Phytochemistry, 1977, Vol. 16, pp. 1507–1510. Pergamon Press. Printed in England.

COMPARISON OF METHODS FOR THE EXTRACTION OF PLANT LIPIDS

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(*Revised received* 18 *April* 1977)

Key Word Index— Lipids: plant tissue: extraction.

Abstract—The ability of a number of different solvent systems to extract lipid from a range of plant tissues was compared by measurement of phospholipid, glycolipid, sterol lipid and total acyl lipid content. A chloroform–methanol extraction method based upon the principles of Bligh and Dyer was considered to be the most efficient system for use with the majority of plant tissues. Cereal seeds were anomalous in that water saturated *n*-butanol was the preferred solvent system due to its superior ability to extract bound lysophospholipids present in large amounts in the endosperm portion of the tissue.

INTRODUCTION

Satisfactory methods for the preparation and purification of lipids from animal tissues have been described by several authors [1–3] and are widely used. These methods are based upon the ability of $CHCl_3$–MeOH mixtures to form monophasic systems with tissue water, disintegrate membrane structures and extract lipid from tissues. The role that components of complex solvent systems play in lipid extractions has recently been discussed in the light of solubility parameter concepts [4].

Apart from certain oil-rich seeds where hydrocarbon solvents are used for the extraction of triglyceride oil, plant tissues are generally extracted by the methods described for animal tissues. However, it has been apparent for a number of years that some form of enzyme denaturation must precede a lipid extraction method if the formation of lipid artifacts caused by enzymic activity is to be avoided. The degradation of plant lipids by hydrolytic and oxidative enzymes has been reviewed by Galliard [5]. Methods commonly used for enzymic deactivation of tissues include the use of boiling 'alcohols' such as *iso*-PrOH [6], MeOH, water saturated *n*-butanol (WSB) [7] or simple heat treatment at 100°. WSB is suspected of giving rise to lysophospholipid artifacts by non-enzymic hydrolysis [7] whilst MeOH may form fatty acid methyl esters through acyl transferase activity [8] or phosphatidyl methanol through transphosphatidylation reactions [9] if the tissue is incompletely 'killed'. *Iso*-PrOH being a secondary alcohol less readily accepts a phosphatidyl or acyl unit and should be regarded as the safest and most efficient solvent for enzyme denaturation.

A survey of relevant literature has shown a lack of conformity in the use of enzyme denaturation reagents and in the choice of extraction solvents coupled with a lack of attention to the potential hazards of artifact formation. This has made it difficult to compare the efficiency of published lipid extraction methods. Where a comparison has been attempted as in the case of wheat seeds [10] objections have been raised concerning the validity of some of the conclusions [7].

For our own studies on the lipid composition of plants we required extraction and analysis methods which could be applied with confidence to different types of plant tissues. The examination in depth of the composition of a large number of plant tissues using a variety of lipid extraction methods would be beyond the scope of this report; we have therefore compared the commonly used methods in an attempt to establish standard methods applicable to plant tissues in general. Plant tissues examined were chosen primarily with regard to their relative starch-water content but also include a green leaf tissue.

RESULTS AND DISCUSSION

Hot *iso*-PrOH was chosen as the means of enzyme denaturation for all $CHCl_3$–MeOH based extractions and hot WSB was used as the first stage of the WSB method. Thus, variations in lipid composition due to enzymic degradation of lipids have been minimized and methods can be judged solely on their ability to extract total lipid. When a full quantitative analysis of individual lipids was carried out on the $CHCl_3$–MeOH and WSB extracted lipids of wheat seed and dried peas, no evidence was found for the occurrence of lipid artifacts associated with enzymic activity [5]. We also found no significant difference between the amounts of LPC (3.2% by wt, phosphorus) and PC (50% by wt, phosphorus) isolated from the phospholipid fractions of pea lipid obtained by either extraction method (Table 1) which is contrary to the suggestion that the use of WSB could lead to the production of small quantities of LPC by non-enzymic hydrolysis of PC [7]. These findings reinforce the present view that the use of boiling 'alcohol' prior to the conventional lipid

Abbreviations—PC, phosphatidylcholine; PE, phosphatidylethanolamine; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; *N*-acyl-PE, *N*-acyl phosphatidylethanolamine.

Table 1. Variation of composition of phospholipids of dried pea and wheat grain with extraction method

Extraction method*:	Wheat		Pea	
	C	D	C	D
	mg P/100g tissue			
LPC	2.8	18.3	2.2	2.2
LPE + PI†	0.8	3.8	7.3	8.0
PC	4.1	4.6	34.3	34.0
PG	—	—	1.3	1.5
PE	1.8	2.3	11.5	11.7
Minor phospholipids	1.9	2.6	2.2	1.9
N-acyl-PE	1.9	3.4	9.8	10.7

* For details see Experimental.
† PI major component of pea and minor component of wheat fraction.

extraction methods effectively denatures potential lipid degrading enzymes.

The yields of total lipid obtained from each plant tissue by the four extraction methods are given in Table 2. Total lipid is expressed in terms of phosphorus, glucose and galactose, sterol and fatty acid methyl ester (FAME) which reflect the yield of phospholipid, glycolipid excepting sulpholipid, sterol lipids and total acyl lipids respectively. Spinach leaf, tomato fruit and potato tuber were all extracted efficiently by $CHCl_3$–MeOH solvents (Methods A–C). $CHCl_3$–MeOH (2:1) was inferior to the Bligh and Dyer extractions with regard to the total acyl lipid and glycolipid content of spinach leaf lipid. The addition of mineral acid to the Bligh and Dyer extraction (Method B) did not release additional bound lipid and a check on extraction efficiency by acid hydrolysis (2N aq. HCl) of the solvent-extracted residues followed by GLC analysis of the fatty acids released, showed on average only 2–3% additional acyl lipid in the residues. In contrast to the $CHCl_3$–MeOH solvents, WSB was markedly inferior in its ability to extract all classes of lipids from spinach, tomato and potato tissue.

The reverse situation apparently occurred with wheat seeds where extraction values for all types of lipids except sterols were significantly greater for WSB than for $CHCl_3$–MeOH solvents. Further separation of wheat total lipid by column chromatography on Si gel to give phospholipid and glycolipid fractions and followed by separation of those fractions into individual lipids by TLC gave the results shown in Tables 1 and 3. The glycolipid content of total lipid is now similar for both Bligh and Dyer and WSB methods; the higher values previously obtained (Table 2) being due to nonlipid contaminants with the same retention times on GLC as glucose and galactose TMSi derivatives. Thus both methods yielded nonlipid sugar contaminants in the extracts although no nonlipid phosphorus was present as shown by the constant phosphorus level

Table 2. Comparison of methods for extracting total lipids from plant tissues

Extraction method†:	Total lipid (mg/100g fresh wt)* A	B	C	D	5% New multiple range test‡
Spinach:					
FAMES§	482 ± 14 (3)	591 ± 42 (3)	577 ± 14 (3)	297 ± 48 (3)	D A C B
Phosphorus	11.1 ± 0.7 (3)	11.8 ± 0.8 (3)	13.1 ± 0.9 (3)	7.5 ± 0.9 (3)	D A B C
Glucose	10.5 ± 0.7 (3)	18.3 ± 0.4 (3)	11.9 ± 0.7 (3)	6.0 ± 0.7 (3)	D A C B
Galactose	124 ± 5 (3)	139 ± 9 (3)	142 ± 4 (3)	78 ± 9 (3)	D A B C
Sterol	23.3 ± 1.8 (3)	24.8 ± 4.0 (3)	29.3 ± 1.9 (3)	18.3 ± 4.3 (3)	D A B C
Tomato:					
FAMES§	45.6 ± 5.3 (3)	45.1 ± 2.7 (3)	44.6 ± 3.8 (3)	35.4 ± 2.9 (3)	D C B A
Phosphorus	1.86 ± 0.13 (3)	1.87 ± 0.13 (3)	1.81 ± 0.07 (3)	1.40 ± 0.10 (3)	D C A B
Glucose	12.3 ± 1.9 (3)	12.7 ± 0.4 (3)	11.7 ± 0.4 (3)	10.2 ± 0.8 (3)	D C A B
Galactose	5.33 ± 1.10 (2)	4.69 ± 0.63 (3)	4.96 ± 0.19 (3)	4.20 ± 0.29 (3)	D B C A
Sterol	9.42 ± 0.53 (3)	9.45 ± 0.64 (2)	9.14 ± 0.23 (2)	7.44 ± 0.31 (3)	D C A B
Wheat:					
FAMES§	1243 ± 35 (3)	1367 ± 31 (3)	1315 ± 21 (2)	1420 ± 10 (3)	A C B D
Phosphorus	8.7 ± 0.2 (3)	13.7 ± 0.4 (3)	13.4 ± 0.3 (3)	33.7 ± 1.3 (3)	A C B D
Glucose	26.7 ± 2.0 (3)	32.6 ± 0.5 (3)	31.5 ± 2.6 (3)	71.7 ± 11.0 (3)	A C B D
Galactose	69.0 ± 2.6 (3)	86.9 ± 3.6 (3)	90.2 ± 3.4 (3)	161.0 ± 19.0 (3)	A B C D
Sterol	32.7 ± 3.1 (3)	38.1 ± 1.2 (2)	39.7 ± 6.4 (3)	38.6 ± 3.1 (3)	A B D C
Potato:					
FAMES§	69.6 ± 1.5 (3)	70.1 ± 5.2 (3)	62.7 ± 4.0 (3)	41.0 ± 0.9 (2)	D C A B
Phosphorus	2.23 ± 0.04 (3)	2.23 ± 0.04 (3)	2.26 ± 0.04 (3)	1.39 ± 0.19 (3)	D A B C
Glucose	4.67 ± 0.70 (3)	4.20 ± 0.62 (3)	4.34 ± 0.78 (3)	2.72 ± 0.06 (3)	D B C A
Galactose	10.2 ± 1.2 (3)	10.2 ± 0.5 (3)	9.4 ± 1.8 (3)	4.0 ± 0.6 (3)	D C A B
Sterol	4.20 ± 0.28 (3)	4.29 ± 0.38 (3)	4.21 ± 0.93 (3)	2.97 ± 0.37 (3)	D A C B

* Mean ± Standard Deviation. Figures in parenthesis refer to number of samples for each mean.
† For details, see Experimental.
‡ Means not underscored by the same line are significantly different. Methods arranged in ascending order of magnitude of mean values
§ Fatty acid methyl ester.

before and after purification (Table 3). The phospholipid compositions (Table 1) show that LPC and LPE were mainly responsible for the increased phosphorus values obtained with WSB extraction of wheat. PC and PE showed a smaller but significant increase (12% and 29% respectively). A sequential extraction of wheat seed by the Bligh and Dyer method followed by WSB yielded a WSB lipid fraction containing 93% phospholipid as LPC and LPE.

The presence of large, additional amounts of choline in the WSB lipid extract compared to the Bligh and Dyer extracts was confirmed by estimating total choline as choline reineckate [11]; 619 μmol/100 g seed of extra choline was present in the WSB lipid sample (an extra 515 μmol/100 g seed of LPC plus PC is present in the WSB lipid, Table 1). The two TLC bands designated as LPC and LPE gave the appropriate positive colour reactions when sprayed with *cis*-aconite [12] and ninhydrin reagents specific for choline and amino acid respectively.

The abnormally high phosphorus values obtained with WSB extracts has been the subject of controversy for some years. Recently de la Roche *et al.* [10] evaluated several solvent systems with regard to efficiency of lipid extraction and concluded that the Bligh and Dyer procedure was the most satisfactory in spite of higher phosphorus and fatty acid values associated initially with crude WSB extractions. The lower yield of fatty acid following purification from nonlipid by a washing procedure was attributed in part to the presence of lipoprotein 'fluff' at the solvent interface. In the present series of extraction procedures, we automatically included a washing step [1] followed by a final purification of lipid on Sephadex G 25. Controlled tests prior to the main experiments showed that under these conditions loss of lipid did not occur and this was confirmed by the high yields of phospholipid obtained from wheat grain with WSB extracts.

Table 3. Phosphorus, glucose and galactose content of total lipid of wheat seed following purification by column and thin layer chromatography

Extraction Method*:	C		D	
	Total Lipid	Purified Lipid	Total Lipid	Purified Lipid†
	mg/100g seed			
Phosphorus	13.6	13.3	35.2	35.0
Glucose	28.5	16.1	79.3	18.8
Galactose	87.9	81.8	167	83.8

* For details see Experimental.
† Purified lipid is the sum of all individual lipid analysed.

The results for wheat seed (Tables 1 and 3) show clearly that WSB is not more efficient than $CHCl_3$–MeOH based methods at extracting most types of lipid. However, in addition to these lipids, large amounts of LPC and LPE are present which are unavailable to $CHCl_3$–MeOH based solvents but are readily extracted by WSB. The reason for this appears to be that the lysophospholipids are associated with the amylose component of starch [13–15]. Recent evidence [16] suggests that LPC occupies the helicoidal structure of amylose and that butanol solvents can enter the amylose helix.

Mature fresh and dried forms of wrinkled peas, similar to wheat seed with regard to a high amylose content [17], were extracted by the Bligh and Dyer and WSB methods. Lipid values for mature, fresh peas were significantly greater for the Bligh and Dyer method whilst no significant difference was noted between extraction methods for FAME, phosphorus or sterol yields obtained from dry peas (Table 4). Thus the phospholipid composition of dried pea (Tables 1 and 4) in contrast to that of wheat is constant in proportion and amount of phospholipids regardless of the extraction method used and the LPC content is low and similar to other plant tissues, such as the potato tuber, which have a high starch but low amylose content [13].

The above results show clearly that the lipids of tissues with high water content (spinach, fresh pea, tomato, potato) are poorly extracted by WSB but efficiently extracted by $CHCl_3$–MeOH solvents. Lipids from materials with low water content (wheat, dried pea), with one exception, are generally extracted equally well by $CHCl_3$–MeOH solvents as by the WSB system which is traditionally used for cereal flour extractions [18]. The exception to this rule concerns the extractibility from cereals of the lysophospholipids associated mainly with the amylose structure of the wheat endosperm.

Recommendations for the extraction of lipid from plant sources are that a Bligh and Dyer solvent system preceded by a boiling *iso*-PrOH stage is the most efficient means of extracting lipid from plant tissues except in the case of cereal seeds where WSB is recommended. In cases where tissues of unknown lipid constitution are being investigated, and where the presence of bound lysophospholipid is suspected, a sequential extraction with the Bligh and Dyer solvents followed by WSB would be a useful procedure. Acid

Table 4. Comparison of methods for extracting total lipids from fresh pea and dried pea

	Fresh pea		Dry pea	
Extraction method*:	C	D	C	D
	Total lipid (mg/100g tissue)†			
FAMES‡	499 ± 34 (3)	312 ± 12 (3)	1980 ± 56 (3)	1910 ± 22 (3)
Phosphorus	20.1 ± 1.0 (3)	12.0 ± 0.8 (3)	66.8 ± 1.5 (3)	67.8 ± 0.2 (3)
Sterol	14.5 ± 1.2 (3)	13.9 ± 0.9 (3)	99.0 ± 8.7 (3)	97.3 ± 2.8 (3)

* For details see Experimental.
† Figures in parenthesis refer to number of samples for each mean.
‡ Fatty acid methyl ester

hydrolysis or saponification of the residue after lipid extraction, followed by fatty acid and sterol analysis should confirm the extraction efficiency.

EXPERIMENTAL

Materials. Tomato (*Lycopersicon esculentum*) fruit and wheat (*Triticum aestivum*) seeds of unknown varieties were purchased locally. Spinach (*Spinacia oleracea*) leaves and fresh mature peas (*Pisum sativum, var.* Onward) were grown at the Institute. Potato tubers (*Solanum tuberosum var.* Desirée) had been stored for 6 months at 5° before use. All solvents were redistilled before use.

Preparation of material. Mature spinach leaves of equal size minus their central ribs were randomized and 10g samples taken for analysis. Tomato fruit were quartered, parenchymatous tissue and seeds together with the inner and radial walls of the pericarp removed, and kept in iced H_2O (0°) until 25g randomized samples were taken for extraction. Potato tubers were peeled, diced into centimetre cubes, washed and held in iced H_2O until 50g samples were taken for analysis. 15g samples of freshly harvested peas were thinly sliced immediately prior to extraction. Pea seed (100g) and wheat seed (100g) were ground in a knife mill and replicate samples (5g) of the sieved flour were used for lipid extraction.

Methods of lipid extraction. All samples were boiled in 150 ml *iso*-PrOH or H_2O-sat *n*-BuOH (WSB) for 10 min before homogenization. Wheat, pea, spinach and tomato samples were homogenized in a top-drive blender and a solvent-type Ultraturax probe used for potato dice. Plant samples were then extracted by one of four different methods: (A) *Iso*-PrOH treated tissue was washed (×3) with 80 ml $CHCl_3$–MeOH (2:1) allowing 3–4 min standing between washes. (B) *Iso*-PrOH treated tissue was blended with 190 ml of $CHCl_3$–MeOH–H_2O (5:10:4) for 2 min, 50 ml $CHCl_3$ and 1 ml conc HCl added and blended for 1 min. The extract was filtered and the residue rinsed with $CHCl_3$–MeOH (2:1). (C) Modified Bligh and Dyer extraction [19]. As method B except that addition of 1 ml conc HCl was omitted. (D) WSB treated tissue was blended twice for 2 min with 200 ml WSB.

Purification of lipid. Bulked filtrates were taken down to dryness on a rotary evaporator at 45°, dried with toluene–EtOH (4:1) and the crude lipid phased in 240 ml $CHCl_3$–MeOH–H_2O (2:1:0.75). 150 ml portions of the lower phase (172 ml) were then purified from non-lipid contaminants [20] by passage through Sephadex G-25 (fine) and the total lipid sample used for analytical determinations or further separation into constituent lipids. Control experiments prior to the main experiment showed no losses of polar lipids to the aqueous phase during purification.

Lipid analysis. Total lipid was divided into a neutral plus glycolipid and a phospholipid fraction by chromatography on Si gel columns [21]. Individual lipids from these fractions were estimated by quantitative TLC on Si gel with the solvent system $CHCl_3$–MeOH–HOAC–H_2O (170:30:20:4). Phospholipids were estimated as inorganic phosphorus [22]. Glycolipids were hydrolysed with 2 ml 0.75 N dry methanolic HCl at 80° for 20 hr followed by GLC estimation of the TMSi derivatives of methylated glucose or galactose with mannitol as an int. stan. [23, 24]. Free sterols were prepared from total lipid extracts by the procedure of ref. [25] and estimated by GLC of the TMSi derivatives of sterols with cholestane as an int. stan. [26]. Fatty acid methyl esters were prepared from total lipid extracts by transmethylation with 5% H_2SO_4 in MeOH at 60° and analysed in the presence of an int. stan., methyl behenate, by GLC on columns of PEGA (10%) at 190° with Ar as carrier gas.

Statistical analysis. Results from the four extraction methods (A, B, C, D) were subjected to a 'one way analysis of variance' followed by Duncan's [27] New Multiple Range Test (5% level).

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