20 min with solvent A. All runs were performed at 293 K. The flow-rate was 1 ml min^{-1}.

Methanol (pro analysi) was purchased from Merck (Darmstadt, Germany), diethyl ether (Normapur) from Prolabo (Paris, France) and dichloromethane and acetonitrile (both HiPerSolv) from BDH (Poole, UK).

3. Results and discussion

Fig. 1 presents a typical chromatogram obtained at 437 nm for pigments extracted from dark-grown 10-day-old bean leaves. Well resolved and clear peak shapes are obtained, even for the first peaks. The absorbance properties of the eluted pigments and their identification are reported in Table 1. It is important to note that the pigment absorbance maxima are blue-shifted when they are eluted in the presence of dichloromethane [41].

On such a non-polar column, the pigments are approximately eluted according to decreasing

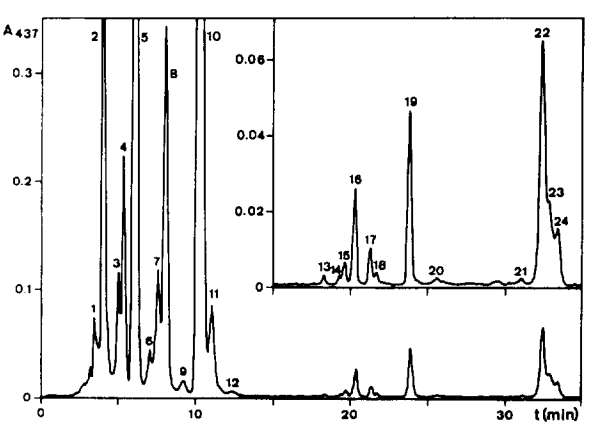

Fig. 1. Reversed-phase HPLC at 437 nm of the pigments extracted from 10-day-old dark-grown bean leaves. For identification of the peaks, see Table 1.

Table 1
Identification and absorbance maxima in the elution mixture of the pigments presented in Fig. 1.

Peak No.	Pigment	Absorbance maxima in the eluent (nm) (±1.4 nm)	Log k' ± S.D. (n = 3)
1	MV-protochlorophyllide	430,580,634	−0.40 ± 0.02
2	MV-protochlorophyllide	430,580,634	−0.28 ± 0.02
3	*trans*-Neoxanthin	413,439,469	0.00 ± 0.02
4	*cis*-Neoxanthin	317,409,434,465	0.06 ± 0.01
5	*trans*-Violaxanthin	412,437,469	0.18 ± 0.01
6	*cis*-Violaxanthin	319,408,433,464	0.20 ± 0.01
7	Lutein-5,6-epoxide	412,437,469	0.34 ± 0.02
8	*trans*-Antheraxanthin	423,444,474	0.47 ± 0.02
9	*cis*-Antheraxanthin	323,413,439,465	0.50 ± 0.02
10	*trans*-Lutein	427,444,474	0.54 ± 0.01
11	*cis*-Lutein	321,406,440,468	0.58 ± 0.01
12	*cis*-Lutein	320,402,442,468	0.60 ± 0.02
13	Chlide *a* geranylgeraniol	432,665	0.74 ± 0.01
14	Unidentified carotenoid	440,470	0.75 ± 0.01
15	PChlide geranylgeraniol	434,634	0.79 ± 0.02
16	*cis*-β-Cryptoxanthin	324,446,474	0.85 ± 0.01
17	PChlide phytatrienol	430,634	0.88 ± 0.01
18	PChlide phytadienol	430,636	0.90 ± 0.01
19	PChlide phytol	430,634	0.93 ± 0.01
20	*cis*-β-Carotene-5,6-epoxide	316,410,434,470	0.94 ± 0.02
21	α-Carotene	416,446,474	1.05 ± 0.01
22	*trans*-β-Carotene	420,454,482	1.06 ± 0.02
23	*cis*-β-Carotene	334,415,418,448,472	1.08 ± 0.02
24	*cis*-β-Carotene	334,415,418,446,474	1.08 ± 0.02

polarities; for instance, the α-carotene derivatives lutein-5,6-epoxide (two hydroxy and one epoxy group) and lutein (two epoxy groups) are eluted successively, whereas α-carotene (devoid of oxygen) is eluted later (see Table 1; for the structures, see Ref. [5]).

The polar molecules such as MV-Pchlide (Mg-2-vinylpheoporphyrin a_5 monomethyl ester, peaks 1 and 2) and the various xanthophylls, neoxanthine [(3*S*,5*R*,6*R*,3′*S*,5′*R*,6′*S*) - 5′,6′ - epoxy - 6,7 - didehydro - 5,6,5′,6′ - tetrahydro - β,β - carotene - 3,5,3′ - triol, peaks 3 and 4], violaxanthine [(3*S*,5*R*,6*S*,3′*S*,5′*S*,6′*S*) - 5,6,5′,6′ - diepoxy - 5,6,5′,6′ - tetrahydro - β,β-carotene - 3,3′ - diol, peaks 5 and 6], taraxanthine (5,6-epoxylutein, peak 7), antheraxanthine (5,6 - epoxy - 5,6 - didehydro-β,β-carotene-3,3′-diol, peaks 8 and 9), lutein [(3*R*,3′*R*,6′*R*) - β,ε - carotene - 3,3′ - diol, peaks 10 and 11] are eluted early. Then come the different esterified Pchlides (peaks 15 and 17–19). The non-polar α- and β-carotene [(6′*R*)-β,ε-carotene and β,β-carotene, peaks 21–23] and the carotenoid precursors (i.e., phytoene and phytofluene), which are more saturated, are eluted at the longest retention times (Tables 1 and 2).

Peak 2 was identified as Mg-2-vinyl-4-ethylpheoporphyrin a_5 monomethyl ester (MV-Pchlide) owing to its absorbance and fluorescence properties and to its co-migration with the authentic standard (data not shown). This result is in agreement with the literature as it has been demonstrated that bean belongs in the dark-

Table 2
Carotenoid precursors found in 20-day-old dark-grown or greening bean leaves

Pigment	log k' ± S.D. (n = 3)	Absorbance maxima (nm)
Phytofluene	1.12 ± 0.01	336,354,374
Phytoene	1.16 ± 0.01	275,288,302

grown monovinyl plant group [42]. Recently, Shioi and Takamiya [39] confirmed this result and indicated that bean leaves also contain minute amounts of Mg-2,4-divinylpheoporphyrin a_5 monomethyl ester (DV-Pchlide [29]). Our assays of cucumber extracts have shown that DV-Pchlide is unfortunately not separated from MV-Pchlide by this method. It must be added that a polyethylene column is generally used for the separation of MV- and DV-Pchlide [39]. The resolution of both pigments using a reversed-phase column has been reported only once but with retention times around 100 min [13]. From its absorbance spectral properties, peak 1 is also identified as MV-Pchlide. It is possible that it corresponds to an epimer of Pchlide or an "X-Pchlide" as proposed by Mukaida et al. [43]. Pheophorbide and/or pheophytins, the well known degradation products of protochlorophyllide and protochlorophyllide esters, were never detected among the pigments of non-illuminated samples, indicating that the extraction and conservation of the sample were satisfactory.

Despite the dark-growth conditions, traces of Chlide *a* geranylgeraniol ester are observed (peak 13). Eskins and Harris [23] also found Chl *a* in dark-grown leaf pigment extracts; they considered it as an artifact due to the green safe light (see Experimental). If we cannot exclude this hypothesis, it is worth noting that the presence of Chl *a* traces has already been reported in dark-grown plants [30,44,45].

Four Pchlide esters are detected (peaks 15, 17, 18 and 19). By comparison with the peaks found for *Cucurbita maxima* Pchl [43,46], we identified them as Mg-2-vinylpheoporphyrin a_5 geranylgeraniol ester (peak 15), Mg-2-vinylpheoporphyrin a_5 phytatrienol ester (peak 17), Mg-2-vinylpheoporphyrin a_5 phytadienol ester (peak 18) and Mg-2-vinylpheoporphyrin a_5 phytol ester (peak 19). This sequence is identical with that of pheophytins [52].

Usually, carotenoid precursors are not observed in wild plants (see Table 1) [5]. In order to accumulate these pigments, one uses chemicals (e.g., norflurazon) which block carotenoid synthesis from the precursors [47,48], but it is time consuming to prepare a sample with both carotenoids and precursors. We found that very old dark-grown leaves contain carotenoids and two precursors, namely phytoene (7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene) and phytofluene (7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene) (Table 2). The stereochemistry could not be determined because the elution solvents do not transmit UV-radiation in the range where the *cis* peak absorbs. By comparison between Tables 1 and 2, it appears that *trans*-β-carotene is partially co-eluted with phytofluene. The two pigments are easily differentiated and quantified, however, using a photodiode-array detector because they have no common absorbance band.

All the xanthophylls are well resolved into their *cis*- and *trans*-isomers (Fig. 1, Table 1). Carotene isomers are poorly separated in the present system, however, and this analysis would need, for instance, the use of a calcium hydroxide column [49,50]. However, good absorption spectra can be obtained from the data for the different carotene isomers (Fig. 2). It should be noted that the relative height of the *cis* peak depends on the *cis*-double-bound position, the more central giving the highest absorbance [24].

The two main tetrapyrrole pigments are Pchlide and Pchlide phytol ester. The main xanthophyll peaks correspond to *trans*-violaxan-

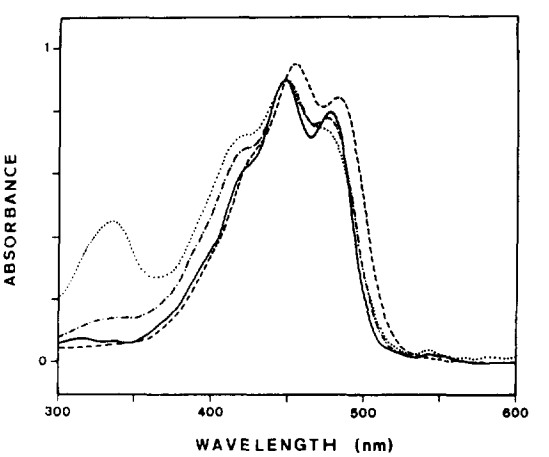

Fig. 2. Absorbance spectra in the elution mixture of (——) α-carotene, (– – – –) all-*trans*-β-carotene, (– · – ·) *cis*-β-carotene and (· · · · · ·) *cis*-β-carotene. The spectra of *cis*-β-carotene do not correspond with that of pure molecules as elution peaks 23–24 overlap.

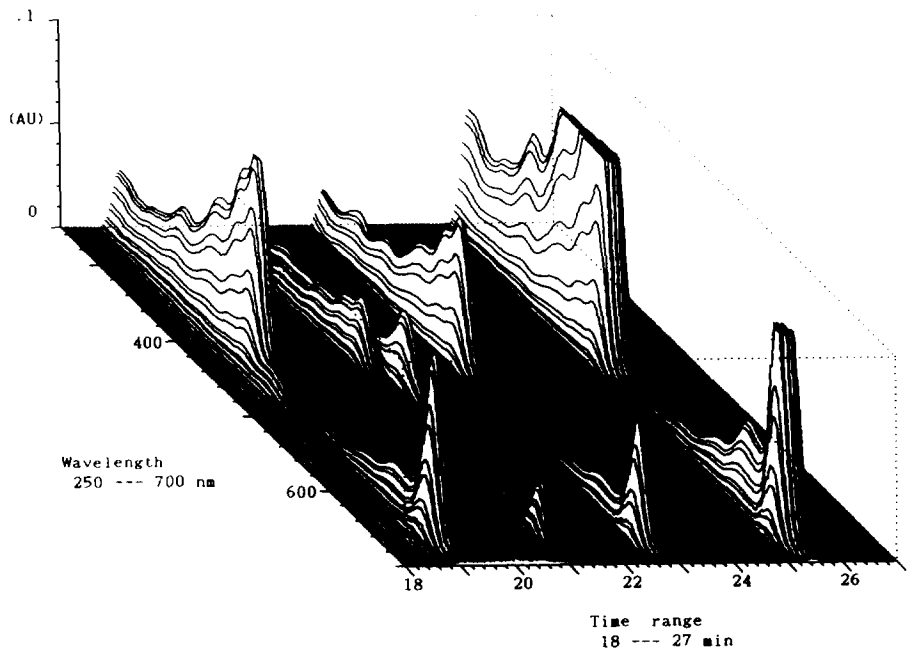

Fig. 3. Three-dimensional plot of the different Chlide *a* esters and chlorophyll *b* from a greening bean leaf. From the left, one can distinguish Chlide *a* geranylgeraniol, Chlide *a* phytatrienol, Chl *b*, Chlide *a* phytadienol and Chlide *a* phytol (Chl *a*).

thin and *trans*-lutein. These results are in agreement with those of Barry et al. [51]. Although the sequence of the peaks presented in Fig. 1 is similar to that of Eskins and Harris [23] for bean leaves, our method gives a better separation and resolution of the pigments, especially for the *cis*/*trans*-isomers of xanthophylls and for the different Pchlide ester forms.

The method was also applied to pigments from several greening higher plants and green algae. The carotenoid pattern is identical with that found for non-illuminated leaves. In illuminated leaves, the Pchlide esters are replaced by Chl(ide) esters. The four Chlide *a* esters, described by Schoch et al. [52], namely Chlide *a* geranylgeraniol, Chlide *a* phytatrienol, Chlide *a* phytadienol and Chlide *a* phytol (chlorophyll *a*) are well separated from Chl *b* (Fig. 3). In green leaves and algae, the main pigments are *trans*-violaxanthine, *trans*-lutein, β-carotene, Chl *a* and Chl *b* (Table 3). The method presented here appears to be very suitable for the separation of photosynthetic pigments and their precursors from a wide range of organisms.

Table 3
Additional pigments found during greening of a 10-day-old bean leaf

Pigment	log k' ± S.D. ($n = 3$)	Absorbance maxima (nm)
Chlide *a*	0.65 ± 0.01	432,665
Chlide *a* geranylgeraniol	0.82 ± 0.02	432,665
Chlide *a* phytatrienol	0.86 ± 0.01	432,665
Chl *b*	0.88 ± 0.01	458,658
Chlide *a* phytadienol	0.90 ± 0.01	432,665
Chlide *a* phytol	0.95 ± 0.02	432,665
Chlide *a'*	0.97 ± 0.01	432,665
Pheophytin *a*	1.08 ± 0.02	409,674

Acknowledgements

This work was supported by a Travel Grant (BV A90/76) from the Belgian French Community Education Ministry and by a grant from the French Embassy in Belgium to B. Schoefs. The presentation of these results at the HPLC'94 Congress by B.S. was supported by the Lefranc Foundation of the University of Liège and the

Agathon de Potter Foundation of the Belgian Royal Academy of Sciences, the Education Department of the French Community of Belgium and by the Organizing Committee.

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