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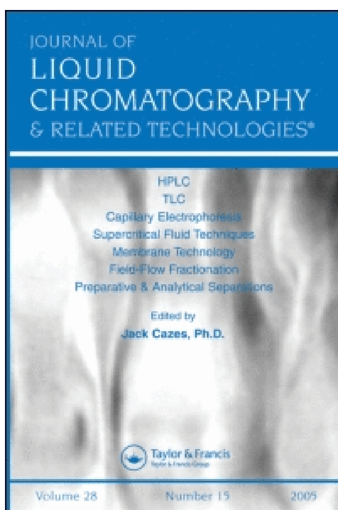

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
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LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF CHLOROPHYLLS, CAROTENOIDS, AND THEIR DERIVATIVES IN FRESH AND PROCESSED VEGETABLES

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Online publication date: 30 May 2002

To cite this Article Gokmen, V. , Bahçeci, S. and Acar, J.(2005) 'LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF CHLOROPHYLLS, CAROTENOIDS, AND THEIR DERIVATIVES IN FRESH AND PROCESSED VEGETABLES', Journal of Liquid Chromatography & Related Technologies, 25: 8, 1201 — 1213

To link to this Article: DOI: 10.1081/JLC-120004019
URL: http://dx.doi.org/10.1081/JLC-120004019

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J. LIQ. CHROM. & REL. TECHNOL., 25(8), 1201–1213 (2002)

LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF CHLOROPHYLLS, CAROTENOIDS, AND THEIR DERIVATIVES IN FRESH AND PROCESSED VEGETABLES

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ABSTRACT

A high-performance liquid chromatographic method has been developed to quantify major chlorophylls and carotenoids in some raw and processed vegetables. Vegetable samples were simultaneously homogenized and extracted with methanol in a homogenizer in two steps. A sample to solvent ratio of 1 : 10 (w/v) was sufficient for a complete extraction of pigments from pea. The extraction yields were determined to be 79.1% and 20.9% for the first and the second extraction steps, respectively. A MikroPak C8 reversed-phase column was used for the separation of chlorophylls and carotenoids in an elution time of 20 min using gradient mixture of methanol : water at a flow rate of 0.75 mL/min. A good separation of chlorophyll a (Chl a) and

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chlorophyll b (Chl b), and of their major derivatives, was achieved. The method has also been shown to be applicable for the separation of yellow (α- and β-carotenes) and orange (xanthophylls) carotenoids in some vegetables. The method was shown to be useful to quantify Chl a and Chl b, and of their Mg-free derivatives of Pheo a and Pheo b, in some fresh and processed peas, as well as to quantify β-carotene in carrots.

INTRODUCTION

Chlorophyll a (Chl a) and chlorophyll b (Chl b) are the major pigments of green vegetables. It has been well established that chlorophylls are susceptible to chemical and physical changes during processing of vegetables. For instance, the bright green appearance of vegetables can be converted to dull olive color as a result of heat treatment. Since color is one of the most important quality attributes of vegetable products, numerous studies have been conducted to investigate the color changes or degradation of chlorophylls during heating. The chlorophyll derivatives, including chlorophyll epimers, chlorophyllides, pheophytins, and pyropheophytins were reported to occur during cooking of vegetables.

The analysis of chlorophylls and their derivatives has been the subject of several investigations. Through the years, a variety of chromatographic techniques including paper chromatography, thin-layer chromatography, adsorption chromatography, and high-performance liquid chromatography have been employed to separate chlorophylls and their derivatives.[1] The introduction of adsorption chromatography for the analysis of leaf pigments allowed the separation of β-carotene, Chl a and Chl b from xanthophylls.[2] Although both normal and reversed phase have been developed, reversed-phase liquid chromatography using C18 packing material has been the method of choice for the analysis of chloroplastic pigments.[3–8] Although several methods for the separation of chlorophylls or carotenoids have been reported, only a few can separate carotenoids from chlorophylls in the same run.[9–12] In these cases, however, pigment resolution is sometimes not good enough to allow accurate quantifications.

This paper describes an improved HPLC procedure that is quick and simple and can separate chlorophylls and their derivatives, as well as some carotenoids, in a single run. The separations were performed using a reversed phase C8 column with gradient elution. The method was found to be applicable for the separation and quantitation of chlorophylls, chlorophyll derivatives, and carotenoids in fresh and processed vegetables with good reproducibility.

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EXPERIMENTAL

Materials

Both chlorophylls a and b standards were purchased from Sigma, and β-carotene from Merck. Stock solutions of Chl a and Chl b were prepared by dissolving 1 mg pure chlorophyll in 250 µL of methanol. Working solutions were prepared by diluting with methanol to final concentrations of 1, 5, 10, 25, 50, and 100 µg/mL. All solvents used were HPLC grade and were from Merck.

Pheophytin a (Phe a) and pheophytin b (Phe b) were prepared by adding 50 µL of 0.1 N HCl to 900 µL methanolic solutions of Chl a and Chl b. These solutions were then stabilized by adding 50 µL of 0.1 N NaOH. C-10 epimers of Chl a and Chl b were formed in vegetable samples by blanching in boiling water for 5 min. The canned pea samples obtained from local sources were used to identify "pyro" derivatives of chlorophylls.

Stock solution of β-carotene was prepared by dissolving 100 mg pure standard in 100 mL of tetrahydrofuran (THF) stabilized with butylatedhydroxy-toluene (BHT). Working solutions were prepared by diluting with THF to final concentrations of 1, 5, 10, 25, 50, and 100 µg/mL.

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Instrumentation

The HPLC instrument consisted of a Varian 9010 solvent delivery system, a Rheodyne 7125 six-way injector with a 10 µL sample loop, a stainless steel column (150 × 4.6 mm i.d.) packed with C8 MikroPak 5 µm particle size, and a Hewlett-Packard 1040A photodiode array detector. HP ChemStation for LC software (rev. A05.01) was used for data processing.

Extraction of Chlorophylls and Their Derivatives

Approximately 25 g of vegetable sample was chopped into ground pieces and the dry weights were measured by placing 5 g vegetable in a hot oven at 105°C for 5 h. 1–5 g of sample was weighed into a homogenizer cup that contained 0.1 g $CaCO_3$ and 1 mL of 1% BHT to stabilize pigments against tissue acids and oxidation, respectively. 25 mL of methanol was added and the mixture was simultaneously homogenized and extracted in a Virtis homogenizer at medium speed for 2 min. After tissue is thoroughly disintegrated, the extract was filtered through a black band filter paper into a 50 mL volumetric flask. The residue and filter paper were returned to the homogenizer cup with additional 25 mL portion of methanol, and the extraction was repeated. The final residue was

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washed with methanol to remove the traces of pigment. The filtrates were combined and completed to volume with methanol. The extraction and filtration steps were carried under low light intensity and at low temperature (+4°C), respectively, to avoid pigment degradations. The methanol extract was kept in a deep freezer (−18°C) until HPLC analysis. The stability of Chl a and Chl b in methanol extract was verified by comparing them with the methanolic standards of Chl a and Chl b kept under the same conditions. The extract was filtered through a 0.45 μm membrane filter and 10 μL was injected into the HPLC column.

Analysis of Chlorophylls and Their Derivatives by HPLC

A solvent system of methanol/water was used to separate chlorophylls and carotenoids and their derivatives. Elution was carried out at room temperature and utilized solvent A as methanol and solvent B as water. The elution program was, at a flow rate of 0.75 mL/min (backpressure of 65 atm), 90% solvent A isocratic for 5 min followed by a 90–95% linear gradient for 5 min with solvent A and holding with 95% solvent A for 5 min, and finally followed by a 95–90% linear gradient with solvent B for 5 min. Chromatograms were recorded simultaneously at 432, 450, 470, 652, and 666 nm, with spectra (400–700 nm) taken continuously throughout the elution.

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RESULTS AND DISCUSSION

Separation of Chlorophylls on C8 Column

A typical chromatogram depicting the separation of Chl a and Chl b and their derivatives, as well as carotenoids of pea, in a C8 column by employing a binary solvent system consisting of methanol and water at a flow rate of 0.75 mL/min, is shown in Figure 1. This chromatogram was recorded after the acidification of methanolic extract of blanched pea with 0.1 N HCl to form Phe a and Phe b and their epimers a′ and b′. The acidified mixture was then neutralized with 0.1 N NaOH and mixed with an equal volume of original methanolic extract. Spectral examination of the chromatogram has shown that a total of 18 chlorophyll derivatives were successfully resolved in an elution time of 20 min. The b form of both chlorophylls and pheophytins eluted faster than those of the a form. Peaks 14, 18, 20, and 23, as shown in Figure 1, were identified as Chl b, Chl a, Phe b, and Phe a, respectively, comparing their retention and spectral data to those of pure standards. Since the epimers were less polar then their parent peaks, the peaks eluted slightly after chlorophylls (peaks 15 and 19) and

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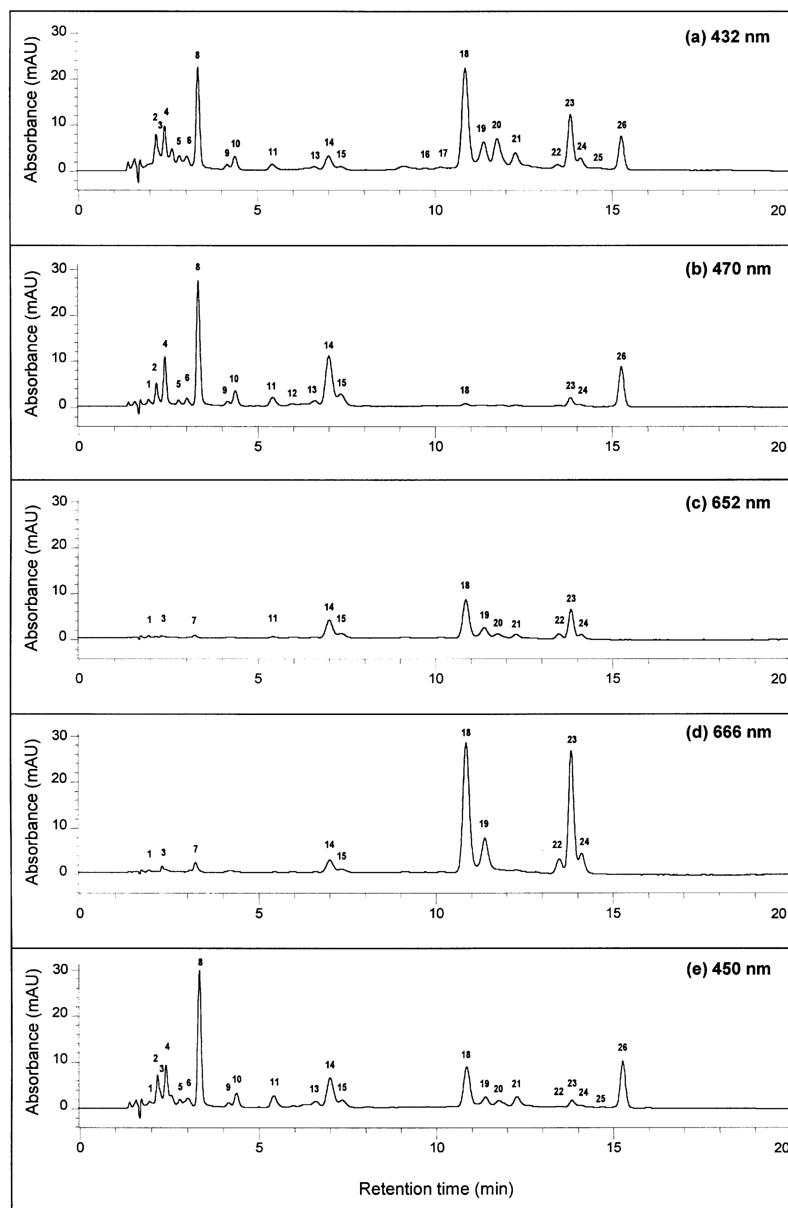

Figure 1. High performance liquid chromatogram of chlorophylls and carotenoids in a pea extract monitored at (a) 432 nm, (b) 470 nm, (c) 652 nm, (d) 666 nm, and (e) 450 nm. See Table 1 for component identification.

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pheophytins (peaks 21 and 24) and were tentatively identified as their corresponding epimers. The spectral characteristics of the peaks assigned as epimers were found to be very similar to their parent peaks. Further efforts are needed to identify other peaks separated in reversed phase C8 columns and assigned as unidentified chlorophyll derivatives in Table 1.

The use of reversed phase C8 columns for the separation of chlorophylls and carotenoids was previously reported.[13,14] However, the isocratic mixture of methanol and water used as the eluent, did not sufficiently resolve early eluting xanthophylls and resulted in band broadening for lately eluting Chl a and Chl b, and β-carotene.

Most of the studies have attempted to use C18 packed columns for the separation of chlorophylls and their derivatives.[6,7,15] However, the separation of certain chlorophyll derivatives from the isomers of carotenoids is still questionable, even if complex ternary or quaternary solvent systems are used.[8] The usual approximation is to use the detection wavelengths that interfering carotenoids do not absorb.[16]

The chromatographic conditions applied here also allowed the separation of yellow (α- and β-carotenes) and orange (xanthophylls) carotenoids in a single run. Since green plants have a high proportion of chlorophylls to carotenoids, the sensitivity of the detection of chlorophylls must be greatly reduced to attain baseline HPLC separation and accurate quantification of carotenoids. When the detection wavelength switched to a range between 430–470 nm, carotenoids in fresh or processed vegetables can be simultaneously identified and quantified together with chlorophylls and their derivatives. Here, the chromatographic separation of carotenoids present in pea extract was depicted in Figure 1e. In this chromatogram, a total of 8 peaks (peak numbers are identical as given in Table 1) were assigned as carotenoids. Only β-carotene was identified comparing its retention and spectral data to that of pure standard. Other carotenoids were tentatively identified as xanthophylls (Table 1), comparing their spectral maxima reported in similar eluent systems by Braumann and Grimme.[17] Here, it is interesting to see a complete resolution of nonpolar β-carotene from Phe a that usually partially coelutes.[8]

The method was also shown to be applicable for the separation and quantitation of β-carotene in fresh carrot (Figure 2). In this case, however, carotenoids were extracted with stabilized THF from carrot, since methanol was not able to extract carrot pigments completely.

Detection Sensitivity of Chlorophylls

Both Chl a and Chl b had two absorption maxima in blue and red regions, as recorded in the mobile phase composition. Their derivatives also exhibited the

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Table 1. Spectral Identification Data for Chlorophylls and Carotenoids of Pea Extract Depicted in Figure 2

Peak No	Component Identification	Retention Time, min	Capacity Factor (k′)	Spectral Maxima, nm[a] This Work (Mobile Phase)	Spectral Maxima, nm[a] Literature[b] (85–95% Methanol)
1	Unidentified Chl der.	1.95	0.40	470, 652	
2	Unidentified carotenoid	2.17	0.56	424, 442, 468	
3	Unidentified Chl der.	2.30	0.65	428, 666	
4	Neoxanthin	2.41	0.73	416, 438, 468	415, 437, 466
5	Antheraxanthin	2.79	1.01	420, 444, 472	421, 447, 473
6	Violaxanthin	3.02	1.16	416, 436, 464	414, 436, 465
7	Unidentified Chl	3.23	1.32	408, 666	
8	Lutein	3.34	1.39	422, 444, 472	421, 445, 473
9	Unidentified carotenoid	4.13	1.98	417, 440, 468	
10	Lutein5,6-epoxide	4.37	2.14	416, 440, 468	417, 440, 469
11	Unidentified Chl der.	5.41	2.89	458, 632	
12	Unidentified Chl der.	5.96	3.29	470, 652	
13	Unidentified Chl der.	6.61	3.76	458, 632	
14	Chlorophyll b	7.00	4.04	470, 652, 666	
15	Chlorophyll b′	7.34	4.28	470, 652, 666	

(*continued*)

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Table 1. Continued

				Spectral Maxima, nm[a]	
Peak No	Component Identification	Retention Time, min	Capacity Factor (k′)	This Work (Mobile Phase)	Literature[b] (85–95% Methanol)
16	Unidentified Chl der.	9.23	5.64	428, 652	
17	Unidentified Chl der.	10.19	6.33	418, 652	
18	Chlorophyll a	10.85	6.81	432, 666	
19	Chlorophyll a′	11.38	7.19	432, 666	
20	Pheophytin b	11.75	7.45	428, 652, 666	
21	Pheophytin b′	12.27	7.83	428, 652, 666	
22	Unidentified Chl der.	13.48	8.70	400, 666	
23	Pheophytin a	13.85	8.96	408, 666	
24	Pheophytin a′	14.11	9.15	408, 666	
25	Unidentified Chl der.	14.57	9.48	408, 666	
26	ß-carotene	15.26	9.98	426, 450, 476	436, 451, 478
	Pyropheophytin a[c]	15.34	10.04	412, 666	

[a]Spectral data used to identify xanthophylls.
[b]See Ref. (17).
[c]Illustrated in the canned pea chromatogram (Figure 3c).

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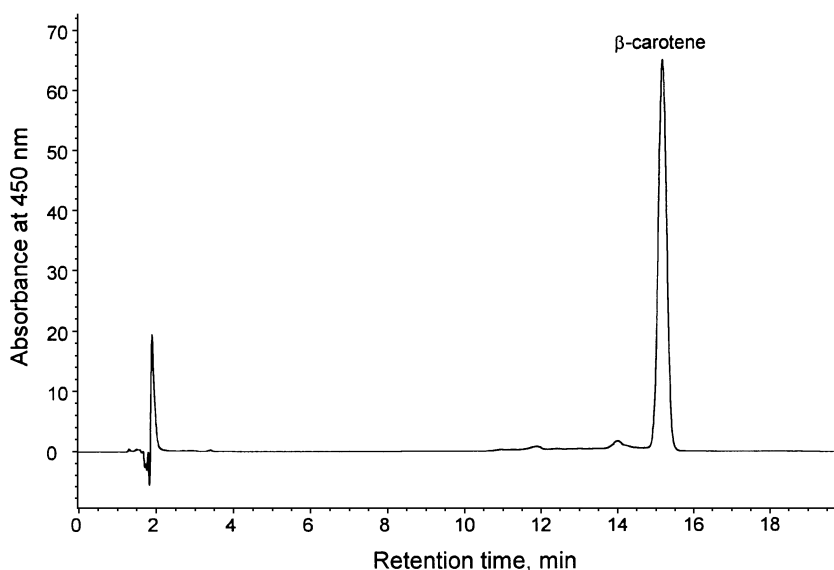

Figure 2. High performance liquid chromatogram of carotenoids in carrot extract.

same characteristic spectra differing from each other only in the shape of absorption spectra. Chl a and Chl a′ had maximum absorption at 432 and 666 nm. Likewise, Chl b and Chl b′ had maximum absorption at 470 and 652 nm. Absorption maxima of Phe a and Phe b in the blue region shifted back to 408 and 428 nm, respectively. However, absorption maxima in the red region remained unchanged for all derivatives. Light absorption values of chlorophylls in blue regions were relatively greater than in red regions. Relative sensitivities of Chl a at detection wavelengths of 432 and 666 nm were 1.0 and 0.97, respectively. Likewise, relative sensitivities of Chl b at detection wavelengths of 470, 652, and 666 nm were 1.0, 0.34, and 0.24, respectively. In this study, the chromatograms recorded at 432 and 470 nm were used to quantify Chl a and Chl b, respectively. The detector response (y) of the a form at 432 nm, over a concentration (x) range of 1 to 100 μg/mL, was linear ($y = 53.189x - 24.269$) with a regression coefficient (r^2) of 0.999. Similarly, the detector response (y) of the b form at 470 nm, over a concentration (x) range of 1 to 100 μg/mL, was also linear ($y = 59.613x + 14.982$) with a regression coefficient (r^2) of 0.9997. Day-to-day variations in the retention times of both forms were found to be less than 1%.

The detection wavelength alone can bring specificity to the method. Monitoring chromatograms at wavelengths between 650–670 nm, for instance 666, may exclude the yellow pigments while enabling the detection of the

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chlorophylls and their Mg-free and phytol-free derivatives. However, the method may fail to detect low amounts of certain forms and derivatives because of loss of detection sensitivity in this region.

Extraction Efficiency

The pigments were extracted with 2×25 mL of methanol from ca. 5 g of pea. The use of $CaCO_3$ and BHT during extraction ensured the stability of pigments against tissue acids and oxidation, respectively. The extraction yields of the first and second steps were 79.1% and 20.9%, respectively. Sample-to-solvent ratio (w/v) of 1 : 10 was found sufficient for a complete extraction of pigments from fresh pea. This ratio must be changed to 1 : 50 (w/v) for the extraction of chlorophylls from fresh spinach.

Polar solvent molecules, such as methanol, interact with chlorophyll molecules causing allomerization and epimerization. A mixture of methanol and petroleum ether has been used to extract chlorophylls because of the ability of petroleum ether to prevent second order reactions of hydrolysis, allomerization, and/or oxidation of pigment.[1] In this study, the stability of methanol extract kept at −18°C was verified by comparing it with methanolic standards of Chl a and Chl b. No significant change was observed in both standard solutions and methanol extracts at −18°C within 2 weeks, which is the longest period taken from extraction to chromatographic analysis.

Chlorophyll Profiles of Fresh and Processed Peas

Fresh peas were found to contain only Chl a and Chl b (Figure 3a). The average concentrations of Chl a and Chl b in fresh peas were 0.96 mg/g and 0.50 mg/g on a dry weight basis, respectively. Blanching peas in boiling water indicated the appearance of Chl a′ and Chl b′, the C-10 epimers of Chl a and Chl b, respectively (Figure 3b). Similar results have been previously published by various researchers for some kinds of green vegetables.[6,7,14,18–20] Here, blanching peas in boiling water for 5 min resulted in a 15% and 8% degradation of Chl a and Chl b, respectively. The major degradation product of the b form was of its epimer, while the a form degraded into both its epimer and pheophytin (Figure 3b). Several studies have shown that the a form degraded faster than the b form.[14,21,22] The configuration of the epimers allows greater interaction with the C8 stationary phase, and, therefore, the epimers have slightly greater retention values relative to their parent compounds. The formation of the isomers does not bring about any change in the color of the green vegetable, since the light absorption properties of the epimers are identical with those of their parent

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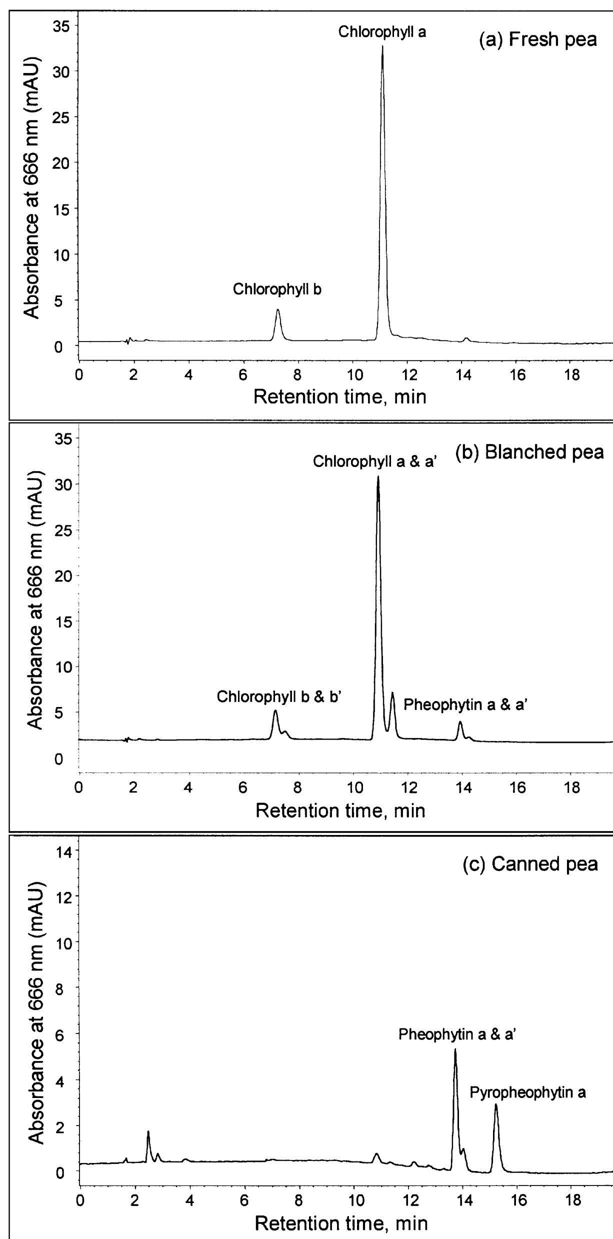

Figure 3. High performance liquid chromatogram of chlorophylls in (a) fresh pea, (b) blanched pea, and (c) canned pea.

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pigments. The most prevalent derivative pathway of the pigments during processing of vegetables is the conversion of the bright green color to dull olive pheophytins.[23] This occurs because of the loss of the central Mg atom, and it is well known that acidic conditions enhance their formation.

The chromatogram of canned peas shows complete destruction of Chl a and Chl b into corresponding pheophytins (Figure 3c). In Figure 3c, the shoulder peak obtained on Phe a is assumed to be its epimer a′. The other major peak was also assumed to be pyropheophytin a, with retention time of 15.34 min. The presence of these compounds in canned spinach has been previously reported.[6] The "pyro" derivatives are formed from the corresponding pheophytins by the loss of the C-10 carbomethoxy group ($-CO_2CH_3$). Removal of the $-CO_2CH_3$ group reduces the polarity of the pheophytin compound and, therefore, a greater retention time on the C8 reversed-phase column would be expected. Furthermore, since the loss of a C-10 chiral center occurred in the formation of pyropheophytins, no epimers are anticipated. This explains the absence of shoulder in the pyropheophytin, a peak compared to the pheophytin peak in which shoulder is assumed to be the respective epimer (Figure 3c).

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CONCLUSION

This paper describes an improved HPLC method using reversed phase C8 columns for the separation and quantitation of chlorophylls and of their major derivatives present in fresh and processed vegetables. Also, the conditions used in this study allow the separation and quantitation of certain carotenoids in a single chromatographic run. The method seems to be useful for separation and accurate quantification of nonpolar pigments, β-carotene and Phe a, that usually coelute.

ACKNOWLEDGMENT

The authors would like to thank The Scientific and Technical Research Council of Turkey (TÜBI TAK), Agriculture, Forestry and Food Technologies Research Grant Committee (TOGTAG) for financial support to this research project (Project no. TOGTAG 2633).

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Received December 20, 2001
Accepted January 20, 2002
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