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ANALYSIS OF MOLECULAR SPECIES OF PLANT POLAR LIPIDS BY HIGH-PERFORMANCE AND GAS LIQUID CHROMATOGRAPHY

CHANTAL DEMANDRE,* ANTOINE TREMOLIERES,† ANNE-MARIE JUSTIN* and PAUL MAZLIAK*

*Laboratoire de Physiologie cellulaire (UA 566), Université P. et M. Curie, Tour 53, 4 Place Jussieu, 75005 Paris, France; †Laboratoire du Phytotron, CNRS, 91190 Gif-Sur-Yvette, France

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Key Word Index—*Solanum tuberosum*; *Nicotiana tabacum*; Solanaceae; phospholipids; galactolipids; high performance liquid chromatography.

Abstract—Total lipid extracts from potato tubers and tobacco leaves are separated into lipid classes by two step HPLC using a silicic column. Elution is first performed for 20 min with a programmed linear gradient of two mixed solvents running from 100% of solution A (isopropanol–hexane, 4:3) to 100% of solution B (isopropanol–hexane–water, 8:6:1.5); the column is then eluted with pure solution B in an isocratic mode for 20 min more. The main polar lipids (MGDG, DGDG, PC, PE, PG) from both plant tissues can be collected and further separated into component molecular species on a simplified HPLC system with a C_{18} column eluted in an isocratic mode with a polar solvent. Molecular species separations are achieved within 35 min; quantifications are made through GLC analysis of attached fatty acids. Three to five main molecular species are thus clearly identified in each lipid class. In potato tuber, phospholipids (PC, PE) 18:2/18:2 species are predominant. In tobacco leaf, six double bond species (18:3/18:3 and 16:3/18:3) are predominant in galactolipids, whereas PC contains a greater number of molecular species varying by their degree of unsaturation (from 18:3/18:3 to 16:0/18:2). Only certain molecular species of PG contain Δ^3-*trans*-hexadecenoic acid.

INTRODUCTION

HPLC is a powerful method of analysis rapidly expanding in biochemical research laboratories [1]. Presently, separations of almost all kinds of cellular constituents can be made routinely by use of instruments with automated controls and sensitive detectors. However, a certain lag in the development of HPLC methods suitable for the analysis of plant membrane lipids can be observed although separations of complex mixtures of phospholipids from animal origin have been proposed [2–13]. The detection procedure employed in these methods was measurement of absorption of eluted substances in the range 200–210 nm, thus enabling the analysis of lipids without chemical modification. When molecular species are analyzed, the published procedures comprise two steps: (1) separation of the complex lipid mixtures into classes (i.e. various phospholipid classes: PC, PE, PI, PS, etc.) by a preliminary preparative chromatography indifferently realized either on a silicic acid column, a preparative thin-layer of silica gel, or directly by HPLC; (2) resolution of each lipid class into molecular species by HPLC utilizing hydrophobic column packings. To our knowledge, only one paper has been published [14] on the separation of galactolipid molecular species (of algal origin) by HPLC. In that work, the two purified lipid classes (MGDG and DGDG), subsequently analyzed by HPLC, had been obtained from chloroplast lipid extracts by combined silicic acid CC and TLC procedures.

The purpose of this paper is to propose a combination of rapid HPLC and GC methods allowing the resolution of complex mixtures of plant phospho- and galactolipids into the various molecular species present in each lipid class.

Abbreviations: DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SL, sulpholipid.

RESULTS

Separation of polar lipid classes by high performance liquid chromatography

Complex lipid mixtures, total lipid extracts from potato tubers (Fig. 1a) or tobacco leaves (Fig. 1b), are first separated into lipid classes by two step HPLC using a silicic acid column: (1) first step: elution with a solvent mixture (*iso*propanol–hexane), progressively enriched in water, for 20 min; (2) second step: elution on the same column, in an isocratic mode, for 20 min by the final solvent mixture of step 1 (see Experimental for details).

The two typical profiles shown in Fig. 1 reveal that, within 40 min, both plant lipid extracts are resolved into at least 12 different classes. For identification, eluant fractions containing lipid classes corresponding to peaks detected at 205 nm, are collected manually; solvents are evaporated under N_2. Concentrated lipids are analyzed again by TLC on silica gel, in the conditions indicated in Experimental. It was possible to establish the following order of elution of lipid classes from the HPLC column, under our experimental conditions: *neutral lipids* (mixed with photosynthetic pigments) are very rapidly (3 min)

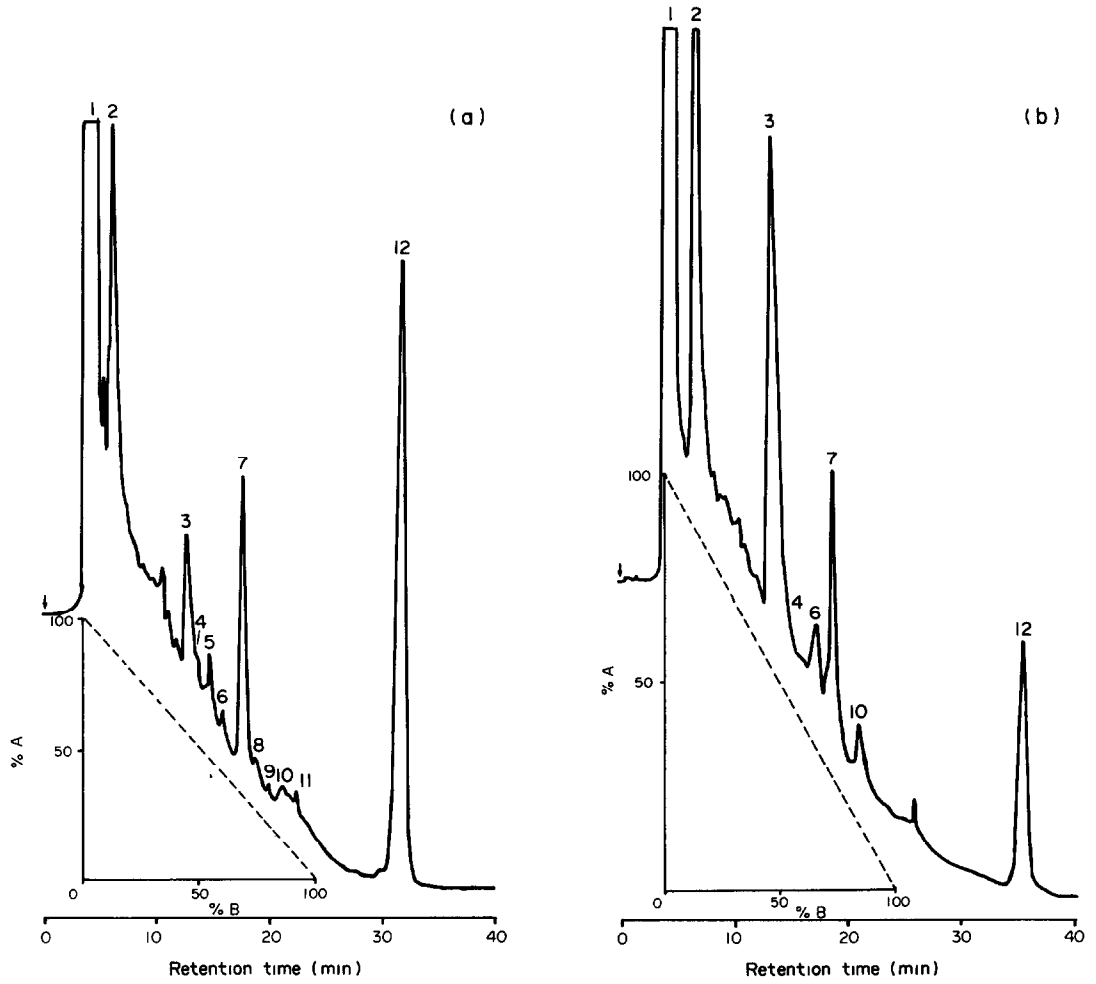

Fig. 1. Separation of polar lipid classes by HPLC—A: potato tuber extract—B: tobacco leaf extract. Peak identifications are discussed in text. The dotted line indicates the percentage of solvent A in eluant.

eluted and form peak 1; the three main plant *glycolipids* (MGDG, peak 2—DGDG, peak 3 and SL, peak 4) are all eluted within 12 min. Sulpholipid cannot be clearly separated from DGDG (because of its small concentration).

Interestingly, in our system, *phospholipids* are eluted after neutral- and glycolipids, in the following order: PA (peak 5), PG (peak 6), PE (peak 7); PS and PI are probably eluted among unidentified lipids (peaks 8, 9, 10 and 11). Peak 12 contains very pure PC, eluted within only 32 min. It is interesting to notice that PC thus separated by HPLC is not contaminated by SL while most TLC systems utilized for plant lipid analysis result in a poor separation of PC from SL [15].

The fatty acid Me esters prepared from the main classes of polar lipids separated by HPLC from potato tubers and tobacco leaves were analyzed by capillary GC. Results are presented in Table 1. The high percentages of triunsaturated fatty acids ($C_{18:3}$ and $C_{16:3}$ in MGDG) found in the galactolipids from tobacco leaves are worth mentioning. As previously found by other methods [16], galactolipids prepared from potato tubers contain high percentages of linoleic acid ($C_{18\,2}$). In the polar lipids extracted from tobacco leaves, a high percentage of *trans*-hexadecenoic acid ($C_{16:1}$ *trans*) can be observed only in PG, which is usual in photosynthetic tissues [17]; however, the presence of a slight amount of $C_{16:1}$ *trans* in the fatty acids prepared from peak 7 (PE) demonstrate some contamination of PE by PG.

Separation of phospholipids and galactolipids into molecular species

We succeeded in the separation of PC, PE, MGDG and DGDG (extracted from potato tuber and tobacco leaves) and PG (from tobacco leaves) into molecular species varying in their fatty acids composition. Separations were achieved rapidly (within 35 min), in a single run, using a C_{18} HPLC column eluted by a polar solvent mixture in an isocratic mode.

Figure 2 shows some typical chromatograms obtained with membrane lipids from potato tubers or tobacco leaves. Three to five main molecular species were clearly identified in each lipid class, giving sharp and symmetrical peaks which could be easily collected for further analysis of esterified fatty acids. Some additional peaks can be detected: for instance PE from tobacco leaves was

Table 1. Fatty acid composition of polar lipid classes of tobacco leaves and potato tubers separated by HPLC

	% Fatty acid by wt							
	C16:0	C16:1 *cis*	C16:1 *trans*	C16:3	C18:0	C18:1	C18:2	C18:3
Tobacco leaves								
MGDG	10.7	4.7	—	10.5	4.2	7.9	5.5	56.5
DGDG	17.7	6.2	—	—	5.6	7.1	4.5	58.8
PG	26.0	2.1	20.4	—	5	11.2	13.3	17.1
PE	29.7	tr.	3.6	—	5.4	15.5	25.4	20.4
PC	24.2	8.8	—	—	9.5	11	20.3	26.2
Potato tuber								
MGDG	20.1	1.7	—	—	6.1	21.8	35	15.2
DGDG	17.6	1.8	—	—	7.2	14.4	44.6	14.4
PE	21.2	0.9	—	—	5.5	2.7	58.2	11.2
PC	23.7	1.1	—	—	6.4	2.0	55.7	11.0

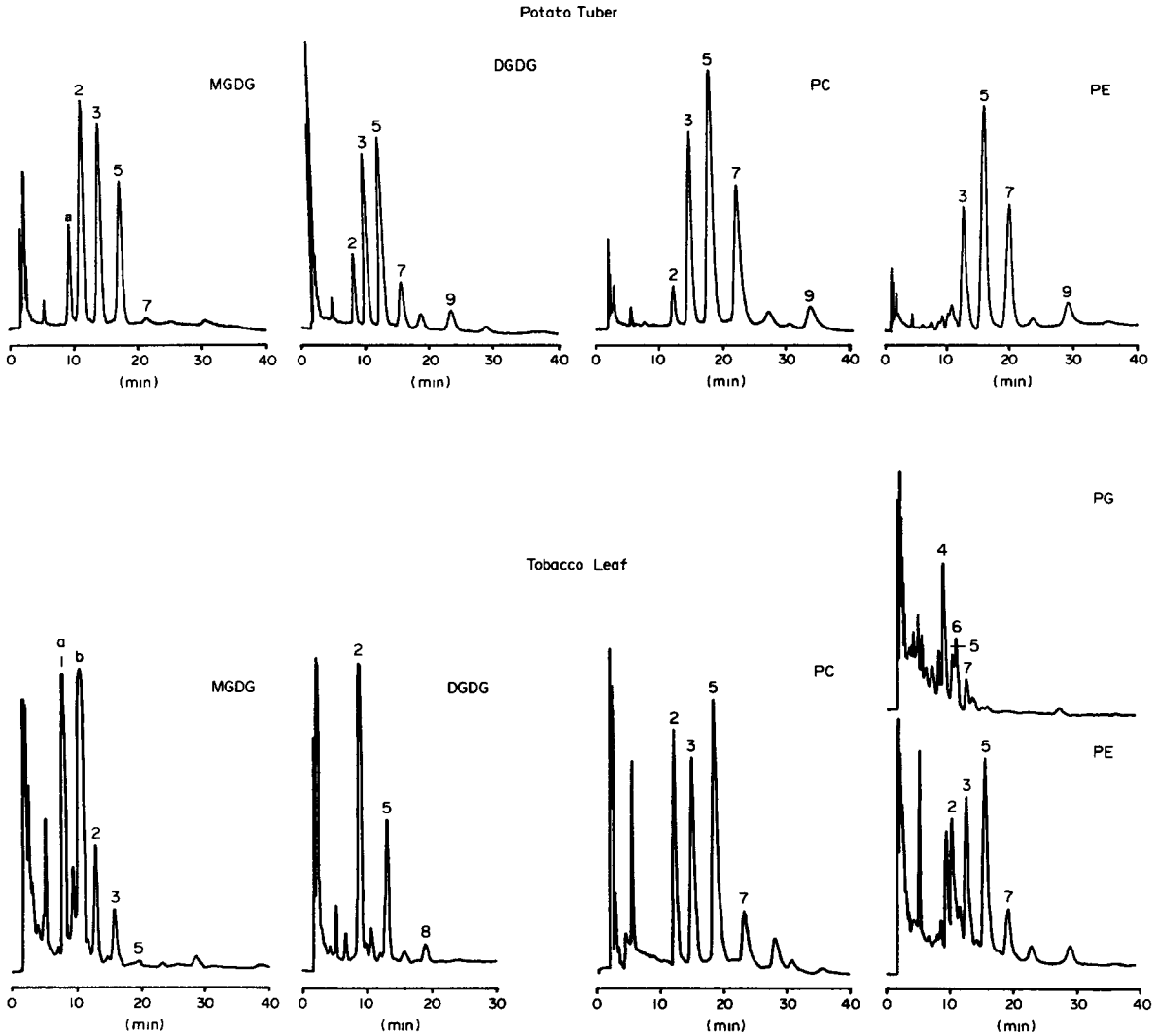

Fig. 2. HPLC separation of molecular species of the main potato tuber and tobacco leaf polar lipids (chromatogram code numbers correspond to the molecular species identified in Tables 2 and 3).

frequently contaminated by PG and, in that case, mixtures of molecular species were eluted from the hydrophobic column. MGDG was also contaminated by pigments (essentially carotenoids). These pigments gave either two additional peaks or were mixed with molecular species on MGDG chromatograms (a, b, Fig. 2). Carotenoids could be clearly detected at 440 nm; these pigments did not interfere in molecular species determination since identification was done by GC of the fatty acid.

The main molecular species eluted from the HPLC column were collected manually: lipids were extracted, transmethylated and fatty acids were analysed by GC, using a Carbowax capillary column (as indicated in Experimental). Results for most lipid classes are given in Tables 2 and 3.

The main potato tuber phospholipids and galactolipids (Table 2) could be separated into six types of molecular species differing by their component C_{16} and C_{18} fatty acids. The more unsaturated species (18:3/18:3) were first eluted; other molecular species were then eluted, in order of decreasing unsaturations. For the same degree of unsaturation, molecular species with C_{16} fatty acids were eluted before species with C_{18} fatty acids. Table 2 shows that the main molecular species of PC, PE, MGDG and DGDG of potato tubers contain two linoleic acid residues (18:2/18:2). Under our experimental conditions, 18:2/18:2 molecular species could not be separated from 16:0/18:3 species.

Phospholipids do not contain 18:3/18:3 species, whereas galactolipids, particularly MGDG, contain some quantities of this six double bond species (17% of total molecular species in MGDG). Potato tuber galactolipids are particularly rich in 18:3/18:2 species. Traces of 18:0/18:3 (peak 6) have been found in DGDG.

Table 3 shows the molecular species composition of the main polar lipids prepared from tobacco leaves. Triunsaturated fatty acids ($C_{16:3}$ and $C_{18:3}$) are the most abundant among galactolipid acyl residues. Three kinds of molecular species are predominant in tobacco galactolipids: 18:3/18:3, 16:3/18:3 and 16:0/18:3. MGDG molecular species (18:3/18:3 and 16:3/18:3) are the most unsaturated; DGDG species are comprised of 75% 18:3/18:3 and 25% 16:0/18:3. Linolenic acid containing molecular species also form high percentages in tobacco leaf phospholipids (PC and PG) which is clearly different from potato tuber phospholipids. Tobacco leaf PG also differs from potato PG by the predominance of two molecular species containing Δ^3-*trans*-hexadecenoic acid: 18:3/16:1t and 18:2/16:1t.

Table 2. Main molecular species found in polar lipids from potato tuber

Peak number Fig. 2	Molecular species	Mol % in each lipid class			
		PC	PE	DGDG	MGDG
2	C18:3/C18:3	traces	—	4.5	16.8
3	C18:2/C18:3	13.7	11.1	21.2	36.4
5	C18:2/C18:2	32.5	34.4	44.8	46.7
	C16:0/C18:3	10.4	9.1	6.5	—
7	C16:0/C18:2	36.3	38.2	13.8	traces
9	C18:0/C18:2	7.1	7.1	9.2	—

Table 3. Molecular species found in polar lipids from tobacco leaves

Peak number Fig. 2	Molecular species	Mol % in each lipid class			
		PC	PG	DGDG	MGDG
1	C16:3/C18:3	—		—	21.6
2	C18:3/C18:3	14.5		74.3	71.9
3	C18:2/C18:3	17.9		—	3.5
4	C18:3/C16:1t	—	49.1	—	—
5	C18:2/C18:2	8.9		—	—
	C16:0/C18:3	42.2	11.6	25.7	3.1
6	C18:2/C16:1t	—	21.9	—	—
7	C16:0/C18:2	17.3	17.3	—	—
8	C18:0/C18:3	—	—	traces	—

DISCUSSION

Classical methods of lipid analysis (TLC and GC) allow only a difficult and time-consuming separation of the molecular species of plant polar lipids. For instance, phospholipids can be hydrolyzed to corresponding diacylglycerols through the use of phospholipase C; the resulting diacylglycerols can be separated either by GC after silylation or by $AgNO_3$–TLC, on the basis of constituent double-bonds. Galactolipid molecular species have been separated directly by $AgNO_3$–TLC [18] on the basis of the number of component double-bonds. In contrast HPLC allows rapid, clean and reproducible separations of the main molecular species of plant phospho- and galactolipids, without any chemical modification.

As a first step, the separation of polar lipid classes by HPLC offers an interesting alternative to classical TLC methods. For routine analyses, HPLC offers many advantages such as clear-cut separations, pure fractions easily collected and recent work with preparative columns, allowed us to collect pure glyco- and phospholipids (in 1 mg quantities) from complex lipid mixtures. The method proposed here has nevertheless to be improved for eliminating some overlaps between MGDG and pigments in the case of leaves, and also to improve the resolution of minor lipid classes such as SL. An improvement in the separation of PG from PE must also be searched for.

In the second step, the separation of the molecular species present in the main plant phospholipids and galactolipids is achieved satisfactorily. With our system, only one pair of molecular species could not be resolved, viz. 18:2/18:2 and 16:0/18:3.

Collecting the separated fractions and analyzing the component fatty acids by GC allows one to get quantitative results. Some difficulties appear, however, for quantitative estimation of the minor molecular species containing for example $C_{18:0}$ and $C_{18:1}$; usually these minor molecular species have not been collected, giving very small peaks at the ends of the chromatograms. Nevertheless it would be important for metabolic studies employing HPLC methods to consider these molecular species which could be implicated as metabolic intermediates.

EXPERIMENTAL

Total lipids were extracted from 20 g of potato tuber or tobacco leaves, following ref. [19] and finally dissolved in 1 ml of $CHCl_3$.

HPLC separation of lipid classes. Two Waters Associates (Milford, Ma, U.S.A.) model 510 pumps were combined with a Model 680 automated gradient controller and a model U6K system injector. Lipid sepn was achieved on a 3.9 × 300 mm column packed with silicic acid (10 μ Porasil). Lipids (200–500 μg) were dissolved in 200 μl *iso*-PrOH–hexane (4:3), filtered through a Millipore (0.5 μm) filter and injected into the column as 20 μl fractions. The elution was performed for 20 min with a prog linear gradient of two mixed solvents, running from 100% of soln A: *iso*-PrOH–hexane (4:3) to 100% of soln B: *iso*-PrOH–hexane–H_2O (8:6:1.5); then the column was eluted with pure soln B in an isocratic mode for 20 min more. The flow rate was 1 ml/min during all analyses. Lipids were detected at 205 nm using a variable wavelength detector fitted with a 20 μl, 1 cm optical path cell.

Identification of lipid classes. Classes eluted from the HPLC system were manually collected and concd by evaporating the solvents under a N_2 stream. TLC on silica gel was performed according to ref. [20]. Classes were identified by comparison with commercial standards and observation of typical coloured reactions after spraying of specific reagents: ninhydrin for NH_2 containing phospholipids [15], α-naphtol for glycolipids [21], Draggendorff's reagent for choline containing phospholipids [22] and Zindzadze reagent for phosphorus containing lipids [21].

HPLC separation of molecular species. This step can be performed on a simplified HPLC system without gradient prog. PC, PE, MGDG and DGDG were separated into molecular species on a 3.9 × 300 mm μ Bondapak C_{18} column in MeOH–H_2O–MeCN (90.5:7:2.5) at a flow rate of 1.5 ml/min as described in ref. [10]. Phospholipids and galactolipids were solubilized in 0.1 to 0.2 ml of EtOH or MeOH and portions of 10–25 μl (50–150 μg) were applied to the column. After elution, lipids were detected at 205 nm as described above. To calibrate the column pure dipalmitoyl PC and dioleoyl PC (Sigma, St. Louis, Mi, U.S.A.) were injected before the first analytical runs.

Fatty acid analysis. Lipids from each fraction collected at the exit of HPLC column were extracted according to ref. [19]. For quantification on a mass basis, an appropriate amount of an intl standard (heptadecanoic acid) was added to the pooled eluate representing each peak. Fatty acid Me esters from each lipid class were prepared by *trans*-methylation according to ref. [23] and analyzed by GC with a Carbowax capillary column (25 m × 0.4 mm). Analysis was carried out isothermally at 170°, with He as carrier gas (flow rate, 40 ml/min); the detection was performed with FID.

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