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SEPARATION AND ESTIMATION OF THE GALACTOLIPID COMPONENTS OF BROAD BEAN LEAVES

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SUMMARY

Lipids extracted from broad bean (*Vicia faba* L.) leaves were purified on a Sephadex LH-20 column. The two major components, the mono- and digalactolipids, were then separated by a simple silica gel column chromatographic method. After separation and acid hydrolysis both were assayed by their galactose content. Glucose from the sulpholipid and sterol glycosides was removed by incubation with yeast. Comparisons of galactolipid and phospholipid contents of broad bean leaves are presented.

INTRODUCTION

Since phospholipids and galactolipids were recognized to be important constituents of photosynthetic tissues[1-9], many workers have undertaken the task of quantitative determination of these components. Much of the early work in lipid analysis involved paper chromatographic techniques and elution[3,10]. Methods employing silica gel-impregnated paper[2,10,11] and thin-layer chromatography[7,10,12,13] have also been reported. All, however, are limited both in the degree of separation obtained and the amount of lipid material which can be analysed.

More recent methods have employed column chromatographic techniques. Silica gel columns have been most widely used. Early attempts in the use of silica gel column chromatography, however, did not give good resolution[5,14]. Since then methods using silica gel columns together with thin-layer chromatography[8,12,15,16] and silicic acid–Celite followed by rechromatography on silica gel columns[17] have been reported.

Many other methods utilize column chromatographic techniques. Cellulose column chromatography combined with quantitative thin-layer chromatography and densitometry has been used by ROUSER *et al.*[18]. ALLEN *et al.*[19] used countercurrent distribution followed by DEAE-cellulose and silica gel column chromatography. O'BRIEN AND BENSON[20] carried out a lipid isolation using a combination of Florisil, DEAE-cellulose and silicic acid column chromatographic procedures. HELMSING[21] recently reported a separation of the galactolipids with Sephadex LH-20. ROUSER *et al.*[12] discuss many of these currently used techniques and the problems with each.

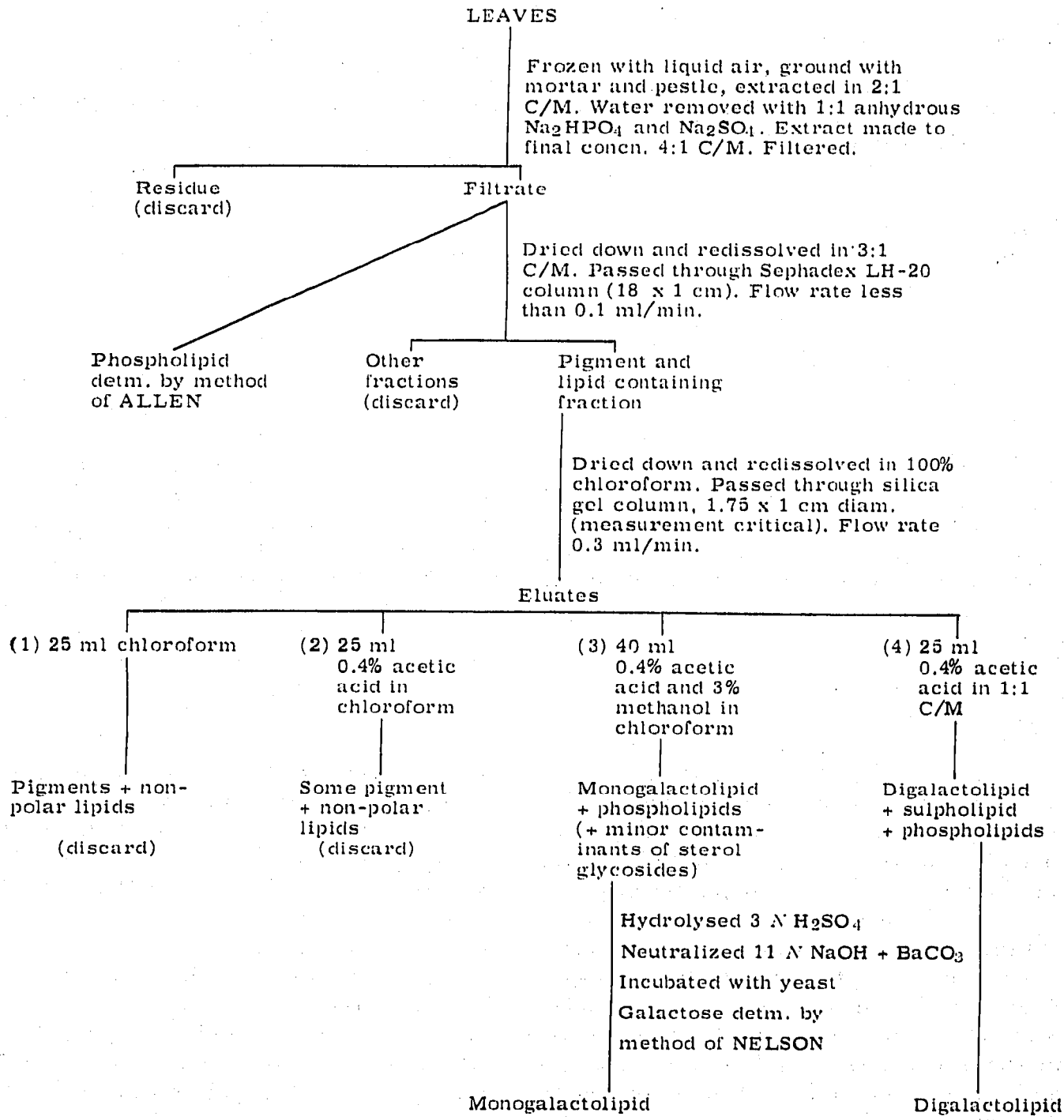


Fig. 1. Flow diagram of the extraction, purification and analysis of the lipid components of broad bean leaves.

Many column methods present a problem in that non-lipid impurities must first be removed from the lipid extracts. This unfortunately has often involved washing the extract with water[3,13,22–25] resulting in subsequent lipid losses[26].

This paper presents a simple column chromatographic method suitable for quantitative separation and estimation of the total phospholipid and the mono- and digalactolipids in unwashed plant lipid extracts.

MATERIALS AND METHODS

Lipid extraction

Mature leaves from 3-week-old broad bean plants (*Vicia faba*, Giant Windsor) were harvested and frozen immediately with liquid air. Lipids were then extracted, after thorough grinding, in chloroform–methanol as previously described[27]. The lipid components of the extract were subsequently analyzed as shown in Fig. 1.

Phospholipid analysis

Aliquots of unpurified extract were evaporated to dryness in 10 ml Kjeldahl flasks and incinerated in 60 % (w/w) perchloric acid until colourless. Phosphorus was determined according to the method of ALLEN[28]. To convert this figure to phospholipid, the experimentally determined values for phosphorus were multiplied by a factor of 25 (ref. 29).

Removal of non-lipid contaminants

Non-lipid sugars were removed by passing an aliquot of lipid extract in 3:1 C/M* through a Sephadex LH-20 column[27].

Silica gel column chromatography

Silica gel (acid silicic, 100 mesh, Malinckrodt Co.) was first washed 4–5 times in distilled water to remove fines and oven dried at 110°. The thoroughly dried powder was then suspended in chloroform and used to prepare a column 1 cm wide by 1.75 cm high. A filter paper disc was placed on top of the gel to prevent any disturbance of the column during solvent additions. The column was then washed with 3–4 column volumes of chloroform. A 1 ml aliquot of lipid extract in chloroform (equivalent to approximately 1 g fresh tissue) was applied to the column and washed in carefully with small quantities of solvent. Fractions 1 and 2 were eluted with 25 ml chloroform and 25 ml 0.4 % acetic acid in chloroform respectively, at a flow rate of approximately 0.3 ml/min.

Fraction 3 was eluted with 40 ml 0.4 % acetic acid and 3 % methanol in chloroform (v/v) and fraction 4 with 25 ml 0.4 % acetic acid in 1:1 C/M. A faster flow rate can be used in the elution of the latter two fractions without interfering with the efficiency of separation.

Thin-layer analysis of fractions

Column fractions were analysed by thin-layer silica gel chromatograms developed in chloroform–methanol–water (65:25:4, v/v) and sprayed with 5 % phosphomolybdic acid in ethanol followed by heating at 105°.

Galactolipid analysis

Galactolipids in fractions 3 and 4 were quantitatively assayed, after acid hydrolysis, by the NELSON method[30]. The eluates were dried down on a rotary evaporator, redissolved in chloroform and aliquots were removed to test tubes for analysis. Chloroform was removed by placing the tubes in a hot water bath. Approx-

* C/M = chloroform–methanol, v/v.

imately 1 ml of methanol and 1 ml 3 N H_2SO_4 were then added to each and hydrolysis carried out for 2 h at 100°. Samples were cooled and partially neutralized with 11 N NaOH. Neutralization (pH paper) was completed by thorough mixing with a small quantity of $BaCO_3$. The $BaCO_3$ was removed from solution by centrifugation. Lipid glucose was removed from the neutralized samples by overnight incubation at 27° with 1 ml of 10 % yeast suspension in distilled water. The yeast was subsequently removed by centrifugation and filtration through a Gelman metricel filter, pore size 0.45 μ. NELSON determinations for galactose were carried out on the filtrate by comparison with a standard curve.

To convert these galactose figures to mono- and digalactolipid, the experimentally determined values were multiplied by factors of 4.3 and 2.7 respectively.

Chlorophyll determination

Chlorophyll was assayed in the extracts according to the method of ARNON[31].

RESULTS AND DISCUSSION

Since only negligible amounts of non-lipid phosphorus have been found in broad been lipid extracts, assays of total phospholipid were done without Sephadex purification. These extracts do, however, contain significant amounts of non-lipid sugars which interfere in assays of sugar-containing lipids. These contaminants were, therefore, removed from the extract by Sephadex column chromatography[27]. This method results in only very small losses of lipid material.

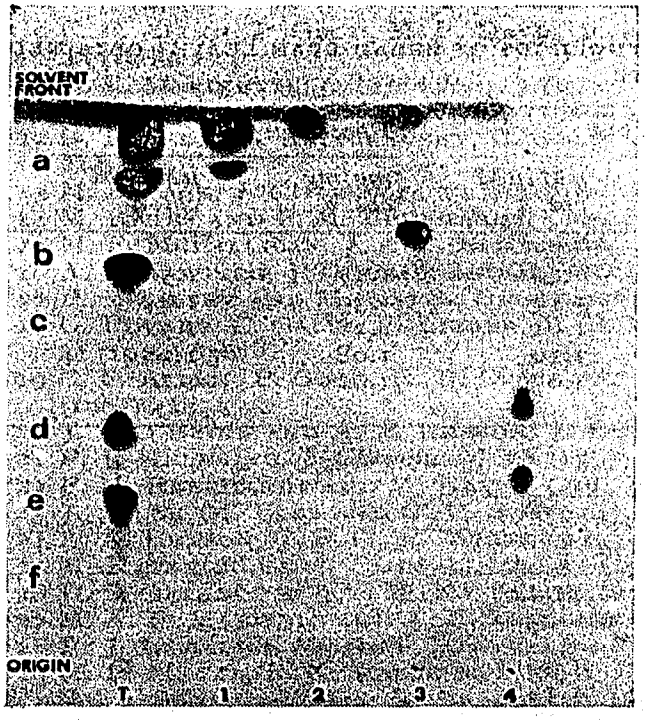
In order to assay the mono- and digalactolipids by their galactose moieties, they must first be separated. This has been done using silica gel column chromatography. The success of this method depends on the use of a very short column (1.75 cm) which eliminates trailing, often a problem with longer columns.

Thin-layer chromatographic analysis of the silica gel column eluates indicated that fractions 1 and 2 contained primarily pigments, non-polar lipids and β-sitosterol. Neither sugar nor phosphorus-containing lipids were detected either by this method or subsequent assays. Fraction 3 contained mainly the monogalactolipid as well as some phospholipid and minor contaminants of sterol glycosides. Fraction 4 contained the digalactolipid, sulpholipid and the majority of the phospholipids.

The separation of the galactolipids into these two fractions was routinely checked by thin-layer chromatography. A typical chromatogram (Fig. 2) of extracts obtained using both Sephadex and silica gel column chromatographic techniques indicates no intercontamination of these compounds. Separation of these lipids has also been carried out on an extract from broad bean leaves supplied with $^{14}CO_2$ in light. The extract was passed through a silica gel column without previous Sephadex purification. Analysis of the fractions by radioautography (Fig. 3) proved that the method gives a clear separation of the mono- and digalactolipids.

These chromatograms also indicated that very little lipid breakdown occurs on the column. Recoveries of sugar-containing lipids from the silica gel column of greater than 99 % based on total sugars applied to and eluted from the column have been obtained.

While the major product of acid hydrolysis of fractions 3 and 4 was galactose, glucose was also produced by the hydrolysis of the sulpholipid[2,3,32] and the sterol



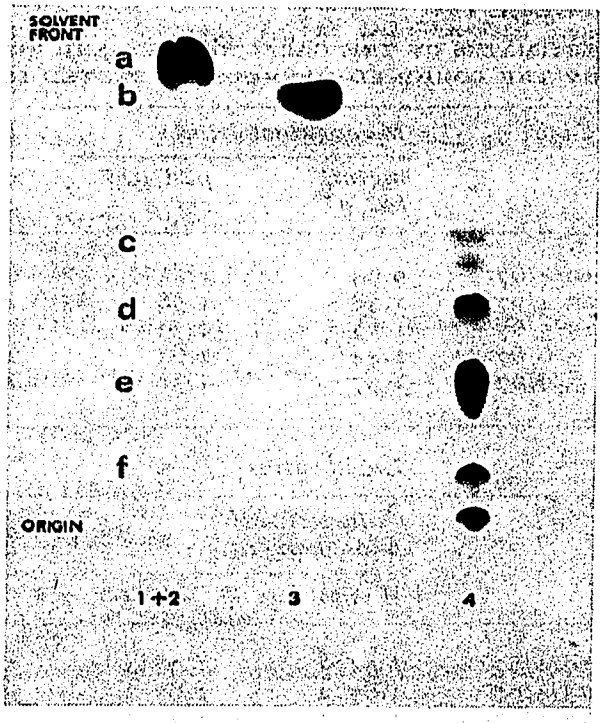


Fig. 2. Thin-layer Silica Gel G chromatogram showing the distribution of broad bean lipid components in the eluate fractions from the silica gel column. The chromatogram was developed in chloroform–methanol–water (65:25:4, v/v) and sprayed with phosphomolybdic acid. a = Pigments and less polar lipids; b = monogalactolipid; c = phospholipids; d = digalactolipid; e = sulpholipid and phosphatidyl choline; f = phospholipids; T = total extract; 1, 2, 3, 4 = fractions from silica gel column (see text).

Fig. 3. Radioautograph of a thin-layer chromatogram showing the distribution of broad bean lipid components in the eluate fractions from a silica gel column. The lipids extracted from leaves supplied with $^{14}CO_2$ had not passed through a Sephadex column. The chromatogram was developed in chloroform–methanol–water (65:25:4, v/v) (lettering as in Fig. 2).

glycosides. Treatment with yeast indicated that the majority of glucose produced by lipid hydrolysis was found in fraction 4. While both fractions 3 and 4 were incubated with yeast, only a negligible change in sugar content was found in fraction 3 but decreases averaging 20 % were found in fraction 4.

The percentage decrease in sugar content after yeast incubation cannot, however, be taken as accurate analysis of sulpholipid content. It is not possible to determine accurately the total sugar present in a mixture of two or more sugars.

Extracts equivalent to 1 g fresh weight of tissue have been separated successfully. This method, therefore, removes the limitation in the amount of material which could be used in methods of separation involving either paper or thin-layer chromatography.

Some preliminary data using these combined methods are presented in Table I. Results from experiments conducted in April of 1967 indicate a content of monogalactolipid slightly higher than digalactolipid and a total galactolipid content approximately twice that of the phospholipids. Subsequent data obtained in June indicate somewhat higher galactolipid/phospholipid ratios.

The mean weight ratio of mono- to digalactolipid (1.12:1) may be compared to the 2.7:1 ratio obtained by WEENINK[33] from red clover and 1:2 ratio obtained by

TABLE I

ESTIMATES OF THE GALACTOLIPIDS AND TOTAL PHOSPHOLIPIDS OF BROAD BEAN LEAVES OBTAINED BY THE COMBINED METHODS PRESENTED IN THE TEXT

Date	*Monogalactolipid*		*Digalactolipid*		*Phospholipids*	
	*mg/mg chl. a**	*μM/μM chl. a*	*mg/mg chl. a*	*μM/μM chl. a*	*mg/mg chl. a*	*μM/μM chl. a*
10/4/67	0.94	1.21	0.85	0.86	1.17	1.50
23/4/67	1.38	1.58	1.22	1.10	1.39	1.64
17/5/67	1.15	1.33	1.12	1.01	1.28	1.51
5/6/67	1.44	1.65	1.21	1.10	1.08	1.28
5/6/67	1.37	1.58	1.14	1.03	1.02	1.20

Lipid ratios

	Galactolipid/phospholipid		*Monogalactolipid/digalactolipid*	
	Weight	*Molar*	*Weight*	*Molar*
10/4/67	1.54:1	1.38:1	1.11:1	1.40:1
23/4/67	1.87:1	1.63:1	1.13:1	1.44:1
17/5/67	1.77:1	1.55:1	1.03:1	1.31:1
5/6/67	2.45:1	2.16:1	1.18:1	1.50:1
5/6/67	2.47:1	2.18:1	1.21:1	1.53:1
Mean	2.01:1	1.78:1	1.12:1	1.42:1

* chl. a = chlorophyll a.

O'BRIEN AND BENSON[20] from alfalfa leaves and *Chorella*. SASTRY AND KATES[17] report a 4:1 ratio in runner beans. While these data have been obtained by widely differing methods and cannot therefore be accurately compared, variations must be attributed mainly to species difference. Data presented in this text also indicate the possibility of seasonal variation.

These methods are now being applied in our laboratory to a study of chloroplast formation in developing broad bean leaves and breakdown during senescence.

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