Journal of Chromatography, 508 (1990) 232–235
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 335

Note

High-performance liquid chromatographic determination of chloroplast pigments with optimized separation of lutein and zeaxanthin

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(First received November 7th, 1989; revised manuscript received February 6th, 1990)

High-performance liquid chromatography (HPLC) is the ideal technique for the determination of the chlorophylls and carotenoids in plants and photosynthetic micro-organisms. The pigments to be analysed have a wide range of polarities and it has not been easy to devise methods that combine rapid elution of the most strongly retained components with separation of closely related compounds, such as the structural isomers lutein and zeaxanthin. Good resolution of these two compounds can be obtained with mixtures of standards[1,2], but in methods designed for the determination of all the major photosynthetic pigments[3–6] the two peaks overlap or are very closely associated. This raises questions about the degree of resolution that would be obtained when attempting to use these methods with column materials from different manufacturers or with different batches of material of the same type. Good resolution is particularly critical in studies of the role of zeaxanthin in the xanthophyll cycle[7], when it frequently has to be measured in the presence of a large excess of lutein.

Our aim here was to develop a method with optimized separation of zeaxanthin from lutein, while still allowing the analysis of the other chlorophylls and carotenoids in a reasonable time. The novelty of the approach lies in the use of gradients of tetrahydrofuran in water rather than the commonly used gradients of ethyl acetate or other non-polar solvents in acetonitrile[3–6]. This allows us to propose a method that combines quantitative measurements of zeaxanthin content with the determination of the other chlorophylls and carotenoids in chloroplast extracts within 20 min.

EXPERIMENTAL

Extraction of pigments

Leaves of field-grown barley plants were frozen in liquid nitrogen and ground in a mortar and pestle in the presence of an equimolar mixture of solid KH_2PO_4 and Na_2HPO_4. Pigments from 2 g of leaf material were extracted with 8 ml of acetone and then re-extracted twice with 4 ml of acetone, the residue being separated by centrifugation after each extraction. Pigments were extracted from isolated spinach chloroplasts in a similar manner.

0021-9673/90/$03.50

HPLC system

An HPLC system consisting of two Kontron 420 pumps, a Kontron 432 UV–visible detector with a halogen lamp set at 450 nm, a Kontron 460 autosampler and a Kontron 450 data system was used. The column was 120 mm × 4 mm I.D. and was packed with octadecylsilica (Shandon Hypersil, 5-μm spherical particles). The column temperature was maintained at 30°C. A mixing chamber was inserted between the two pumps and the column.

Chromatographic separation

Separation of pigments was achieved in a gradient of tetrahydrofuran and water. The flow-rate was 1 ml/min. At the time of injection the solvent was tetrahydrofuran–water (51:49). After 5 min at this composition, the tetrahydrofuran content was increased linearly until the ratio at 13 min after injection was 90:10. During repetitive measurements the composition was then decreased linearly to 51:49 over the next 5 min, and then maintained at this level for 3 min (total analysis time 21 min).

Tetrahydrofuran was of HPLC grade (Rathburn). Solvents were degassed by ultrasonic treatment under vacuum and the solvent reservoirs were bubbled with helium during chromatography.

Calibration

Calibration was carried out using pigments isolated from spinach using reversed-phase thin-layer chromatography as described by Henry *et al.*[8]. Concentrations were determined using data provided by Davies[9] and Lichtenthaler[10]. Authentic samples of lutein and zeaxanthin were kindly provided by Dr. Jan Lundquist (Roche, Hvidovre, Denmark).

RESULTS AND DISCUSSION

Preliminary experiments using simple gradients of tetrahydrofuran and water showed satisfactory separation of all the pigments of interest in chloroplast extracts, except for the isomers lutein and zeaxanthin. We therefore investigated the effect of solvent composition on the separation of mixtures of the purified compounds under isocratic conditions. The results (Fig. 1) showed that optimum resolution (1.44) of the two compounds was obtained at 51% tetrahydrofuran. The resolution of neoxanthin and violaxanthin was better than that of lutein and zeaxanthin over the whole concentration range studied. The time required to elute the xanthophylls was much increased at lower concentrations of tetrahydrofuran. Elution of the slowest component, lutein, required 6.2 min with 51% tetrahydrofuran and 26 min with 45% tetrahydrofuran.

An example of the isocratic separation of mixtures of lutein and zeaxanthin using 52% tetrahydrofuran is given in Fig. 2. The optimized separation can be incorporated in a procedure to determine all the major photosynthetic pigments in higher plant chloroplasts by combining isocratic elution of the xanthophylls with a gradient of tetrahydrofuran in water to elute the less polar components. Fig. 3 shows the retention times for seven components with isocratic elution at various tetrahydrofuran concentrations for 5 min followed by a linear gradient up to 90% for the next 8 min. The slope of this gradient was chosen so as to obtain a good separation of

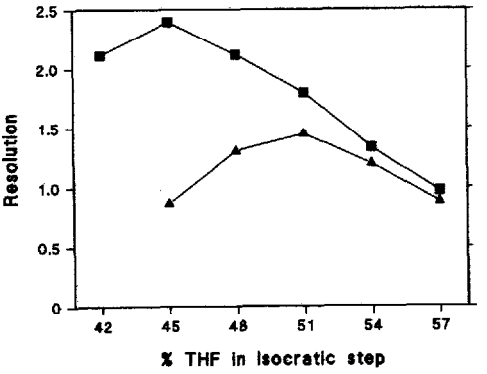

Fig. 1. Effect of concentration of tetrahydrofuran (THF) in water on the resolution of pairs of xanthophylls under isocratic conditions. ▲ = Separation of mixture of purified lutein and zeaxanthin; ■ = separation of neoxanthin and violaxanthin in spinach chloroplast extract.

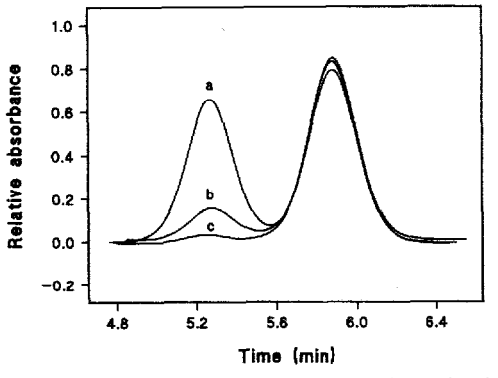

Fig. 2. Separation of lutein and zeaxanthin using isocratic elution with tetrahydrofuran–water (52:48). (a) 1:1 Mixture; (b) zeaxanthin–lutein (1:10); (c) lutein standard containing a trace of zeaxanthin. Other conditions as described in the text.

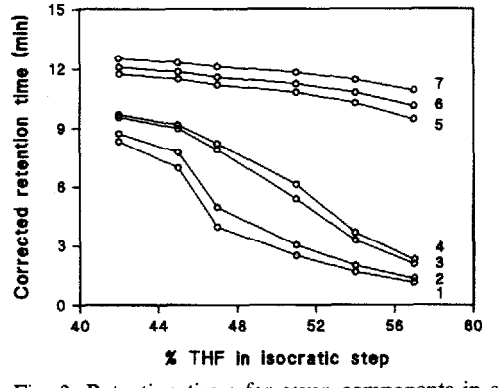

Fig. 3. Retention times for seven components in spinach chloroplast extracts with added zeaxanthin. Isocratic elution with the concentration of tetrahydrofuran (THF) shown was followed after 5 min by a linear gradient up to 90% THF after 13 min. 1 = Neoxanthin; 2 = violaxanthin; 3 = zeaxanthin; 4 = lutein; 5 = chlorophyll *b*; 6 = chlorophyll *a*; 7 = *β*-carotene.

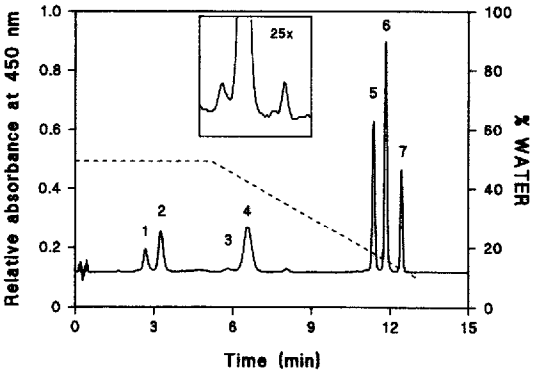

Fig. 4. Separation of the pigments of barley leaves supplemented with a trace of purified zeaxanthin (about 4% of the concentration of lutein) using the method described in the text. Peaks: 1 = neoxanthin; 2 = violaxanthin; 3 = zeaxanthin; 4 = lutein; 5 = chlorophyll *b*; 6 = chlorophyll *a*; 7 = β-carotene. The inset shows the separation of zeaxanthin and lutein on an expanded scale. The small peak after lutein is an unidentified component.

chlorophylls *a* and *b* and β-carotene whilst allowing a reasonably short analysis time. Fig. 3 shows that optimized separation of the xanthophylls is not at the expense of resolution of the less polar chlorophylls and carotenes.

Fig. 4 shows a chromatogram of the pigments from barley leaves using this approach with an isocratic step using 51% tetrahydrofuran. This demonstrates that the same protocol can be used both to measure the concentrations of the major pigments and to determine small amounts of zeaxanthin, as is necessary in studies of the xanthophyll cycle.

ACKNOWLEDGEMENTS

This research was supported by the Danish Natural Sciences Research Council. The HPLC equipment used was partially funded by a grant from the Carlsberg Foundation. We are grateful to Roche, Hvidovre, for the gift of standard carotenoids.

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