SEPARATION OF CHLOROPHYLLS *a* AND *b* AND RELATED COMPOUNDS BY THIN-LAYER CHROMATOGRAPHY ON CELLULOSE

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Chloroplast pigments have been separated by thin-layer chromatography on a mixture of kieselguhr G, silica gel, calcium carbonate, calcium hydroxide and ascorbic acid[1]; on kieselguhr G impregnated with triglycerides[2,3]; and by a lengthy development on silica gel[4]. This report describes a simpler system, using thin layers of cellulose, by means of which mixtures of chlorophylls *a* and *b* and some of their derivatives can be separated. Sucrose and cellulose are frequently employed as adsorbents in column or paper chromatography of chloroplast pigments[5], and the separation of these pigments by thin-layer chromatography on sucrose has been reported[6] since the work on cellulose was started.

EXPERIMENTAL AND RESULTS

Cellulose powder (Macherey Nagel MN 300*, 12 g) and distilled water (68 ml) were mixed with a fast electric stirrer for 4 min. Powders made by Pleuger** (83 M 086, 12 g) and Whatman (CC 41, 25.5 g) were also satisfactory. The slurry was applied with a Desaga spreader* as a 250 μ layer to grease-free glass plates, 20 cm × 20 cm, and allowed to partially dry at room temperature for 25 min. The plates were then placed in a ventilated oven at 105°, horizontally for 10 min and vertically for 25 min. After the plates had cooled, the sample was applied as a spot or streak in dim light and the chromatogram was developed in the dark at room temperature with petroleum spirit (A.R., boiling range 60–80°)–acetone (A.R.)–*n*-propanol (90:10:0.45, v/v/v). This mixture was usually added to the tank, which was lined with chromatography paper, about 20 min before inserting the plate. Development was over a distance of 15 cm and took less than 30 min.

The R_F values for chlorophylls *a* and *b* and some of their derivatives are given in Table I. It must be stressed that these are only approximate values, and that they vary with, for example, the make of cellulose powder and the load, or when the pigments are applied as a mixture rather than as separate spots. The running speeds of the chlorophylls and chlorophyllides, in particular, increase with the load; thus the R_F value for chlorophyll *a* rises to 0.65 for a spot containing 0.6 μg, and to 0.72 for 2.0 μg. The limit of easy detection of the various pigments by daylight was about 0.1 μg, but viewing under ultraviolet light increased the sensitivity for most of the compounds.

* U. K. Distributor: Camlab (Glass), Ltd., Cambridge.
** U. K. Distributor: Townson and Mercer, Ltd., Croydon.

TABLE I

APPROXIMATE R_F VALUES FOR CHLOROPHYLLS AND DERIVATIVES ON THIN LAYERS OF CELLULOSE
The compounds were applied as 0.2 μg spots; the developing solvent, petroleum spirit (60–80°)–acetone–*n*-propanol (90:10:0.45, v/v/v), was allowed to ascend 15 cm beyond the origin.

Compound	R_F	*Compound*	R_F	*Colour of spot (both compounds)*
Pheophytin *a*	0.93	Pheophorbide *a*	0.18	Grey
Pheophytin *b*	0.80	Pheophorbide *b*	0.07	Yellow-brown
Chlorophyll *a*	0.60	Chlorophyllide *a*	0.03	Blue-green
Chlorophyll *b*	0.35	Chlorophyllide *b*	0.02	Yellow-green

In spite of considerable tailing of chlorophylls *a* and *b*, there was a clear zone between these two pigments. Very little tailing occurred with the pheophytins and pheophorbides. Although overloading of the chlorophylls or chlorophyllides resulted in overlapping of some of the pigments, these could be distinguished by their colours (see Table I). Application of the sample as a streak rather than as a spot gave particularly clear results. Small blue-green and yellow-green zones were sometimes observed just ahead of and almost separated from chlorophylls *a* and *b*, respectively, and were presumably the *a'* and *b'* isomers. Protochlorophyll, obtained from the inner coats of marrow seeds, ran between chlorophylls *a* and *b* but tailed badly into *b*. The separation of carotenoids was not studied in detail, but with an extract of bean leaves β-carotene ran between pheophytin *a* and the solvent front, and major xanthophyll components were approximately level with pheophytin *b* and pheophorbide *a*.

Care was needed in the choice of solvent for the pigments when applying them to the cellulose layers. With acetone solutions containing 20 % of water, much chlorophyll remained on the baseline after development, and could have been mistaken for chlorophyllide. This difficulty did not arise with water-free acetone solutions, with which conversion of chlorophylls to pheophytins was also minimised. Water should therefore be removed from acetone extracts of leaves before applying them to the cellulose. This can be done by repeated evaporation at low pressure and room temperature, with addition of further acetone (A.R.) after each distillation, until the residue is dry, or by shaking with ammonium acetate (1 g/5 ml of extract) and diethyl ether (1 vol.) and allowing the phases to separate. The possibility of a similar source of error in paper chromatography and in thin-layer chromatography on other supports must be borne in mind. Preliminary tests with silica gel G indicated that much chlorophyll *a* remained on the baseline when spotted from a water-free solvent rather than from one containing water.

Chromatography on microscope slides

Cellulose layers on microscope slides were found to be suitable for rapid qualitative work. A mixture of cellulose powder and water was applied to the microscope slide and smoothed with a glass rod; the layer was dried horizontally at 105° for 25 min. A small covered cylindrical vessel lined with filter paper was used for development. The R_F values of the pheophorbides and chlorophylls were higher than with large plates run for 15 cm, and the pheophytins were less well separated, particularly

when using the Whatman powder. Even smaller amounts of the pigments were visible, however, and development took less than 5 min.

Quantitative chromatography

The possibility of using thin-layer chromatography on cellulose to estimate mixtures of chlorophylls and pheophytins quantitatively was also investigated. Purified samples of each of the four pigments were dissolved in petroleum spirit–acetone–*n*-propanol (90:10:0.45, v/v/v), and aliquots (80 μl) of the solutions were mixed together. The mixture was applied as a streak about 16 cm long to a 20 cm × 20 cm plate; an applicator suitable for this has been described recently[7]. After development of the chromatogram in the dark, over a distance of 15 cm, the chlorophyll *a* band was scraped off and mixed immediately with acetone (A.R., 10 ml) in a stoppered centrifuge tube. This was repeated as soon as possible with chlorophyll *b*, using 5 ml of acetone, and then with the pheophytins. After gentle shaking for about 1 min, the mixtures were centrifuged and the supernatant fluids were removed by pipette for spectrophotometric estimation. Absorbancies were measured at 700 mμ and at the wavelength of maximum absorption in the red region of the spectrum, and the difference between each pair of figures was noted. Use was made of the absorption maximum in the red region because any carotenoid contamination would affect the absorption in the blue region; the reading at 700 mμ was deducted to allow for light-scattering by cellulose still suspended in the supernatant fluid. Absorbancy differences were measured also on aliquots of the original pure pigment solutions, diluted with acetone but not subjected to chromatography; losses during the chromatographic procedure could then be calculated.

Oxidative bleaching, especially of the chlorophylls, occurs readily and is increased by illumination or by exposure of the pigments on a dry chromatographic surface. It was therefore essential to do all the operations rapidly in the minimum of light, speed being particularly important during removal of the pigment zones from the plate. Reflected light from a partly shaded 25 W tungsten lamp in a dark-room was found satisfactory when illumination was necessary. Table II shows the recoveries obtained under these conditions. In experiments 5, 7 and 9, in which the pheophytins were not included, the area between chlorophyll *a* and the solvent front was scraped off and eluted with acetone to test for the presence of pheophytins. The absorbancy of the eluate was equivalent to 0.5 ($\pm$ 0.4) μg of pheophytin *a* or 0.7 ($\pm$0.6) μg of pheophytin *b*, or to even smaller amounts of a mixture of the two. This suggests that very little chlorophyll is converted to pheophytin during chromatography. Examination in each of the experiments of the spectrum of the chlorophyll *b* eluate indicated that only about 1% of the chlorophyll *a* tailed into the chlorophyll *b* zone.

The method appears to be suitable for quantitative analysis of mixtures of chlorophylls and pheophytins, and perhaps also of pheophorbides and chlorophyllides, although practice in rapid working was necessary before recoveries of chlorophyll *a* were adequate. Alternative methods of estimation have been reviewed recently by HOLDEN[5]. They include the procedure of VERNON[8], who used direct spectrophotometry on plant extracts before and after complete conversion of chlorophylls to pheophytins, and that of TAN AND FRANCIS[9], who obtained excellent recoveries from sugar-starch columns. Comparison with other thin-layer and paper chromatographic methods is difficult, because recoveries are not usually quoted. The cellulose powder

TABLE II

PERCENTAGE RECOVERIES FOR MIXTURES OF CHLOROPHYLLS AND PHEOPHYTINS SUBJECTED TO THIN-LAYER CHROMATOGRAPHY ON CELLULOSE

Experiment No.	*Chlorophyll a*		*Chlorophyll b*		*Pheophytin a*		*Pheophytin b*		*Type of cellulose*
	Load (μg)	*Recovery* (%)	*Load* (μg)	*Recovery* (%)	*Load* (μg)	*Recovery* (%)	*Load* (μg)	*Recovery* (%)	
1	49	93	19	93	—	—	—	—	Pleuger
2	49	89	20	96	—	—	—	—	M. Nagel
3	17	94	7	104	—	—	—	—	Pleuger
4	18	90	7	98	—	—	—	—	Whatman
5	42	91	19	90	—	—	—	—	Pleuger
6	44	90	20	100	24	96	4	97	Pleuger
7	43	89	22	96	—	—	—	—	Whatman
8	46	90	20	96	24	97	4	102	Whatman
9	45	91	25	95	—	—	—	—	M. Nagel
10	44	90	20	88	24	94	4	100	M. Nagel
Averages		91		96		96		100	

used for thin-layer chromatography probably allows sharper separations than would be obtained with paper; thus chromatography under the same conditions as above on Whatman No. 3 paper, the most suitable of several Whatman grades tried, resulted in about 2.5 % of the chlorophyll *a* tailing into the chlorophyll *b* zone.

Acetone was used for elution in preference to diethyl ether because of the volatility and ease of peroxide formation of the latter. To calculate pigment concentrations in acetone, the approximate specific absorption coefficients shown in Table III

TABLE III

APPROXIMATE SPECIFIC ABSORPTION COEFFICIENTS (α) OF CHLOROPHYLLS AND PHEOPHYTINS IN ACETONE (A. R. GRADE, < 1 % OF WATER)

Compound	λ_{max} (red) ($m\mu$)	$\alpha\lambda_{max}$ (red) — $\alpha_{700\ m\mu}$ ($l/g \cdot cm$)
Chlorophyll *a*	663	90
Chlorophyll *b*	645	52
Pheophytin *a*	667	53
Pheophytin *b*	654	34

could be used. These values are based on the absorption coefficients in diethyl ether[9–11] and a comparison of the absorptions of the purified pigments in diethyl ether and acetone; on quantitative conversion of the chlorophylls to pheophytins and a comparison of their absorptions in acetone; and on reports by MACKINNEY[12] and VERNON[8]. They are probably not in error by more than 8 l/g·cm.

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SUMMARY

Chlorophylls *a* and *b*, pheophytins *a* and *b*, and pheophorbides *a* and *b* can be separated from each other and from chlorophyllides *a* and *b* on thin layers of cellulose. With precautions, recoveries of chlorophylls and pheophytins exceed 90 %, and it is suggested that the method is suitable for quantitative analysis of mixtures of the pigments. Cellulose layers on microscope slides are useful for rapid qualitative work.

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