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# Improved liquid chromatographic method for the analysis of photosynthetic pigments of higher plants

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**Abstract**

The paper presents an improved reversed-phase LC method for the separation of the pigments from green leaves. A good separation of carotenoids and of their *cis*- and *trans*-isomers was achieved, especially for the separation of *trans*-lutein, zeaxanthin, *cis*-lutein, which are usually not well separated. No perfect separation of α-carotene, β-carotene and pheophytin *a* was possible, but conditions for a perfect coelution of pheophytin *a* with either β-carotene or α-carotene were established. Simultaneous equations allowing the determination of pheophytin *a* and α-carotene or pheophytin *a* and β-carotene are also given. 

*Keywords:* Higher plants; Photosynthetic pigments; Carotenes; Pheophytin *a*

## 1. Introduction

The study of pigments in plants started as early as 1800 [1]. The introduction of adsorption chromatography for the analysis of leaf pigments allowed the separation of β-carotene, and chlorophyll *a* and *b* from xanthophylls [2]. This technique has continuously been improved and nowadays, reversed-phase liquid chromatography (RPLC) is the method of choice for the analysis of chloroplastic pigments. Concomitantly, the number of detected pigments increased, although their function remained sometimes unclear until recently. This is especially true for the *cis*-isomers of carotenoids [3,4], lutein-5,6-epoxide [5], pheophytin *a* [6] and chlorophyll *a′* [7]. Recently it was reported that the *trans*-carotenoid isomers are located in the light-harvesting complexes, whereas pheophytin *a* and *cis*-isomers of β-carotene are only bound in the reaction center II [3,6]. The violaxanthin de-epoxidation upon illumination is also accompanied by a *trans–cis* isomerization [4,8]. It was also reported that a new type of xanthophyll cycle, involving the conversion of lutein-5,6-epoxide and lutein occurred in higher plants [5]. In addition, data supporting that the *cis*-to-*trans* ratio changes during plant greening have been published [9]. Therefore, continuous efforts in the separation of the chloroplast pigments are necessary.

Although several methods for the separation of chlorophylls [10–12], carotenoids [13–15], or both carotenoids and chlorophylls [16–18] have been reported, only a few can separate carotenoid isomers from chlorophylls in the same run [19–21]. In these cases, however, pigment resolution is sometimes not

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good enough to allow accurate quantifications. For instance, α-carotene, β-carotene and pheophytin *a* usually partially coelute. This was also the case for the pigments involved in the xanthophyll cycle, i.e., violaxanthin, antheraxanthin and zeaxanthin [22]. In this case, the difficulty mainly originates from the fact that zeaxanthin elutes between the lutein isomers.

In this work an improved RPLC method was obtained by modifying the elution programme. In addition, the effect of the proportion of dichloromethane on the separation of α-carotene and β-carotene and pheophytin *a* was studied. Although the perfect separation of these three pigments was not possible at any dichloromethane proportion, conditions allowing perfect coelution of pheophytin *a* with either β-carotene or α-carotene were found. On the basis of the absorbance properties of these pigments, we derived simultaneous equations which should be used for the determination of the individual amount of the pigments coeluting.

## 2. Experimental

### 2.1. Preparation of the samples

The experiments were performed on fully developed green leaves of horseweed [*Erigeron canadensis* syn. *Conyza canadensis* (L.)]. The plants were cultivated in a greenhouse under natural daylight. The leaves were exposed to high-light irradiation (1000 μmol $m^{-2}$ $s^{-1}$ PAR) for 30 min in order to induce violaxanthin conversion to antheraxanthin and zeaxanthin. Then the leaves were immediately ground into liquid nitrogen and the photosynthetic pigments were extracted according to Schoefs et al. [20]. All the extraction steps were performed at low temperature (0–4°C) and under a weak light intensity to avoid photosynthetic pigment degradations as recommended by Bertrand and Schoefs [23].

The pigment standards were prepared by thin-layer chromatography (TLC) using the method described by Bratt et al. [24]. The colored bands were scraped off and the pigments were eluted into ethanol (Merck, Damstadt, Germany, HPLC quality). The pigments were characterized on the basis of their absorption spectra recorded with a Unicam spectrophotometer and identified by comparison with literature data [25,26].

### 2.2. HPLC setup and pigment analysis

Pigment separations were carried out by RPLC as described by Schoefs et al. [20] using a $C_{18}$ column (Zorbax, DuPont, Willmington, DE, USA) (particle size: 4.65 μm; 25 cm×4.6 mm I.D.). The eluent was composed of acetonitrile, methanol and dichloromethane, as in the original method (see Ref. [20]) but the programme was modified as follows: from 0 to 10 min, acetonitrile–methanol (85:15, v/v) was run isocratically, followed by a 5 min linear gradient until the proportion of acetonitrile–methanol–dichloromethane (63:17:20, v/v/v) was reached. This composition was held for 7 min (i.e., until chlorophyll *a* elution). In order to improve the separation of α-carotene, β-carotene and pheophytin *a*, different proportions (20–50%) of dichloromethane were tested at the end of the elution programme. The final dichloromethane proportion was reached after a 3 min linear gradient and held until 30 min. During this step the proportion of methanol was kept at 18%. Then the column was re-equilibrated for 20 min with the solvent mixture used initially. All RPLC analyses were made at room temperature (25±2°C). The flow-rate was 1 ml $min^{-1}$. All solvents were of HPLC grade and purchased from Merck.

## 3. Results and discussion

The aim of this work was to improve the separation of the photosynthetic pigments present in higher plant green leaves. Different solvent proportions were tested, but only the best programmes are presented (see Experimental and Fig. 1). Fig. 1 presents a typical chromatogram for the pigments, extracted from green leaves previously illuminated with a high-light irradiation in order to trigger zeaxanthin formation through the operation of xanthophyll cycle [22]. In the main panel of Fig. 1, the final dichloromethane proportion was 45% whereas in the insert it was 28% (see below). Absorbance properties of the eluted pigments and $k'$

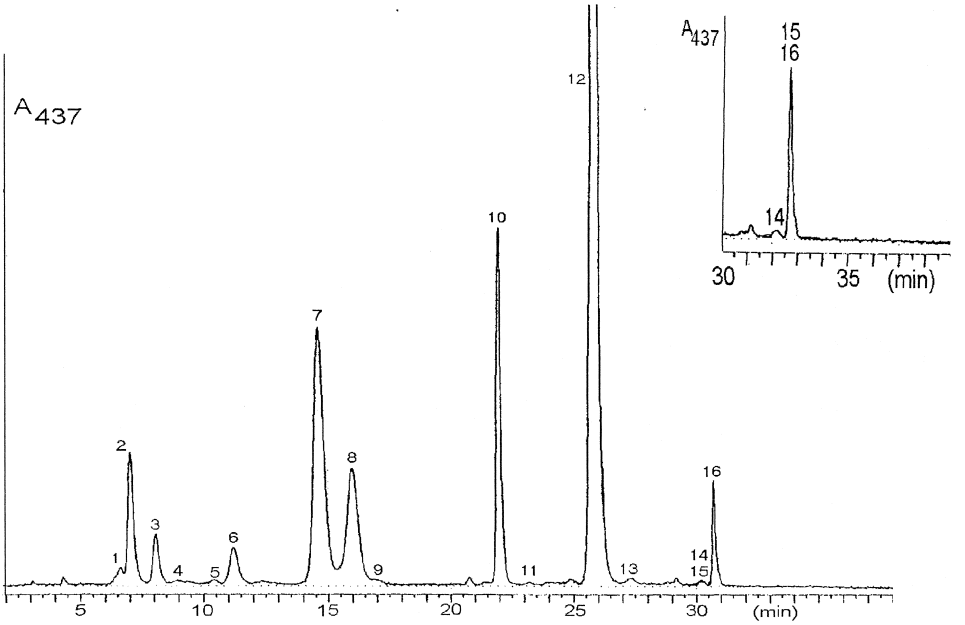


Fig. 1. Reversed-phase RPLC chromatogram recorded at 437 nm of the pigments extracted from illuminated green leaves of horseweed (*Erigeron canadensis*). In the main panel, the final dichloromethane proportion is 45% whereas in the insert it is 28%. For identification of the peaks, see Table 1.

values of the pigment standards were used for the identification of the eluted pigments (see Table 1).

The elution order of the pigments is identical to that reported in Ref. [20]. However, when using the new separation programme, the polar pigments were eluted a bit slower and the nonpolar ones somewhat quicker than in Ref. [20] (Table 1).

As illustrated in Fig. 2, the separation of *cis*- and *trans*-neoxanthin (peaks 1 and 2) is still not complete (the resolution is lower than 1) but better than in Ref. [20]. *Trans*-neoxanthin, *cis*- and *trans*-violaxanthin (peak 3, peak 4) are well separated from each other. A good separation of lutein-5,6-epoxide (peak 5) and antheraxanthin (peak 6) as well as as *trans*-lutein (peak 7), zeaxanthin (peak 8) and *cis*-lutein (peak 9) is necessary for their accurate quantification, which is used for the determination of the interconversion of violaxanthin to zeaxanthin (via antheraxanthin) through the operation of the xanthophyll cycle and for the study of the new xanthophyll cycle involving lutein and lutein-5,6-epoxide [5,26]. Using the present method, the resolution is always significantly higher than 1 (Fig. 2). We have to remark that when zeaxanthin is missing, for example in dark-adapted leaves, the elution peaks of the *trans*-lutein and *cis*-lutein are somewhat closer (log $k'=0.59\pm0.02$ for *trans*-lutein, log $k'=0.65\pm0.02$ for *cis*-lutein; $R=1.30\pm0.12$) than when zeaxanthin is present (compare with log $k'$ data in Table 1).

A frequent problem encountered during the separation of plant pigments by RPLC is the separation of α-carotene, β-carotene and pheophytin *a*. To obtain a better separation of these nonpolar pigments, proportions of dichloromethane from 20 to 50% were tested at the end of the programme (see Experimental). No perfect separation was achieved in any case (Fig. 3). Coelution of pheophytin *a* with either β-carotene or α-carotene was obtained at 28% or 45% of dichloromethane (Figs. 1 and 3). It is important to note that the separation order of the nonpolar pigments changes depending on the proportion of dichloromethane. When it is higher than 28% pheophytin *a* is eluted before β-carotene [27]. On the other hand their separation order also changes when only the last part of the programme is used to elute the pigments, i.e., the recent history of the column strongly influences the separation of the pigments.

The absorbance spectra of α-carotene, β-carotene

Table 1
Identification and absorbance maxima in the elution mixture of the pigments presented in Fig. 2

| Peak No. | Pigments | Log $k' \pm$SD ($n=5$) | |
|---|---|---|---|
| 1 | *trans*-Neoxanthin | 0.09±0.02 | |
| 2 | *cis*-Neoxanthin | 0.15±0.02 | |
| 3 | *trans*-Violaxanthin | 0.25±0.02 | |
| 4 | *cis*-Violaxanthin | 0.31±0.01 | |
| 5 | Lutein-5,6-epoxide | 0.44±0.01 | |
| 6 | Antheraxanthin | 0.46±0.01 | |
| 7 | *trans*-Lutein | 0.60±0.02 | |
| 8 | Zeaxanthin | 0.65±0.02 | |
| 9 | *cis*-Lutein | 0.68±0.02 | |
| 10 | Chlorophyll *b* | 0.83±0.02 | |
| 11 | Chlorophyll *b'* | 0.84±0.02 | |
| 12 | Chlorophyll *a* | 0.90±0.02 | |
| 13 | Chlorophyll *a'* | 0.93±0.02 | |
| | | Proportion of dichloromethane | |
| | | 28% | 45% |
| 14 | α-Carotene | 1.02±0.02 | 0.96±0.01 |
| 15 | Pheophytin *a* | 1.02±0.02 | 0.96±0.01 |
| 16 | β-Carotene | 1.03±0.02 | 0.97±0.01 |

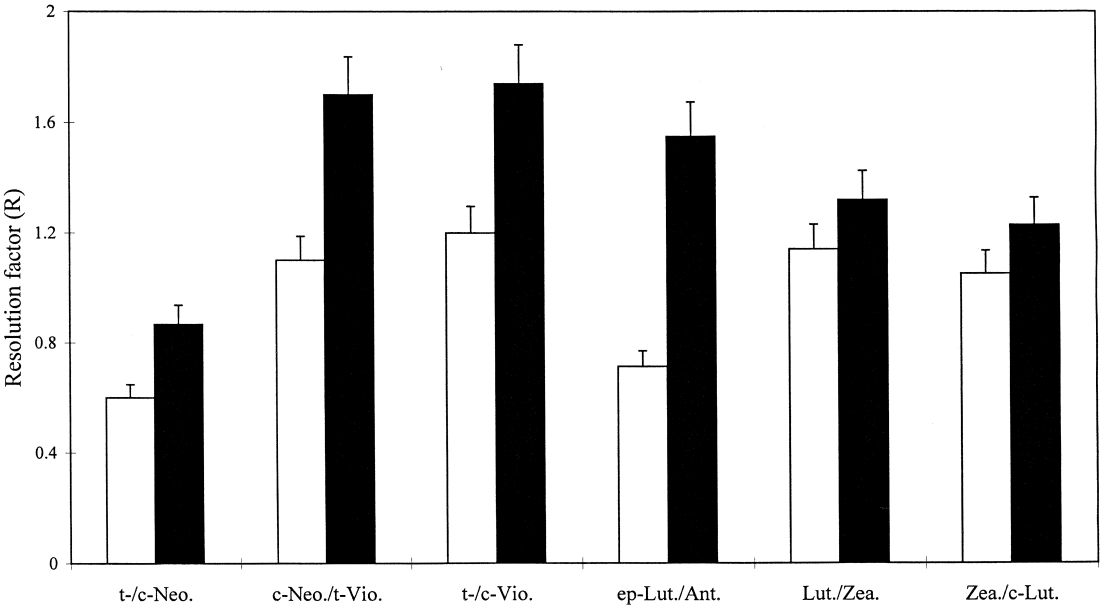


Fig. 2. Comparison of the resolution factors for the pigments eluted using the original (white bar) and present (dark bar) elution programmes. Ant = antheraxanthin; ep-Lut = Lutein-5,6,-epoxide; c-Lut = *cis*-lutein; Lut = lutein; c-Neo = *cis*-neoxanthin, t-Neo = *trans*-neoxanthin; c-Vio = *cis*-violaxanthin, t-Vio = *trans*-violaxanthin; Zea = zeaxanthin.

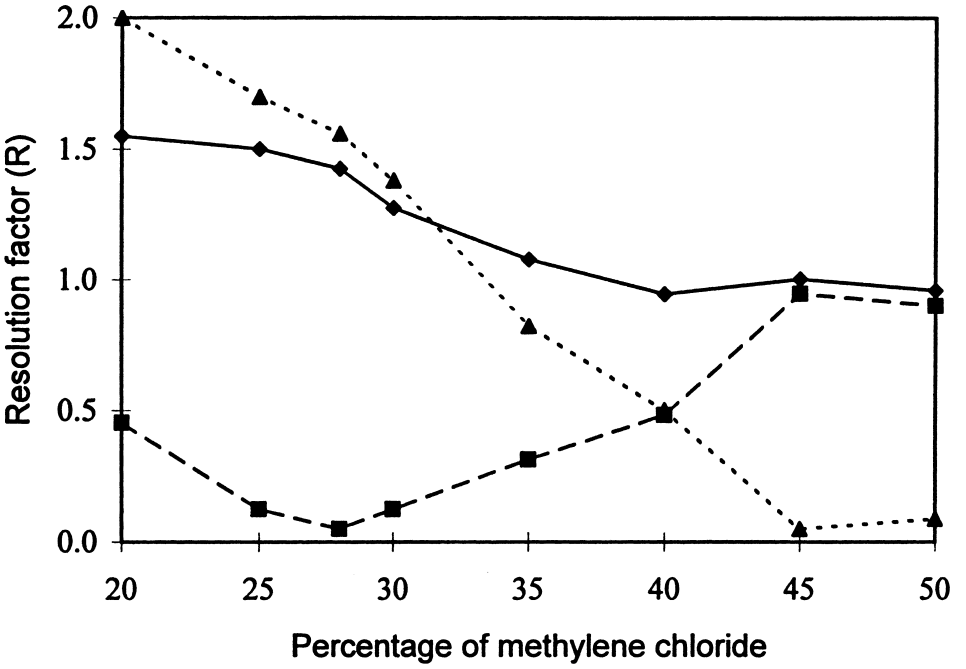


Fig. 3. Resolution factors of α-carotene and pheophytin *a* (close triangle), α-carotene and β-carotene (close losange), and β-carotene and pheophytin *a* (close squares) at different dichloromethane proportions.

and pheophytin *a* are compared in Fig. 4. Accordingly, pheophytin *a* can be quantitated on the basis of its absorbance in the red region (at 666 nm). When coelution occurs, quantification of the carotenes is not possible directly because of the overlapping of the absorption spectra of pheophytin *a* and the carotenes in the blue region. Therefore, we derived equations taking into account the contribution of pheophytin *a*. They should be used for accurate determination of α-carotene (Eq. (1)) and β-carotene (Eq. (2)) when they are coeluted with pheophytin *a*.

$$A_{\alpha\text{-carotene, 450 nm}} = A_{\text{measured, 450 nm}} - 0.040A_{\text{pheophytin } a\text{, 666 nm}} \quad (1)$$

$$A_{\beta\text{-carotene,457 nm}} = A_{\text{measured, 457 nm}} - 0.047A_{\text{pheophytin } a\text{, 666 nm}} \quad (2)$$

where $A$ is the area of the elution peaks corresponding to α-carotene, β-carotene and pheophytin *a* as measured at the indicated wavelengths.

In Eqs. (1) and (2), the constants are the ratio between pheophytin *a* measured at 450 nm or 457

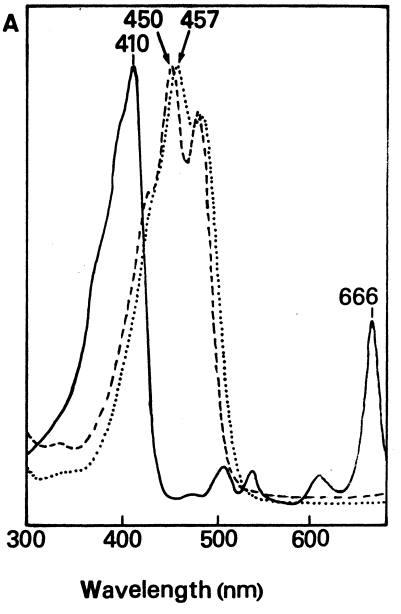


Fig. 4. Absorption spectra of the standards of α-carotene, β-carotene and pheophytin *a* using the elution programme with 45% dichloromethane.

nm and 666 nm in the adequate solvent mixture. Purified pheophytin *a* was prepared by TLC according to Ref. [24].

When photodiode array detection is not available, the quantification of the three pigments is not possible in a single run. In this case, we recommend the use of an elution programme ending with 28% dichloromethane since the absorbance of pheophytin *a* is minimum in the region of 450–460 nm, where the absorbance maxima of α-carotene and β-carotene are found (Fig. 4). Using photosystem II reaction center preparations, which only contain chlorophyll *a*, pheophytin *a* and β-carotene and where no α-carotene can be found, we propose the elution programme ending with 45% of dichloromethane, since under this condition, pheophytin *a* and β-carotene are fully separated. Alternatively, methods especially developed for such separations can be used (e.g., see Ref. [28]).

## 4. Conclusions

This paper describes an improved RPLC method for the determination of the photosynthetic pigments usually present in green plant material. The method is especially suitable for the separation of *cis*- and *trans*-carotenoid isomers, *trans*-lutein, zeaxanthin, *cis*-lutein. Accurate quantification of the not well separated non-polar pigments, α-carotene, β-carotene and pheophytin *a* is also discussed.

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