Separation of Chlorophyll Compounds and Their Polar Derivatives by High-Performance Liquid Chromatography†

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Chlorophyllides *a* and *b* were produced by incubation of spinach (*Spinacia oleracea*, cv. Melody) leaves in hot water (65 °C for 30 min). Chlorophylls and chlorophyllides *a* and *b* and their degradation products were separated and identified by using a high-performance liquid chromatographic (HPLC) technique including a gradient solvent system and a reversed-phase C-18 column or a normal-phase separation with a silica column. Of the two separation methods, the reversed-phase technique was rapid (25–30 min) and capable of resolving 12 different chlorophyll derivatives. Peak identification was based on ultraviolet–visible absorption spectra by using a photodiode array detector and confirmed by using fast atom bombardment mass spectrometry.

INTRODUCTION

The separation of chlorophylls and their derivatives (C-10 epimers, pheophytins, pyropheophytins, chlorophyllides, pheophorbides, and pyropheophorbides) has been studied extensively (Strain and Svec, 1969; Strain and Sherma, 1972; Hager, 1957; Perkins and Roberts, 1962; Jones, 1963; Bacon, 1965). The decomposition of chlorophylls in foods and analysis of chlorophyll derivatives have also been the subject of numerous investigations because of the importance of these pigments in the maintenance and quality of color in fruit and vegetable products.

Mild heat treatments of vegetables induce the formation of chlorophyll C-10 epimers (prime derivatives). During prolonged heating chlorophylls degrade to olive-colored pheophytins and pyropheophytins. A variety of chromatographic techniques for analyzing these compounds have been used. In recent years HPLC techniques have been of paramount importance in the separation of these photosynthetic pigments and their derivatives (Evans et al., 1975; Eskins et al., 1977; Jacobsen, 1978; Shoaf, 1978; Rebeiz et al., 1978; Iriyama et al., 1978; Schwartz et al., 1981; Watanabe et al., 1984). HPLC methods permit rapid separation with high resolution and detection sensitivity. An added advantage is also the ability to collect samples as they are eluted from the chromatographic column (Schwartz and Lorenzo, 1990).

Willstatter and Stoll (1928) studied the production of the water-soluble chlorophyllides from chlorophylls in plant tissue by activation of existing chlorophyllase enzyme without prior purification. Weast and Mackinney (1940) studied different treatments and showed that the hydrolysis of fresh spinach leaf chlorophylls in hot water had an optimum temperature of 75 °C (95% of the chlorophyll was hydrolyzed) or of 50 °C if the leaves had been previously frozen (90% of the substrate was hydrolyzed). Incubation of the leaves in acetone was also studied, concluding that at higher acetone concentration lower incubation temperatures were required (25 °C). These results indicated that the substrate must first be available before the enzyme can become active. Further work on this enzyme in plant tissues has not been forthcoming partly because of the need for a simple rapid method to monitor and measure the production of polar chlorophyll compounds.

Both normal- and reversed-phase methods have been developed for the analysis of chlorophylls and some of their derivatives (Yoshiura et al., 1978; Watanabe et al., 1984; Shoaf, 1978; Schoche et al., 1978; Braumann and Grimme, 1979; Abaychi and Riley, 1979). Most successfully reversed phase modes have been widely used with an octadecyl-bonded stationary phase (Suzuki et al., 1987). Schwartz et al. (1981) used a reversed-phase C-18 column to separate chlorophylls and derivatives including the pyro forms. Few techniques have been developed to separate the polar chlorophyllides from chlorophylls. Shioi et al. (1983, 1984) and Bidigare et al. (1985) also used a reversed-phase column for this separation. However, further developments in HPLC methodology are needed to rapidly resolve all the chlorophyll compounds as well as their polar derivatives from a single sample extract. Methodology for this analysis has been hampered by difficulty in producing detectable quantities of the polar chlorophyll derivatives (chlorophyllides, pheophorbides and pyropheophorbides) in biological tissues.

The objective of this study was to develop a rapid and sensitive HPLC method to separate and identify both *a* and *b* types of chlorophylls, chlorophyllides, pheophorbides, pyropheophorbides, pheophytins, and pyropheophytins.

MATERIALS AND METHODS

Preparation of Sample. Fresh spinach leaves (*Spinacia oleracea*, cv. Melody) from local markets were used. The spinach was washed, drained, and chopped (approximately 1 cm^2). Thirty grams of chopped leaves was incubated in either 70 g of water or 70 g of acetone. Incubation was performed at various temperatures (30–65 °C) and times (20–120 min) in a water bath (Haake A81, Berlin, West Germany) to activate the enzyme chlorophyllase which is present naturally in spinach leaves. After incubation, the leaves were cooled to room temperature (using fresh water at room temperature), the mixture was blended for 4 min, and the puree was deaerated under reduced pressure.

Extraction of Pigments. Five grams of spinach puree was weighed into a 25-mL Erlenmeyer flask, and 18.8 mL of acetone was added. The mixture was homogenized in a Tekmar tissumizer, Model TR-10Z (Tekmar Co., Cincinnati, OH) for 2 min and filtered through Whatman No. 1 and No. 42 filter papers and brought to volume in a 25-mL volumetric flask. Before the sample was injected onto the HPLC system, the extract was

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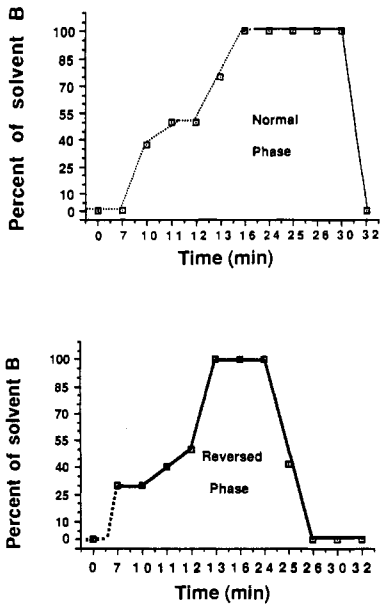

Figure 1. Normal- and reversed-phase gradient elution profiles. Normal-phase flow rate (- - -) 1.6 mL/min; (—) 2.5 mL/min. Reversed-phase flow rate (- - -) 1.3 mL/min; (—) 1.5 mL/min.

filtered through a 3-mm diameter, 0.45-μm syringe nylon filter unit (Micron Separations Inc., Westboro, MA).

Preparation of Various Chlorophyll Derivatives. Chlorophylls *a* and *b* were obtained from 10 g of fresh spinach leaves after extraction with 36 mL of acetone and brought to volume in a 50-mL volumetric flask. Pheophytins *a* and *b* were prepared by acidification of the chlorophyll extract (Schwartz et al., 1981). Hydrochloric acid (1 N) was added dropwise to 15 mL of the chlorophyll extract in a test tube, and the solution was stirred after each addition. The conversion was reached once the color of the extract changed completely from green to olive brown. Ethyl ether was used to separate the pheophytins from the aqueous layer (pheophytins are soluble in the organic layer). The excess acid was removed with 5 mL of water (washing four or five times). The organic layer was dried over anhydrous sodium sulfate, the ethyl ether was removed under a stream of N_2, and the pheophytins were dissolved in acetone. Pyropheophytins were extracted from spinach puree that had been heat processed at 145 °C for 7 min.

Chlorophyllides were obtained after incubation of fresh spinach leaves (30 g) in 70 mL of hot water (65 °C for 30 min), followed by extraction as outlined above. Pheophorbides were prepared from the chlorophyllide extract by acidification with HCl using the same procedure utilized to form pheophytins.

HPLC Apparatus. An HPLC system consisting of U6K injector and two Model 510 pumps controlled by a Model 680 gradient controller (Waters Associates, Milford, MA) was used. Both a Waters Model 440 detector and a Linear UVIS Model 203 detector (Anspec Co., Inc., Ann Arbor, MI) were used to detect the peaks at 658 nm.

Normal-Phase Separation. A silica column 4.6 mm i.d. × 250 mm (MacMod Analytical Inc., Chadds Ford, PA) was used with a gradient system of hexane/2-propanol with the following proportions: solvent A, 98.3/1.7, and solvent B, 75/25 (v/v). Initial conditions consisted of 100% of solvent A (flow rate, 1.6 mL/min) followed by the gradient profile illustrated in Figure 1.

Reversed-Phase Separation. Separation of chlorophyll derivatives was achieved on an octadecylsilane column (C-18), 5 μm particles 4.6 mm i.d. × 250 mm (MacMod Analytical). The solvent system used was ethyl acetate/methanol/water with the following proportions: solvent A, 15/65/20, and solvent B, 60/30/10 (v/v/v). Initial conditions consisted of 100% solvent A at a flow rate of 1.3 mL/min. The gradient was applied after 6 min under isocratic conditions as shown in Figure 1. Final conditions consisted of 100% solvent B at a flow rate of 1.5 mL/min. After 10 mins, the solvent composition was returned to the initial proportions.

Peaks Area Calculation. Chromatographic data were collected on an Apple II+ computer equipped with an ADALAB data acquisition card and CHROMATOCHART, chromatographic software for the integration of the peak areas (Interactive Microware, State College, PA).

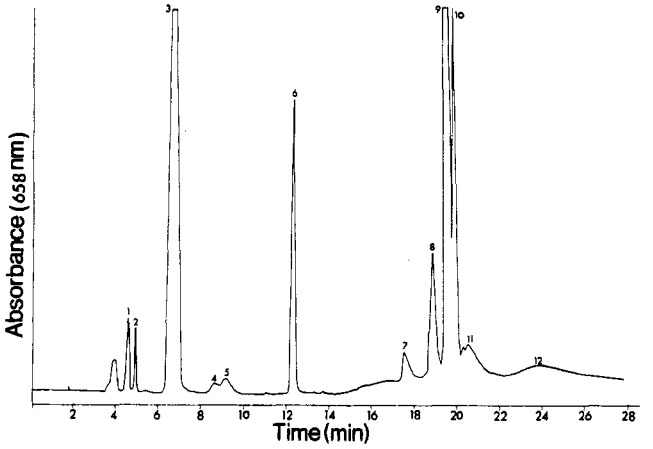

Figure 2. Chromatographic separation of a mixture of chlorophyll components; normal-phase silica with a gradient mobile phase of hexane/2-propanol. Peak 1, chlorophyll *a'*; peak 2, pheophytin *a*; peak 3, chlorophyll *a*; peak 4, pheophytin *b*; peak 5, chlorophyll *b'*; peak 6, chlorophyll *b*; peak 7, pheophorbide *a'*; peak 8, chlorophyllide *a'*; peak 9, chlorophyllide *a*; peak 10, pheophorbide *a*; peak 11, chlorophyllide *b'*; peak 12, chlorophyllide *b*.

Identification of Chlorophylls and Derivatives. *Absorption and Mass Spectra.* Chlorophylls and their derivatives were identified from their absorption spectra by using a Waters 990 photodiode array detector equipped with NEC APCIV computer and chromatography software (Waters Associates). Spectral data for identification were compared to available standards as well as literature values (Goedheer, 1966). Identification of the samples was confirmed by fast atom bombardment (FAB) mass spectrometry (van Breemen et al., 1991). Each compound was collected as it eluted from the chromatographic column. Approximately five to seven injections were needed to collect enough sample for the mass spectrometry analysis (25-μL samples, concentration 1 μg/mL). Water was eliminated from the collected mixture by extracting the pigments with ethyl ether and concentrating the ether extract under nitrogen. Chlorophylls and derivatives were stored in ethyl ether, while chlorophyllides and pheophorbides were freeze-dried and dissolved in acetone. All extracts were handled in the dark throughout the injection, collection, and concentration steps.

RESULTS AND DISCUSSION

The highest conversion levels of chlorophylls to chlorophyllides were found at 65 °C for 30 min in hot water. If the heat treatment was prolonged, the concentration of degradation compounds (pheophytins and pheophorbides) increased, and there was a significant decrease in the initial concentration of chlorophylls and chlorophyllides. Thus, to maximize the conversion into polar derivatives, care must be exercised to first optimize chlorophyllide content and minimize other decomposition reactions. On the other hand, lower incubation temperatures (25–30 °C) were required to produce chlorophyllides in acetone. Presumably acetone promotes access to the chlorophyll substrate for enzymatic phytol cleavage. It was also observed that chlorophyll *b* was transformed more rapidly than chlorophyll *a*, suggesting that the *b* form is a better substrate for chlorophyllase enzyme action. Spinach leaves of the Melody variety were found to have a high chlorophyllase activity, while there appeared to be less transformation for other varieties tested (data not shown). Further work is needed to confirm these observations.

Figure 2 shows a normal-phase HPLC profile of a spinach extract containing both polar and nonpolar chlorophyll compounds. The retention times increased in the order pheophytin *a'* < chlorophyll *a'* < pheophytin *a* < chlo-

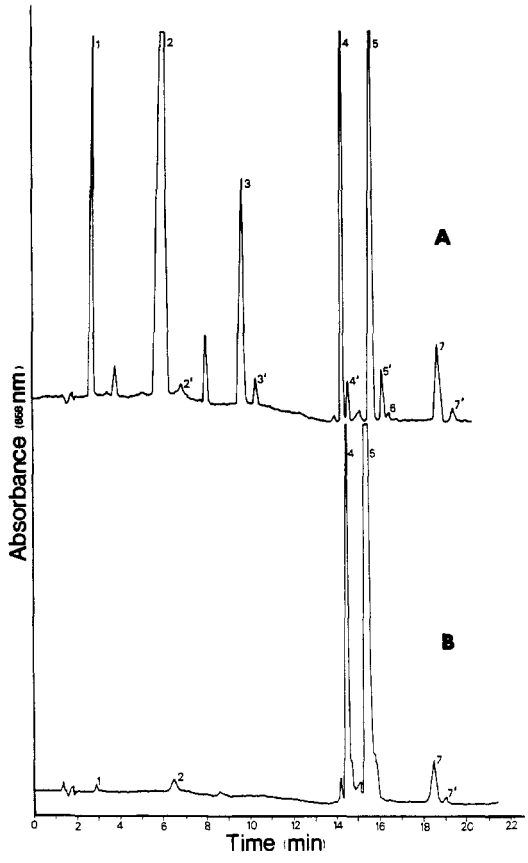

Figure 3. Chromatographic separation of a spinach extract on reversed-phase C-18 column with a gradient mobile phase of ethyl acetate/methanol/water. (A) Extract of spinach leaves after incubation (65 °C, 30 min) in water: peak 1, chlorophyllide *b*; peak 2, chlorophyllide *a*; peak 2′, chlorophyllide *a*′; peak 3, pheophorbide *a*; peak 3′, pheophorbide *a*′; peak 4, chlorophyll *b*; peak 4′, chlorophyll *b*′; peak 5, chlorophyll *a*; peak 5′, chlorophyll *a*′; peak 6, pheophytin *b*; peak 7, pheophytin *a*; peak 7′, pheophytin *a*′. (B) Fresh spinach extract: peak 4, chlorophyll *b*; peak 5, chlorophyll *a*; peak 7, pheophytin *a*; peak 7′, pheophytin *a*′.

rophyll *a* < pheophytin *b* < chlorophyll *b*′ < chlorophyll *b* < pheophorbide *a*′ < chlorophyllide *a*′ < chlorophyllide *a* < phoephorbide *a*′ < chlorophyllide *b*′ < chlorophyllide *b*. These results are in agreement with those reported by Watanabe et al. (1984), who used an isocratic normal-phase HPLC technique for their separation of chlorophylls and pheophytins. The technique reported here additionally separates and detects the polar derivatives.

Figure 3A shows the chlorophyll derivatives formed after the incubation procedure in hot water at 65 °C for 30 min. Chromatogram B illustrates a typical reversed-phase HPLC chromatogram of the chlorophyll derivatives present in a sample extract of fresh spinach puree. The formation of chlorophyllides and pheophorbides can be observed. Following the described gradient program, excellent resolution of the chlorophyll derivatives was obtained. For both the *a* and *b* series of compounds, the retention times increased in the order chlorophyllides < pheophorbides < chlorophylls < pheophytins. Also, the C-10 epimers (prime derivatives) were resolved eluting immediately after the parent peaks. This agrees with the results reported by Suzuki et al. (1987), who used thin-layer plates and open-column chromatography.

The reversed-phase HPLC separation method is able to resolve and quantify all the major chlorophyll derivatives including the more polar chlorophyllides. In addition, it involved a simple extraction step (acetone/water). When the normal-phase separation is used, an extra step is needed to eliminate water from the extract and to transfer the components from acetone solution into hexane since water

Table I. Absorbance Maxima[a] of Chlorophyll and Derivatives

compd	max absorbance	red band	other absorbance	
chlorophyll *a*	432, 417	666	618	
chlorophyll *b*	464	650	600	
chlorophyllide *a*	432, 418	667	618	
chlorophyllide *b*	468	658	602	
pheophytin *a*	409	668	608	506, 535
pheophytin *b*	436	653	597	530
pheophorbide *a*	409	667	608	506, 537
pheophorbide *b*	439	653	600	530
pyropheophytin *a*	411	666	609	509, 539
pyropheophytin *b*	437	653	597	529
pyropheophorbide *a*	411	666	608	509, 539
pyropheophorbide *b*	439	653	600	530

[a] Photodiode array detector accuracy ±1.4 nm.

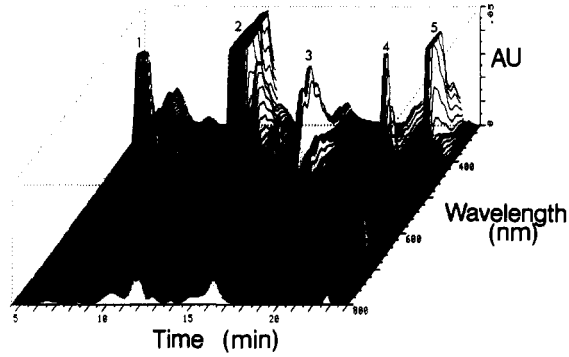

Figure 4. Three-dimensional photodiode array chromatogram of pheophorbides and pheophytins from spinach puree extract (reversed-phase separation). Peak 1, pheophorbide *b*; peak 2, pheophorbide *a*; peak 3, xanthophylls; peak 4, pheophytin *b*; peak 5, pheophytin *a*.

in the extract caused problems in chromatographic resolution as well as varying retention times. In addition, this technique was not quantitative because of the difficulty in transferring all the polar derivatives into a hexane layer. On the other hand, an advantage of the normal-phase method is its ability to resolve the magnesium chlorophyll compounds from other metal-chelated chlorophyll derivatives (Canjura and Schwartz, unpublished data).

The identification of the chromatographic peaks was based on ultraviolet–visible absorption and mass spectrometry results. Table I lists the major absorbance bands for the 12 chlorophyll derivatives obtained with the photodiode array detector. The data were obtained in the mobile phase as the compounds were eluted from the column. Both the chlorophyllides and chlorophylls presented essentially the same absorbance spectra. This agrees with the literature data (Jackson, 1976). On the other hand, pheophorbides showed similar, but slightly different, spectra from those of pheophytins. Pyropheophytins and pyropheophorbides presented the same spectral properties but differ slightly from their precursors pheophytins and pheophorbides. This agrees with the data published by Pennington et al. (1964). In addition, the C-10 epimers that were resolved showed spectra identical with those of their parent compounds as expected (Pennington et al., 1964; Schwartz et al., 1981). Any differences between the experimental values and the literature values may be due to the solvents in which thespectra were determined. Also, the accuracy of the photodiode array detector is ±1.4 nm.

Figure 4 illustrates a three-dimensional chromatogram (using the reversed-phase separation technique) for a heat-treated sample where both pheophorbides and pheophytins are present. This plot was very useful in the

Table II. Molecular Weights of Chlorophyll Compounds Determined by Fast Atom Bombardment Mass Spectrometry

chlorophyll derivative	exptl molecular weight,[a] mass units	calcd molecular weight, mass units
chlorophyll *a*	893.0	893.5
chlorophyll *b*	906.9	907.5
chlorophyllide *a*	614.3	613.5
chlorophyllide *b*	627.9	627.5
pheophytin *a*	871.0	871.2
pheophytin *b*	885.0	885.2
pheophorbide *a*	592.4	591.7
pheophorbide *b*	607.4	606.0
pyropheophytin *a*	813.5	813.2
pyropheophytin *b*	827.0	827.2

[a] The precision of the JEOL mass spectrometer is approximately $^1/_{10}$ of a mass unit.

identification of each compound because it allowed observation of the full absorption spectra of each peak as it eluted from the chromatographic column. Thus, detection of other compounds was easily noted, and coeluting components could be observed. For example, at approximately 16 min (peak 3) the separation of xanthophyll pigments can be observed.

The mass spectrograms and B/E linked scans were obtained for each chlorophyll derivative except pyropheophorbides, which were not collected because of the limited quantities present. From the linked scan of each compound, precursor ions and molecular fragmentation patterns were analyzed in detail, and the results are reported elsewhere (van Breemen et al., 1991). Molecular ions corresponded to the molecular weight of each compound, confirming the identity of the isolated component (Table II). The observed molecular weights of the chlorophyll derivatives were found in excellent agreement with their calculated values.

CONCLUSIONS

This study demonstrates a rapid, sensitive reversed-phase HPLC method for resolution and analysis of both polar and nonpolar chlorophyll derivatives present in leafy tissues. Also, a normal-phase separation for the major chlorophyll derivatives is shown. This will permit further research in the area of chlorophyll biosynthesis, metabolism, and catabolism as well as investigations involving these compounds in edible plant tissues.

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