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A New Reversed Phase-HPLC Method Resolving All Major Higher Plant Photosynthetic Pigments[1]

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ABSTRACT

A new reversed phase-high performance liquid chromatography method has been developed to analyze the full complement of higher plant photosynthetic pigments (*cis*-neoxanthin, neoxanthin, violaxanthin, taraxanthin, anteraxanthin, lutein, zeaxanthin, *cis*-lutein, chlorophyll *b*, chlorophyll *a*, α- and β-carotene). The separation is carried out on a C_{18} column in about 10 minutes, using a single high-pressure pump and three different mobile phases in three isocratic steps. This method introduces a major improvement in higher plant photosynthetic pigment analysis, resolving in only 10 minutes all photosynthetic pigments while achieving good separation of lutein from its isomer zeaxanthin. Zeaxanthin is involved in the xanthophyll cycle, which recently has been proposed to play a significant role in the protection of the photosynthetic apparatus from photoinhibitory conditions (Demmig *et al.* [1987] Plant Physiol 84: 218–224).

In most plant physiology laboratories, the analysis of photosynthetic pigments is still carried out spectrophotometrically, using the formula proposed by Arnon (1) from coefficients calculated by MacKinney (13) almost 50 years ago. Since Arnon's and other similar methods (8) can be used only for Chl, Lichtenthaler and Wellburn (12) devised a new formula to calculate the total amount of carotenoids from the absorbance of the extract at 470 nm. The fact that the different carotenoids and Chl are located within different pigment-protein complexes in the photosynthetic apparatus (17) makes it highly desirable to have a method for quantifying the amounts of the individual pigments. The obvious choice for this method is rp-HPLC,[3] but no such method is widely used among researchers. Major reasons for this are incomplete resolution of carotenoids, long analysis time, and the need for an expensive, dedicated analytical system.

The first attempts to separate carotenoids and Chl from plants by rp-HPLC used methanol/water-based mobile phases (2, 6, 7, 19). These systems eluted slowly the less polar carotenoids (*i.e.* β-carotene), and consequently required long separation times (30–75 min for β-carotene). This fact made it difficult, for laboratories having a large number of samples to process, to adopt these methods. It has been recently shown that analysis time can be shortened to acceptable values (less than 10 min per sample) by using eluent gradients and high rates of solvent flow (18). The major drawback still remaining in methods using methanol/water mixtures is their inability to separate the positional isomers, lutein and zeaxanthin. This limitation can be specially important in studies involving photoinhibition caused by environmental stresses such as excess light and drought (4, 5), where significant amounts of zeaxanthin are present in the leaves.

One approach to resolve positional isomers was to use eluting systems based in acetonitrile. Although not all acetonitrile-based rp-HPLC systems can separate lutein from zeaxanthin in plant extracts (3, 9), some methods (10, 11, 14, 16) can achieve this separation. However, none of these methods separated Chl *a* and *b*, and they also were unable to separate some carotenoids, such as α- and β-carotene (10, 11) or neoxanthin and violaxanthin (11, 14). These methods eluted β-carotene in 18 to 32 min. A second approach toward the separation of lutein and zeaxanthin has been the use of two analytical (C_{18}) columns in series. Prenzel and Lichtenthaler (15) resolved lutein and zeaxanthin with separation times of 25 and 25.5 min, respectively, and Wright and Shearer (20) achieved a similar separation from phytoplankton extracts in 20 min.

The aim of the present work was to develop a simple HPLC method to resolve all major photosynthetic pigments that could be used as a routine method by laboratories not specifically devoted to HPLC studies. Desirable characteristics for this method should include (a) clear separation of all major pigments, including the positional isomers zeaxanthin and lutein; (b) short analysis time, permitting the analysis of large number of samples; and (c) minimal requirements of equipment.

MATERIALS AND METHODS

Pigment Extraction

Pigments were extracted from *Populus nigra* sun leaves. Discs were taken with a cork borer and ground in a mortar with acetone (about 1 cm^2 of leaf tissue per milliliter of solvent) in the presence of sodium ascorbate. The extract was kept in the darkness at −30°C until analyzed.

Zeaxanthin Induction

Zeaxanthin formation was induced by illuminating leaves with a fiber optic light guide for 3.5 h (white light from a 150-W halogen lamp, filtered through KG 1 and KG 3 Schott filters). Light intensity at the leaf surface was 3000 $\mu Em^{-2} s^{-1}$.

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[3] Abbreviations: rp-HPLC, reversed-phase HPLC.

HPLC

Chromatography was carried out on a 100 × 8 mm Waters Novapak C_{18} radial compression column (4-μm particle size). Samples were injected with a Rheodyne 7010 injector with a 20-μL loop, and mobile phases were pumped by a Waters M45 high pressure pump at a flow of 2 mL/min. Peaks were detected at 450 nm by a Shimadzu UV-VIS detector and integrated with a Shimadzu CR3 A integrator. The column was equilibrated prior to injecting each sample by flushing with acetonitrile:methanol (7:1, v/v, mobile phase A) for 7 min. The sample was injected into the column and mobile phase A was pumped for another 2 min. A mixture of acetonitrile:methanol:water:ethyl acetate (7:0.96:0.04:2, by vol; mobile phase B) was then pumped for 1 min to achieve the resolution of lutein and zeaxanthin. Finally acetonitrile:methanol:water:ethyl acetate (7:0.96:0.04:8, by vol; mobile phase C) was pumped until β-carotene was eluted (about 7 min). Typical working pressures with solvent flows of 2 mL/min were around 300 psi. HPLC solvents were from LabScan (Dublin, Ireland). Pure zeaxanthin was from Roche Laboratories. Peaks were identified by standard methods described previously (18).

RESULTS AND DISCUSSION

The major improvement introduced by this method in higher plant photosynthetic pigment analysis over previous systems (18) is that it resolves, in a short time, all major photosynthetic pigments, including lutein and its isomer zeaxanthin. A chromatographic separation of the pigments extracted from a *Populus nigra* healthy sun leaf is shown in Figure 1A. We achieved separation of the full complement of higher plant photosynthetic pigments, *i.e.* neoxanthin, *cis*-neoxanthin, violaxanthin, taraxanthin, anteraxanthin, lutein, zeaxanthin, *cis*-lutein, Chl *b*, Chl *a*, and β-carotene, in about 10 min. This method is also able to separate α-carotene when present (*i.e.* in *Citrus* and some *Prunus* species, data not shown), but this carotenoid was absent in *P. nigra*.

Lutein and zeaxanthin separated in the chromatogram at 4.1 and 4.5 min, respectively. One of the problems often encountered in HPLC when the peaks are very close is the lack of peak resolution when one of the compounds is present in much larger amounts than the other. We achieved good separation even in the extracts obtained from the untreated control leaves, in which the molar ratio of lutein-zeaxanthin was as high as 15 (Fig. 1A). When standard zeaxanthin was added to the *P. nigra* plant extract, it separated at the same retention time to that assigned to native zeaxanthin (Fig. 1B).

To calibrate the method, pigments were first isolated by HPLC. Isolated fractions were dried under nitrogen and dissolved in the appropriate solvent, and pigment concentrations were determined using the coefficients of extinction indicated in Val *et al.* (18). The method was calibrated by injecting known amounts of pure pigments, and plotting peak area (integrator counts) *versus* mass of pigment injected. Coefficients ($C_{pigment}$) shown in Table I were the actual values used for quantification in our system. We found no significant differences between the contents of Chl *a*, Chl *b*, and total carotenoids measured by HPLC and those measured spectrophotometrically (1, 8) (results not shown), confirming that the coefficients were accurate. It is obvious that any modification in the system, *i.e.* a different detector or integrator, will result in changes in $C_{pigment}$. However, considering that the extent of the change will be practically the same for all coefficients, they can be normalized by dividing by one of them, for instance C_{lutein}, and the resulting normalized values (Table I) will be the same irrespective of the system used. To calibrate a different system, a calibration with a single pigment, *i.e.* lutein, must be carried out first to obtain C_{lutein} in the new system. Then the product of each normalized coefficient (Table I) and C_{lutein} can be used to quantify the different pigments.

An example of the use of this method for the study of the

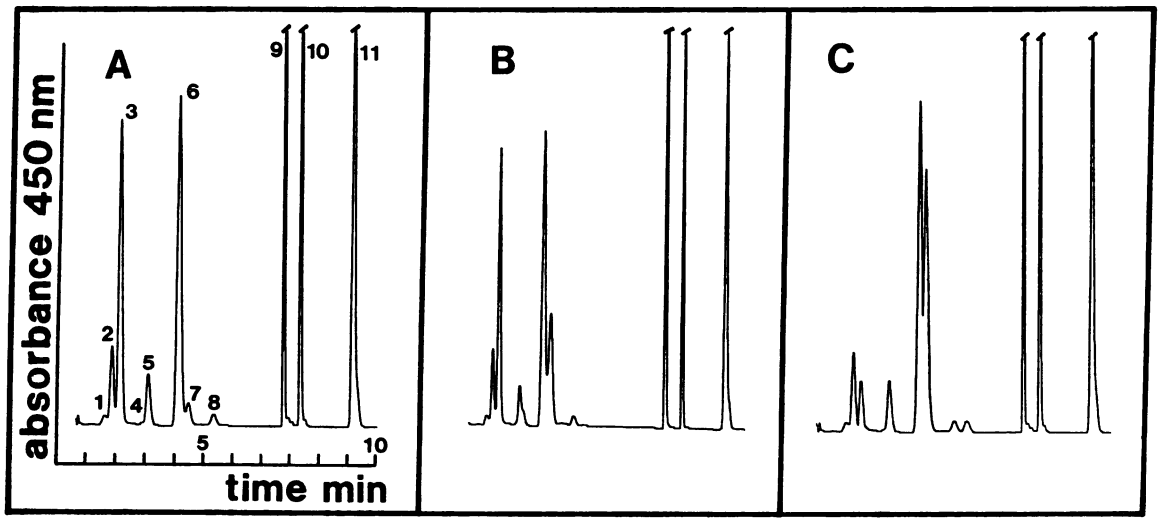

Figure 1. Separation of higher plant photosynthetic pigments by reversed phase-high pressure liquid chromatography. Pigments are neoxanthin (1), *cis*-neoxanthin (2), violaxanthin (3), taraxanthin (4), anteraxanthin (5), lutein (6), zeaxanthin (7), *cis*-lutein (8) Chl *b* (9) Chl *(a)* (10), and β-carotene (11). A, extract from a control leaf; B, extract from a control leaf supplemented with pure zeaxanthin; C, extract from a control leaf after illumination with a strong light (see text for details).

Table I. *Coefficients Used To Quantify the Method*

Coefficients ($C_{pigment}$) are given in nanomoles of pigment per peak unit area (in integrator counts). Normalized coefficients are the result of dividing each $C_{pigment}$ by C_{lutein}.

Photosynthetic Pigment	Coefficients ($C_{pigment}$)	Normalized Coefficients ($C_{pigment}/C_{lutein}$)
Neoxanthin	332[a]	1.21
Violaxanthin	296	1.08
Anteraxanthin	274	1.00
Lutein	274	1.00
Zeaxanthin	260	0.95
cis-Lutein	274	1.00
Chl *b*	332	1.21
Chl *a*	2112	7.71
β-Carotene	268	0.98

[a] All coefficients are 10^{-9}.

Table II. *Pigment Concentrations per Unit Area in a P. nigra Sun Leaf*

Samples were taken from the same leaf before (control) and after illumination with a strong light (photoinhibited) as described in Materials and Methods.

Photosynthetic Pigment	Control	Photoinhibited
	$nmol \cdot cm^{-2}$	
Neoxanthin	1.20	1.15
Violaxanthin (V)	3.25	0.41
Anteraxanthin (A)	0.67	0.49
Lutein	4.31	4.30
Zeaxanthin (Z)	0.32	3.31
cis-Lutein	0.18	0.23
β-Carotene	4.20	4.22
Chl *b*	6.54	6.88
Chl *a*	25.87	26.52
V + A + Z	4.24	4.21

changes caused by environmental factors on the function of the photosynthetic apparatus is given in Figure 1C. The same *P. nigra* leaf whose pigments were analyzed in Figure 1A was illuminated for 3.5 h with strong light, thought to cause photoinhibition (3000 $\mu Em^{-2} s^{-1}$). When pigments were analyzed at the end of the illumination period, a decrease of violaxanthin and an increase of zeaxanthin could be readily seen in the chromatogram. In this experiment, prior to illumination, leaves had 3.2, 0.7, and 0.3 $nmol \cdot cm^{-2}$ violaxanthin, anteraxanthin, and zeaxanthin, respectively (Table II). After illumination, leaves had 0.4, 0.5, and 3.3 $nmol \cdot cm^{-2}$ violaxanthin, anteraxanthin, and zeaxanthin (Table II), indicating a quantitative interconversion of about 87% of the violaxanthin into zeaxanthin.

An important feature of the new reversed phase-HPLC method presented here is the minimal requirement of equipment. The method uses a single high-pressure pump and three different mobile phases in three isocratic steps, making unnecessary expensive pieces of equipment such as gradient formers and a second high-pressure pump. Furthermore, this method optimizes solvent consumption and column life by using lower flow rates and lower column pressures than previous systems (18).

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