Extraction, Purification, and Characterization of Chlorophylls from Spinach Leaves

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Crude chlorophyll was extracted from fresh spinach leaves by use of methanol and acetone as two separate solvents. The crude chlorophyll was partially purified by successive precipitation using a mixture of dioxane and water. The partially purified chlorophyll was subjected to column chromatography with DEAE-Sepharose CL-6B. Chlorophylls *a* and *b* were obtained by further purification of a Sepharose CL-6B column chromatography. The recovery of chlorophyll fractions was determined, and the degree of their purity was demonstrated by spectrophotometric scanning and by HPLC analysis. The extraction of 100 g of fresh spinach leaves with methanol and subsequent purification procedures resulted in 38 mg of chlorophyll *a* (30.1% purity) and 13 mg of chlorophyll *b* (27.5% purity). Extraction with acetone and subsequent purification procedures resulted in 61 mg of chlorophyll *a* (44.5% purity) and 29 mg of chlorophyll *b* (32.4% purity), respectively. The purities of commercial samples of chlorophylls *a* and *b* were 36.0% and 32.4%, respectively.

INTRODUCTION

Chlorophylls are the pigments of photosynthesis. Chlorophylls are found in living organisms almost exclusively as chlorophyll–proteins complexes. Chlorophylls are of interest to agriculture and ecology, where they are indicators of the health status of individual plants and communities, and are often used as a quantitative reference in physiological research. They are also permitted as food colors (Humphrey, 1980). Last, but not least, chlorophylls have recently attracted interest as phototherapeutic drugs (Scheer, 1991).

Since the discovery of chlorophylls of Pelletier and Caventou in 1818, several methods have been used for their extraction and purification. Chlorophylls have been extracted from plant tissue using various organic solvents; however, aliphatic and aromatic hydrocarbons are not used because they do not extract chlorophylls (Schwartz and Lorenzo, 1990). A good extraction procedure must bring all chloroplast into solution with little or no alteration to the chlorophyll (Svec, 1991). Acetone and methanol are the most commonly used solvents for the extraction of plant pigments in general (Holden, 1976). Iriyama et al. (1974) suggested that the use of a mixture of dioxane/water for the extraction of chlorophylls is based on the fact that the dioxane interacts with chlorophylls to form selective chlorophyll–dioxane adducts which can precipitate in a microcrystalline form, leaving the bulk carotenoid in the solution.

The analysis of chlorophylls and their derivatives and the use of highly purified chlorophylls as reference materials for these analysis have been the subject of studies by biochemists as well as food chemists. However, some difficulties, e.g., the appropriate procedure of extraction and purification, still remain as far as preparation of substantial quantities of highly purified chlorophylls *a* and *b* is concerned.

Thin-layer chromatography (TLC) has been used both quantitatively and qualitatively for separation of chlorophylls and associated pigments, their derivatives and products of degradation, in many species of photosynthetic bacteria, algae, and higher plants (Sestak, 1967, 1982).

A range of liquid chromatographic techniques has been used for chlorophyll purification. Column chromatography, using a cellulose-based stationary phase, has been used to purify chlorophylls *a* and *b* (Strain et al., 1971). Although chlorophylls *a* and *b* were successfully separated by chromatography with carbohydrate powders as stationary phase, there were some difficulties associated with the use of this column; a high degree of technical skill, a large volume of solvent, and a long time of elution are needed for complete elimination of carotenoid from chlorophyll (Omata and Murata, 1983). Omata and Murata (1980, 1983) obtained pure chlorophylls *a* and *b* from acetone and dioxane/water precipitation, according to the method described previously by Iriyama and Shiraki (1979). Liquid chromatography was used for the purification of chlorophylls using a combination of ion-exchange chromatography and size exclusion chromatographies such as DEAE-Sepharose CL-6B and Sepharose CL-6B (Omata and Murata, 1980) and DEAE-cellulose and Sephadex LH-20 (Sato and Murata, 1978).

High-performance liquid chromatography (HPLC) techniques are now considered to be among the quickest, simplest, and most reproducible methods for analyzing complex mixtures of pigments in foods and other substances (Bushway, 1986). Chlorophylls can be characterized by comparison of their retention times during their separation by liquid chromatography. The major chlorophylls found in green vegetables are chlorophylls *a* and *b*, which have been separated on C_{18} reversed-phase HPLC (Khachik et al., 1986). Isocratic (Shioi et al., 1983; Shoaf, 1978) as well as gradient HPLC techniques (Schwartz et al., 1981; Eskins et al., 1977) have been developed for the separation of chlorophylls. Shioi et al. (1983) reported the separation of chlorophylls and their derivatives by C_{18} reversed-phase HPLC using an octadecyl silica column and 100% methanol as the eluting solvent; Canjura and Schwartz (1991) used somewhat similar conditions except that the eluting system was a gradient of mixtures of two variable concentrations of hexane/2-propanol.

The aim of this study was to compare the efficiency of two different organic solvents, acetone and methanol, for the extraction of chlorophylls from fresh spinach leaves. Another objective of this work was to demonstrate the degree of purity of purified chlorophylls, using successive liquid chromatographic techniques, by spectrophotomet-

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Table I. Purification Scheme for Chlorophylls Extracted from Fresh Spinach Leaves and Determined by Spectrophotometry

fraction	recovery[a] via extraction method		degree of purity[b,c] via extraction method	
	I[d]	II[e]	I[d]	II[e]
solvent extract	1.890	1.706	12.70	17.70
first dioxane/water precipitation	0.296	0.470	21.20	24.00
second dioxane/water precipitation	0.180	0.204	25.00	29.00
DEAE-Sepharose CL-6B	0.091	0.127	37.60	40.60
Sepharose CL-6B				
chlorophyll *a*	0.038	0.061	30.10	44.50
chlorophyll *b*	0.013	0.029	27.50	32.40

[a] Recovery is defined as percent, by weight, of chlorophyll per 100 g of fresh spinach leaves. [b] Degree of purity is defined as the relative percent of chlorophyll area peak to the total area of peaks determined by a spectrophotometric scanning from 380 to 700 nm. [c] The degree of purity of commercial chlorophyll *a* is 36.0%; the degree of purity of commercial chlorophyll *b* is 32.4%. [d] Method I indicates the use of methanol as a solvent of the extraction of crude chlorophylls. [e] Method II indicates the use of acetone as a solvent of the extraction of crude chlorophylls.

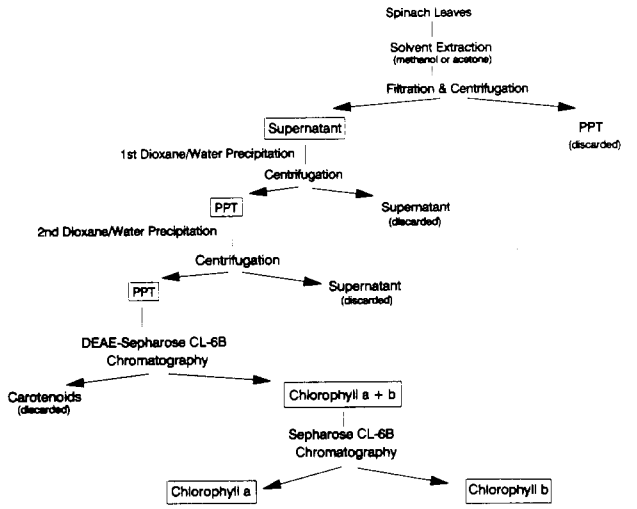

Figure 1. Diagram of extraction and purification of chlorophylls from spinach leaves.

ric scanning and by HPLC analysis. This could result in suggestions for an alternative procedure for the extraction and purification of chlorophylls leading to higher degrees of recovery and purity.

MATERIALS AND METHODS

Preparation of Chlorophyll Crude Extract. Leaves of fresh spinach (*Spinacia oleracea*) were purchased from the local market. A modification of the extraction procedure described by Iriyama et al. (1974) was used (Figure 1). The extracted clear suspension containing crude chlorophylls was subjected to further purification.

Partial Purification of Chlorophylls Extract. The crude extract obtained was partially purified according to the procedure described by Iriyama et al. (1974). The dioxane/water precipitate, considered to be the partially purified chlorophylls, was subjected for further purification by successive ion-exchange and size exclusion liquid chromatographies.

DEAE-Sepharose CL-6B Ion-Exchange Chromatography. Ion-exchange liquid chromatography was performed according to the procedure described by Omata and Murata (1983) using DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals).

Sepharose CL-6B Size Exclusion Chromatography. Size exclusion chromatography was performed using Sepharose CL-6B (Pharmacia Fine Chemicals). The gel was prepared by

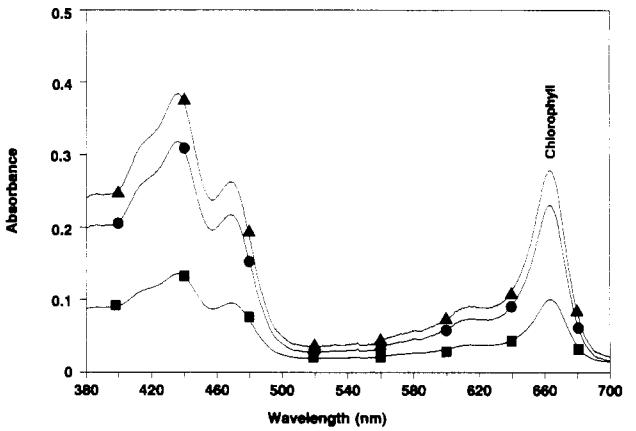

Figure 2. Absorption spectrum of chlorophylls extracted by methanol (■) and precipitated with first addition of dioxane/water mixture (●) and precipitated with second addition of dioxane/water mixture (▲).

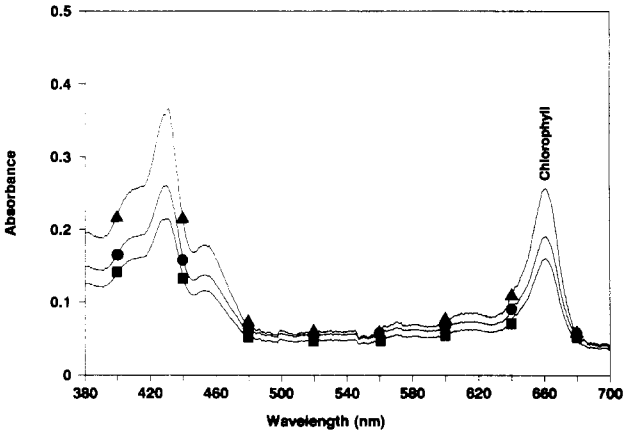

Figure 3. Absorption spectrum of chlorophylls extracted by acetone (■) and precipitated with first addition of dioxane/water mixture (●) and precipitated with second addition of dioxane/water mixture (▲).

washing with deionized water followed with acetone. The Sepharose CL-6B was subjected to further successive washing using acetone/hexane (2:1 v/v), acetone/hexane (1:2 v/v), hexane/2-propanol (10:1 v/v), and hexane/2-propanol (20:1 v/v). The unseparated chlorophylls *a* and *b* obtained from the ion-exchange chromatography were dissolved in hexane/2-propanol (1 mL) and applied to a 2.5 × 35-cm column of Sepharose CL-6B equilibrated with the same solvent. The elution was performed at a flow rate of 8 mL/min. Chlorophyll *a* was eluted with 175 mL of hexane/2-propanol (20:1 v/v) and the elution of chlorophyll *b* with 200 mL of hexane/2-propanol (10:1 v/v).

Characterization of Chlorophylls. The chlorophyll fractions were characterized by spectrophotometric scanning and by high-performance liquid chromatography. Commercial purified chlorophylls *a* and *b* were also characterized at the same time. The degree of purity was defined as the relative percent of chlorophyll area peak to the total area of peaks determined either by a spectrophotometric scanning from 380 to 700 nm or by HPLC using absorbance measurements at the red region (654 nm).

Spectrophotometric Scanning. The chlorophyll fractions were scanned using a Beckman DU-65 spectrophotometer equipped with Beckman data leader graphics spectrophotometer software. The spectrophotometric measurements of chlorophylls *a* and *b* were performed directly after their elution from the Sepharose CL-6B column. Triplicate scanning was performed in a 3-mL quartz cell in the range 380–700 nm.

High-Performance Liquid Chromatography. High-performance liquid chromatography (HPLC) was carried out with a gradient Beckman Gold System (Beckman Instruments, Inc., San Ramon, CA) using a C_{18} reversed-phase column (250 × 4.6 mm, 5 mm, Econosil, Alltech Associates, Inc.); all solvents were of HPLC grade (BDH Inc.). The chlorophyll samples were

Table II. Purification Scheme for Chlorophylls Extracted from Fresh Spinach Leaves and Determined by High-Performance Liquid Chromatography

fraction	degree of purity[a,b]			
	extraction method I[c]		extraction method II[d]	
	chlorophyll *a*	chlorophyll *b*	chlorophyll *a*	chlorophyll *b*
solvent extract	28.10	42.22	48.80	36.32
first dioxane/water precipitation	34.47	44.95	50.67	38.92
second dioxane/water precipitation	44.98	45.56	55.49	40.29
DEAE-Sepharose CL-6B	40.66	48.35	56.20	23.17
Sepharose CL-6B				
chlorophyll *a*	100.0	0.0	50.21	31.20
chlorophyll *b*	23.17	64.82	0.0	92.13

[a] Degree of purity is defined as the relative percent of chlorophyll area peak to the total area of peaks determined by high-performance liquid chromatography using absorbance measurement at 654 nm. [b] Commercial chlorophyll *a* contained 50.66% chlorophyll *a* and 45.56% chlorophyll *b*; commercial chlorophyll *b* contained 16.23% chlorophyll *a* and 81.14% chlorophyll *b*. [c] Method I indicates the use of methanol as a solvent of the extraction of crude chlorophylls. [d] Method II indicates the use of acetone as a solvent of the extraction of crude chlorophylls.

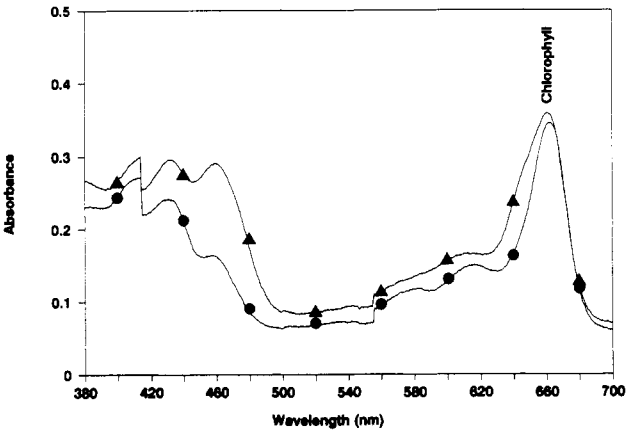

Figure 4. Absorption spectrum of chlorophylls separated by ion-exchange liquid chromatography on a DEAE-Sepharose CL-6B column, extracted by methanol (▲) and acetone (●).

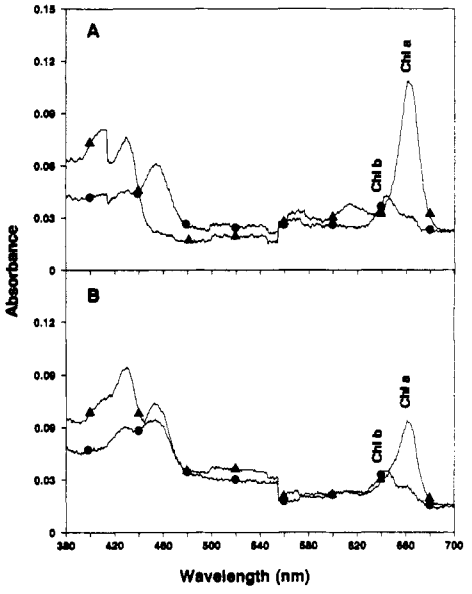

Figure 5. Absorption spectrum of chlorophylls *a* (▲) and *b* (●) separated by size exclusion liquid chromatography on a Sepharose CL-6B column, extracted by (A) methanol and (B) acetone.

filtered through a 0.22-μm type GV filter (Millipore Corp). The procedure of separation was performed according to the method of Schwartz et al. (1981). The absorbance of the eluate was monitored at 654 nm using a visible light detector (Beckman Model 166). The chromatographic data were collected by means of a Packard Bell personal computer (Packard Bell Electronic, Inc., Chatsworth, CA). Integration of the peaks and presentation of the chromatograms were carried out with the aid of the Gold system PC software, version 5.1 (Beckman Instruments).

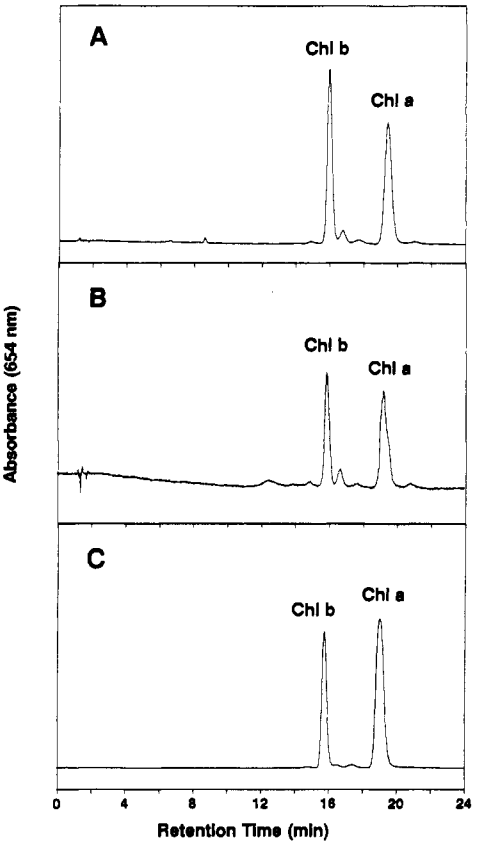

Figure 6. Chromatograms of HPLC analysis of chlorophylls extracted by methanol (A) and precipitated, successively, with first (B) and second (C) additions of dioxane/water mixture.

RESULTS AND DISCUSSION

The use of spectrophotometric scanning for the determination of the degree of purity of chlorophyll fractions (Table I) indicated that the crude and the partially purified chlorophyll fractions obtained by acetone and successive dioxane/water precipitations (Figure 2) have higher degrees of purity (17.7%, 24.0%, and 29.0%, respectively) than those (12.7%, 21.2%, and 25.0%, respectively) obtained by methanol and successive dioxane/water precipitations (Figure 3). The spectrophotometric scanning also demonstrated the presence of appreciable amounts of yellow pigments, which has been reported by other workers (Strain and Svec, 1966; Strain et al., 1971); however, Iriyama et al. (1974) pointed out that most yellow pigments present in the chloroplast and other carotenoids were removed from the crude extract during the first precipitation using the dioxane/water mixture. In addition, HPLC analysis (Table II) followed by absorbance

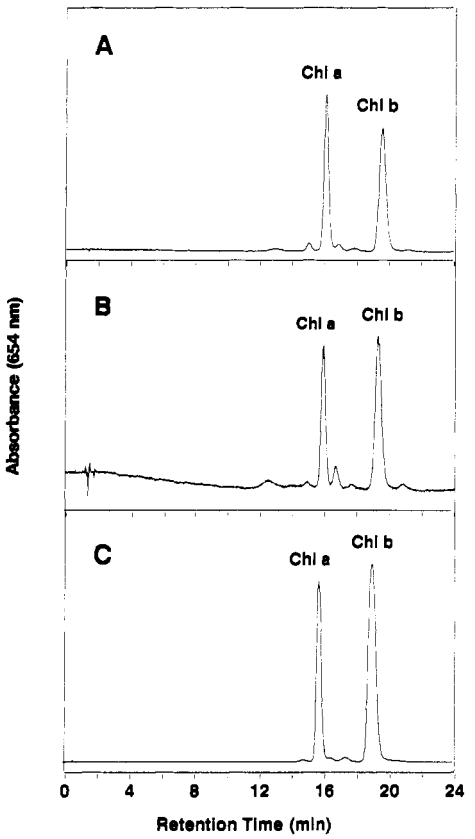

Figure 7. Chromatograms of HPLC analysis of chlorophylls extracted by acetone (A) and precipitated, successively, with first (B) and second (C) additions of dioxane/water mixture.

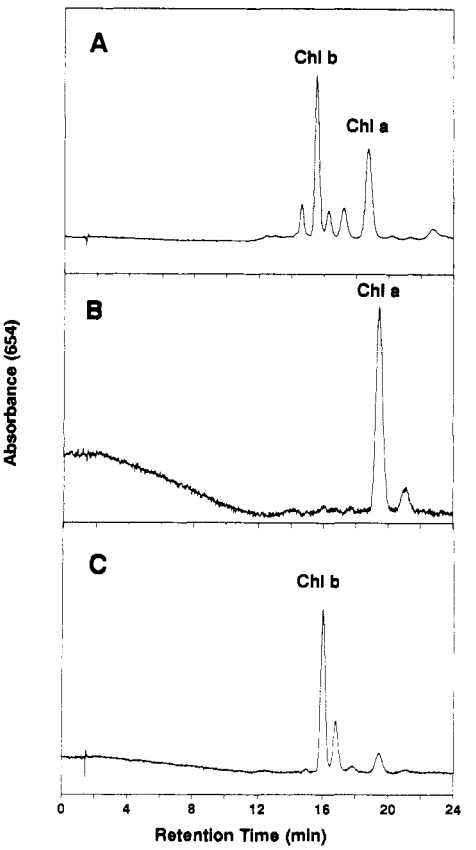

Figure 8. Chromatograms of HPLC analysis of chlorophylls extracted with methanol and purified by successive liquid chromatography columns of DEAE-Sepharose CL-6B (A) and Sepharose CL-6B (B) for chlorophyll *a* and (C) for chlorophyll *b*.

measurements at 654 nm were used to demonstrate the relative degree of purity of extracted and purified chlorophyll fractions. Acetone and methanol are the most common solvents used for the extraction of plant pigments; their efficiency is related to their capacity to disrupt the linkage between pigments and proteins (Holden, 1976). However, acetone is preferred by some researchers because of the likelihood of allomerization in methanol (Holden, 1976; Terpstra, 1980).

Further purification of chlorophyll fractions by DEAE-Sepharose CL-6B ion-exchange chromatography resulted in an increase in the degree of the purity (40.6% for the acetone fraction and 37.6% for the methanol fraction; Table I). However, spectrophotometric scanning of the purified chlorophylls (Figure 4) demonstrated the presence of carotenoid contaminants (400–500 nm). These results are different from those of Omata and Murata (1980), who reported that column chromatography with DEAE-Sepharose CL-6B completely eliminated carotenoids and pheophytin from chlorophyll. Further fractionation of chlorophyll by size exclusion chromatography on Sepharose CL-6B resulted in chlorophylls *a* and *b*, with a higher degree of purity for fractions obtained by acetone extraction compared to those obtained by methanol extraction (Figure 5). However, spectrophotometric scanning of purified chlorophylls *a* and *b* again demonstrated the presence of carotenoid contaminants (400–500 nm). The differences in findings between our work and those reported by Omata and Murata (1980) could be related to the fact that those authors based their interpretations solely on chromatographic analysis, whereas our suggestions are supported by data from both liquid chromatography and spectrophotometric scanning.

Our results (Table I) indicate that the purified chlorophyll *a* showed a higher degree of purity (44.5%) when compared to that of the commercial chlorophyll *a* (36.0%). The purified chlorophyll *b* showed the same degree of purity (32.4%) when compared to that of the commercial chlorophyll *b*.

The results of the recovery of chlorophyll fractions (Table I) show that the ratio of chlorophyll *a* to chlorophyll *b* is 2.8:1 for those obtained by the acetone extraction and 3:1 for those obtained by the methanol extraction. These ratios are very similar to those (2.9:1) reported by Iriyama et al. (1981); Omata and Murata (1980, 1983) reported a ratio of 3.7:1. The ratio of 3:1 for chlorophylls *a*:*b* in higher plants is generally considered to be acceptable (Lichtenthaler, 1987); however, growth conditions and environmental factors, particularly high light and sun exposure, can modify this ratio (Schwartz and Lorenzo, 1990).

Figure 6 shows the separation of partially purified chlorophylls by C_{18} reverse-phase column HPLC analysis. These results show that well-separated chlorophylls were obtained by methanol extraction (Figure 6A), followed by precipitation with successive dioxane/water additions (Figure 6B,C). Chlorophylls obtained with acetone extraction (Figure 7A), followed by precipitation with successive dioxane/water additions (Figures 7B and 8C), gave similar chromatograms which indicate adequate separation of the chlorophylls.

The purification of chlorophylls into well-separated fractions of chlorophylls *a* and *b* was achieved by size exclusion chromatography on Sepharose CL-6B; the chromatograms of HPLC analysis are shown in Figures 8B,C and 9B,C. These results (Table II) suggest that the methanol extraction method could be more appropriate for the purification of chlorophyll *a*, whereas the acetone extraction method could be more appropriate for chlo-

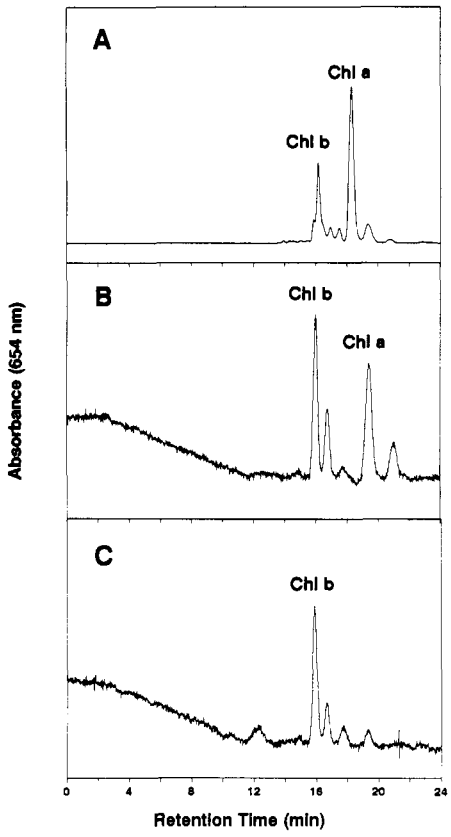

Figure 9. Chromatograms of HPLC analysis of chlorophylls extracted with acetone and purified by successive liquid chromatography columns on DEAE-Sepharose CL-6B (A) and Sepharose CL-6B (B) for chlorophyll *a* and (C) for chlorophyll *b*.

rophyll *b* purification. However, spectrophotometric scanning (Figure 5) demonstrated that, generally, chlorophylls *a* and *b* obtained by acetone extraction had relatively higher degrees of purity when compared to those of chlorophylls obtained by methanol extraction. These differences between HPLC analysis and spectrophotometric scanning may be due to the fact that the spectrophotometric analysis was able to demonstrate the presence of carotenoid pigment contaminants (Strain et al., 1971) in the chlorophyll samples, whereas the HPLC analysis, using fixed wavelength, did not detect such contamination. The HPLC analysis also suggested that, regardless of the type of solvent used in the extraction, there was a reciprocal contamination in both purified chlorophylls *a* and *b*. Sato and Murata (1978) reported that chlorophyll *b*, obtained from spinach leaves and purified by successive liquid chromatography columns of Sephadex LH-20 and DEAE-cellulose, was also contaminated with chlorophyll *a*. The chromatograms of HPLC analysis of purified chlorophylls *a* and *b* (Figures 8B,C and 9B,C) also demonstrate the presence of isomer contaminants. These isomers could be identified as chlorophylls *a'* and *b'*, which were not detected in the fresh acetone or methanol crude extract of leaves but were formed when chlorophylls *a* and *b* were adsorbed to the DEAE-Sepharose CL-6B column (Omata and Murata, 1983). Chlorophylls *a* and *b* have been purified by preparative C_{18} reversed-phase high-performance liquid chromatography (Bidigare et al., 1991); these workers showed the presence of minor amounts of chlorophyll alteration products. Canjura and Schwartz (1991) separated chlorophyll compounds and their polar derivatives by HPLC and characterized them by spectrophotometric absorbance. The minor contaminants chlorophylls *a'* and *b'* (Figures 8B,C and 9B,C), which are, in all likelihood, C_{10} epimeric isomers, have also been reported by Schwartz et al. (1981) in the separation of chlorophylls and their derivatives in fresh and processed spinach. The formation of these contaminant isomers is promoted when the solutions of the natural green pigments are permitted to stand at room temperature (Strain and Svec, 1966). Since conversion of chlorophylls *a* and *b* into chlorophyll epimeric isomers *a'* and *b'* is faster at higher temperature and slower at lower temperature, it has been suggested that the purification procedure should be performed at a low temperature such as 0 °C (Omata and Murata, 1983) and should be done as quickly as possible (Omata and Murata, 1980).

CONCLUSION

The data gathered in this study indicate the use of two different organic solvents for the extraction and purification of chlorophylls from green leaves as well as the use of simultaneous spectrophotometric and HPLC analyses of extracted and purified chlorophyll fraction. A systematic quantitation of the recovery has also been performed. The extraction and purification protocols include a successive solvent extraction and liquid chromatography purification steps. Spectrophotometric scanning and HPLC analysis of purified chlorophylls and commercial chlorophylls, based on the relative percent of area of peak to the total area of peaks of separated samples, indicate that the degree of purity of chlorophylls prepared in our laboratory is close to that of commercial chlorophylls.

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