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Note

The purification of chlorophyll *a* from spinach leaves by partition chromatography on Bio-Glas columns

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Procedures for the chromatographic isolation of chlorophyll *a* and *b* from green plant materials have been reviewed by Strain and Svec[1]. The method most commonly adopted is that described by Strain *et al.*[2] which utilises sucrose columns and a petroleum eluent containing a little *n*-propanol.

The rather laborious and complex operations associated with the preparative procedures in the literature have encouraged us to examine a more direct approach which employs partition chromatography. Porous glass is used as an inert support for a column of the lower phase from an equilibrated mixture of petroleum (b.p. 60–80°)–cyclohexane–dimethylformamide–water (30:30:20:1). The upper phase from this mixture is used as the mobile phase. We found that this solvent system, in association with the procedure described below, allows chlorophyll *a* and *b* to be separated from each other and the accompanying pigments in one chromatographic pass and isolated in high yields.

Chlorophyll *a*, which has been our main objective, can be obtained directly from the column free from other pigments and with a purity of at least 88% by this method and, if necessary, the overall operation can be completed in less than 8 h. Further purification to purities of greater than 98% has been achieved by conventional methods. The loadings given are recommended for good separations of chlorophyll *a* and *b* but the column is capable of handling substantially larger quantities of pigment when very pure materials are not required. Furthermore, the column can be readily stripped and re-equilibrated without removing the supporting glass beads.

MATERIALS AND METHODS

Materials

"Birds-Eye" frozen spinach leaves were purchased locally and kept in a deep-freezer until required. Bio-Glas-2500 (Bio-Rad Labs., Richmond, Calif., U.S.A.) was washed with acetone and dried at 70° under vacuum for 6 h before use.

Solvents were freshly distilled and de-aerated with nitrogen before use. The partition solvent system was prepared from petroleum (b.p. 60–80°) (600 ml), cyclohexane (600 ml), dimethylformamide (400 ml) and water (20 ml) by shaking the mixture in a 3-l separating funnel, which had been flushed with nitrogen, and allowing

the upper phase, which we term solvent A, and the lower phase, solvent B, to equilibrate for at least 1 h before use.

Column preparation

Bio-Glas-2500 (200 g) was placed in a flask, covered with solvent B, and thoroughly de-gassed at room temperature. The excess solvent was decanted and the support was washed several times with solvent A before being transferred in small portions as a slurry in solvent A to a glass column (32 mm I.D.). Each portion was firmly tamped down with a perforated PTFE disc to ensure efficient packing and the resulting 360-mm column was irrigated with solvent A to remove final traces of non-adsorbed solvent B.

Extraction of pigments

Throughout the extraction the amount of light and oxygen that reached the chlorophylls was kept to a minimum. Frozen leaf spinach (120 g) was allowed to come to room temperature before excess water was removed by pressing the leaves between filter papers. Stalk material was removed manually. The leaves were extracted by blending with 3 × 200 ml cold aqueous acetone* (acetone–water, 4:1) and the combined extracts were transferred to petroleum (700 ml). The aqueous phase, which contained much of the xanthophyll component, was discarded. The petroleum solution was washed with aqueous acetone (2 × 500 ml) and 0.5% sodium bicarbonate solution (2 × 500 ml) before being briefly dried over magnesium sulphate and evaporated to dryness under reduced pressure at 40°. The extracts were finally dried at room temperature and at 0.5 mmHg for 3 h. A typical yield was 0.25 g.

Chromatography of the pigments

This operation was carried out in a darkened room. The dried extract was dissolved in the minimum volume (*ca.* 5 ml) of solvent A and carefully transferred to the top of the column by means of a peristaltic pump. The column was then eluted with solvent A at a flow-rate of 1–1.5 ml/min.

The pigments were eluted in well defined zones. The order was carotenes (40–65 ml), chlorophyll *a* (130–200 ml), and chlorophyll *b* (420–550 ml). The xanthophylls, which moved only slowly, were normally stripped off with solvent B. Occasionally the chlorophyll *a* was preceded by a small amount of pheophytin *a*.

The fractions containing the chlorophylls were diluted with equal volumes of cyclohexane and washed with saturated solutions of magnesium sulphate to remove the dimethylformamide. They were evaporated to dryness at 40° under reduced pressure and residual traces of solvent removed at 0.5 mmHg, at room temperature. Typical yields for chlorophyll *a* and *b* were 60 and 20 mg, respectively.

Estimation and further purification of chlorophyll a

Ethereal solutions of the chlorophyll *a* from the column showed the absorption maxima reported in the literature. Values for ε_{660} and $\varepsilon_{429}/\varepsilon_{660}$ were 76,800 and 1.30, respectively; the former figure indicating a purity of 89% based on the value given by

* Extraction with hot acetone removed substantial amounts of colourless material which did not separate from chlorophyll *a* on the column.

Strain *et al.*[2]. This material was dissolved in the minimum volume of ether and precipitated with 2,2,4-trimethylpentane to give chlorophyll *a* with a purity of 98%.

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