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ANALYSIS OF MAJOR CLASSES OF PLANT LIPIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLAME IONIZATION DETECTION

Abstract—Although HPLC has been successfully used to separate most types of biomolecules, the lack of a quantitative detection technique has prevented the application of this powerful separation tool for the direct analysis of classes of plant lipids. The commercial availability of a reliable flame ionization detector (FID) has now opened up this field. We have developed a ternary gradient system (isooctane–isopropanol–water) which will separate all of the common classes of nonpolar lipids, galactolipids and phospholipids present in most plant tissues. The lower limits of detection utilizing this system are *ca* 1 μ g of each lipid component per injection. Standards of 23 common plant lipids could be resolved with this new technique. The HPLC-FID system was used to provide a quantitative analysis of the lipid classes in potato leaves, potato tubers, corn roots, tobacco leaves and spinach leaves.

INTRODUCTION

Several laboratories have reported HPLC techniques for the separation of lipid classes from animal tissues [1–3]. The two previous reported HPLC determinations of plant lipid classes [4, 5], were qualitative rather than quantitative because the UV detectors employed essentially can only measure the degree of unsaturation of sample peaks. In the last four years, a flame ionization detector (FID) designed for HPLC, has become commercially available. Some of our colleagues have published preliminary results using HPLC-FID with normal phase columns to separate standards of several different lipid classes [6]. A similar HPLC-FID system was also recently used to separate and quantify cholesterol oxidation products [7]. G. A. Thompson's laboratory [8–10] also developed an HPLC-FID system with reversed-phase columns to separate and quantify the molecular species of several plant phospholipids and galactolipids. The current study was undertaken to investigate and optimize conditions for the separation of polar and nonpolar lipid classes from several leaf and nongreen plant tissues using HPLC-FID.

RESULTS AND DISCUSSION

Separation of lipid standards

Preliminary investigations were performed utilizing lipid class standards in order to optimize HPLC separations. Three isocratic mobile phases were tested for their ability to resolve different lipid classes with a LiChrosorb Si 60 column (Table 1). The first two isocratic mixtures,

40% A/55% B/5% C (the compositions of A–C are shown in Table 1) and 40% A/53% B/7% C, were able to resolve PE, PI and PC, although the PC peak at 44 min in the first isocratic run was too broad to be integrated accurately. Unfortunately, none of the isocratic systems tested could also successfully separate the complete range of lipids typically present in plant tissues (including nonpolar lipids, galactolipids and phospholipids). With each of the three isocratic conditions all of the nonpolar lipids and the galactolipids had very short R_s and were not clearly resolved from each other.

We then explored the possibility of using the isooctane–isopropanol–water gradient system reported by Christie [1] to separate plant lipids. Because this system was designed to separate lipids from animal sources it required several modifications for plant lipid analyses. The major difference between the lipid classes in animal tissues and plant tissues is that animals lack mono- and digalactosyldiacylglycerols which are intermediate in polarity between nonpolar lipids and phospholipids. After exploring several types of gradient shapes and durations, the gradient system in Table 2 was adopted as optimal for the LiChrosorb Si 60 column. A 22 min re-equilibration between the end of one gradient and the injection of the next sample was crucial, even though it added considerable time to each analysis. With either shorter or longer time periods between injections, the R_s of nonpolar lipids and galactolipids varied considerably from one analysis to the next. However, strict adherence to the conditions described in Table 2 eliminated these problems.

Using the ternary gradient system, a mixture of nine lipid standards was clearly resolved by the HPLC-FID system (Fig. 1). There was a small increase in the baseline starting at *ca* 14 min. This increasing baseline did not

Table 1. Retention times of phospholipid standards in three isocratic systems

Composition of mobile phase			Retention times (min)			
%A	%B	%C	NPL	PE	PI	PC
40	55	5	0.93	3.65	10.78	44.29
40	53	7	0.90	2.10	4.64	14.33
40	51	9	1.02	1.23	1.39	3.04

For abbreviations see Table 3.

A = isooctane-tetrahydrofuran (99:1) B = Isopropanol. C = water.

Table 2. Ternary gradient system for lipid class separation, linear gradients were programmed between the indicated time points

Composition of mobile phase			
Time (min)	%A	%B	%C
0	100	0	0
5	95	5	0
10	85	15	0
15	40	60	0
33	40	51	9
48	40	51	9
53	40	60	0
58	100	0	0
80	100	0	0

See Table 1 for composition of components A, B and C.

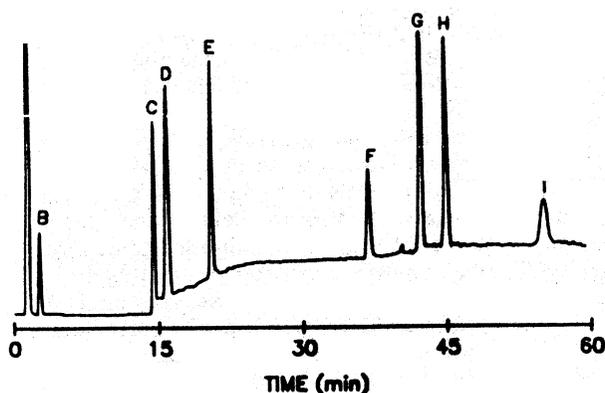


Fig. 1. Separation of standards of lipid classes. The standard mixture contained: (a) 1.30 mg cholesteryl stearate, (b) 0.50 mg tripalmitin, (c) 0.92 mg cholesterol, (d) 1.02 mg palmitic acid, (e) 0.50 mg monogalactosyldiacylglycerol, (f) 0.65 mg phosphatidylethanolamine, (g) 1.32 mg phosphatidylserine, (h) 1.05 mg phosphatidylcholine and (i) 0.86 mg lyso-phosphatidylcholine in a total volume of 1 ml chloroform.

appear to be due to incomplete removal of solvents and did not interfere with the detection or integration of peaks. Two other types of silica columns (ChromSphere Si, 5 μ , and Spherisorb Si, 5 μ , both supplied by Chrom-pack in 3 \times 100 mm glass cartridges) were also evaluated

with this gradient system but were found to be inferior to LiChrosorb Si 60 because they produced a higher background and often produced several artifact peaks per gradient. For this reason, the LiChrosorb Si 60 column was used for all subsequent analyses.

A total of 23 lipid standards were injected and their R_s are recorded in Table 3. Several concentrations of plant lipids were then injected to obtain standard curves of lipid mass versus detector response (Fig. 2). Among the five lipids tested, monogalactosyldiacylglycerol and PC gave the highest signal responses followed by β -sitosterol, triacylglycerol and finally free palmitic acid. The calibration curves were linear in the range of 1–200 μ g. These results are consistent with those of Maxwell *et al.* [6] who reported linear detector responses for 1–200 μ g of PE or PC. Accordingly the HPLC-FID system can be used to accurately quantify from 1 to 200 μ g of each lipid class. Although it would be possible to obtain similar calibration curves for all lipid classes being analysed, we chose at this point to express the analytical data for plant extracts in terms of area %. This was done because it did not seem necessary at this stage to obtain calibration curves for each of the 23 plant lipid classes which were analysed in the complex mixtures of plant lipids.

Analysis of lipids in potato leaves

Lipids were extracted from potato leaves, separated by HPLC and measured by four different detection methods (Fig. 3). Detection with FID yielded a rather complex chromatogram, mainly due to the presence of many pigment peaks which eluted between 14 and 20 min. Integration of FID data yielded lipid analyses (Table 4) which were very similar to those obtained using conventional TLC chromatographic techniques. The galactolipid and phospholipid peaks occurred at 20–32 min and 36–46 min, respectively, and were quite well resolved. The only polar lipids which may not have been clearly resolved were PG and SQDG, and PA and PS (Table 3). UV detection at 205 nm was nearly as sensitive as FID for most of the peaks, but because 205 nm essentially measures the level of unsaturation of lipid classes, it is not considered to be a quantitative method. Visible detection at 440 nm revealed β -carotene at ca 1 min and chlorophyll and carotenoids at 14–20 min. Visible detection at 600 nm revealed the chlorophylls at 14.7 and 15.5 min and an unidentified peak at ca 14.0 min. It was noted that when lipid samples containing high concentrations of chlorophyll were injected, a small amount of green residue began to build up in the silica packing of the glass cartridge-type HPLC columns. Preliminary experiments

Table 3. Separations of lipid standards on a Lichrosorb Si 60 column with the gradient system described in Table 2

Peak No.	Lipid class	Abbreviation	Retention time (min)
1	Sterol esters and β -carotene	StE	1.16
2	Triacylglycerol	TAG	1.98
3	Phytol	Phyt	7.26
4	Diacylglycerols	DAG	13.80
5	Free sterols	St	14.11
6	Chlorophyll A	ChlA	14.71
7	Chlorophyll B	ChlB	15.55
8	Free fatty acid	FFA	16.36
9	Carotenoids	Car	16.80
10	Acylated sterol glycoside	ASG	17.83
11	Cerebrosides	Cer	20.27
12	Monogalactosyldiacylglycerol	MGDG	20.80
13	Sterol glycoside	SG	27.68
14	Digalactosyldiacylglycerol	DGDG	31.03
15	Cardiolipin	DPG	33.27
16	Phosphatidylethanolamine	PE	36.25
17	Phosphatidylglycerol	PG	38.41
18	Sulfoquinovosyldiacylglycerol	SQDG	38.99
19	Phosphatidylinositol	PI	40.60
20	Phosphatidic acid	PA	41.15
21	Phosphatidylserine	PS	42.20
22	Phosphatidylcholine	PC	44.65
23	Lysophosphatidylcholine	LPC	54.70

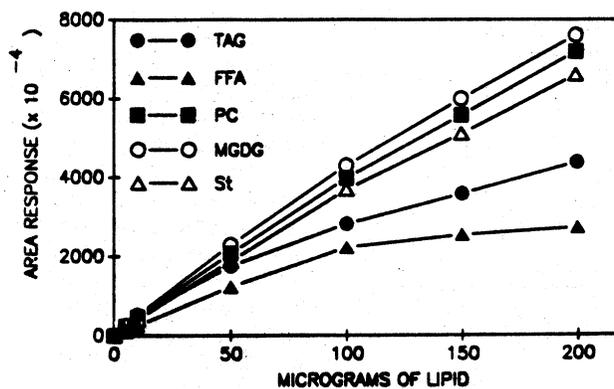


Fig. 2. Calibration curves of mass of lipids injected versus detector response (integrated areas of each peak). Each point represents the mean of at least three determinations. The standards were tristearin, palmitic acid phosphatidylcholine (dipalmitoyl), monogalactosyldiacylglycerol (from spinach), and β -sitosterol.

revealed that this green material was not retained by a silica guard column, so the use of a guard column was discontinued. This phenomenon was previously attributed to the breakdown of chlorophyll catalysed by the silica itself, and for this reason it has been recommended that silica columns should not be used for the quantitative analysis of plant pigments [11]. Even though lipid samples from green tissues did cause this problem, the quality of the separations was still quite good even after more than 100 injections on a column. The main problem that limited the life of the columns was a gradual increase in pressure (eventually exceeding the 2500 psi limit recommended by the manufacturer of this cartridge system)

after an extended number of injections (usually more than 100). An HPLC procedure utilizing reversed phase HPLC has been developed to quantitatively separate and quantify plant pigments [11], but because reversed phase columns only separate molecular species of lipids [4, 8, 9], it is unlikely that reversed phase columns could also be used for lipid class separations. We have also considered using 'bonded phase' (polar-bonded-phase silica gel) columns to try to separate lipid classes without causing the accumulation of chlorophyll breakdown products on the column. Unfortunately, others have attempted to separate plant lipid classes on 'bonded phase' columns, and have reported them to be useful for galactolipids and

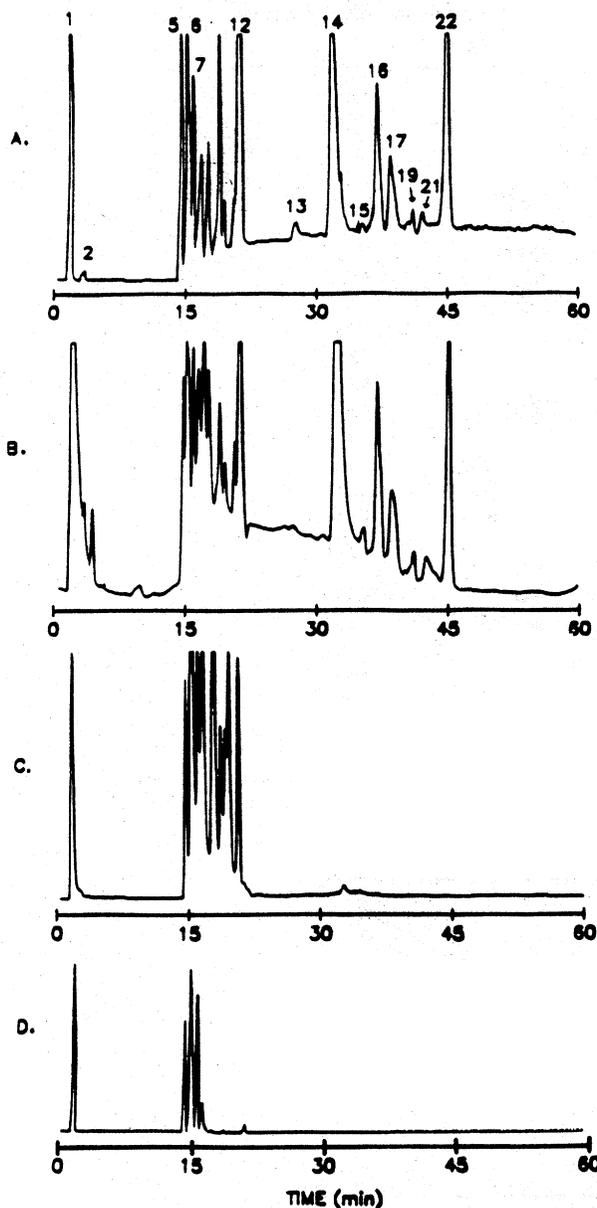


Fig. 3. Separation of lipid classes from potato leaves and comparison of various types of detection: (a) FID, (b) UV detection at 205 nm, (c) visible detection at 440 nm and (d) visible detection at 600 nm. The numerical symbols of lipid classes are shown in Table 3, quantitative analysis of FID data in Table 4. The units for the y axes are relative response for chromatogram A, and 0.5 A full scale for chromatograms B–D.

certain phospholipids, but several acidic lipids (PG, PI and free fatty acids) were not eluted from the columns [5].

Analysis of lipids in potato tubers

A similar analysis of lipids from potato tubers yielded less complex chromatograms (Fig. 4). Detection with FID revealed 13 major peaks and the integrated values are shown in Table 4. Detection at 205 nm again closely paralleled the FID peaks. An advantage of this gradient system is that each of the solvents used does not absorb at

205 nm, so for workers not possessing an FID, it could be very useful to monitor preparative or qualitative separation of unsaturated lipids. However, it must be used cautiously because saturated lipids are not detected at 205 nm. Although the lipid extract was quite yellow in colour, detection at 440 nm revealed two small peaks, a β -carotene peak at *ca* 1 min and carotenoid peak at *ca* 17 min (probably violaxanthin) which only registered as a minute peak with FID.

Analysis of lipids in other plant tissues

Lipids were also extracted from corn roots, tobacco leaves and spinach leaves. The chromatograms of the lipids in each of these tissues were similar to those shown in Figs 3 and 4. The integrated values of each lipid class are presented in Table 4. The major peaks in the chromatograms from corn roots were PC, sterol esters, PE and PA, in decreasing order. The sterol ester peak should be interpreted with caution because it may contain nonpolar lipids other than sterol esters. This peak also contains β -carotene, and we have additional evidence that it may contain squalene and other hydrocarbons. The leaves of spinach and tobacco contained very high amounts of mono- and digalactosyldiacylglycerols, as is to be expected from green tissue. A unique feature of the tobacco leaves is that they contained a much higher proportion of triacylglycerol than the other two leaf samples.

CONCLUSIONS

The advantages of the HPLC-FID technique are that it is sensitive (minimum limits of detection of each lipid class are *ca* 1 μ g), it is the first HPLC technique which analyses all of polar and nonpolar plant lipids in a single injection, and it is convenient (once the lipid extracts are prepared, all other steps in the analyses are automated). The traditional techniques for the analysis of galactolipids and phospholipids require separation of lipids on TLC, quantification of galactolipids with a spectrophotometric assay such as the phenol-sulphuric acid method [12], and quantification of the phospholipids by digesting the carbons with perchloric acid and measuring the remaining phosphorous with another type of spectrophotometric assay [13]. Although HPLC-FID has previously been used to separate and quantify molecular species (lipids with the same head group and different fatty acids on the *sn*-1 and *sn*-2 positions) of plant lipids [8, 9], this is the first time it has been employed to quantify lipids classes from plants. Both the FID and another new type of detector, an evaporative light scattering detector (ELSD), such as that used by Christie [1], are considered to be 'mass detectors'. In the future, we intend to compare the response of the FID and ELSD for the quantitative analysis of plant lipids.

EXPERIMENTAL

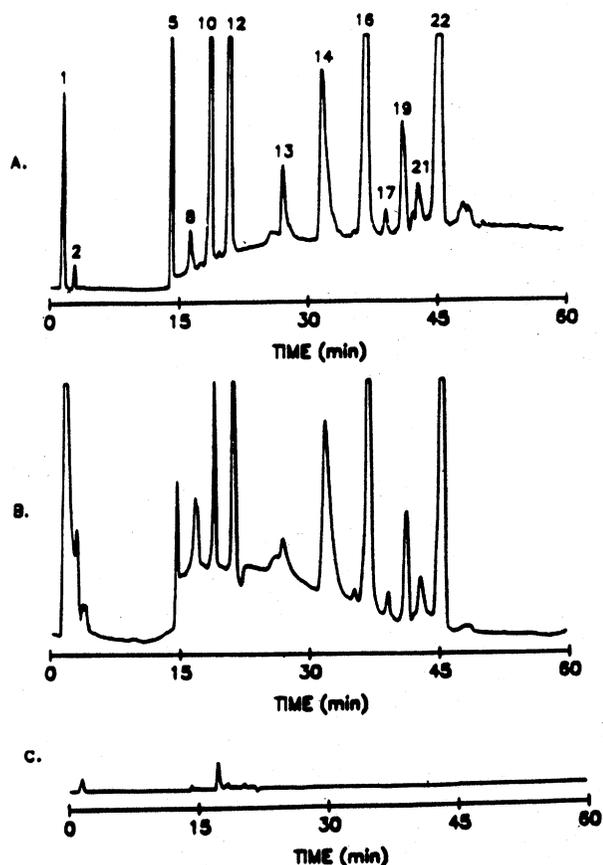
Materials. Standards of most of the various lipid classes were obtained from Sigma. Sulphoquinovosyldiacylglycerol was isolated from spinach leaves as previously described [8]. HPLC grade solvents were used and *iso*PrOH was redistilled on a weekly basis. Potato (*Solanum tuberosum* cv Kennebec) seed tubers were stored at 4° and potato plants were grown as previously described [14]. Tobacco (*Nicotiana tabacum* cv

Table 4. Lipid composition of various plant tissues as determined by HPLC-FID

Lipid class	Area %				
	Potato leaf	Potato tuber	Corn root	Spinach leaf	Tobacco leaf
StE	9.0	2.0	19.2	9.6	7.8
TAG	0.2	0.2	6.9	0.8	16.7
Phyt	nd	nd	nd	0.8	0.7
DAG	nd	nd	nd	nd	nd
St	5.8	3.2	tr	4.3	7.7
ChIA	5.7	nd	nd	4.2	1.1
CHIB	2.8	nd	nd	1.6	3.6
FFA	3.4	0.8	1.2	2.0	2.5
Car	4.5	tr	nd	1.4	0.6
ASG	5.5 (7.4)	23.2 (20.8)	2.2	1.3	2.2
MGDG	35.5 (33.3)	8.1 (9.3)	7.2	24.4	21.4
SG	0.8	2.4	nd	nd	nd
DGDG	9.7 (12.3)	6.2 (8.0)	0.7	14.9	14.0
DPG	nd	nd	0.3	1.7	1.6
PE	4.3 (3.8)	33.6 (31.7)	15.9	8.6	3.5
PG	2.9	0.4	tr	7.4	3.7
SQDG	tr	nd	2.7	nd	3.6
PI	0.6	2.5	tr	2.9	0.9
PA	nd	nd	13.5	nd	2.3
PS	0.6	1.4	nd	nd	nd
PC	7.9 (7.1)	15.9 (17.2)	30.2	14.0	6.3
LPC	1.0	nd	nd	nd	nd

Numbers in parentheses are the lipid composition ($\mu\text{g}/100 \mu\text{g}$ total lipid) obtained by conventional TLC techniques (see Experimental).

nd = Not detected.



Turk) plants were grown similarly. Corn (*Zea mays* WF9 \times Mo17) seedlings were grown on damp filter paper for 5 days at 25°. Spinach (*Spinacea oleracea*) was purchased locally.

Lipid extractions. Lipids were extracted with hexane-isoPrOH as previously described [15].

HPLC. Analyses were performed with an Isco Model 2350 HPLC Pump and Isco Model 2360 Gradient Programmer equipped with a Valco Model C6W injector and a 10 μl sample loop. For most studies, the chromatographic column was a 10 cm \times 3.0 mm Chrompack ChromSep 7 micron LiChrosorb Si 60 silica cartridge system and no guard column (see explanation in Results and Discussion section), with a flow rate of 0.5 ml min⁻¹. Two other silica columns (Chrompack 5 micron ChromSphere Si and Chrompack 5 micron Spherisorb Si) were also evaluated as described in the text. The detector was a Tracor Model 945 Flame Ionization LC Detector (Tracor Instruments, Austin, TX) operated at 190°. For most analyses, a ternary gradient (Table 1) was employed. However, for preliminary studies the gradient programmer was employed to mix the three solvents in isocratic mobile phases. Samples were inj. in CHCl₃.

TLC. Lipid samples were spotted on silica gel G, developed in CHCl₃-MeOH-NH₄OH (30:15:2) and air-dried for 30 min. Plates were then developed in a second direction in

Fig. 4. Separation of lipid classes from potato tubers and comparison of various types of detection: (a) FID, (b) UV detection at 205 nM and (c) visible detection at 440 nm. The numerical symbols of lipid classes are shown in Table 3, quantitative analysis of FID data in Table 4. The units for the y axes are those described in Fig. 3.

CHCl₃-MeOH-HOAc-H₂O (170:30:20:7), air-dried and visualized with I₂. Spots which co-chromatographed with authentic PC and PE were removed and quantified as previously described [13]. Spots which co-chromatographed with MGDG, DGDG and ASG were quantified by the method of ref. [12].

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