THE SEPARATION AND IDENTIFICATION OF PLANT PHOSPHOLIPIDS AND GLYCOLIPIDS BY TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY*

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INTRODUCTION

Recent development in thin-layer chromatography and its application to the analysis of lipids have proved to be very effective for the separation of animal phospholipids and cerebrosides[1,2]. In contrast, plant lipids contain other distinctive glycolipids of similar physical properties such as galactosyl glycerides, digalactosyl glycerides[3,4], polygalactosyl glycerides[5], phosphatidyl glycerol as well as the sulfolipid of BENSON[6]; their separation by chromatography is consequently a much more difficult operation. Nevertheless, clean separations of these plant lipids have been achieved by two-dimensional thin-layer chromatography. This communication sets forth the technique and its application to the fractionation of complex mixtures of polar plant lipids. Identification techniques and the value of thin-layer chromatography in autoradiography are also described.

EXPERIMENTAL

Lipid extracts

Total lipids were extracted from alfalfa leaves, potato leaves and tubers, and ^{14}C-containing *Chlorella* by homogenizing the tissue in hot 80 % ethanol for 2 min and extracting the residue with acetone, followed by a mixture of chloroform–methanol (2 : 1). The combined extracts were filtered, concentrated *in vacuo* and the lipid concentrate taken up in chloroform and washed thoroughly with water to remove non-lipid material. Stock solutions of lipid in chloroform were adjusted so that 2.5 μl solution was the equivalent of 1 mg fresh tissue.

Commercial soya lecithin, egg lecithin and animal kephalin were also used as reference material in chloroform–methanol solution at a concentration of 10 mg/ml.

Apparatus and solvents

Glass plates, 20 cm × 20 cm, were coated to a depth of 0.25 mm with Silica Gel G (Merck, Darmstadt) applied in a suspension of 25 g gel in 55 ml distilled water. The

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plates were activated by a final drying at 100° for 30 min. The chromatography jars were lined with filter paper to ensure saturation of the enclosed space with solvent vapors.

Three different solvent systems were used for the development of thin-layer chromatograms:

Solvent A: chloroform–methanol–water (65:25:4)[1].
Solvent B: diisobutyl ketone–acetic acid–water (80:50:10)[7].
Solvent C: hexane–diethyl ether–acetic acid (90:10:1)[8].

Spotting and development

For one-dimensional thin-layer chromatograms, 1–10 μl quantities of lipid extract were spotted in the conventional manner and allowed to develop in equilibrated jars until the solvent front had climbed a distance of 17 cm. The times required for development were: 2 h for solvent A, 3 h for solvent B and only 1 h for solvent C.

Two-dimensional thin-layer chromatograms were prepared by applying the lipid sample as a single spot at the right-lower corner of the plate and using solvents A and B as developing systems. At the end of the first run in solvent A, the plates were removed from the chromatography jars, dried for 30 min and then placed in the second solvent after clockwise rotation through 90°.

Detection of spots

After the developing solvents were removed, the lipids were detected on the chromatogram in the following manner.

A. General methods

(1) *Iodine vapor*[9]. The spots were revealed by iodine vapors and were outlined with a pencil. The iodine was then removed *in vacuo*, and the plates used again with more specific reagents.

(2) *Aqueous 20% perchloric acid.* The chromatoplates, after preheating at 100° for 5 min, were sprayed with an aqueous 20% perchloric acid solution. Most lipids appeared as brown spots, which could be intensified by further heating. Two spots, U_1 and U_2, tentatively designated as esterified sterol glycoside and sterol glycoside respectively gave atypical reddish spots.

B. More specific methods

(1) *Ninhydrin.* The chromatoplates were stained with 0.2% ninhydrin in 99% *n*-butanol and 1% pyridine for phosphatidyl ethanolamine and phosphatidyl serine. When the chromatoplates were heated at 100° prior to spraying, no further heating was necessary for the appearance of the spots.

(2) *Modified Dragendorff reagent*[10]. Choline-containing phospholipids appeared immediately on spraying with this reagent. Galactolipids also yield orange spots, but these appeared more slowly.

(3) *Perchloric acid–Schiff reagent*[11]. The chromatoplates were damply sprayed with an aqueous 0.5% sodium periodate solution. After 5 min, the chromatoplates,

while still wet, were exposed to SO_2 gas to remove excess periodate and then stained with an aqueous 0.5% *p*-rosaniline solution, freshly decolorized by bubbling SO_2 gas. After full development of the color (about 1 h), the background can be lightened by spraying with a 1% perchloric acid solution. All phospholipids and glycolipids appeared as well defined blue and purple spots on a yellowish background.

The sequence of sprays that we have followed was: the chromatoplates were first exposed to iodine vapors. The spots were outlined, iodine removed and the chromatoplates stained with periodate–Schiff reagents to detect glycolipids. Alternatively, after the iodine vapor, the chromatoplates were sprayed with ninhydrin to detect the amino-phospholipids, then with Dragendorff reagent to detect choline-containing phospholipids and finally with perchloric acid to char all the lipid spots.

C. Method for radioactive lipids

Radioactive lipids gave excellent autoradiograms when the chromatoplates were placed powder-side down on Kodak, single coated, X-ray film. No damage to the plate or loss of radioactive lipid was encountered. Because the spots on thin-layer plates are so compact, an exposure of only 2 days was required for clear pictures of spots containing about 2 mμC ^{14}C.

Identification by alkaline hydrolysis

A modified Dawson procedure[5,12] using toluene–pyridine (10:1) as solvents and 0.1 *N* methanolic potassium hydroxide followed by neutralization with Dowex 50 (H^+), was used to deacylate lipids that could not be fully identified on thin-layer plates by spray reagents. Preparative, one-dimensional, thin-layer chromatography in solvents A or B furnished material for the hydrolysis. Two-dimensional paper chromatography[12] was employed to separate the glycotic portions of the unknown lipids. Spots were identified by a comparison of R_F values[13–15].

RESULTS AND DISCUSSION

One-dimensional thin-layer chromatography of the plant lipid extracts gave only incomplete resolution of the components. This inferior performance was confirmed by hydrolysis and paper chromatography of allegedly pure spots. Resolution of all mixtures into single components was, however, achieved by two-dimensional chromatography using solvents A and B (Fig. 1). Phospholipids separated suitably in solvent A, but travelled much less rapidly in solvent B. The latter solvent was, however, more effective for the separation of glycolipids and sterol glycosides. This two-dimensional system effected therefore a clean separation of all lipid components.

It has not been possible to identify all components on the chromatograms with the various chemical sprays. All lipids were located either with iodine vapors, 20% perchloric acid or periodate–Schiff spray reagents, but these reagents lacked specificity for distinguishing phosphatidyl inositol from phosphatidyl glycerol or phosphatidic acid, and the monogalactosyl glyceride from the digalactosyl glyceride and the polygalactosyl glycerides. Only phosphatidyl ethanolamine, phosphatidyl serine and phosphatidyl choline were identified with certitude with the aid of ninhydrin or Dragendorff reagents. Because of these limitations it has been necessary to insure

unambiguous identification through hydrolysis and comparison of R_F values on paper chromatography of their deacylated products.

The above scheme has been used to separate potato tuber lipids into 17 components of which 12 have been identified. Their R_F values, relative intensities and re-

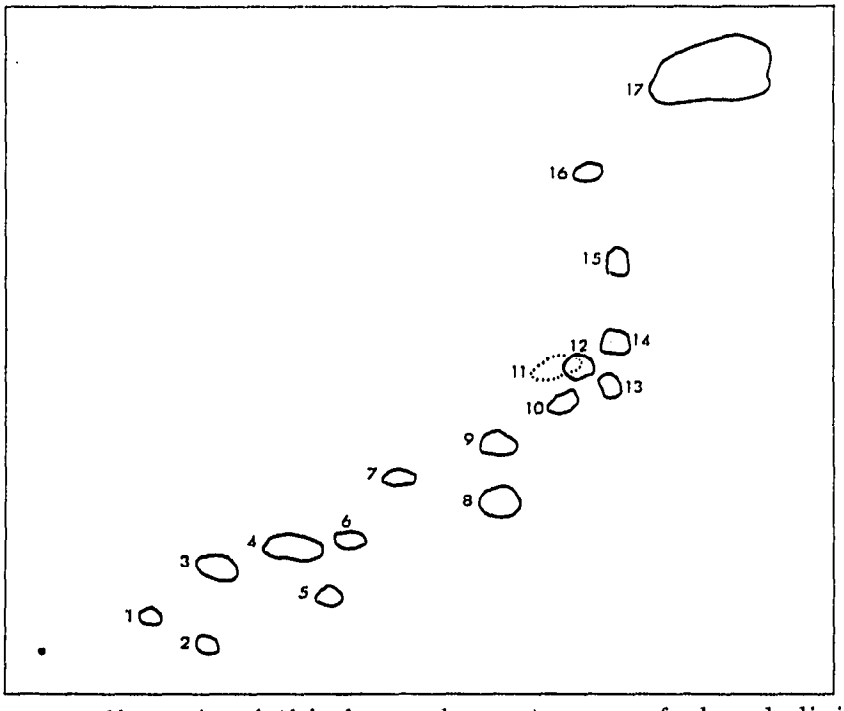

Fig. 1. Tracing of a two-dimensional thin-layer chromatogram of phospholipids and glycolipids from potato tuber extracts. The chromatogram was developed in chloroform–methanol–water (65:25:4) (solvent A) in the x-direction and then in diisobutyl ketone–acetic acid–water (80:50:10) (solvent B) in the y-direction. The spots were detected with iodine, followed by staining with 20% perchloric acid. The tracing was made from a contact photograph. Numbers refer to those in Table I.

sponse to various chemical sprays are indicated in Table I. Iodine was found to be the most sensitive chemical to detect all spots on chromatograms, but it failed to detect compound 12. Perchloric acid, although generally less sensitive than iodine, produced a reddish color, characteristic of sterols, with compounds 12 and 15. Hydrolysis of compound 15 in 0.1 N methanolic potassium hydroxide produced fatty acids and a derivative with the same R_F value as compound 12. This suggests that compound 15 is similar to compound 12, but has in addition a fatty acid portion. Further degradation of compound 15 by acid hydrolysis freed sterols and sugars. On the basis of these observations, compounds 12 and 15 have been tentatively identified as sterol glycoside and esterified sterol glycoside respectively. Details of their chemical characterization will be reported later.

When the above mixture of plant lipids was analysed by the procedure of KIDMORE AND ENTENMAN[2], only 12 of the 17 compounds were separated.

SUMMARY

The thin-layer chromatographic properties of plant phospholipids and glycolipids have been determined. Two-dimensional thin-layer chromatography, using chloro-

TABLE I

R_F VALUES AND RELATIVE INTENSITIES OF PLANT PHOSPHOLIPIDS AND GLYCOLIPIDS

No.	Components	$R_F \times 100$ in solvents[a]		Spray reagents[b]					Rel. intensity[c]	$R_F \times 100$ in PW[d]	
		A	B	A_1	A_2	B_1	B_2	B_3		Found	Reported[4]
1	Phosphatidyl serine	14	6	+	+	+			w		
2	Unidentified	21	2	+	+	—		+	w		
3	Phosphatidyl inositol	23	14	+	+	—		+	m	9	12
4	Phosphatidyl choline	33	18	+	+	—	+	+	s		
5	Unidentified	36	9	+	+			+	w		
6	Sulfolipid	42	22	+	+	—	—	+	m	18	18
7	Phosphatidyl glycerol	48	30	+	+	—		+	m	42	36
8	Digalactosyl glyceride	62	25	+	+	—	+	+	vs	48	46
9	Phosphatidyl ethanolamine	62	35	+	+	+	—	+	s		
10	Unidentified	71	41	+				+	m		
11	Unidentified	70	47					+	vw		
12	Unknown U_2[e]	73	48	—	r	—	—	+	s		
13	Unidentified	77	44		+	—		+	m		
14	Monogalactosyl glyceride	77	51	+	+	—	+	+	vs	62	62
15	Unknown U_1[f]	78	65	+	r	—	—	+	s		
16	Phosphatidic acid	74	79	+	+	—	—	+	m		
17	Neutral lipids	92	97	+	r	—	—	+	vs		

[a] R_F values of intact lipids.
[b] + = positive spot; — = negative spot; r = reddish spot.
[c] vw = very weak; w = weak; m = medium; s = strong; vs = very strong. This is applicable to all spray reagents.
[d] R_F values of compounds after deacylation. PW = phenol saturated with water.
[e] Tentatively identified as sterol glycoside.
[f] Tentatively identified as esterified sterol glycoside.

form–methanol–water (65:25:4) and diisobutyl ketone–acetic acid–water (80:50:10) solvent systems, yielded separation of all the major lipids, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl glycerol, phosphatidyl inositol, digalactosyl glyceride, monogalactosyl glyceride and the sulfolipid, as well as other minor lipids. Two partially characterized glycolipids, probably sterol glycosides, were found in the potato lipid extracts.

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