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Note

Preparation and reversed-phase high-performance liquid chromatography of chlorophylls

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Many experiments in photosynthesis require pure chlorophylls. The most frequently used separation method for extracts of the chloroplast pigments is adsorption chromatography on sugar–starch mixtures as the stationary phase[1]. Other stationary phases, for example polyamide[2], do not give the simultaneous separation of the carotinoids, xanthophylls and pheophytins from chlorophylls (chl) *a* and *b*. Moreover, adsorbents such as magnesium oxide or silica gel rapidly degrade chl *a* and *b*[3]. Inactive stationary phases tested during the last few years include Sephadex LH-20[4,5], microcrystalline cellulose[6], DEAE-cellulose[7,8] and polyethylene powder[9].

The precipitation of chlorophylls from 2-propanol as polymeric 1,4-dioxane complexes[10–12] prior to liquid chromatography gives very pure chl *a* and chl *b* preparations[13] if the separation is carried out on Sephadex HP ultrafine (particle size 17–23 μm) as stationary phase with cyclohexane–tetrahydrofuran (1000:8) as eluent. Sephasorb HP ultrafine comprises rigid, porous beads of hydroxypropyl-substituted cross-linked dextran, a polysaccharide. Except for a very small amount of diastereoisomers, no other compounds could be detected in the chlorophyll preparations. Instead of the chlorophyll–dioxane complex, the analogous complexes formed with 1,4-pyrazine[14,15] and 1,4-diazabicyclo[2.2.2]octane (DABCO)[10,14,15] can be used for the precipitation, but in our experience they do not offer any obvious practical advantage.

The composition of the chlorophylls purified by the above method has now been determined by high-performance liquid chromatography (HPLC) on C_{18} reversed-phase material, which easily separates chl *a'* and *b'* from chl *a* and *b*. Mobile phases containing methanol promote the allomerization reactions of the chlorophylls and therefore should be avoided.

EXPERIMENTAL

The solvents were obtained from E. Merck (Darmstadt, G.F.R.). Cyclohexane, technical grade, was distilled. Tetrahydrofuran, analytical grade, was freed from peroxides by column chromatography on neutral alumina (Woelm, Eschwege, G.F.R.). All other solvents were analytical grade and were used without further purification.

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Preparation of the chlorophyll extract

An 800-g amount of fresh spinach (*Spinacia oleracea*) was blended in a commercial mixer and extracted three times with 400 ml 2-propanol. Fresh spinach is preferred because the deep-frozen material contains significant amounts of chl *a'* and *b'*.

Precipitation of the chlorophyll–dioxane complex

A 165-ml volume of dioxane was added to the 1200 ml of filtered extract in 2-propanol. To the stirred solution, 270 ml water were added dropwise. After *ca.* 1 h at 4°C the chlorophyll–dioxane complex precipitated quantitatively. The black precipitate was centrifuged and the yellow supernatant rejected. The precipitate was dissolved in 270 ml of 2-propanol–dioxane (7:1) and the chlorophyll–dioxane complex was reprecipitated with 130 ml water as described above.

Centrifugation and separation of the precipitate should be carried out in the dark. Degassing of the solvents used does not increase the purity. The precipitate was dried *in vacuo* at room temperature. Small amounts of xanthophylls, carotinoids and pheophytins are detectable in the precipitate by thin-layer chromatography using a reversed-phase technique with cellulose as carrier[16]. The impurities are easily separated by chromatography on Sephasorb HP.

The 800 g of spinach give *ca.* 700 mg of chlorophyll *a*/*a'*–chlorophyll *b*/*b'*–dioxane complex containing traces of xanthophylls, carotinoids and pheophytins.

Packing of the preparative column

A 400-g amount of Sephasorb HP ultrafine (Pharmacia, Uppsala, Sweden) was suspended in 400 ml of mobile phase [cyclohexane with 0.8% tetrahydrofuran (THF)]. This slurry was poured into the column with the aid of a pump and a filling pipe. At mobile phase flow-rates of up to 4 ml/min the pressure remains below 1 bar. According to the manufacturer, glass columns of diameter 4 cm are safe at a maximum pressure of 4.5 bar. Column lengths of up to 1 m keep the resistance well below the maximum pressure load.

Preparative liquid chromatography on Sephasorb HP

The chlorophyll–dioxane precipitate was dissolved in high purity carbon tetrachloride to a concentration of *ca.* 30 mg/ml. A 1–2 ml volume of this solution was injected directly onto the preparative column. The low pressure chromatographic system used is presented in Fig. 1. A cross-section of the connections at the end of the preparative column is shown in Fig. 2. The advantage of this separation system is the lack of parts which can be worn out such as seals and gaskets. It is completely leak-proof to all organic solvents owing to the use of Teflon® for the connections. After the separation the obtained fractions containing chl *a* and *b* were pulverized and the purity was tested with HPLC and thin-layer chromatography (TLC). The conditions for the preparative separation are listed in Table I, while those for the HPLC separation are in Table II. The manufacturers of the components of the low pressure chromatographic system are listed in Fig. 1. The fittings for the preparative assembly were manufactured in our workshop. A chromatogram of the chlorophyll–dioxane complexes separated on Sephasorb HP ultrafine is shown in Fig. 3.

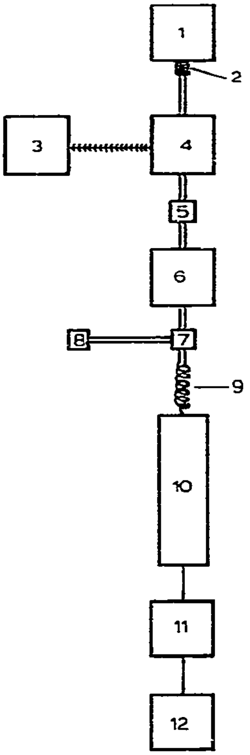


Fig. 1. Scheme of the low pressure preparative column. 1 = Solvent reservoir (Jenaer Glaswerk Schott & Gen., Mainz, G.F.R.); 2 = filter of sintered stainless steel (Waters GmbH, Königstein/Ts., G.F.R.); 3 = thermostat for solvent degassing unit (Haake, Karlsruhe, G.F.R.); 4 = solvent degassing unit (Otto Fritz GmbH, Hofheim/Ts., G.F.R.); 5 = glass filter, type G3; 6 = controlled volume pump (instrument minipump; Dosapro Milton Roy, Neu-Isenburg, G.F.R.); 7 = three-way cock (Otto Fritz GmbH); 8 = injection system (Latek, Heidelberg, G.F.R.); 9 = sample loop, 1/8 ft. Teflon tubing; 10 = separation column, 40 x 4 cm I.D. (QvF Glastechnik GmbH, Wiesbaden, G.F.R.); stationary phase: Sephasorb HP ultrafine (Pharmacia, Uppsala, Sweden); 11 = photometer-detector (PM2DL; Zeiss, Oberkochen, G.F.R.); 12 = fraction collector (7000 Ultrarac; Kontron, Munich, G.F.R.).

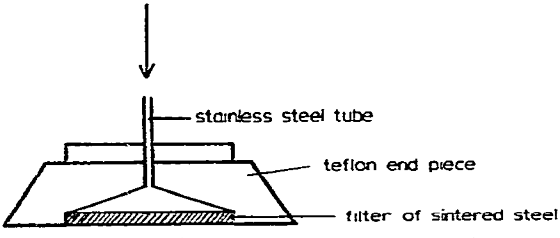


Fig. 2. Cross-section of the connective assembly.

HPLC of chlorophylls

HPLC of chlorophyll on a 5-μm RP-C_{18} column is an excellent method for testing the purity of the isolated fractions with regard to their content of chl *a* and *b* and of the isomeric chlorophylls. By-products, most probably allomerized chloro-

TABLE I
CONDITIONS OF PREPARATIVE SEPARATION

Column	See Fig. 1
Stationary phase	Sephasorb HP ultrafine (Pharmacia, Uppsala, Sweden)
Eluent	Cyclohexane–tetrahydrofuran (1000:8 v/v)
Flow-rate	25 ml/h
Temperature	20°C
Head pressure	Below 1 bar

TABLE II
CONDITIONS FOR HPLC OF CHLOROPHYLL *a* AND *b*

Column	Stainless steel, 120 × 4.5 mm (Knauer, Berlin, G.F.R.), packed with 5-μm LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R.)
Column efficiency	34,500 plates per m (chl *a*)
Eluent	Acetonitrile–water (94:6 v/v)
Flow-rate	3 ml/min
Pressure	138 bar (2000 p.s.i.)
Temperature	25°C
Pump	Type M 6000 (Waters Assoc., Königstein/Ts., G.F.R.)
Detector	Spectrophotometer Type LC 55 (Perkin-Elmer, Überlingen, G.F.R.) at 436 nm

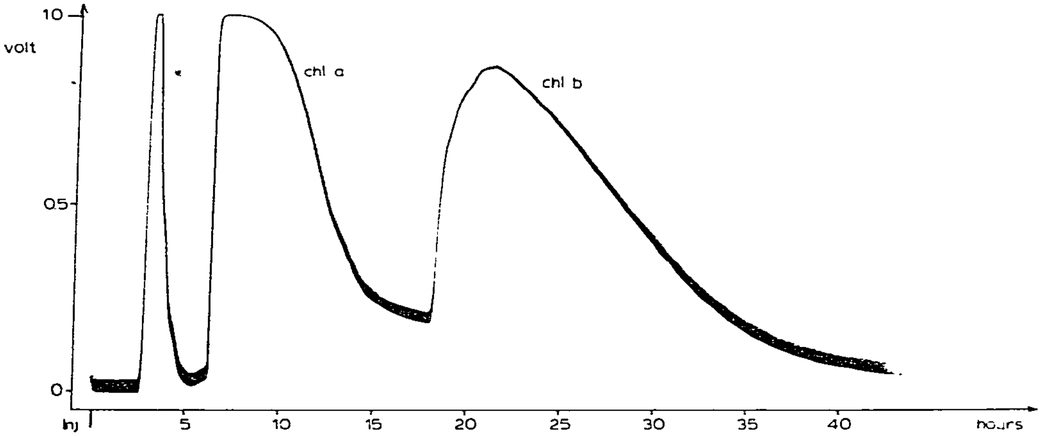


Fig. 3. Chromatogram of the chlorophyll–dioxane complexes separated on Sephasorb HP ultrafine. The asterisk denotes carotinoids, xanthophylls and pheophytins.

phylls[17], can be detected by using a mobile phase based on methanol (Fig. 4). Formation of these products can be avoided with a mobile phase based on acetonitrile. Satisfactory separations were achieved using acetonitrile–water (94:6) or acetonitrile–water–tetrahydrofuran (93:6:1). The efficiency of both these mobile phases is about

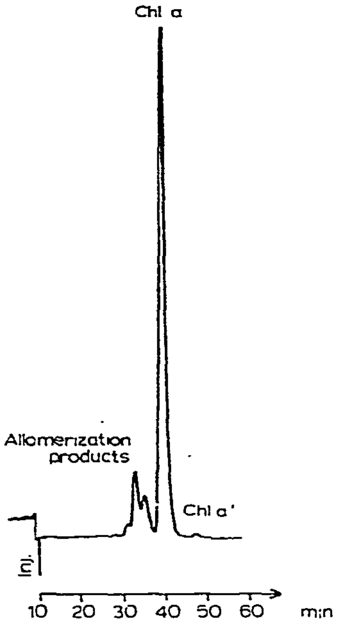


Fig. 4. HPLC chromatogram of chl *a* prepared according to the method described here using methanol–water (95:5 v/v) instead of acetonitrile–water (94:6 v/v).

the same. The chlorophylls in the mixture were identified by comparison of their retention times with those of the pure compounds. Note that small differences in the retention times for chl *a*, chl *a′*, chl *b* and chl *b′* (see Figs. 4–7) result from small variations in the water content of the mobile phase. Unfortunately, the purest commercial chlorophylls are of limited purity (Fig. 5). Chl *a′* and chl *b′* were identified after isolation and equilibration with chl *a* and chl *b*, respectively (Fig. 6). At the detector wavelength employed, 436 nm, small amounts of impurities, such as the xanthophylls and pheophytins, are not detected. However, the simultaneous separation and detection of all compounds of interest can be achieved by TLC on cellulose using a reversed-phase technique[16].

RESULTS AND DISCUSSION

The method described offers the following advantages compared to other preparative methods for chl *a* and *b*:

(1) Precipitation of the chlorophylls as magnesium coordination polymers, *e.g.*, as dioxane or 1,4-pyrazine complexes, offers a very effective and specific preseparation. The subsequent column chromatographic separation can thus be done with a highly precleaned material.

(2) The expenditure of work per separation run is low, because the columns can be used for many runs. The handling of this low pressure chromatographic system is simple and can be automated.

An HPLC chromatogram of the crude chlorophyll–dioxane precipitate is shown in Fig. 7. The small traces of xanthophylls and pheophytins found in this

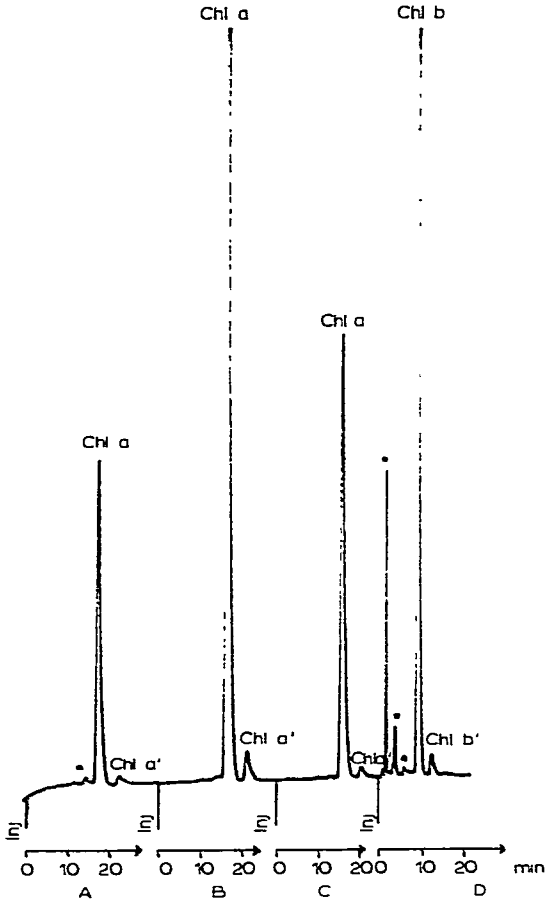


Fig. 5. HPLC chromatograms of pure chl *a* separated on a sugar column (asterisk probably indicates an allomerization product) (A), of pure chl *a* prepared according to the method described here (Table I) (B), of pure chl *a* from Sigma (Munich, G.F.R.) (C) and of pure chl *b* from Sigma (D).

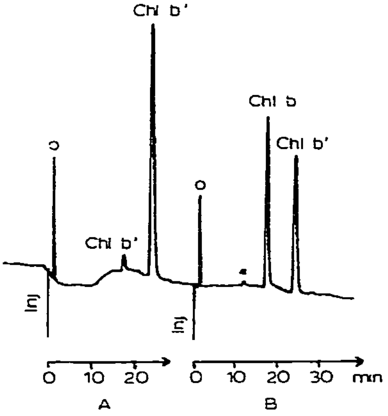
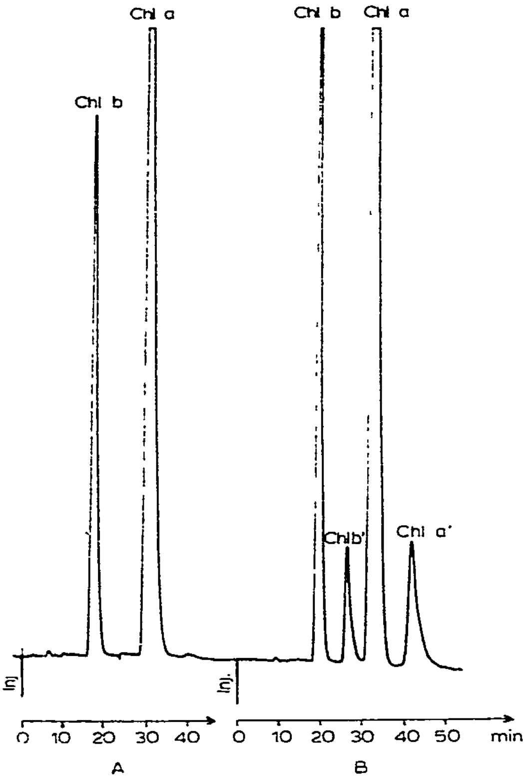


Fig. 6. HPLC chromatograms of enriched chl *b'* (A) and of the same probe after heating the carbon tetrachloride solution for 20 min at 50°C (B). The change of chl *b'* into chl *b* can be seen. Solvent peaks are indicated by circles, and the asterisk denotes an unknown component.

precipitate were not detected by the LC–UV detector operating at 436 nm but by TLC. No glyco- and phospholipids were detected in the solution of the dioxane precipitate using the TLC test of Pohl *et al.*[18].

The HPLC test shows (Fig. 5) that the separation of chl *a* and *b* from one another by the preparative method described is nearly complete. In the chl *b* only a very small amount of chl *a* is detectable (Fig. 8), while chl *a* is completely free from chl *b* (Fig. 5). The efficiency of the separation depends to a large degree on the tetrahydrofuran content in the cyclohexane. The efficiency of the chl *a*–chl *b* separation increases when the amount of tetrahydrofuran is lowered to *ca.* 0.4%, so it is also possible to prepare chl *b* absolutely free from chl *a*. If only very pure chl *a* is needed, a higher tetrahydrofuran content of *ca.* 0.8% in the cyclohexane should be chosen for a



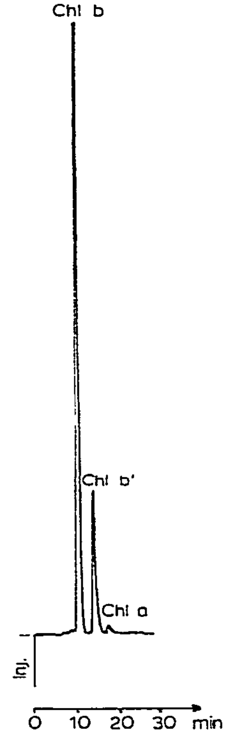


Fig. 7. HPLC chromatograms of the chlorophyll–dioxane precipitate from fresh spinach (A) and from deeep-frozen spinach (B).

Fig. 8. HPLC chromatogram of chl *b* prepared according to the method described (Table I).

shorter retention time. No isomerization of chl *a* and *b* to the diastereoisomers chl *a'* and *b'* on the preparative column is detectable.

Experiments with a semipreparative column packed with 5-μm C_{18} reversed-phase material have shown that it is possible to separate larger quantities of the two diastereoisomers chl *a'* and *b'* with the mobile phase acetonitrile–water (94:6), for example from an extract of deep-frozen spinach.

CONCLUSIONS

Although generally presented as a purity criterion[8,13,19], absorption spectra give only crude information about the purity of chlorophylls, since it is not possible to detect impurities below the 5% level. Only the analytical characterization of these compounds by highly effective separation methods such as HPLC and an improved type of reversed-phase TLC can be used to determine the purity of chlorophylls. Since the method described here combines a specific enrichment step —complexation of the central magnesium of chlorophylls— with a highly effective separation of the chlorine moiety, it seems feasible to analyse for chlorophylls other than the known ones.

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