Journal of Chromatography, 585 (1991) 168–172
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 566

Short Communication

Determination of carotenoid pigments in several tree leaves by reversed-phase high-performance liquid chromatography

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(First received August 22nd, 1990; revised manuscript received June 11th, 1991)

ABSTRACT

A rapid and simple reversed-phase high-performance liquid chromatographic method has been used to analyse all carotenoid pigments from leaves of five different trees. The pigments were separated on an octadecylsilane radial compression column, using a mobile phase mixtures of acetonitrile, methanol–water and ethyl acetate in three isocratic steps. This method resolves all higher plant photosynthetic pigments, carotenoids and also chlorophylls in less than 15 min, while achieving a relatively good separation of *trans*-lutein —the major carotenoid— from its isomers zeaxanthin and *cis*-lutein.

INTRODUCTION

Carotenoids are essential for the survival of photosynthetic organisms. No green plants which occur in nature lack carotenoid pigments since they have several significant functions in photosynthesis [1,2]. Thus, these coloured octaprenoids act as photoprotective agents, protecting against light-induced destruction by molecular oxygen (photo-oxidative damage). They also function in plants as accessory light-harvesting pigments, allowing autotrophic organisms to utilize light energy over a wider spectral range. A third function —specific of xanthophylls— is to act as carriers of light-induced reductive deoxygenations [3]. The first of these functions is essential for living organisms, and it must be quite clearly stated that without carotenoids there would be no photosynthesis in the presence of oxygen [4].

Specific separation and quantitative determination of each photosynthetic pigment (chlorophylls and carotenoids) of plants are essential to determine the function and role of each of these pigments in envelope and thylakoid chloroplastic membranes. In most plant physiology laboratories the analysis of photosynthetic pigments is still mainly concentrated on chlorophyll analysis (chlorophyll *a* and *b*) and it is carried out spectrophotometrically [5]. This method of determination is also usually used to determine the total carotenoid content; however, a differentiated estimation of the quantitative molecular pattern of each carotenoid pigment is needed in order to study in depth the role of carotenoids in photosynthetic organisms and the changes in their stoichiometry under ecological, physiological or biochemical influences.

The carotenoids of all functional chloroplasts in higher plants and green algae include β-carotene, lutein, violaxanthin and neoxanthin as major and regular components of the photochemically active thylakoids. The xanthophylls antheraxanthin, ta-

0021-9673/91/$03.50

raxanthin and zeaxanthin are regular but minor carotenoid components [5]. Therefore, it can be seen that a good analytical chromatographic system for these pigments must allow separated elution of compounds of widely divergent polarity: from more polar xanthophylls (like neoxanthin, 5′,6′-epoxi-3,5,3′-triol) to non-polar carotenoids (β-carotene, which has no oxygenated group).

This paper describes the use of a rapid, simple and sensitive reversed-phase high performance liquid chromatographic (RP-HPLC) method for the analytical separation and quantitative determination of all major carotenoids contained in the green leaves of five different deciduous trees. An accurate quantitative determination of each carotenoid present in these leaves was obtained from HPLC analyses, which also provided interesting data about the specific carotenoid composition of different leaves. The pigments were separated on an octadecylsilane radial compression column, using as mobile phase mixtures of acetonitrile, methanol–water and ethyl acetate in three isocratic steps. Detection of the pigments, after chromatographic separation, was carried out using a photodiode array detector.

EXPERIMENTAL

Leaf collection and pigment extraction

Pigments were extracted from sunlit leaves of five common deciduous trees: *Populus alba, Populus nigra, Acer pseudoplatanus, Quercus robur* and *Tilia tomentosa*. All species were grown in local gardens at Lejona (Vizcaya, Spain) (latitude 43°20′ north, < 100 m altitude), under well watered conditions. The leaves used for experiments were collected in spring —late May or early June— and were sunlit leaves with a photon flux density (PFD) incident from 1600 to 2000 μmol photon m^{-2} s^{-1}, and with a daylength range from 14 to 15 h.

Several clean leaves (4–8) stripped of main stems and veins were cut into small pieces for pigment extraction, and 1 g fresh weight was placed in a mortar and ground to a fine powder with acetone in the presence of sodium ascorbate as an antioxidant. The grinding and extracting were repeated with several volumes of acetone until no more colour could be eluted (the total volume of acetone used was about 50 ml/g of leaf tissue).

All the extractions were conducted in the cold (at 4°C) and in subdued artificial light (dim light). Reagents and solvents were of analytical grade. Before use, acetone was treated with sodium bicarbonate to neutralize any remaining acidity.

The acetonic extracts were filtered twice through a 0.45-μm HVLP Millipore filter to remove any insolublc particles, and were kept in darkness at −30°C until they were analysed. Pigments in the acetonic solution were analysed by injecting 20-μl aliquots onto the HPLC column.

Liquid chromatography

The HPLC system used for the pigment analyses consisted of a high-pressure pump (Waters M-45), a Rheodyne injector (7010-7011) fitted with a 20-μl loop, and a Shimadzu UV-visible detector (SPD-6AV) set at 450 nm and connected to a Shimadzu C-R3A integrator. A radial compression column, NovaPak C_{18} (Waters Rad-PakA, 100 × 8.0 mm I.D., with 4-μm spherical particles of octadecylsilane), was used with this chromatograph. The column was contained in a radial compression module (Wates RCM 8 × 10) and protected by an RCSS Guard-Pak (Waters, 10 × 8.0 mm I.D., 10 μm particle size) and a pre-column filter. A Waters 990 UV–visible photodiode array detector, covering the range 300–600 nm and connected to an NEC APC III computer, was used in some analyses for determination of the spectra of each peak separated by HPLC (Fig. 2).

The mobile phase composition was: (A) acetonitrile–methanol (7:1) (2 min); (B) acetonitrile–methanol–water (7:0.96:0.04) with 20% ethyl acetate (1.5 min); and (C) acetonitrile–methanol–water (7:0.96:0.04) with 50% ethyl acetate (8 min) if there was only β-carotene or (C′) acetonitrile–methanol–water (7:0.96:0.04) with 40% ethyl acetate (10 min) for separation of α- and β-carotene.

Reagents

HPLC-grade solvents, methanol, isopropanol, acetonitrile, ethyl acetate, tetrahydrofuran, dichloromethane and chloroform (Scharlau, France), were used. They were filtered through a 0.45-μm membrane filter (Fluoropore, Millipore, USA, for ethyl acetate and Ultipor NX, Millipore, for the other solvents) and degassed with a stream of helium before use.

Reference samples of carotenoids for calibration were purified from leaves according to published procedures. Purified standards of all-*trans*-β-carotene and zeaxanthin were provided by Hoffman-La Roche (Basle, Switzerland) and by Dr. G. Britton (University of Liverpool, UK).

Identification and quantitative evaluation

Peak identification was based on the comparison of retention times in HPLC with those of known standards, and on the isolation of each peak and the comparison of its absorbance maxima in the visible spectrum in different solvents (ethanol, acetone, chloroform and hexane) with standards values [6–9].

In order to carry out quantitative analysis of the data, peaks were monitored at 450 nm. The method was calibrated as by De Las Rivas *et al.* [10]. Most of the coefficients used for quantification of the pigments were the same as those previously reported [10]. However, some of them were modified after more accurate calibration. For these the new normalized coefficients were: taraxanthin, 1.03; anteraxanthin, 1.04; zeaxanthin, 1.01; α-carotene, 0.96; β-carotene, 0.93.

RESULTS AND DISCUSSION

The present work shows the quantitative determination of carotenoid composition in green spring leaves of five different trees, obtained by using the RP-HPLC method [10] modified as described in ref. 11. The analysis with this simple and reproducible RP-HPLC method allowed the resolution and quantification of all major photosynthetic pigments, with separation and detection of low levels of carotenoid isomers: zeaxanthin and α-carotene. The HPLC separation of the leaf pigments obtained with this method is shown in Fig. 1. This figure presents two chromatograms of the total green leaf pigment extract from two trees (*Tilia tomentosa* and *Quercus robur*), showing the separation of ten carotenoids (neoxanthin, *cis*-neoxanthin, violaxanthin, taraxanthin, antheraxanthin, lutein, zeaxanthin, *cis*-lutein, α-carotene and β-carotene) and two chlorophylls (chlorophyll *a* and *b*).

The short test-time (11.5–13.5 min) permitted analysis of a large number of samples, and the data shown in Table I are average values of eight different leaf samples for each species assayed. The analysed species were all deciduous-leaf trees, and trees very common in gardens and heaths. Proper coefficients for quantitative analysis of each carotenoid were calculated as in ref. 10, in order to provide an accurate quantitative evaluation of these pigments. The carotenoid composition (%, w/w) of the green leaves of the five deciduous trees analysed is shown in Table I.

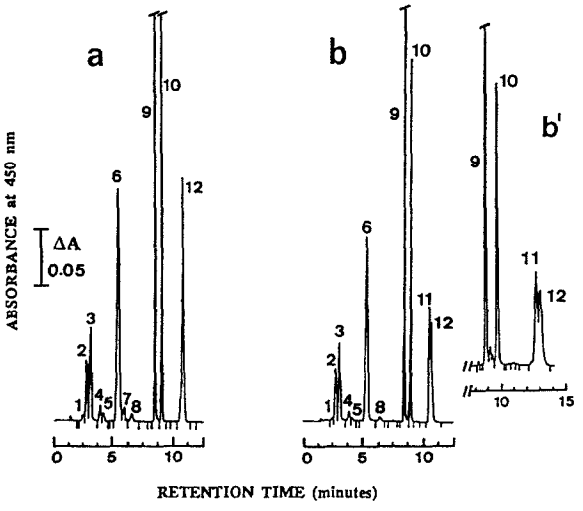

Fig. 1. Reversed-phase HPLC absorbance (450 nm) profiles of the pigment extracts from green leaves of the trees *Quercus robur* (a) and *Tilia tomentosa* (b), using the solvent system constituted by three isocratic phases: (A) acetonitrile–methanol (7:1) (2 min); (B) acetonitrile–methanol–water (7:0.96:0.04) with 20% ethyl acetate (1.5 min); and (C) acetonitrile–methanol–water (7:0.96:0.04) with 50% ethyl acetate (8 min). (b') Separation of the same sample of *Tilia tomentosa* using C' as the third solvent [C': acetonitrile–methanol–water (7:0.96:0.04) with 40% ethyl acetate (10 min) for better separation of α- and β-carotene]. Flow-rate, 2.00 ml/min; column, NovaPak C_{18}, 4 μm (100 × 8.0 mm I.D.); detection, 450 nm; volume injected, 20 μl; injection solvent, acetone. Peaks: 1 = neoxanthin; 2 = *cis*-neoxanthin; 3 = violaxanthin; 4 = taraxanthin; 5 = antheraxanthin; 6 = lutein; 7 = zeaxanthin; 8 = *cis*-lutein; 9 = chlorophyll *b*; 10 = chlorophyll *a*; 11 = α-carotene; 12 = β-carotene.

This accurate pigment determination has provided interesting figures about the normal carotenoid composition of the leaves of the trees studied, and about the specific difference that appears for each plant when precise quantitation is obtained. Moreover, the use of a photodiode array detector allows a quick identification of the peaks eluted from the

TABLE I

CAROTENOID COMPOSITION OF THE GREEN LEAVES FROM FIVE DECIDUOUS TREES AND RETENTION TIMES FOR EACH PIGMENT RESOLVED

Pigment	Retention time[a] (mean ± S.D.) (min)	Carotenoid composition of leaves (%, w/w)				
		Populus alba	*Populus nigra*	*Tilia tomentosa*	*Acer pseudoplatanus*	*Quercus robur*
Neoxanthin	2.60 ± 0.11	10.1	9.4	13.7	10.4	12.6[b]
cis-Neoxanthin	2.87 ± 0.11					
Violaxanthin	3.19 ± 0.11	19.7	17.2	15.4	26.6	14.7
Taraxanthin	3.97 ± 0.18	–	–	1.9	–	2.3
Antheraxanthin	4.29 ± 0.22	1.1	0.8	0.6	2.4	1.3
Lutein	5.68 ± 0.29	40.8	41.6	40.9	34.4	39.8
Zeaxanthin	6.19 ± 0.22	–	–	–	1.2	1.7
cis-Lutein	6.60 ± 0.26	0.8	1.1	1.1	0.8	1.4
Chlorophyll *b*	8.57 ± 0.18					
Chlorophyll *a*	9.12 ± 0.18					
α-Carotene	10.39 ± 0.26	–	–	13.8	–	1.3
β-Carotene	10.70 ± 0.22	27.5	29.9	12.5	25.0	26.1

[a] Retention time of each pigment using solvent systems A, B and C (see Experimental section). S.D. = Standard deviation of the retention times (mean of fifteen samples).

[b] Percentage of neoxanthin and *cis*-neoxanthin.

HPLC column, since it provides the visible absorption spectra of each pigment eluted in the mobile phase. The spectra of the main pigments present in a green extract obtained with this detector (shown in Fig. 2) were consistent with the spectra of the standards dissolved in similar solvents.

Lutein is the major component in all the leaves analysed, showing a percentage composition which is very constant, within the range of 39.4–41.6% (w/w). Leaves from *Acer pseudoplatanus* show slightly less lutein because of an increase in other xanthophylls, but the absolute amount is very similar to the other trees. Neoxanthin, the most polar carotenoid and the only one which presents *cis* configuration [12], represents around 12% (10.4–13.7%) of total carotenoid content. The xanthophyll cycle pool (violaxanthin, antheraxanthin and zeaxanthin) presents more variations: from 16.0% in *Tilia tomentosa* to 32.0% in *Acer pseudoplatanus*. This difference could mean a difference in the capacity of each plant to adapt to high-intensity light, in accordance with the recently proposed function for the xanthophyll cycle of dissipation of excess excitation energy, by formation and accumulation of zeaxanthin [13]. The data obtained show that the normal green leaves have very low levels of zeaxanthin (Table I), usually less than 3%, indicating that no particular situation of stress had affected the fresh mature green leaves from the trees studied.

The quantitation of carotenoids from leaves of *Tilia tomentosa* and *Quercus robur* show significant differences in the content of carotenes, since the former contains appreciable amounts of α-carotene (13.8%) and the latter has only 1.3% α- and 26.1% β-carotene. Not many quantitative data have been previously reported concerning these specific differences in the carotenoid content of extracts from chloroplasts. Recently, Thayer and Björkman [14] also reported the significant presence of α-carotene in leaves of some species: *Monstera deliciosa, Calycanthus occidentalis* and *Asarum caudatum*. These authors demonstrated that α-carotene is an important carotenoid constituent of shade plants [14]. This carotene is also present in significant quantities in gymnosperms [15].

The focus of this work, using an RP-HPLC method for analytic determination of carotenoids from leaves, is a first step to elucidating the role of each carotenoid in the photosynthetic apparatus of plants, which is not yet well known. The carotenoid content data given in this paper (Table I) reveal characteristic differences in the leaves of each spe-

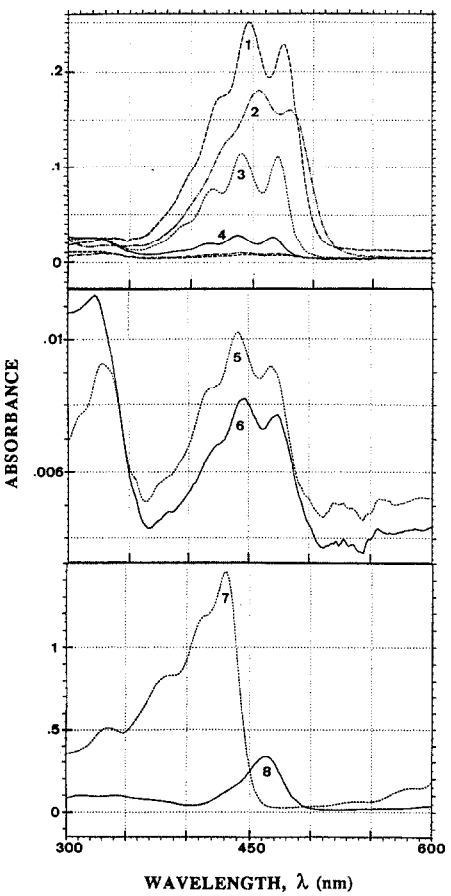

Fig. 2. Photodiode array absorption spectra between λ = 300 and 600 nm of the major pigments (carotenoids and chlorophylls) of green leaves separated by RP-HPLC. Sample: extract from *Populus alba* green leaves. The spectra correspond to the pigments in the eluent obtained at the outlet of the HPLC column with the photodiode array detector; therefore the pigments are in the mixture of acetonitrile, methanol and ethyl acetate that corresponds to the mobile phase where each one elutes. The concentration of the green extract injected was aproximately 40 μg of chlorophyll per ml. Peaks: 1 = lutein; 2 = β-carotene; 3 = violaxanthin; 4 = *cis*-neoxanthin; 5 = *cis*-lutein; 6 = antheraxanthin; 7 = chlorophyll *a*; 8 = chlorophyll *b*.

cies. Further studies involving more species and different growing conditions will be needed to establish the presence and function of each xanthophyll and carotene in leaves, chloroplasts, thylakoids and protein complexes of photosynthetic membranes.

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