Separation and quantitation of molecular species of plant phosphatidylcholine by high-performance liquid chromatography with flame ionization detection[1]

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Summary A method is described for the direct quantitation of phosphatidylcholine molecular species by reverse phase high-performance liquid chromatography employing flame ionization detection. The method is shown to be applicable to plant phosphatidylcholine. The molecular species are separated with a C_{18} column eluted in an isocratic mode. Detection by a commercially available flame ionization detector overcomes the problems of detecting underivatized naturally occurring lipids using ultraviolet detectors, and allows direct and rapid mass determination of the resolved molecular species. Detection limits for quantitation are defined. —**Norman, H. A., and J. B. St. John.** Separation and quantitation of molecular species of plant phosphatidylcholine by high-performance liquid chromatography with flame ionization detection. *J. Lipid Res.* 1986. **27:** 1104–1107.

Supplementary key words plant phospholipids

Previously reported separations of phosphatidylcholine (PC) molecular species by high-performance liquid chromatography (HPLC) have employed ultraviolet (UV) detection at short wavelengths (1–4). There are inherent problems in utilizing this method of detection of naturally occurring lipids. In particular, since the observed absorption of underivatized lipids at these wavelengths results primarily from the double bonds present in the fatty acid constituents, the more saturated molecular species present in naturally occurring lipid mixtures may not be detected even in the 200–205 nm region, while molecular species containing highly unsaturated fatty acids show a relatively strong absorbance. Thus direct quantitation is not possible.

Different applications of flame ionization detection (FID) have been described in HPLC analyses (5–7). Recent reports employed a commercially available mass detector utilizing a moving belt of quartz fibers and FID for the quantitative analysis of plant galactolipid (8), phosphatidylglycerol (PG) (8), and diacylglyceryltrimethylhomoserine (9) molecular species resolved by HPLC. In this report we describe the application of this FID with reverse phase HPLC for the determination of plant PC molecular species.

MATERIALS AND METHODS

Materials

PC standards were obtained in 99% purity from Sigma Chemical Company (St. Louis, MO). HPLC grade solvents were obtained from Fisher Scientific (Medford, MA). Spinach (*Spinacea oleracea*) was purchased locally. *Arabidopsis thaliana* (L.) Heynh plants were grown in previously defined conditions (10). The leaves were pulse-labeled for 1 hr with [^{14}C]oleic acid (sp act 52.6 mCi/mmol; New England Nuclear, Boston, MA) (10).

Lipid analysis

PC was recovered from spinach and *Arabidopsis* leaf lipid extracts by chromatographic procedures (10). TLC was performed on silica gel H with chloroform–acetic acid–methanol–water 75:25:5:2.2 (v/v/v/v). PC standards (Sigma Chemical Company) were also chromatographed in this system. Solvent vapors were allowed to dissipate, and PC (detected by brief exposure of the plate to iodine vapor) was eluted from the silica gel using chloroform–methanol–water 3:5:1 (v/v/v). The lipid extracts were then adjusted to give proportions of chloroform–methanol–water 2:3.5:4 (v/v/v), and the lower phase was dried under nitrogen.

HPLC analyses were conducted with a Waters Associates (Milford, MA), Model 6000A solvent delivery system equipped with a Model U6K universal injector. The chromatographic column was a 25 cm × 4.6 mm Rainin Microsorb (5 μm) reverse-phase column. A Tracor 945 Flame Ionization LC Detector (Tracor Instruments, Austin, TX) operated at a block temperature of 160°C was used for detection. The HPLC solvent was acetonitrile–methanol–acetic acid–water–1-ethylpropylamine 89.8:6.8:1.5:1.0:0.9 (v/v/v/v/v) delivered at a flow rate of 1.1 ml/min without eluate splitting. The pump flow rate was increased to 1.9 ml/min in analyses where 45% of the column eluate was split away for collection. For compositional analyses, the individual molecular species collected were dried under nitrogen and fatty acid methyl esters

Abbreviations: PC, phosphatidylcholine; HPLC, high-performance liquid chromatography; UV, ultraviolet; FID, flame ionization detector; PG, phosphatidylglycerol; TLC, thin-layer chromatography; GLC, gas–liquid chromatography. In the shorthand numbering system used to identify fatty acids, the number preceding the colon represents the number of carbon atoms and that following the colon indicates the number of double bonds present.

[1] Mention of companies or commercial products does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.

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were prepared with boron trifluoride–methanol (Sigma Chemical Company, St. Louis, MO) (11). The methyl esters were identified with a Hewlett Packard (Model 5880A) gas chromatograph using the procedures of Lynch and Thompson (12). The positional distribution of fatty acids was determined by hydrolyzing the eluted lipids with phospholipase A_2 from *Crotalus adamanteus* venom (Boehringer, Mannheim) (10). Lipid phosphorus was determined using the methods of Bartlett (13) as modified by Marinetti (14). Radioactivity of eluted lipids, after evaporation of solvents under nitrogen, was determined using a Packard Tri-Carb. (Model 2925) liquid scintillation counter.

RESULTS

The reverse phase HPLC separation and FID detection of spinach leaf PC molecular species utilizing acetonitrile–methanol–acetic acid–water–1-ethylpropylamine 89.8:6.8:1.5:1.0:0.9 (v/v/v/v/v) as the mobile phase is shown in **Fig. 1.** The fatty acid composition of the resolved peaks is recorded in **Table 1.** Initially, commercial PC standards were used to develop this optimal mobile phase by modification of the mobile phase used for separation of *Dunaliella* PG molecular species (8). In addition to identification by retention times, the identity of molecular species was confirmed by fatty acid analysis of individual components recovered after HPLC separation. In order to accomplish this, the column effluent was split prior to FID detection and an approximate 45% portion of each peak was collected (8). Stream splitting necessitated the pump flow rate to be increased from 1.1 ml/min to 1.9 ml/min in order to maintain the minimum flow rate to the FID required to provide uniform application of the mobile phase to the revolving belt. The retention times of different molecular species increased with the sum of carbon atoms in both fatty acyl chains and with a decrease in the number of double bonds. Under these conditions, 16:0/18:3 PC could not be separated from 18:2/18:2 PC, and 16:0/18:2 eluted with the 18:1/18:2 molecular species.

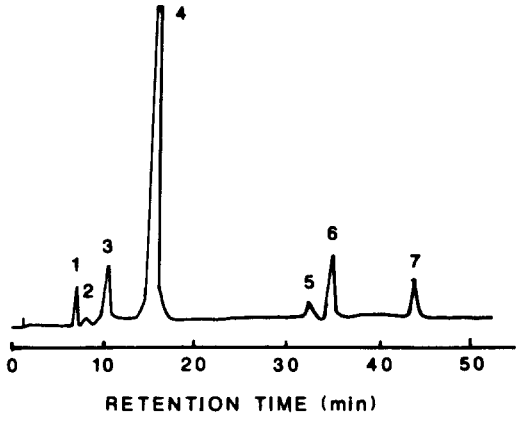

Fig. 1. HPLC separation of molecular species of spinach leaf PC. Detection was by FID. Peaks are numbered in sequence of elution and are identified in Table 1.

TABLE 1. Molecular species composition of PC from spinach leaf

Peak No.	Fatty Acid Composition[a]	% of Total Molecular Species[b]
1	18:3/18:3	3.5 ± 0.4
2	18:3/18:2	0.9 ± 0.2
3	18:2/18:3	7.9 ± 0.6
4	[18:2/18:2 16:0/18:3][c]	70.2 ± 2.3
5	18:1/18:3	2.9 ± 0.3
6	[18:1/18:2 16:0/18:2]	10.9 ± 1.0
7	18:1/18:1	3.7 ± 0.4

[a] The fatty acids separated by a slash represent the components in the *sn*-1 and *sn*-2 positions, respectively, of the molecular species.
[b] Values are expressed as mean percent of total sample weight ± SEM of four analyses. Molecular species were quantified by integration of peaks detected by FID/HPLC.
[c] Brackets signify that the included molecular species were not fully resolved.

The FID used here responds on the basis of mass (8) and thus relative amounts of the component PC molecular species could be directly determined by integration of the peaks detected. Positional distribution of fatty acids was determined by hydrolysis of eluted lipids with phospholipase A_2 from *Crotalus adamanteus* venom (10). To confirm the accuracy of the FID/HPLC quantitation, the fatty acid distribution was calculated from the % distribution of molecular species shown in Table 1. (All of the peaks in which a particular fatty acid was present were totalled and then divided by 2.) By GLC analysis it was determined that 18:2/18:2 PC (unresolved from 16:0/18:3 PC) contributed about 16% of the total sample weight, and 16:0/18:3 about 54%. Peak 6 was composed of approximately equal proportions of 16:0/18:2 and 18:1/18:2 PC. The overall fatty acid composition calculated was compared with the fatty acid analysis of an aliquot of total PC determined directly by GLC. The agreement between the calculated and directly determined values was good (**Table 2**). The major component of spinach PC was the 16:0/18:3 species. 18:2/18:3 PC contributed about 8% of the total weight. This was clearly separated from the 18:3/18:2 species which was a minor component (Table 1).

The lower detection limits for quantitation of PG and plant galactolipid molecular species with the Tracor 945

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TABLE 2. Comparison of the fatty acid composition of PC as determined by direct GLC analysis and after FID/HPLC

Fatty Acid	% of Total PC[a]	
	GLC Analysis[b]	HPLC Analysis[c]
16:0	27.7 ± 1.5	29.8 ± 2.0
18:1	6.1 ± 0.9	7.9 ± 1.0
18:2	28.4 ± 2.1	25.8 ± 1.5
18:3	37.8 ± 1.9	36.5 ± 3.0

[a] Values are expressed as mean percent ± SEM of four analyses.
[b] Fatty acid distribution determined by GLC of an aliquot of total PC before HPLC.
[c] Fatty acid distribution calculated from the % distribution of PC molecular species (Table 1).

FID used here have been defined as approximately 1.2–1.4 nmol (8). Reproducible quantitation of component molecular species of spinach leaf PC was obtained when samples of 0.1–3 μmol of total lipid were injected. The minimum level of 0.1 μmol indicated that the quantitation limit of minor molecular species contributing only about 1% of total PC mass (Table 1) was approximately 1.0 nmol. It was confirmed that the relative peak area occupied by 0.1–3 μmol of a 16:0/18:2 PC standard (Sigma Chemical Co.) was proportional to the total mass of lipid injected.

The recovery of lipids in the column eluate was determined using a PC sample containing [^{14}C]-labeled 18-carbon fatty acids extracted from leaves of *Arabidopsis thaliana* pulse-labeled with [^{14}C]oleic acid. Rapid desaturation of this substrate leads to the accumulation of ^{14}C-labeled 18:1, 18:2, and 18:3 in PC (10). Determination of the radioactivity in 1.2-ml samples of total column eluate collected continuously between 3 and 50 min after injection showed the recovery to be 93–95%.

DISCUSSION

In previous reports, UV has been predominantly used in analyses of underivatized phospholipid molecular species by HPLC (1–4). Inasmuch as UV absorption results primarily from double bonds present in the fatty acid moieties, this method presents problems with both detection and direct quantitation. The separated fractions detected by UV can be collected and component fatty acids can be analyzed by GLC after the addition of an internal standard in order to achieve quantitative estimation (4). Alternatively, phospholipids can be hydrolyzed with phospholipase C and the resultant diacylglycerols can be converted to UV-absorbing derivatives for HPLC quantitation (15, 16).

The HPLC method described here for the analysis of underivatized molecular species of PC is an improvement over alternative procedures described previously because the use of FID permits direct mass determinations of the molecular species resolved. The successful application of the revolving disc conveyor FID for quantitation of the molecular species of other plant lipid classes has been recently reported (8, 9), and the mobile phase developed for analysis of PC is a modification of that utilized for resolution of PG molecular species (8). Preliminary findings show that the same mobile phase can be used to separate the molecular species of naturally occurring phosphatidylethanolamine (Norman, H. A., and J. B. St. John, unpublished data). The lower detection limits for quantitation were found to be approximately 1.0 nmol for a single molecular species, and recoveries were 93–95%. The method should be applicable for analyzing PC composition of plant membrane preparations in studies of the metabolism of this major lipid component.

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