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Note

High-performance liquid chromatography of chlorophylls and carotenoids from vegetables

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The photosynthetic pigments, chlorophylls and carotenoids, belong to the group of isoprenoid plant lipids called prenyl lipids. Several procedures have been developed for the separation of these pigments, including paper (PC), column (CC), thin-layer (TLC) and more recently high-performance liquid chromatography (HPLC)[1]. The simultaneous separation and detection of chlorophylls and carotenoids have been achieved by HPLC with stepwise gradient elution systems on reversed-phase columns[2–7]. These systems, however, were either time consuming or very expensive. The isocratic separation of prenyl pigments by adsorption and reversed-phase HPLC have also been reported[8]. Apart from polar xanthophylls and β-carotene, only chlorophyll *a* and *b* could be separated. The chromatographic determination of chlorophyll degradation products required a second elution step with a stronger solvent mixture on another stationary phase.

There are many possibilities for increasing the efficiency of HPLC techniques for the separation of photosynthetic pigments under isocratic conditions, *e.g.* the use of a suitable chromatographic adsorbent and a mobile phase that increases the separability of a wide variety of pigments. The objective of this work was to devise an HPLC method for the simultaneous separation of chlorophylls and carotenoids and their derivatives on a C_{18} column under isocratic conditions.

EXPERIMENTAL

Preparation of pigments

Pigments of green vegetables were extracted with methanol and carbon tetrachloride as described previously[9]. Chlorophylls and pheophytins were prepared from raw, blanched and cooked spinach leaf extracts by TLC on cellulose sheets (MN-300; Merck, Darmstadt, F.R.G.) developed with two solvent systems[10].

Saponification of chlorophylls and pheophytins to chlorophyllids and pheophorbides was carried out by refluxing the extracted pigments for 10 min with 30 ml of 0.5% methanolic potassium hydroxide solution at the boiling point of methanol in the presence of ascorbic acid. It should be noted that saponification of chlorophylls by a concentrated alkaline solution leads to the formation of highly polar pigments that cannot be recovered by water-immiscible solvents. The saponified pigments were

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then extracted from methanol by shaking gently with benzene or chloroform. Following removal of the organic solvent under vacuum, the pigments were separated on a cellulose layer, developed with *n*-heptane–pyridine (7:3, v/v)[10].

Oxygen-containing carotenoids were separated and prepared on cellulose layers eluted with different mobile phases[11].

Identification of pigments

Chlorophyll and carotenoid-type pigments and their derivatives thus prepared were identified according to their R_F values obtained from TLC analysis and their visible absorption characteristics compared with those in the literature[4,6,12]. The identified pigments were scraped off the TLC plate and eluted with suitable solvents. After evaporating the solvent, the residues were dissolved in the minimum volume of the HPLC eluent and applied to the column for retention time measurement and to scan their absorption maxima between 200 and 700 nm (Table I). β-Carotene was identified and quantified by using the standard pigment (Sigma, St. Louis, MO, U.S.A.). Chlorophyllides and pheophorbides were further identified by comparison with authentic standards prepared by a method that included chlorophyllase-[13] and acetic acid-catalysed[14] reactions. The tentative identification of the oxidation products of chlorophylls (peaks X and Y in Table I) was achieved by special experiments

TABLE I

SPECTRA AND CHROMATOGRAPHIC PROPERTIES OF PIGMENTS ISOLATED FROM GREEN VEGETABLES AND SEPARATED BY HPLC

Eluent, acetonitrile–methanol–ethyl acetate (53:40:7); flow-rate, increased from 0.5 to 2 ml/min at the 22nd min of elution.

Peak No.	*Pigment*	$\lambda_{max.}$ *(nm)*	*Retention time (min)*	*Capacity factor (k')*	*Peak area* $(mm^2)^a$	*C.V.* *(%)*[b]
1	Chlorophyllid *b*	433, 660	4.27	0.58	2.2	1.6
2	Chlorophyllid *a*	408, 675	4.70	0.75	17.5	1.4
3	Neoxanthin	417, 443, 472	5.61	1.11	23.4	0.8
4	Violaxanthin	422, 447, 475	6.38	1.38	52.5	0.7
5	Lutein epoxide	417, 446, 477	7.15	1.66	16.3	1.2
6	Lutein	426, 453, 478	8.50	2.16	220.4	1.0
7	Neolutein	415, 439, 468	9.07	2.37	43.5	0.8
8	Chlorophyll *b*	462, 660	14.26	4.30	415.6	0.6
9	Chlorophyll *b'*	460, 660	14.86	4.50	46.7	1.2
10	Chlorophyll *a*	432, 668	20.61	6.51	1170.2	1.8
11	Chlorophyll *a'*	428, 667	22.30	7.28	230.8	2.3
12	Pheophytin *b*	436, 659	25.90	8.62	34.6	0.9
13	Pheophytin *a*	415, 669	29.05	9.70	9.2	1.5
14	β-Carotene	456, 477	30.38	10.29	96.4	0.4
15	Pyropheophytin *a*	414, 668	33.67	11.52	242.3	0.7
16	Unidentified	415, 669	34.75	13.91	46.8	0.9
X	Chlorophyll *b* oxidation product	460, 663	12.73	3.73	45.2	1.7
Y	Chlorophyll *a* oxidation product	430, 669	16.96	5.30	60.4	1.9

[a] Detection was carried out at 430 nm for carotenoids and 660 nm for chlorophylls.

[b] Coefficient of variation for six runs of the same sample.

including incubation of the extracted pigments with soybean lipoxygenase and linoleic acid as described previously[15].

High-performance liquid chromatography

The extracts (20 μl) were injected into a Beckman liquid chromatograph equipped with a Model 114 M solvent-delivery module pump, a Model 420 controller, a Model 340 organizer and a Model 165 variable-wavelength detector. The detector signals were electronically recorded with a Shimadzu Type GR3A integrator.

Separations were performed on a Chromsil C_{18} (10 μm spherical particles) stainless-steel column (25 cm × 4.6 mm I.D.) (Labor-MIM, Estergom, Hungary). The mobile phase was acetonitrile–methanol–ethyl acetate (53:40:7). The flow-rate was 0.75 ml/min at the beginning of run and then elevated to 2 ml/min as shown on the figures. Detection was carried out at different wavelengths.

Reagents

Acetonitrile, methanol and ethyl acetate were of HPLC grade (Reanal, Budapest, Hungary). They were used after redistillation, filtration and degassing. Benzene and chloroform were of technical grade and were used without purification.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of a methanol–carbon tetrachloride extract obtained from raw green pea. The mobile phase was acetonitrile–methanol–water (50:45:5) and the pigments were eluted at a flow-rate of 1 ml/min. This elution system gave a good separation of the polar components, which eluted first on a reversed-phase column, but failed to resolve the pigments of low polarity such as β-carotene and pheophytins, with retention times of less than 50 min. The appearance of broad peaks for β-carotene and pheophytins made their detection and determination difficult and unsatisfactory under the conditions used.

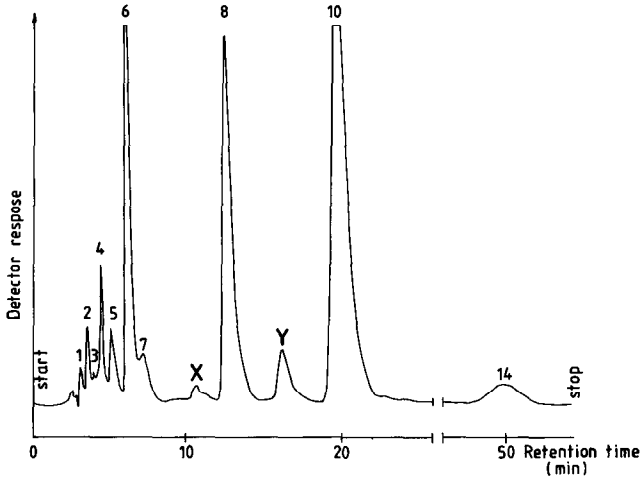

Fig. 1. HPLC profile of green pea extract on a Chromsil C_{18} column eluted with acetonitrile–methanol–water (50:45:5) at a flow-rate of 1 ml/min. Detection at 438 nm.

Addition of water to the elution mixture greatly extends the elution time[2]. Therefore, we attempted to improve the resolution of the apolar pigments by replacing water with ethyl acetate. This modification resulted in a satisfactory separation of most of the chlorophylls and carotenoids with symmetrical peaks, as shown in Fig. 2. Similar resolution profiles of some vegetable extracts have been obtained with gradient elution systems that take a long time for a complete run[2,4].

Another modification was the programmed increase in flow-rate, which shortened the retention time of the apolar pigments and increased their detectability even when they were present at relatively low concentrations.

Chlorophylls and their derivatives could be distinguished from other pigments by detecting the separated components at 650 nm (Fig. 3). Unesterified derivatives of chlorophylls such as chlorophyllides eluted first. The ratio of chlorophyllid *a* to *b* differs in the different vegetables. One-step separation and detection of such metabolites opens up a new possibility for using HPLC in the rapid analysis of enzyme- and non-enzyme-catalysed hydrolysis of chlorophylls to phytyl alcohol and unesterified derivatives.

As the coefficients of variation (C.V.) between runs for each chlorophyll- and carotenoid-type pigment did not exceed 2%, the method can be considered to be precise and accurate for the analysis of a wide variety of components (Table I). The relatively high C.V. for some xanthophylls, chlorophylls *a* and oxidation products indicate their susceptibility to degradation by oxidation and isomerization processes.

Detection of the separated pigments at different wavelengths indicated that 415–420 nm was the best range for the simultaneous detection of xanthophylls, carotenes, chlorophylls and pheophytins. The most familiar derivatives of chlorophylls

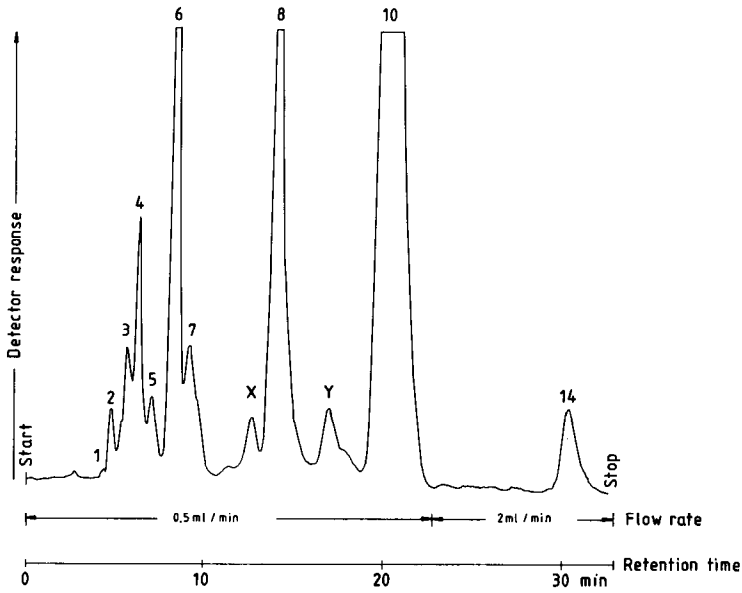

Fig. 2. HPLC profile of celery leaf extract on a Chromsil C_{18} column eluted with acetonitrile–methanol–ethyl acetate (53:40:7) with increase in flow-rate. Detection at 438 nm.

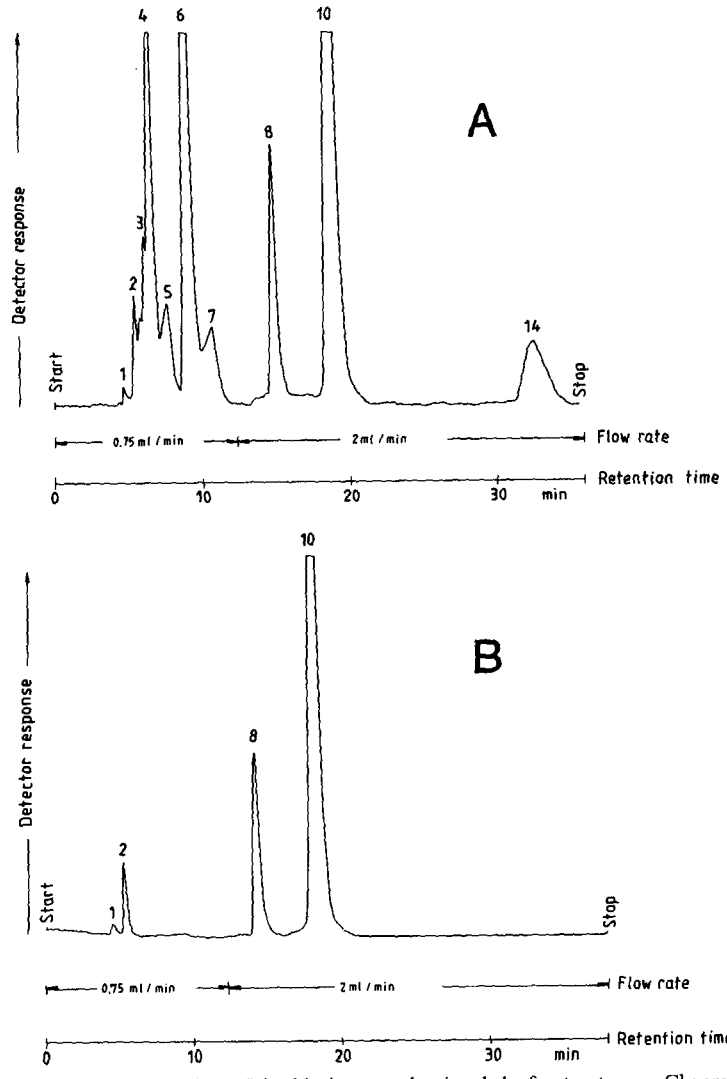

Fig. 3. HPLC profiles of freshly harvested spinach leaf extract on a Chromsil C_{18} column eluted with acetonitrile–methanol–ethyl acetate (53:40:7). Detection: (A) at 438 nm; (B) at 650 nm.

are pheophytins and pyropheophytins *a*. Their detection in the above range could be achieved even when they were present at relatively low concentrations. Shorter detection wavelengths have been reported for the chlorophylls, pheophytins and pheophorbides *a*[6,16].

Fig. 4 shows the chromatograms of chlorophylls and carotenoids in raw and blanched celery leaves. Bleaching for 2 min at 100°C resulted in the appearance of chlorophyll *a'* and *b'*, the C-10 epimers of chlorophyll *a* and *b*, respectively. These isomers had higher retention values than their parent pigments. Such HPLC behaviour has been observed for the same compounds extracted from raw blanched spinach[17].

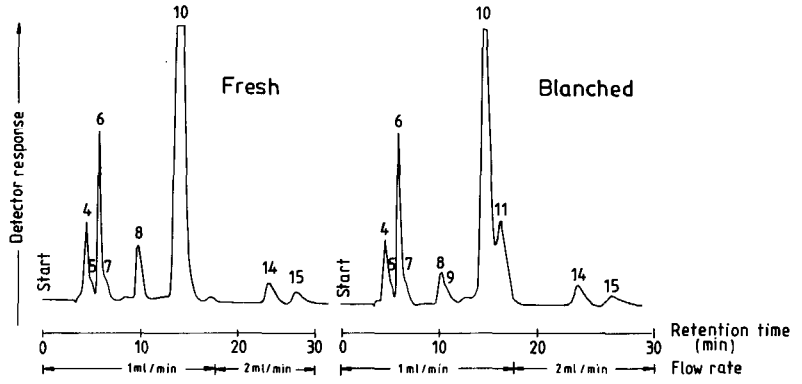

Fig. 4. Separation of chlorophylls and carotenoids of fresh and blanched celery leaves on a Chromsil C_{18} column eluted with acetonitrile–methanol–ethyl acetate (53:40:7). Detection at 415 nm.

Cooking of vegetables with brine or an acidic solution leads tot the formation of pheophytins from chlorophylls. As chlorophyll *a* is more susceptible to degradation than chlorophyll *b*, the extract of cooked green pea distributed mostly pyropheophytin a (Fig. 5). The magnesium-free derivatives were eluted with retention values around that of β-carotene without overlapping. It should be mentioned that the chromatograms were monitored at 650 nm to exclude the carotenoid pigments while assisting in detecting the chlorophylls and their derivatives.

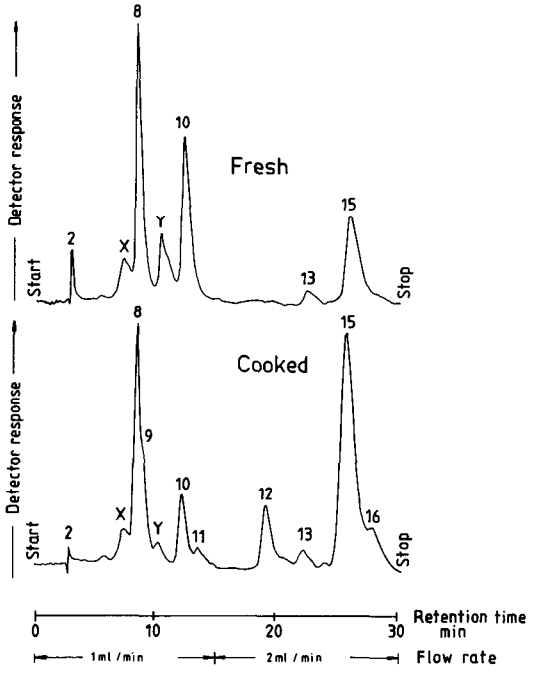

Fig. 5. Separation of chlorphyll-type pigments of fresh and cooked green pea on a Chromsil C_{18} column eluted with acetonitrile–methanol–ethyl acetate (53:40:7). Detection at 650 nm.

TABLE II

β-CAROTENE CONTENTS (mg PER 100 g OF EDIBLE FOOD) OF VEGETABLES DETERMINED BY HPLC

Vegetable	*No. of determinations*	*β-carotene (mean ± S.D.)*	*C.V. (%)*
Green pea (Jof)	4	0.22 ± 0.01	5.0
Green pepper:			
Bell-boy	5	0.88 ± 0.03	3.4
Szegedi-20	5	1.44 ± 0.04	2.8
Angeli emlike	5	1.56 ± 0.07	4.4
Cucumber (Aminex F-1)	4	1.15 ± 0.03	2.6
Lettuce (local)	6	3.12 ± 0.16	5.1
Spinach (local)	6	6.11 ± 0.20	3.3
Celery (local)	6	15.45 ± 0.62	4

Table II shows the determination of β-carotene in vegetables. The results indicate that the β-carotene content of different vegetables varies over a fairly wide range, depending on variations attributable to cultivars and growing location. It should be mentioned that the relatively high content of β-carotene in celery leaves indicates the high nutritive value of this product. The quantitative distribution of xanthophylls, chlorophylls and carotenes in several raw and processed vegetables will be described in another paper.

CONCLUSION

The simultaneous separation and detection of the major carotenoids, chlorophylls, and magnesium- and phytol-free derivatives of chlorophylls was achieved under isocratic conditions with an elution time of less than 35 min by using a Chromsil C_{18} column with acetonitrile–methanol–ethyl acetate (53:40:7) as the eluent with a stepwise increase in flow-rate. This method is simple and applicable to the determination of a wide variety of photosynthetic pigments.

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