High-Performance Liquid Chromatography of Chlorophylls and Their Derivatives in Fresh and Processed Spinach

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Chlorophylls *a* and *b* and their derivatives were separated by high-performance liquid chromatography (LC). The method involves a gradient elution reverse-phase separation. The two solvents were a methanol–water mixture and ethyl acetate. Compounds were detected at 654 nm. Twelve chlorophylls and derivatives were resolved. The method was applied to monitor changes in chlorophyll during processing of spinach. Chlorophylls *a* and *b* were the only pigments detected in fresh spinach. Blanching for 2.5 min at 100 °C showed the appearance of chlorophylls *a'* and *b'*. Blanching for 10 min increased the relative amounts of the *a'* and *b'* isomers. Frozen spinach contained chlorophylls *a*, *a'*, *b*, and *b'* and pheophytin *a*. In canned spinach, almost all chlorophylls were converted to pheophytins and pyropheophytins. Pyropheophytins were not previously reported to be present in canned spinach. They were identified by their visible absorption spectra, LC retention times, and NMR spectra.

The analysis of chlorophylls and their derivatives has been the attention of several investigations. The light absorbance characteristics of chlorophylls have made possible the development of both qualitative and quantitative analyses. Vernon (1960) developed a quantitative spectrophotometric method for the determination of chlorophylls *a* and *b* and pheophytins *a* and *b*. The method utilizes specific absorptivities and light absorptions at the maximum for each pigment in 80% acetone. The equations developed to quantify each component were applied to model solutions and to measure chlorophylls in several vegetables. A similar procedure was used by Jones et al. (1962) to determine chlorophylls *a* and *b*, pheophytins *a* and *b*, and pheophorbides *a* and *b* in an ether extract to study pigment changes in the brining and storage of cucumbers. White et al. (1963) expanded this quantification of chlorophyll derivatives to include the chlorophyllides *a* and *b*. The sensitivity of the method was increased by employing a fluorometric analysis (White et al., 1972).

High-performance liquid chromatography (LC), coupled with absorption spectrophotometers as a detector, has also been employed by several workers to investigate chlorophylls and other photosynthetic pigments. Eskins et al. (1977) developed a preparative LC method for chlorophyll separation using a reverse-phase column and a stepwise gradient system starting with 80% methanol and ending with an ether–methanol mixture. Iriyama et al. (1978) developed a microscale method for the separation of chlorophylls using a silica gel column. These techniques were time consuming, requiring 75 min or longer for completion. Braumann and Grimme (1979) shortened the time needed for analysis and resolved more than 10 different pigments from the green algae *Chlorella fusca* by using a C_{18} reverse-phase column and a step-gradient technique. Rebeiz et al. (1978) separated cucumber chlorophylls *a* and *b* and pheophytins *a* and *b* using a C_{18} column with a ternary solvent system consisting of methanol–acetone and water. These above described methods work well for the intended purpose of monitoring chlorophyll in fresh plant material. There is a need for a sensitive and rapid LC method to determine changes in chlorophyll composition during the processing of foods where alterations in pigment composition may occur due to extended heat treatment.

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Table I. Apparatus and Conditions for the Separation of Chlorophyll Pigments and Their Derivatives by LC

column	μBondapak C_{18} (Waters Associates, Milford, MA)
pumps A and B	Waters Associates, Model 6000A
solvent A	75:25 CH_3OH–H_2O
solvent B	ethyl acetate
initial condition	100% solvent A
final condition	50% solvent A–50% solvent B
gradient	curve 7 (solvent programmer, Model 660, Waters Associates) for a duration of 10 min
flow rate	2.0 mL/min
detector	Perkin-Elmer Model 55, fitted with an 8-μL flow cell
injector	Waters Associates, Model U6K
sample size	20 μL
detection wavelength	654 ± 2 nm

Color changes during processing of green vegetables has been reviewed by Clydesdale et al. (1970).

The objective of this study was to establish a method to resolve spinach chlorophylls and their derivatives by LC and monitor qualitative changes in chlorophyll pigments after blanching and heat processing.

MATERIALS AND METHODS

Sample Preparation. Fresh and frozen spinach was obtained from local sources. Samples were blanched in boiling water for 2.5 and 10 min. Canned samples consisted of 400 g of blanched spinach packed in 303-size cans with 2% NaCl solution as the brine. The samples were heated to 77 °C before sealing and processed at 122 °C for 55 min in a still retort. Fresh, blanched, frozen, and canned samples were analyzed for pigment composition immediately. In addition, canned samples were analyzed after 2.5 months of storage at room temperature.

Extraction of Pigments. Five grams of spinach was blended with 50 mL of acetone for 2 min and filtered and the residue washed with an additional 50 mL of acetone. Seventy-five milliliters of ether was added, and the mixture transferred to a separatory funnel and washed with 50 mL of 5% Na_2SO_4 to form two phases. The aqueous phase was removed and extracted with ether until the lower layer was

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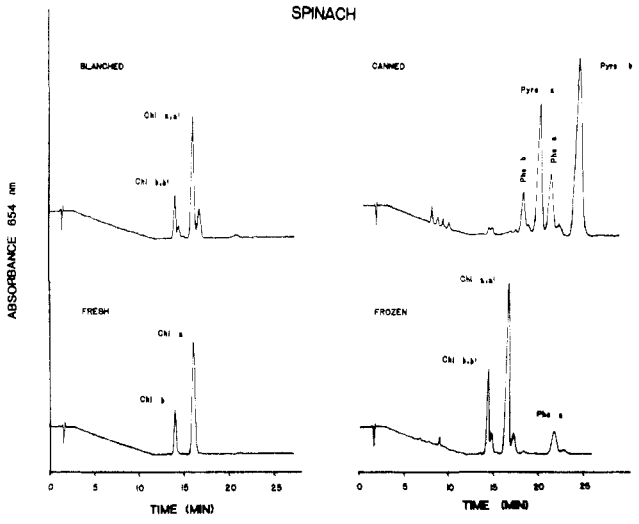

Figure 1. LC chromatograms of chlorophylls and derivatives in fresh, blanched, frozen, and canned spinach.

colorless. The combined ether extracts were diluted to 150 mL and dried over anhydrous Na_2SO_4. For LC analysis, a 2-mL aliquot was removed, the ether was evaporated under a stream of N_2, and the pigments were dissolved in 1 mL of acetone prior to injection. The apparatus and conditions for the LC analysis are outlined in Table I.

Preparation and Identification of Chlorophyll Pigments and Derivatives. Chlorophylls *a*, *a'*, *b*, and *b'* were obtained by reverse-phase thin-layer chromatography using the midrange system developed by Daley et al. (1973). Pheophytins *a* and *b* were obtained from their respective chlorophylls by adding 13% HCl to a chlorophyll–ether solution (Hynninen and Ellfolk, 1973). Spectral data and retention times of these compounds were compared with those found in the LC chromatograms of the spinach extracts. For NMR work, pheophytin *a* was prepared from chlorophyll *a* obtained from Sigma Chemical Co. (St. Louis, MO) following the procedure outlined by Hynninen and Ellfolk (1973). After removal of the solvent, an NMR spectrum was taken in $CDCl_3$ on a Bruker FT 270-MHz instrument. Pyropheophytin *a* was prepared by a method similar to that described by Kenner et al. (1973). One milligram of pheophytin *a* was placed in a small test tube, dissolved in 1.0 mL of dry collidine, and capped with an airtight septum. The solution was boiled rapidly by using a Bunsen burner while flowing N_2 through the system and venting the flammable vapors. The dry residue was allowed to cool, and the residue dissolved in acetone for LC analysis. An NMR spectrum was taken in $CDCl_3$. Chemical shifts are reported in ppm relative to $CHCl_3$ (δ = 7.23).

Chlorophyllides *a* and *b* were prepared by treating an acetone extract of frozen spinach leaves with chlorophyllase enzymes from beet leaves (Holden, 1961). The completion of the conversion to phytol-free derivatives was checked by their insolubility in petroleum ether. The chlorophyllase-treated pigments were chromatographed by LC and identified by their absorption spectral data.

Pheophorbides *a* and *b* were prepared and identified in the same manner as the chlorophyllides, except for an additional acidification in ether solution prior to chromatography.

RESULTS AND DISCUSSION

The chromatograms of chlorophylls and their derivatives in fresh, blanched, frozen, and canned spinach are shown in Figure 1. The chromatograms were monitored at 654 nm to exclude the yellow pigments while enabling the detection of the chlorophylls and their Mg and phytol-free derivatives. Fresh spinach was found to contain only chlorophylls *a* and *b*. Blanching for 2.5 min at 100 °C indicated the appearance of chlorophylls *a'* and *b'*, the C-10 epimers of chlorophylls *a* and *b*, respectively. Similar results were found by Strain (1954) after boiling mallow leaves for 1–2 min. Bacon and Holden (1967) reported a 10% conversion of chlorophylls *a* and *b* to the *a'* and *b'* isomers after disks of *Heracleum sphondylium L.* were heated in boiling water after 5 min. Heating spinach for 10 min increases the isomer content of blanched spinach. The configuration of the epimers allows greater interactions with the C_{18} stationary phase, and therefore the epimers have greater retention values relative to their parent compounds. The formation of the isomers does not bring about any change in color of the spinach since the light absorption properties of the epimers are identical with those of their parent pigments. Freezing and frozen storage brings about greater alterations in the pigment content. The LC analysis indicates the presence of chlorophylls *a*, *a'*, *b*, and *b'* and pheophytin *a*. The most prevalent degradative pathway of the pigments during processing of foods is the conversion of the bright green chlorophylls to dull olive pheophytins (Tan and Francis, 1962). This occurs because of the loss of the central Mg atom, and it is well-known that acidic conditions enhance their formation. Only trace amounts of pheophytin *b* were found in the frozen spinach samples. Schanderl et al. (1962) reported that chlorophyll *a* is 5 times more susceptible to pheophytin formation than chlorophyll *b*. This may account for the low levels of pheophytin *b* in spinach extracts.

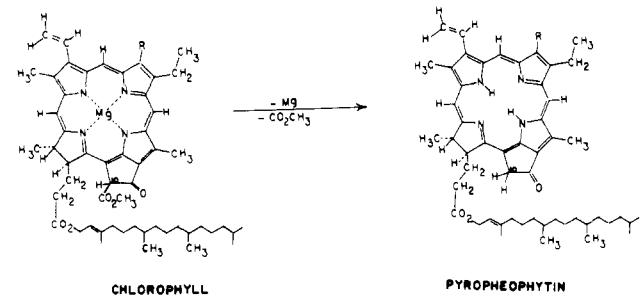

Figure 2. Formation of pyropheophytin from chlorophyll.

The chromatogram of canned spinach shows essentially complete destruction of chlorophylls *a* and *b*. Pheophytins *a* and *b* and two other major peaks contributing to the color of the canned samples are indicated in the LC chromatogram (Figure 1). The shoulder peaks obtained on the pheophytins are assumed to be the pheophytin isomers *a'* and *b'*. The other two major peaks were identified as pyropheophytins *b* and *a* with retention times of 19.3 and 24.0 min, respectively. These compounds have not been previously reported to be present in canned food products. There was no observable change after 2.5 months of storage at room temperature. The "pyro" derivatives are formed from the corresponding pheophytins by the loss of the C-10 carbomethoxy group [$-CO_2CH_3$ (Figure 2)]. Failure of their detection in canned spinach may be attributed to the lack of resolution of other methods used and the identical absorption characteristics of pheophytin and pyropheophytin. The evidence for their identification is as follows. Removal of the $-CO_2CH_3$ group reduces the polarity of the pheophytin compound and therefore a greater retention time on the C_{18} reversed-phase column would be expected. Furthermore, since the loss of a C-10 chiral center occurred in the formation of pyropheophytins *a* and *b*, no epimers are anticipated. This explains the absence of shoulders in the pyropheophytin

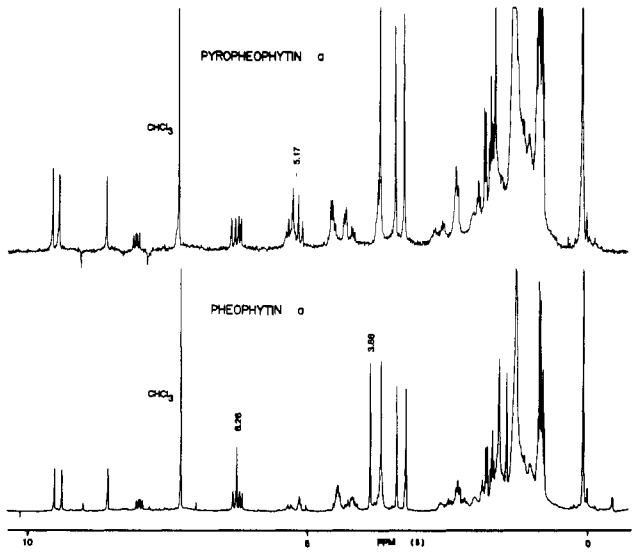

Figure 3. NMR spectra of pyropheophytin *a* and pheophytin *a*.

peaks (Figure 1) compared to the pheophytin peaks in which the shoulders are assumed to be the respective epimers.

Pennington et al. (1964) formed pyro derivatives from purified chlorophyll *a*, methylchlorophyllide *a*, pheophytin *a*, and methylpheophorbide *a* by heating these compounds in pyridine at 100 °C for 24 h in a sealed tube under reduced pressure. In the present study, when pheophytins *a* and *b*, obtained from fresh spinach after acidification, were treated similarly, two additional peaks, representing pyropheophytins *a* and *b*, were found in the chromatogram. The retention times of the pyro derivatives peaks were identical with those of the peaks identified as pyro derivatives in canned spinach. Heating of chlorophylls in pyridine to remove the C-10 carbomethoxy group has given rise to pyro derivatives as well as decomposition products. To eliminate this possibility, Kenner et al. (1973) has suggested to produce pyro derivatives in quantitative yields by refluxing chlorophylls in collidine. Applying this technique to pheophytins *a* and *b*, obtained from spinach pigment extracts after acidification, two peaks were formed having identical retention times to those observed as pyropheophytins *a* and *b* in canned spinach.

The NMR spectra of pheophytin *a* and pyropheophytin *a* were obtained to ensure further identification and are shown in Figure 3. These spectra correspond well with previously reported NMR data for these compounds (Pennington et al., 1964; Closs et al., 1963). The difference between the two spectra is the absorption at $\delta = 3.88$, corresponding to the three methyl protons at C-10b, in pheophytin *a* which is no longer apparent in the spectrum for pyropheophytin *a*. Another characteristic feature is the shift of the C-10 proton resonance at $\delta = 6.26$ in pheophytin *a* to the absorption centered at $\delta = 5.17$ in pyropheophytin *a*. Excellent resolution of pheophorbides *a* and *b* and chlorophyllides *a* and *b* with retention times of 8.7, 7.6, 6.6, and 3.8 min, respectively, was also achieved with the method described, but they were not present in any of the spinach samples analyzed.

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