Phytochemistry, Vol. 22, No. 11, pp. 2359–2363, 1983.
Printed in Great Britain.

0031–9422/83 $3.00+0.00

THE PROTEIN, LIPID AND CARBOHYDRATE COMPOSITION OF PROTEIN BODIES FROM *LUPINUS ANGUSTIFOLIUS* SEEDS

ADRIAN R. PLANT and KEITH G. MOORE

Plant Biology Group, School of Biological Sciences, University of Bath, Claverton Down, Bath BA2 7AY, U.K.

(*Revised received* 18 *March* 1983)

Key Word Index—*Lupinus angustifolius*; Leguminosae; blue lupin; seeds; protein bodies; lipids; sugars; proteins.

Abstract—Protein bodies isolated from dehulled seeds of *Lupinus angustifolius* (cv New Zealand Bitter Blue) contained 73% protein, of which 78% were globulins, 5% albumins and 17% comprised a high MW, sodium hydroxide-soluble protein fraction with an amino acid composition distinct from that of the globulins. Soluble sugars (mostly sucrose, raffinose and stachyose) accounted for 4% and lipids for 6–19% of the dry weight of the protein body. The lipid fraction contained 68% neutral lipid (largely triglycerides and diglycerides) and 27% polar lipid (of which 56% was phosphatidylcholine, 22% phosphatidylethanolamine and 23% an unidentified component, possibly *lyso*-phosphatidylethanolamine). A single glycolipid accounted for 8% of the total lipid. Some differences between the fatty acyl substituents of the neutral, polar and glycolipids were detected.

INTRODUCTION

The seeds of *Lupinus* species contain substantial amounts of storage globulins [1], together with some lipid [2] of which triglycerides form a large proportion [3]. The seeds lack starch but contain massive cell walls which are thought to function as carbohydrate reserves during germination [4].

The major storage globulins in *L. angustifolius* are the glycoproteins α-, β- and γ-conglutin [5, 6], which are thought to be deposited within protein bodies (PBs). Studies of legume PBs have shown that small, but sometimes quite significant, amounts of a variety of other substances including carbohydrates and lipids often accompany the proteins [7]. Little information is available on the composition of PB-associated lipids and although the protein-bound carbohydrates of the storage glycoproteins have been described [1, 6], the nature of the free sugars in the PB is largely unknown.

In the present study we have identified some of the free sugars and have carried out a preliminary characterization of the lipid and protein components of a PB preparation from dry seeds of *L. angustifolius*. Subfractionation of the PB proteins suggested that although the globulins were the major component, a high MW protein fraction with an amino acid composition distinct from that of the major globulins was also present.

RESULTS AND DISCUSSION

Proteins

Although *L. angustifolius* seed meal contained only 16.5% (w/w) protein, 70–75% of PB dry weight could be accounted for as protein after extraction of total protein with sodium hydroxide (Table 1), confirming that this organelle is a major site of deposition of seed protein in this species as in others [7].

Subfractionation using the Osborne criteria [8] of differential solubility in water, dilute salts, aqueous ethanol and alkaline solutions (Table 2) showed that little water-soluble protein was present and that ethanol-soluble proteins were virtually absent. In subsequent experiments the use of ethanol, a potential protein denaturant, was omitted.

The albumin fraction was not resolved by cellulose acetate electrophoresis (CAE), appearing as a diffuse zone over a wide area of the electrophoretogram, and it seems

Table 1. Composition of *Lupinus* protein bodies

Fraction	Mean (*n*)	Max-min range
Total NaOH-soluble protein	73.2 (2)	70.8–75.6
Total lipid	12.5 (2)	6.0–19.0
Soluble carbohydrate	4.3 (1)	—
Insoluble residue	7.6 (3)	6.3– 8.4

Values taken from independent analyses of protein body preparations and expressed as per cent of protein body dry weight. Number of observations = *n*.

Table 2. Components of *Lupinus* protein body proteins

Fraction	Mean (*n*)	Max–min range
NaCl dialysis ppt. (globulin)	57.0 (3)	55.8–58.0
NaCl dialysis supernatant (albumin)	4.0 (2)	3.9– 4.0
EtOH extract (prolamine)	Trace (2)	—
NaOH extract	12.6 (3)	9.3–18.4
Total	73.6	

Values derived from fractions obtained by sequential extraction of protein body preparations and expressed as per cent of protein body dry weight. Number of observations = *n*.

clear that this fraction was heterogeneous. The largest proportion of the PB protein was globulin, which was resolved by CAE into three distinct bands co-electrophoresing with purified preparations of conglutin α, β and γ (Fig. 1A). Estimates by densitometry of the relative proportions of the conglutins in seed extracts indicated that the globulin fraction was composed of approximately 51% α-conglutin, 42% β-conglutin and 7% γ-conglutin. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of DTT-treated PB globulin revealed at least eight major and eight minor bands (Fig. 1B), of which the major bands were found to have MWs ranging from 8500 to 70000.

A substantial proportion of the PB dry weight (9–18%) was recovered in a protein fraction which was insoluble in 0.3 M sodium chloride but could be extracted in 0.5 M sodium hydroxide (Table 2). Since a similar yield of this fraction could be extracted directly from glycerol prepared PBs from which albumins and globulins had been removed by treatment with 0.3 M sodium chloride, it is clear that the fraction was not an artefact of ethanol treatment. Both globulin and sodium hydroxide-soluble fractions were also obtained when proteins were extracted directly from seed meal, which suggests that the sodium hydroxide-soluble fraction already existed in the dry seed, and had not arisen as a result of the PB isolation procedures. When the fraction was isolated in the presence of 2-mercaptoethanol, either from protein bodies or seed meal, recovery was only slightly increased (by 13.0 and 11.9%, respectively) compared with when 2-mercaptoethanol was absent. This suggests that if disulphide cross-linking is an important factor in the formation of the fraction, then it must have occurred *in vivo* rather than during isolation.

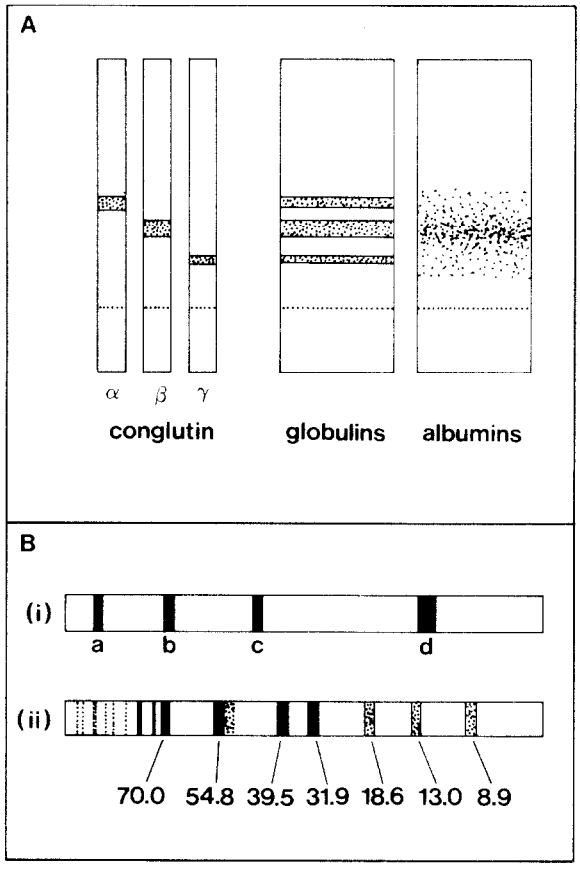

Fig. 1. Electrophoresis of protein body proteins. (A) Cellulose acetate electrophoresis of the water-soluble (albumin) and sodium chloride-soluble (globulin) fractions and conglutin standards. (B) SDS-PAGE (i) of MW standards and (ii) of the globulin fraction. The estimated MW of major bands is expressed in kilodaltons. a = aldolase, b = bovine serum albumin, c = hen albumin, d = cytochrome *c*.

Amino acid analysis (Table 3) confirmed that the sodium hydroxide-soluble fraction was proteinaceous. Although the amino acid composition of the PB globulin fraction closely resembled published values for *Lupinus* seed globulins [9, 10], that of the sodium hydroxide-soluble fraction was clearly different, containing less glutamate and greater amounts of alanine and valine. In addition, it also contained relatively low levels of bound carbohydrate (1.46%) compared with published values for the globulins in this species [6]. When the fraction was dissolved in 0.1 M sodium hydroxide and slowly acidified by addition of solid 2-(*N*-morpholino)ethane sulphonic acid, a turbid suspension formed at pH 9.2–9.0. The suspension persisted as the pH was decreased until at pH 5.5–3.5 flocculation occurred and the protein became readily sedimentable by centrifugation. The low solubility of the sodium hydroxide-soluble fraction at moderate pH prevented its resolution in the buffer systems in which the globulin fraction was successfully resolved by CAE (pH 6.8) or SDS-PAGE (pH 6.6), yet despite its improved solubility at high pH it did not migrate from the origin on CAE (pH 9.7) and would not enter 7.5% SDS-PAGE gels at pH 9.5. This suggests that the fraction was composed of very high MW proteins.

After gel filtration at pH 9.5 on Sephadex G200 (Fig. 2A) and Sepharose CL-6B (Fig. 2B), two major protein peaks (I, II) emerged from both gels close to the blue dextran marker (MW 2×10^6) indicating that a substantial proportion of the protein was of similarly high molecular size.

Table 3. Amino acid composition of globulins and sodium hydroxide-soluble proteins isolated from *Lupinus angustifolius* protein bodies

	Composition (residues/100 residues)	
Amino acid	Globulin	NaOH-soluble protein
Lys	3.2	4.2
His	2.0	3.1
Arg	9.6	7.2
Asp	9.8	10.7
Thr	3.1	4.8
Ser	6.6	7.1
Glu	22.9	12.7
Gly	6.3	8.6
Ala	4.4	7.9
Val	2.9	5.8
Ile	3.3	4.1
Leu	6.4	8.4
Tyr	2.3	2.4
Phe	2.8	3.4

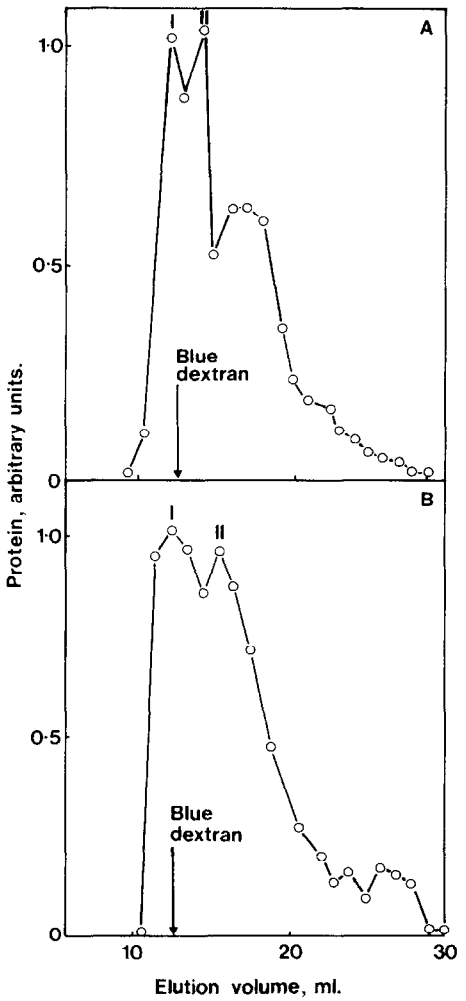

Fig. 2. Resolution of the sodium hydroxide-soluble protein fraction by gel filtration on (A) Sephadex G200 and (B) Sepharose CL-6B.

Carbohydrates

Phenol–sulphuric acid determinations of ethanol extracts of PBs showed that 4.3% of their dry weight was accounted for by free carbohydrates (Table 1) compared with 8.0% of whole seed meal. (Interestingly, calculations based on the PB globulin content and the data reported in ref. [6] indicate that the sugar moieties of the conglutin glycoproteins represent *ca* 3–4% of PB dry weight.)

TLC of the free carbohydrates revealed that sucrose, raffinose and stachyose were the predominant sugars, with an unknown substance remaining at the origin of the plates. Judging by the intensity of the spots, the levels of stachyose and raffinose were low compared with sucrose. The cotyledons of *L. angustifolius* cv Blusa also contain these sugars [11] but additionally contain significant levels of the raffinose homologues verbascose and ajugose. The material remaining at the origin during TLC may have been such long chain galactosides which the solvent system was incapable of resolving.

Sucrose and the galactose residues of raffinose homologues are rapidly utilized in respiration early in seed germination [12]. We have found galactosidases in *Lupinus* PBs which are capable of releasing galactose from stachyose and raffinose [13], showing that the first step in galactoside mobilization could occur within the organelle.

Lipids

Lipid accounted for 6–19% (w/w) of the PB (Table 1) compared with a value of 8.6% for whole cotyledons of *L. angustifolius* cv Uniwhite [3]. A PB preparation containing 6% total lipid was used for subfractionation of the lipids and was found to contain 65.2% neutral lipid, 8.4% glycolipid and 26.5% polar lipid.

Of the neutral lipids, 83.7% were triglycerides (TG), 12.1% diglycerides (DG) and 4.3% monoglycerides (MG) or free fatty acids (FFA). The relatively higher proportion of polar and glycolipid classes in PB compared with the seed lipids from *L. angustifolius* cv Uniwhite [3] suggests that the PB lipid is enriched in membrane components at the expense of storage lipids. However, the presence of substantial amounts of triglyceride does indicate that spherosomes may have constituted a significant source of contamination. Spherosomes are often closely associated with the PB membrane in many seeds [14], and their presence in varying amounts in preparations could account for the variation in lipid values which have sometimes been reported [7].

The relative proportions of the fatty acid chains in PB lipid (Table 4) were generally similar to those reported for whole seed lipid [3]. Of the neutral lipids, DGs were enriched with 16:0 and the MG/FFA fraction with 18:2. Low levels of longer chain length and/or more unsaturated fatty acids were also detected in the neutral lipids but were not accurately quantifiable due to their long retention on GLC.

One glycolipid was detected by TLC, resolving midway between *Pisum* chloroplast monogalactosyl- (MGDG) and digalactosyl-diacylglycerol (DGDG), and contained notably low levels of 18:3 acyl chains (Table 4).

Three major polar lipids were detected, phosphatidylcholine (54.5%), phosphatidylethanolamine (22.3%) and an unknown lipid with an R_f similar to *lyso*-phosphatidylethanolamine (23.2%). The fatty acyl composition of this lipid was very different from that of any of

Table 4. Fatty acid profiles of the lipid classes from *Lupinus* protein bodies

	Fatty acid				
Lipid class	16:0	18:0	18:1	18:2	18:3
Neutral lipid					
TG	11.3	5.2	28.1	49.3	6.1
DG	27.0	6.3	25.0	37.2	4.5
MG/FFA	7.6	3.9	23.7	57.3	7.6
Glycolipid	17.4	12.2	25.2	44.6	0.6
Polar lipid					
PC	12.4	5.8	32.6	44.6	4.7
PE	17.7	6.6	26.0	47.2	2.5
Lyso-PE	22.7	15.8	16.6	38.7	6.7

Values for each fatty acid are expressed as per cent of the total fatty acid content of each lipid class.

the other lipids, being enriched in 16:0 and 18:0 but with less 18:1 and 18:2 (Table 4), and may have arisen by phospholipase action.

Insoluble residue

After extraction of the other fractions from the PB, a glutinous solid always remained, accounting for 6.3–8.4% of PB dry weight (Table 1). TLC separation of acid hydrolysates of this residue and of a crude cell-wall preparation from lupin showed that although traces of cell-wall derived sugars (glucose, cellobiose) could be detected, cell-wall material apparently constituted only a minor part of the residue fraction. The identity of the residue was not investigated further.

Conclusions

This work has confirmed that conglutins are the major components of *L. angustifolius* PBs, but has also demonstrated a hitherto unreported high MW, possibly aggregated protein insoluble at physiological pH within the organelle. This fraction was apparently not an artefact of isolation conditions, and its amino-acid composition suggests that it was not derived from the major PB globulins.

Although the PB is largely a protein storage site, it is interesting that it also contains carbohydrates and lipids. In *Lupinus* species the carbohydrate reserves are mostly deposited at the cell walls [4] and it is possible that PB carbohydrate has only a transient involvement in respiration early in germination, before the major reserves have been mobilized. In germinating seeds of *L. albus*, mobilization of spherosomal lipid begins at *ca* day 4 [4], yet it is evident from the dramatic increase in PB membrane surface area during imbibition and germination [4] that there is a requirement for new components to be inserted into the expanding membrane at an earlier stage and it is possible that PB lipids could be involved in this process.

EXPERIMENTAL

Protein body isolation. PBs were isolated from dehulled, ground dry seeds of *Lupinus angustifolius* (cv New Zealand Bitter Blue) using the non-aq. glycerol method as described previously [13, 15]. Light microscopy revealed that the preparation was a dense suspension of protein bodies, and calcofluor staining showed that little cell-wall contamination was present. After removal of adherent glycerol by 3 × low speed centrifugation in Me_2CO and separation on a $CHCl_3$–*n*-hexane density gradient, 96% of PB preparation dry wt was recovered within a density range (1.23–1.31 g/mol) similar to that reported for the PB of other species. PB dry wt was determined after drying Me_2CO-washed samples to constant wt at 105°.

Protein fractionation. All protein isolations were performed at 4° using glycerol PB preparations without Me_2CO washing. Crude protein fractions were extracted from 0.15 g PB samples by stirring for 3 hr in 50 ml of 50 mM Tris–HCl–0.3 M NaCl, pH 8.0, and centrifuged at 35 000 *g* for 30 min. The supernatant was dialysed against 3 × 3 l. H_2O adjusted to pH 5.6 with HOAc yielding a ppt. (globulin) and a supernatant which was concd against polyethyleneglycol (PEG) to give the albumin fraction. The 35 000 *g* pellet was extracted with 2 × 10 ml 50% (v/v) EtOH for 30 min, centrifuged at 77 000 *g* for 30 min and the combined supernatants were concd against PEG to yield the prolamine fraction. The 77 000 *g* pellet was extracted with 15 ml 0.5 M NaOH for 15 hr, recentrifuged leaving an 'insoluble residue' in the pellet and a supernatant which was dialysed as above to give a ppt. designated the NaOH-soluble fraction. Bulk quantities of NaOH-soluble protein were extracted directly from either PB or seed meal, after prior removal of NaCl-soluble proteins. Mercaptoethanol (0.01 M) was used in some extractions during both NaCl and NaOH steps.

Cellulose acetate electrophoresis (CAE). CAE of albumins and globulins was performed with a running buffer of 0.05 M NaPi, pH 6.8, basically as in ref. [5] using cellulose acetate membranes impregnated by flotation on 0.15 M NaPi buffer, pH 6.8. Protein samples and conglutin standards (prepared as in ref. [5]) were dissolved to saturation in 0.15 M buffer containing bromophenol blue as front marker and 5–10 μl applied to the membranes. For six 12 × 5 cm membranes, a current of 25 mA (220 V) was applied until the front marker had migrated 5 cm. The NaOH-soluble protein fraction was dissolved in 0.15 M gly–NaOH buffer, pH 9.7, and electrophoresed as above using 0.05 M gly–NaOH, pH 9.7, as running buffer. After electrophoresis, proteins were fixed in 5% (w/v) TCA for 5 min, stained with Coomassie blue (2% w/v) in 7% (v/v) HOAc for 10 min and destained with 7% HOAc.

SDS-PAGE. SDS-PAGE was performed using preformed 10 cm Biorad 'Bio Phore' 7.5% gels employing 0.205 M Tris–0.205 M HOAc–0.1% (w/v) SDS, pH 6.6, buffer for globulins and the NaOH-soluble protein and 0.188 M cyclohexylaminopropane sulphonic acid/2-amino-2-methyl-1,3-propanediol (CAPS-AMPD) buffer, pH 9.5, containing 0.1% SDS for NaOH-soluble protein only. Buffers were introduced into gels by overnight pre-electrophoresis at low power (0.3–0.5 W/tube). Samples were prepared in appropriate buffer (diluted 1 : 4 with H_2O) and made 1% with SDS, 0.001 M EDTA and 0.04 M DTT and incubated for 15 hr at 37°. A front marker of bromophenol blue was added and samples (containing 25–35 μg protein in 30 μl) were introduced to the gels at 4 mA/gel at pH 6.6 and 1 mA/gel at pH 9.5. Samples at pH 6.6 were run for 6–8 hr at 7 mA/gel and those at pH 9.5 for 40 min at 2 mA/gel during which time the front marker had migrated 8–9 cm. MW standards were cytochrome *c*, hen albumin, bovine serum albumin and aldolase. After electrophoresis, gels were fixed for 1 hr in *iso*-PrOH–HOAc–H_2O (4 : 1 : 5), stained overnight with 0.5% (w/v) Coomassie blue in HOAc–*iso*-PrOH–H_2O (1 : 1 : 8) and the position of bands was determined after destaining with HOAc–*iso*-PrOH–H_2O (1 : 1 : 8).

Gel filtration. NaOH-soluble protein (1.8 mg) was stirred into 0.8 ml of 10 mM AMPD–HCl, pH 9.5, for 3 hr, centrifuged at 10 000 *g* for 15 min and 0.5 ml of the supernatant passed through 15 × 1.5 cm equilibrated columns of either Sephadex G-200 or Sepharose CL-6B at a flow rate of 0.45 ml/min and the eluates were assayed for protein.

Protein determinations. Protein was assayed throughout by the Hartree modification [16] of the Lowry method, using bovine serum albumin as standard.

Amino acid analysis. Amino acid analysis was performed on a Rank Hilger Chromospek J180 amino acid analyser using 40 μg samples of protein hydrolysed *in vacuo* for 24 hr with 6 M HCl. The limits of reproducibility varied from 4 to 8%.

Carbohydrate determinations. Carbohydrates were qualitatively determined in acid hydrolysates of the insoluble residue and a crude cell-wall fraction remaining after exhaustive extraction of dehulled seeds with 0.1 M NaOH. Samples of each were taken up in a small vol. of 2 M HCl, sealed in a glass capillary tube and maintained at 95° for 20 hr. Hydrolysis products were separated by impregnated silica gel TLC and detected with aniline diphenylamine–Me_2CO–H_3PO_4 [17].

Qualitative determinations of soluble sugars were performed by homogenizing 0.5 g of PB in 2 × 15 ml of 80% (v/v) EtOH at

90° for 1 hr. After centrifugation at 1000 *g* for 10 min, the supernatant was concd to half vol. and 10 μl aliquots were applied to TLC plates as described above. D-Glucose, D-mannose, D-fucose, *N*-acetyl-D-glucosamine, arabinose, xylose, sucrose, cellobiose, raffinose and stachyose were used as standards.

Quantitative sugar estimations of the EtOH extracts used the PhOH–H_2SO_4 method [18] with D-glucose as standard and employing a small correction factor to allow for the presence of glycerol in the PB fraction (the wt of glycerol adhering to PBs was calculated by weighing the preparation before and after Me_2CO washing and the correction factor determined by assaying a control containing the appropriate amount of glycerol).

Measurement of bound carbohydrate was performed on 80% EtOH-washed samples of the NaOH-soluble protein fraction dissolved at 2.8 mg/ml in 15 mM gly–NaOH, pH 9.7. Aliquots of 0.5 ml were assayed directly with PhOH–H_2SO_4.

Lipid extraction. Total lipids were extracted from PBs (0.49 g/ml in 50 mM Tris–HCl, 0.3 M NaCl, pH 8.0) using the solvent partition procedure of Bligh and Dyer [19]. Lipid classes were resolved from total lipid extract using silicic acid CC [20]. A slurry of silicic acid in $CHCl_3$ was loaded into a 2.0 ml Pasteur pipette, washed with 10.0 ml $CHCl_3$ and 250 μl of total lipid extract layered on the column. Neutral, glyco- and polar lipids were successively eluted with 5.0 ml vols. of $CHCl_3$, Me_2CO and MeOH, respectively, and the identities confirmed by comparison with authentic standards on TLC (see below).

TLC of lipids. A known vol. of silicic acid column eluate of each lipid class was streaked onto 0.2 mm × 25 cm × 25 cm silica gel 60 TLC plate (Merck 5553). Neutral lipids were developed in petrol (60–80°)–Et_2O–HOAc (80:20:1), polar and glycolipids in $CHCl_3$–MeOH–H_2O (65:25:3.5) and the identity of each band confirmed by reference to authentic standards of tripalmitin (TGs); pentadecanoic acid (FFA); *Pisum* chloroplast MGDG and DGDG (glycolipid); PC, PE, PS, PI, *lyso*-PE and phosphatidic acid (polar lipid) and visualized by exposure to I_2. When GLC of lipid fatty acyl Me esters was to be performed, the bands were scraped from the plates and directly methylated as described below.

GLC of lipids. The total lipid silicic acid column eluates and TLC subfractions were analysed by GLC of their fatty acyl Me esters. Samples of 1.0 ml of each fraction (to which a 15:0 internal standard had been added) were methylated with methanolic BF_3 [21] and their Me esters dissolved in 30 μl petrol (60–80°). Approximately 5 μl of this was injected onto a 2.0 m column of 10% (w/w) polyethyleneglycol adipate maintained at 195° (N_2 carrier gas at 40 ml/min), and the emergence of peaks monitored by FID and quantified with reference to the internal standard. Identification was by reference to the Me esters of 16:0, 18:0, 18:1, 18:2 and 18:3 fatty acids.

Acknowledgements—This work was financed by SERC research grant No. GR/A/7116.5. We would like to express our thanks to Mrs. Sheila Kendall for the isolation of the conglutin standards, Mr. R. Edwards for his assistance in conglutin densitometric measurements, and Dr. M. P. Percival for his help with the GLC of fatty acid methyl esters.

REFERENCES

1. Derbyshire, E., Wright, D. J. and Boulter, D. (1976) *Phytochemistry* **15**, 3.
2. Gladstones, J. S. (1980) in *Advances in Legume Science* (Summerfield, R. J. and Bunting, A. H., eds.), p. 603. Min. Agric. Fish. and Food.
3. Hansen, R. P. and Czochanska, Z. (1974) *J. Sci. Food Agric.* **25**, 409.
4. Parker, M. L. (1975) Ph.D. Thesis, University of Wales, Bangor.
5. Blagrove, R. J. and Gillespie, J. M. (1975) *Aust. J. Plant Physiol.* **2**, 13.
6. Eaton-Mordas, C. A. and Moore, K. G. (1978) *Phytochemistry* **17**, 619.
7. Pernollet, J.-C. (1978) *Phytochemistry* **17**, 1473.
8. Osborne, T. B. (1924) *The Vegetable Proteins*, 2nd edn. Longmans, Green, London.
9. Blagrove, R. J. and Gillespie, J. M. (1975) *Aust. J. Plant. Physiol.* **2**, 13.
10. Duranti, M., Restani, P., Poniatowska, M. and Cerletti, R. (1981) *Phytochemistry* **20**, 2071.
11. Beroard, J. C. and Veral, A. F. (1980) *Z. Lebensm. Unters-Forsch.* **171**, 281.
12. Pazur, J. H., Shadaksharaswamy, M. and Meidel, G. E. (1962) *Arch. Biochem. Biophys.* **99**, 78.
13. Plant, A. R. and Moore, K. G. (1982) *Phytochemistry* **21**, 985.
14. Vick, B. and Beevers, H. (1978) *Plant Physiol.* **62**, 173.
15. Yatsu, L. Y. and Jacks, T. J. (1968) *Arch. Biochem. Biophys.* **124**, 466.
16. Hartree, E. F. (1972) *Analyt. Biochem.* **48**, 422.
17. Hansen, S. A. (1975) *J. Chromatogr.* **107**, 224.
18. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Analyt. Chem.* **28**, 350.
19. Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911.
20. Rouser, G., Kritchevsky, G. and Yamamoto, A. (1976) in *Lipid Chromatographic Analysis* (Marrinetti, G. V., ed.), Vol. 3, p. 713. Marcel Dekker, New York.
21. Metcalfe, I. J. and Schmitz, A. A. (1979) *Analyt. Chem.* **33**, 363.