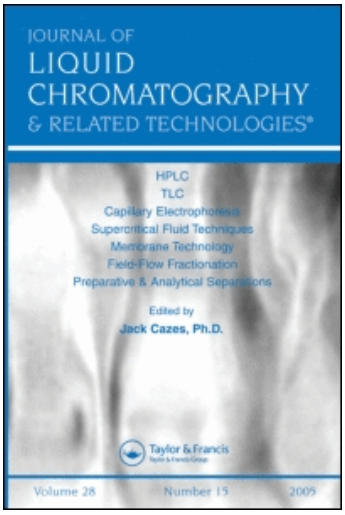

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An Improved Method Fòr Extraction, Partial Purification, Separation and Isolation of Chlorophyll from Spinach Leaves

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AN IMPROVED METHOD FOR EXTRACTION, PARTIAL PURIFICATION, SEPARATION AND ISOLATION OF CHLOROPHYLL FROM SPINACH LEAVES

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ABSTRACT

Powdered sugar column chromatographic separation and isolation of chlorophyll were studied. A wet column was employed using a Pharmacia R25 chromatographic tube connected, through a UV monitor, to a fraction collector. Chlorophyll extracted from spinach leaves with acetone was selectively precipitated with dioxane and water, and then was washed with 80 % (v/v) aqueous methanol. Use of the further purified chlorophyll as a starting material for chromatographic separation and isolation of chlorophyll, made column chromatographic procedures become extremely effective and easier. Thin-layer chromatographic and high-performance liquid chromatographic tests revealed that chlorophyll preparations in this study did not contain any other photosynthetic pigments and their degradation products.

INTRODUCTION

The need for highly purified chlorophyll is now increasing in many diverse area of investigation. However, some difficulties still remain in preparation of chlorophyll. Preparatory methods for chlorophylls "a" and "b" have already been reviewed elesewhere (1-3). The published procedures may be divided into at least three steps. The first step involves extraction of chlorophyll, with appropriate solvents, from photosynthetic living bodies. The second step is to eliminate most yellow pigments and most of the colorless substances from the extract prior to chromatography. In the third step, column chromatography is used to separate and to isolate the pigments. Purification of chlorophyll should be accomplished by the third step. However, we experienced considerable difficulties in separating and isolating chlorophyll via column chromatography with mild adsorbents, such as cellulose, starch, and powdered sugar (1-3). One of the difficulties in the separation and isolation via column chromatography results from the existence of migrating yellow pigments, which prevent sufficient separation and isolation. Therefore, we developed a method for partial purification of chlorophyll extracted from photosynthetic living bodies before chromatographic separation and isolation and reported the method briefly (4,5). Following to the method (4,5), all the yellow pigments except for the nonsorbed carotenes, (which did not disturb the separation and isolation of chlorophylls from one another with a powdered sugar column) were eliminated from leaf extracts before chromatography, and thus, the chromatographic procedures become extremely effective;

it is then easier to collect each of the pigment fractions eluted from the column. Details of these procedures will be presented in this paper. The method for separation and isolation of chlorophyll with various kinds of adsorbents will be also discussed.

EXPERIMENTAL

Materials. All the solvents used here were reagent grade, purified further, by a method described elsewhere (6), to remove traces of undesirable contaminants. Powdered sugar, used for preparing the chromatographic columns, was commercial confectioner's sugar containing 3 % of corn starch, by weight. The powdered sugar was dried over night in an oven at 80°C. Fresh spinach was used as a test material. All other chemicals, of reagent grade, were used without further purification.

Glassware. All glassware was treated with KOH-saturated aqueous ethanol containing 3% distilled water for 6 hrs at room temperature, rinsed well with distilled water, and dried in an oven at 60°C for at least 12 hrs.

Identification of pigments. Visible absorption spectra of the pigments were measured in diethyl ether using a Hitachi ESP-3T spectrophotometer. The molar extinction coefficients of Comar and Zscheile (7) were used for determining chlorophylls "a" and "b". In addition, the purity and stability of chlorophyll molecules were tested by thin-layer chromatography (8), and high-performance liquid chromatography (9,10), as it has been already described (2) and these authors also accepted that the spectroscopic observation should be supplemented by chromatographic tests to demonstrate that

chlorophyll molecules were not altered. It was possible to detect the pigments qualitatively to the order of 10^{-8}g and 10^{-10}g by using of the thin-layer chromatographic method (8) and the high-performance liquid chromatographic method (9,10), respectively. The chromatographic elution patterns of the pigments were monitored at a wavelength of 380 nm using JASCO UVIDEC-100, because all the pigments studied here exhibit absorption at this wavelength. Further, all developing solvents used have no absorption in this region.

Preparation of the partially purified chlorophyll. In this study, chlorophyll was handled either in complete darkness or under a green-dim light to prevent the photodegradation of chlorophyll. The partially purified chlorophyll was collected as a precipitate by adding dioxane and water to the acetone extract of chlorophyll from spinach leaves according to the method of Iriyama et al (4); the crude chlorophyll was then washed with 80 % (v/v) aqueous methanol (5). Details follow: Spinach leaves (100 g fresh weight) were homogenized for 3 min in a Waring blendor with 500 ml acetone. The green extract thus obtained was filtered through a pad of cotton and the filtrate was centrifuged at 10,000xg for 5 min. The deep-green supernatant solution (Acetone-Extract, ca. 490 ml) was mixed with about 70 ml of dioxane. Then distilled water (80-100 ml) was added dropwise, with stirring, until turbidity developed. The mixture was placed in an ice box for 1 hour to allow sedimentation. The top portion was decanted and the lower thick mass of dark green sediment was collected by centrifugation at 10,000xg for 5 min. The crude mass of chlorophyll thus obtained (Ppt I) was dissolved in about 150 ml of acetone : dioxane mixture (7 : 1, v/v) and precipitated again by dropwise addition of about

25 ml of water to yield Ppt II. Ppt II was dissolved in 500 ml of methanol containing 125 ml of petroleum ether (b.p. 20-40°C) and then 250 ml of distilled water was added into the solution with gentle stirring, to bring about a transfer of the chlorophyll from the methanol to the petroleum ether layer. The upper petroleum ether layer was then washed several times with 80 % (v/v) aqueous methanol to eliminate the residual yellow pigments from the solution. After washing with the aqueous methanol several times, the petroleum ether solution containing chlorophylls "a" and "b" was evaporated under reduced pressure and the product was dried in a vacuum desiccator. Dark-green microcrystals were thus prepared (Ppt III; further purified chlorophyll). Ppt I, Ppt II, and Acetone-Extract were also evaporated and dried in the same way. Figure 1 shows a flow sheet of the procedures used.

Preparation of the powdered sugar columns. The powdered sugar columns are usually prepared by packing succesively small portions of dry powder with a plunger as described by Strain and Sherma (11). In this study, a wet column was employed using a Pharmacia R25 chromatographic tube (2.5x50 cm) connected, through the UV monitor (JASCO UVIDEC-100), to a fraction collector (SF-160K, Toyo). The column was prepared as follows: 150 ml of petroleum ether was poured into the chromatographic tube and 40 g of powdered sugar was then added to the tube; the suspension was then pressed by a slight pressure applied to the top of the column to pack the sugar tightly and uniformly. The procedure was repeated until the final amount of powdered sugar packed into the tube was about 130 g, providing a column about 35 cm high. This column had a capacity of about 50 mg of

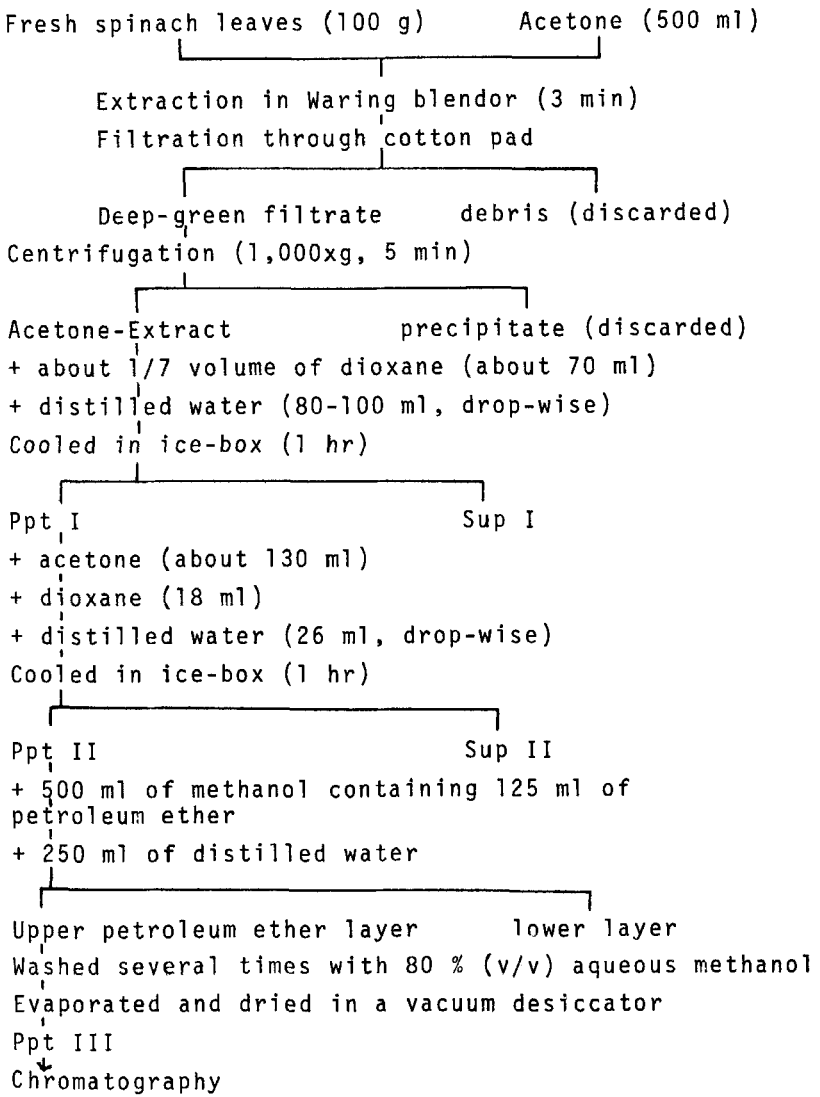

FIGURE 1

Flow sheet of the improved method for extraction and partial purification of chlorophyll.

chlorophyll "a" + "b" without being overloaded. The columns thus prepared were employed for the separation and isolation of pigments in this study.

Powdered sugar column chromatography. The chromatograms in this study were usually developed with the solvent program reported by Perkins and Roberts (12). Percolation of the pigment solutions and the developing solvents was accelerated by slight pressure from a rotor pump (SJ-1210, Mitsumi; flow speed: about 20 ml/hr/cm^2) connected to the top of the chromatographic tube by way of a teflon tube. Each of the crude masses of prepared chlorophyll was dissolved completely in a minimum volume of diethyl ether; petroleum ether was then added to each of the solutions until the final petroleum ether concentration was 90 % (v/v). This solution was then added to the top of the column and then the column was washed with petroleum ether. After the nonsorbed carotenes were eluted and collected, the column was washed with 10 % (v/v) diethyl ether in petroleum ether until all the pigments were adsorbed on the column and then the blue-green zone (chlorophyll "a"), which followed the grey zone (pheophytin "a") and the second yellow zone (xanthophylls), reached nearly to the bottom of the column. At this stage, 0.5 % (v/v) isopropyl alcohol in petroleum ether was poured into the column to separate all the pigments remaining in the column from each other and to elute the blue-green zone, the third yellow zone (zeaxanthin plus lutein), the yellow-green zone (chlorophyll "b"), and the fourth yellow zone (neoxanthin plus violaxanthin) in sequence.

RESULTS AND DISCUSSION

Figure 2 shows the separation of the leaf pigments contained in the acetone extract. Development on the

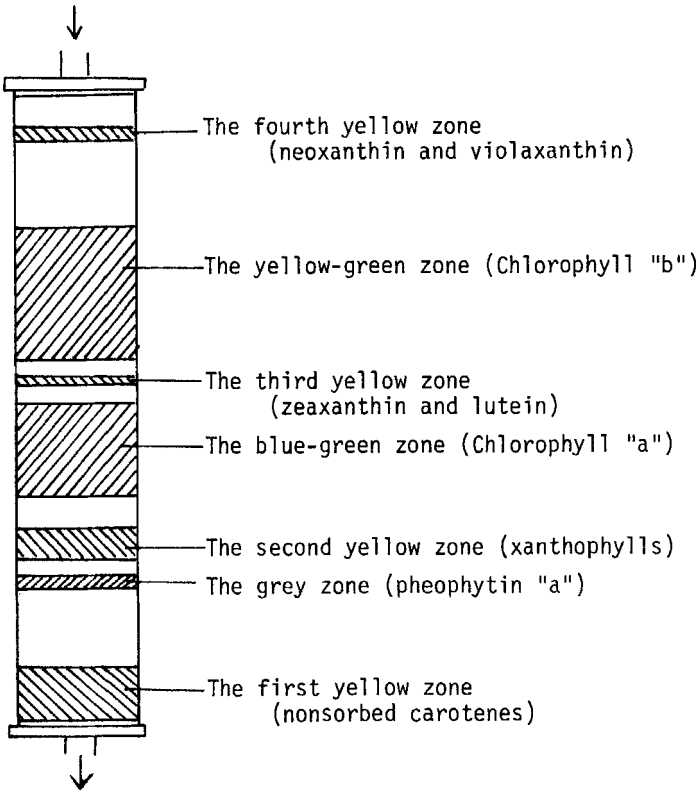

FIGURE 2

Leaf pigments in Acetone-Extract* separated in a column of powdered sugar equilibrated with 0.5 % (v/v) isopropyl alcohol in petroleum ether (b.p. 20-40°C).
*For the explanation, see Fig. 1.

sugar column with 0.5 % (v/v) isopropyl alcohol in petroleum ether yields separated zones of the leaf pigments in the sequence; the first yellow zone (consisting of the nonsorbed carotenes on the column), the grey

zone (consisting of pheophytin "a"), the second yellow zone (consisting of xanthophylls), the blue-green zone (consisting of chlorophyll "a"), the third yellow zone (consisting of zeaxanthin and lutein), the yellow-green zone (chlorophyll "b"), and the fourth yellow zone (consisting of neoxanthin and violaxanthin). The formation of pheophytins in Acetone-Extract was observed. They are produced via degradation of chlorophylls when the acetone extract was allowed to stand for a long period (longer than 2 hrs). Acetone is a more effective solvent than methanol for extracting chlorophylls "a" and "b" from spinach leaves. Since the resolution was generally poor, except between the first and second yellow zones, multiple-zoning and tailing often occurred with high pigment loadings, ie., more than 20 mg of total pigment weight on the column. It is very difficult to check the migration of yellow pigments in chlorophyll preparations by a spectroscopic method alone. For example, commercial chlorophyll "a" was reported (13) to contain xanthophylls and to be free from chlorophyll "b". In fact, we have experienced that thin-layer and high-performance liquid chromatographic tests revealed the migration of xanthophylls, lutein and/or zeaxanthin into the chlorophyll "a" and "b" fractions, although the "complete" separation among the pigments zones were performed visually and spectroscopic tests of each of the chlorophyll fractions showed no significant differences of visible absorption spectra from literature values. Therefore, we tried to eliminate xanthophylls, lutein and zeaxanthin from acetone extract before chromatographic separation and isolation, and then developed a method for partial purification of chlorophyll extracted from spinach leaves prior to chromatographic separation and isolation (4,5)

by the procedure which was described in the experimental section in this paper. The method (4) is principally based on the fact (14, 15) that dioxane interacts with chlorophyll to form chlorophyll-dioxane adducts selectively and that the adducts are precipitated in microcrystalline form, leaving the bulk carotenoids in solution.

The effectiveness of the partial purification of chlorophyll before chromatography was examined by thin-layer chromatography (8) and high-performance liquid chromatography (10). Figure 3 (a) shows the thin-layer chromatogram of the acetone extract developed in solvent system (*tert*-butyl alcohol : pentane = 1 : 9, v/v). In the chromatogram, lutein, zeaxanthin, and xanthophylls were located in a same spot. Figure 3 (b) shows the thin-layer chromatogram of Ppt I developed in the same solvent system of Fig. 3 (a). Thin-layer chromatographic analysis revealed that Ppt I did not contain even trace amounts of violaxanthin, neoxanthin, zeaxanthin or lutein. Xanthophylls gave a minor spot between chlorophyll "a" and "b" spots. High-performance liquid chromatography of Ppt II showed that the second dioxane treatment could not eliminate xanthophylls from Ppt I completely. Figure 4 shows the high-performance liquid chromatograms of the acetone extract and Ppt II. In some cases, thin-layer chromatographic analyses of Ppt II could not detect any extraneous visible spots corresponding to xanthophylls. However, it should be noted that repeated precipitations of chlorophyll by using of dioxane were not always effective to eliminate the second yellow zone (xanthophylls) completely, although the amount of xanthophylls was decreased gradually by repeated dioxane treatments. 50 mg of Ppt I was chromatographed on the sugar column to elucidate

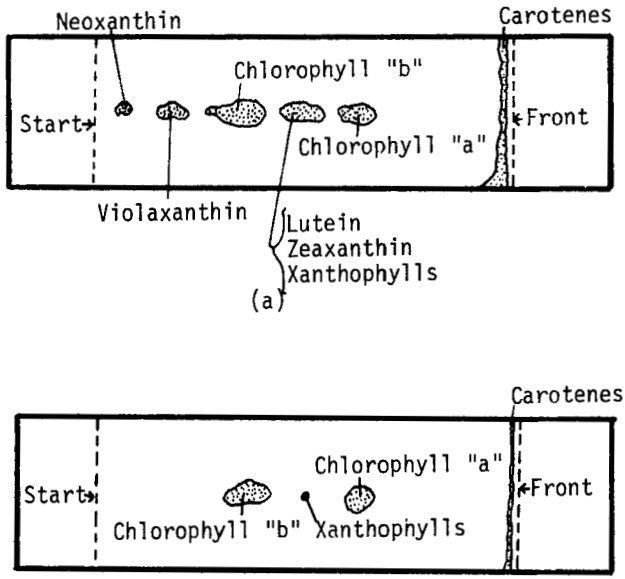

FIGURE 3

Thin-layer chromatograms (a) for the acetone extract* and (b) for Ppt I* on silica gel layers developed in solvent system (*tert*-butyl alcohol : pentane = 1 : 9, v/v). 2.5x10 cm of commercial silica gel sheets (silica gel "Spotfilm" without fluorescence indicator, Tokyo Kasei LTD, Tokyo, Japan) were used. Solutions of test materials in diethyl ether were spotted with 2 µl micropipette 1.5 cm from the lower edge.
*For the explanations, see the text.

possible minor contamination of the pigments contained in the second and third yellow zones into the chlorophyll "a" and "b" fractions. The elution pattern is presented in Fig. 5 (a). The chromatogram shows that chlorophyll "a" was mixed with xanthophylls. Xanthophylls, however, should be removed from Ppt II before chromatographic

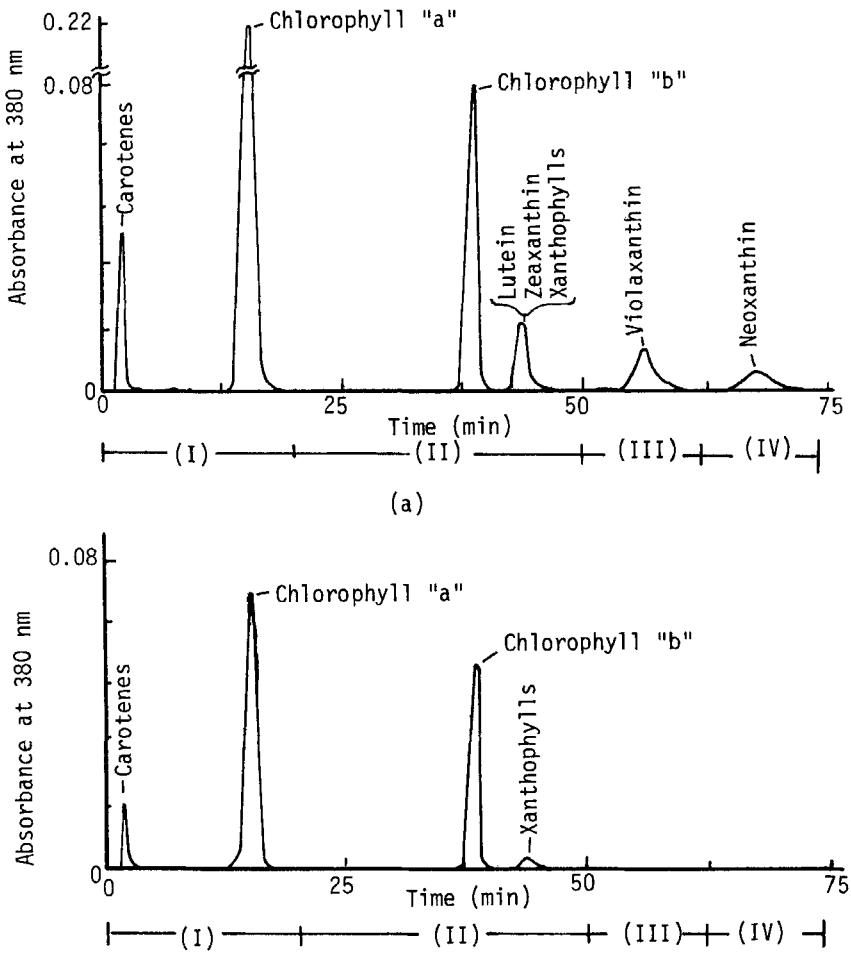

FIGURE 4

High-performance liquid chromatograms (a) for the acetone extract* and (b) Ppt II*. High-performance liquid chromatography was carried out with a Familic-100 instrument (Japan Spectroscopic, Tokyo, Japan) with 65x0.5 mm I.D. PTFE tube packed with silica gel powder SS-05 (particle size 0.5 µm, Japan Spectroscopic). Elution patterns were monitored at 380 nm with Uvidec-100 (Japan Spectroscopic). The light path-length was 0.5 mm. (I) 1 %, (II) 2 %, (III) 5 % and (IV) 10 % isopropyl alcohol in hexane were eluted at a flow-rate of 16 µl/min at room temperature.
*For the explanations, see the text.

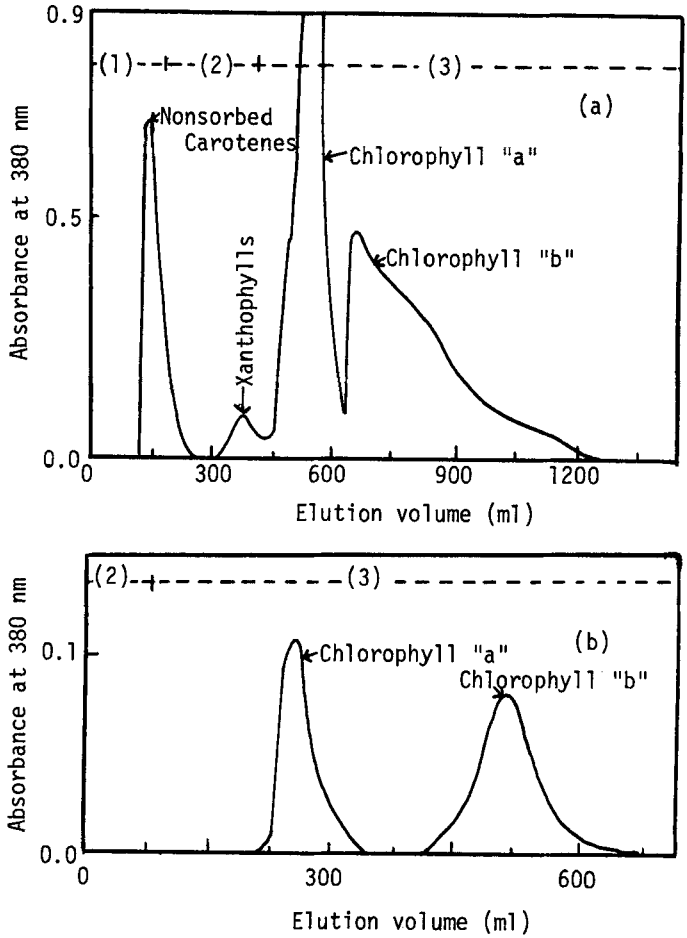

FIGURE 5

Chromatogram of chlorophylls in powdered sugar columns monitored at 380 nm with a flow-rate of 20 ml/hr/cm^2. Fractions of 15 ml were collected. (a) Ppt II* (50 mg), and (b) fractions from 600 ml to 670 ml presented in (a).
Eluents: (1) petroleum ether, (2) 10 % (v/v) diethyl ether in petroleum ether, and (3) 0.5 % (v/v) isopropyl alcohol in petroleum ether.
*For the explanation, see the text.

separation and isolation because xanthophylls disturb the separation and isolation of chlorophylls from one another with a powdered sugar column. The fractions from 600 ml to 675 ml were collected and were evaporated under reduced pressure. The resultant precipitates after drying were then re-chromatographed. The elution pattern is presented in Fig. 5 (b). This pattern also supported the fact that Ppt I did not contain any trace amounts of lutein and zeaxanthin.

On the other hand, aqueous methanol was used for separating yellow pigments from the leaf extracts in petroleum ether solution (1,3). Strain and Svec (2) reported in their article that xanthophylls were removed from petroleum ether solutions of chlorophyll by washing with 80 % (v/v) aqueous methanol. To eliminate xanthophylls from Ppt II, it was washed with 80 % (v/v) aqueous methanol several times to yield Ppt III. 30 mg of Ppt III was then chromatographed on the sugar column; the elution pattern is shown in Fig. 6. It can be seen that xanthophylls were absent from Ppt III. Trace pheophytins were not detected in fresh Ppt III preparations. Each of the pigment fractions was analyzed by thin-layer chromatography. These analyses supported the column chromatographic observations. Thin-layer chromatographic analyses of the column chromatographic fractions from 375 ml to 600 ml yielded only one spot corresponding to chlorophyll "a". In the fractions (375-600 ml), a shoulder was observed. The shoulder might be derived from the exchanging of the developing solvents from petroleum ether to 10 % (v/v) diethyl ether in petroleum ether.

Pigment composition of the acetone extract, Ppt I, Ppt II, and Ppt III is represented in Fig. 7. Pigment composition of the acetone extract in this study does

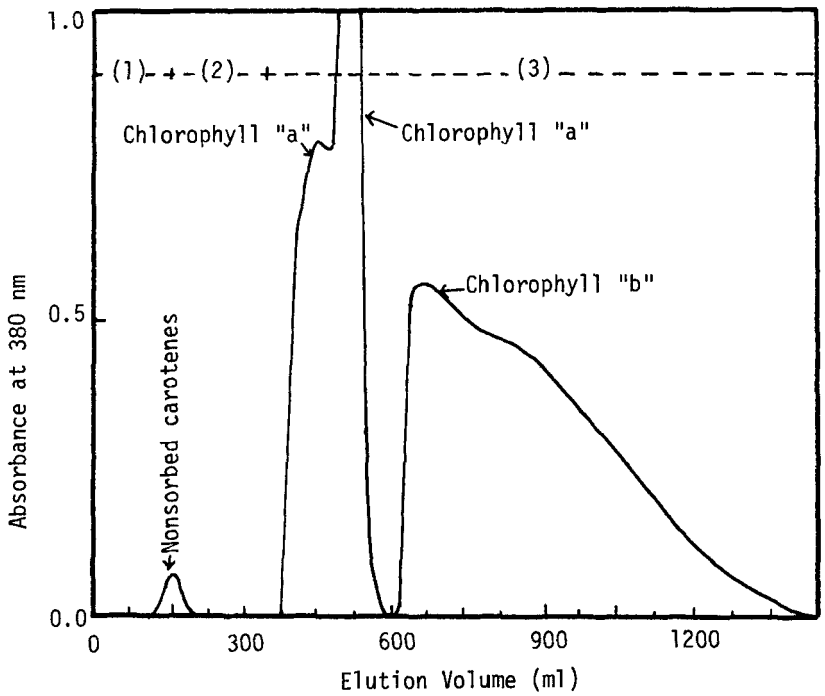

FIGURE 6

Chromatogram of crude chlorophylls in Ppt III* (30 mg). Other conditions were the same as presented in Fig. 5. Thin-layer chromatographic analyses of the column chromatographic fractions from 375 ml to 600 ml yielded only one spot corresponding to chlorophyll "a". *For the explanation, see the text.

not reflect the true pigment composition in the leaves. The amount of chlorophyll "a" is lower in the spinach leaves, although, in general, the ratio of occurrence in higher plants is about 1 molecule of chlorophyll "b" to 3 molecules of chlorophyll "a". However, this presents no problem for the preparative work described elsewhere (16).

As mentioned above, all the yellow pigments, which interfere with the purification of chlorophylls "a" and

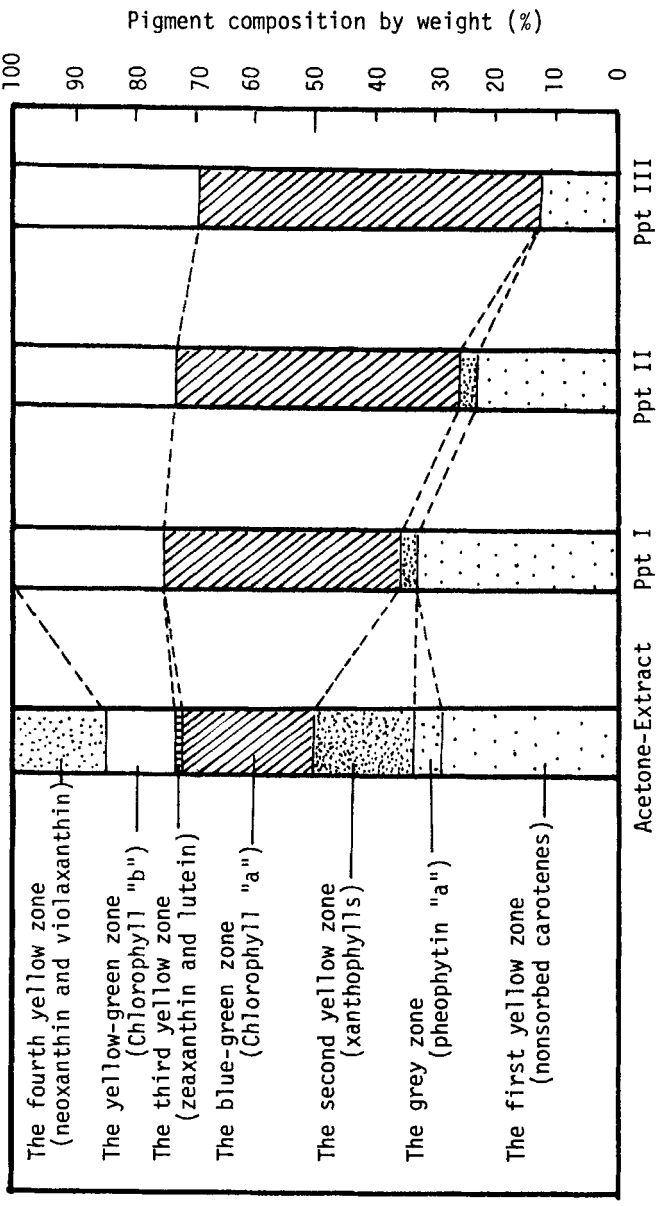

FIGURE 7

Pigment composition of crude chlorophyll preparations by weight %. For the explanations of Acetone-Extract, Ppt I, Ppt II, and Ppt III, see the text.

"b" via the powdered sugar column method of Perkins and Roberts (12) were eliminated completely from the acetone extract of spinach leaves by dioxane treatment and successive washings with 80 % (v/v) aqueous methanol prior to chromatography.

The sugar columns prepared here were very effective for the separation of pigments in Ppt III, and good reproducibility was observed. With this sysyem, 80 mg of Ppt III could be passed through the column, without over-loading, to purify chlorophylls "a" and "b". Thin-layer chromatographic tests of each of freshly prepared pigments yielded one spot. These observations showed also that chemical degradation of the pigments did not occure during the course of separation and isolation by the column chromatography in this study. There have been few detail reports concerned with the preparatory method for purification of chlorophylls "a" and "b" by using of a fraction collector to collect each of the pigment fractions.

Column chromatography with low-cost powdered sugar has been widely used to prepare chlorophyll (2-5, 11, 12, 17); sugar is an ideal adsorbent for the separation isolation of chlorophylls "a" and "b" without inducing chemical degradation. However, preparation of sugar columns is not easy, because powdered sugar has a tendency to adsorb atmospheric moisture; there is difficulty in packing powdered sugar into a tube uniformly and with moderate packing density. Therefore, powdered sugar containing 3 % starch by weight is usually employed as adsorbent. Strain and Sherma (11) recommended the use of powdered sugar containing 10 % by weight starch as an adsorbent in very humid weather, especially if the packed columns are to stand more than a few hours before use. The uniform shapes of the pigment zones on

the column in development depend not only upon the adsorbed moisture, but also upon the packing uniformity of density of powdered sugar in the chromatographic tube. It was effective against the humidity to pack the powdered sugar into the tube uniformly and tightly according to the method of Strain and Svec (2), but we have experienced considerable difficulty to pack the powdered sugar in a sufficient reproducibility. Thus, the procedure described in the experimental section was employed to pack the powdered sugar into a Pharmacia column; a good result was then obtained. The sugar columns used in this study were always observed to be effective for separation and isolation of chlorophylls "a" and "b" and we were able to use them repeatedly (for at least 10 times) for purification of the pigments.

Various kinds of adsorbents have been tested by many investigators; these are reviewed elsewhere (2,3). Strain and Sato (18) found that the pigment zones were better defined on sugar columns than on cellulose. Recently, Sato and Murata (19) developed a preparative method for chlorophyll via column chromatography with diethylaminoethylcellulose. They collected chlorophylls as precipitates according to the method of Iriyama et al (4). The crude chlorophyll precipitates were applied to a Sephadex LH-20 column with chloroform as the eluent and then to a diethylaminoethylcellulose column with a chloroform/methanol mixture (49 : 1, v/v) as the eluent. However, the separation and isolation of chlorophylls from one another was not sufficient to collect each of the pigment fractions. Re-chromatography was required for further purification. It was found to be effective to prepare chlorophyll "a" by using of blue-green algae as starting materials. Polystyrene

powder was also effective as an adsorbent to separate chlorophylls. However, these synthetic adsorbents, such as polystyrene powder, diethylaminoethylcellulose and Sephadex LH-20, contain unknown colorless impurities whch can be dissolved into the developing solvents used in separation and isolation of chlorophylls by column chromatography. Therefore, we are now attempting to develop a procedure how to wash the synthetic adsorbents. Most recently, we developed a method for purification of chlorophyll "a" without any chromatographic procedures (20).

All the procedures for the preparation of chlorophylls "a" and "b" should be accomplished smoothly and swiftly to obtain highly purified pigment preparations, because chlorophylls "a" and "b" molecules in solution are very unstable and are chemically converted to their degradation products (e.g., chlorophyll "a'", chlorophyll "b'", pheophytin "a", pheophytin "b" etc.). Since Ppt III can be stored at -20°C, in darkness, for at least 3 days without any significant chemical change, the preparation of Ppt III should be performed within 6 hrs. In addition, we would recommend examination of the purity and stability of chlorophylls "a" and "b" in an acetone extract, Ppt I, Ppt II, and Ppt III by the thin-layer chromatography (8) before proceeding to next procedure. When the thin-layer chromatographic tests reveal the formation of degradation products of chlorophyll in Ppt III, it should be precipitated again by dioxane treatment and washed with 80 % (v/v) aqueous methanol and then the crude chlorophyll preparation should be chromatographed. Otherwise, the presence of degradation products of chlorophyll may prevent separation and isolation of the pigments with sugar column chromatography in this study.

As we have seen, chromatographic methods are effective for micro-scale analysis of chlorophylls as well as for preparation of the pigments. The purity of chlorophyll preparations should be determined spectroscopically after chromatographic tests. It has been confirmed thin-layer chromatographically that about 20 % of chlorophyll "a" in diethyl ether solution placed in complete darkness at room temperature were converted to chlorophyll "a'" in 4 hrs. It has been recognized (21-23) that chlorophyll "a" and chlorophyll "a'" are very similar spectroscopically as well as interconvertible. Hence, spectroscopic tests alone provide no indication of the purity of chlorophyll "a". Recently, we developed a micro-method for the qualitative and quantitative analysis of photosynthetic pigments using high-performance liquid chromatography (9,10). This method may be useful for chlorophyll studies, because there is no other method to analyze photosynthetic pigments and their degradation products at levels of 10^{-9}g, quantitatively.

Thus, the procedures in this study are recommended as a routine method for preparation of chlorophylls "a" and "b" in the laboratory.

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