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ISOLATION AND QUANTITATIVE ANALYSIS OF PHOSPHATIDYLGLYCEROL AND GLYCOLIPID MOLECULAR SPECIES USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLAME IONIZATION DETECTION

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SUMMARY

Conditions are described for the quantitative analysis of phosphatidylglycerol and plant glycolipid molecular species by reversed-phase high-performance liquid chromatography employing a commercially available flame ionization detector. Direct detection on a mass basis overcomes the problem of poor detectability found with most natural lipids. Effective mobile phases composed primarily of volatile solvents are described. Splitting of the column eluate stream allows a portion of each individual molecular species to be recovered for other types of analysis.

INTRODUCTION

Although the potential of high-performance liquid chromatography (HPLC) for resolving complex mixtures of biological compounds is abundantly documented, application of HPLC towards lipids has been hindered due to the poor detectability of these compounds in column eluates. Of those HPLC analyses of underivatized lipids that have been reported, UV detection in the 200–205-nm range was typically the method of choice[1–4]. This type of detection has severe limitations. First, UV-absorbing mobile phases may not be used. Second, even in the 200-205-nm region, only lipids containing unsaturated alkyl chains or other less common absorbing groups can be detected. As most natural lipid classes are composed of a mixture of molecular species* differing in their degree of saturation, those lipid classes which include a large proportion of highly unsaturated molecular species would show a stronger absorbance than those which do not. For this reason, quantification of natural lipid mixtures using UV detection is impractical.

The determination of molecular species of individual phospholipid classes raises special problems as the more saturated molecular species may not be detected by UV at all. To circumvent this problem, the few successful HPLC quantitative

* Molecular species within a certain lipid class are identical except for having different combinations of fatty acids bound at the *sn*-1 and *sn*-2 positions of their glycerol moiety.

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analyses reported have involved conversion of lipids under study to colored derivatives for improved detectability[2]. A different type of detection system, therefore, is clearly needed if the potential resolving power of HPLC for lipid analyses is to be fully utilized.

Fortunately, there have been several successful attempts at designing a more universal detector. One such success, described by Charlesworth, is a mass detector based on light scattering detection[5], and several reports applying this detector to lipid separations show great promise for its future use[6–8]. An alternative type of mass detection features deposition of the column eluate on a moving wire or belt which, after evaporation of the mobile phase, is passed on for mass spectrometry or flame ionization detection (FID)[9]. The FID response has been shown to be proportional to the mass of the compounds eluted[10]. A slightly modified system utilizing a moving belt of woven quartz fibers has also been described[11]. In this system, the column eluate is applied to the circular quartz belt as a fine jet stream. The elevated temperature of the belt housing removes the solvent, leaving the sample solute behind to pass through the detector flame. The belt than passes through a hotter oxygen–hydrogen flame for cleaning prior to application of additional column eluate. Recently, this FID system has become commercially available at a cost comparable with that of other HPLC detectors.

In this report, the application of the revolving belt flame ionization detector towards analyzing the molecular species composition of membrane lipids is examined. Detection limits and the amount of sample needed to conduct a typical analysis are presented, and the ability to utilize eluate splitting techniques for sample collection and/or independent analysis, the limitations imposed by the use of this detector, and its potential for use in lipid analysis are discussed.

EXPERIMENTAL

Isolation of phosphatidylglycerol and glycolipid classes

Phosphatidylglycerol (PG), digalactosyldiglyceride (DGDG), and monogalactosyldiglyceride (MGDG) were isolated from *Dunaliella salina* chloroplast lipid extracts by silicic acid column chromatography and thin-layer chromatography (TLC) procedures previously described[12]. TLC was performed on silica gel H using chloroform–acetic acid– methanol–water (75:25:5:2.2, v/v/v/v) as the developing solvent. After dissipation of solvent vapors under nitrogen, the resolved components were eluted from the silica gel using chloroform–methanol–water (3:5:1, v/v/v). MGDG and DGDG extracts were adjusted to give proportions of chloroform–methanol–0.1 *M* potassium chloride (2:3.5:4). The lower phase was then dried under a stream of nitrogen. PG extracts were purified in the same way but replacing potassium chloride with water in the wash. Sugar analyses were conducted using the method of Dubois *et al.*[13] and lipid phosphorus was determined using the method of Bartlett[14] as modified by Marinetti[15].

HPLC analysis

HPLC analyses were conducted utilizing a BAS Model PM 30-A dual piston pump, a Rheodyne Model 7125 syringe-loading sample injector with a 20-μl loop, and a 25 cm × 4.6 mm I.D. Rainin Microsorb (5 μm) reversed-phase column. A

0.5-μm pore size prefilter was placed between the injector and the column. Molecular species were detected by a Tracor 945 flame ionization LC detector (Tracor Instruments, Austin, TX, U.S.A.) at a block temperature of 160°C, and elution profiles were recorded and integrated by an HP 3390A integrator (Hewlett-Packard, Avondale, PA, U.S.A.). HPLC grade methanol, acetonitrile, water (Fisher Scientific), 1-ethylpropylamine and acetic acid (Aldrich) were utilized in mobile phases. A Milton Roy spectromonitor D variable-wavelength UV detector (Milton Roy, Riviera Beach, FL, U.S.A.) was also employed at a wavelength of 205 nm where stated for certain HPLC applications. In analyses where 60% of the column eluate was split away for collecton, a pump flow-rate of 1.9 ml/min was utilized to maintain a solvent delivery of 0.76 ml/min to the revolving quartz belt. A pump flow-rate of 0.8 ml/min was used for analyses without eluate splitting.

Gas chromatographic analysis

Gas chromatographic (GC) analyses were conducted on a Varian 3700 gas chromatograph equipped with an SP 2330 capillary column (10 m × 0.25 mm I.D.) (Sulpelco, Bellefonte, PA, U.S.A.) and a flame ionization detector. Integration of GC peaks was performed utilizing a CSI-204 integrator (Columbia Scientific, Austin, TX, U.S.A.). Molecular species resolved by HPLC were concentrated under nitrogen, and fatty acid methyl esters of these lipids were prepared directly with boron trifluoride–methanol (Sigma, St. Louis, MO, U.S.A.)[16]. These were analyzed isothermally at 170°C. The analysis of PG molecular species was conducted on the trimethylsilyl (TMS) derivatives of diacylglycerols prepared by phospholipase C hydrolysis according to Lynch and Thompson[17].

RESULTS

HPLC with FID

A primary requirement of the revolving belt flame ionization detector is evaporative removal of mobile phase from the belt without loss of the sample solute. Although this requirement would suggest that the detector would be more suitable in normal-phase applications where organic mobile phases are employed, reversed-phase applications can be obtained for lipids if methanol, acetonitrile, and other relatively volatile solvents are the primary constituents in the mobile phase. Evaporation must be accomplished within the 8 sec time span required for the belt to transport eluate from its application point to the detecting flame, and even though the temperature of the belt during this transport can be adjusted between 80 and 210°C, we have found that no mobile phase components should have boiling points greater than 100°C. Furthermore, satisfactory baselines can be obtained only if components with boiling points close to 100°C are present in concentrations that are less than 20% of the total volume. This mobile phase volatility requirement, then, is restricting in that a number of buffers, ion pairing agents, or salts normally used in reversed-phase applications cannot be used with this detector. For example, we have found that 30 m*M* choline chloride and potassium phosphate buffer, both of which have been previously employed in HPLC separations of lipids[3,18] are not suitable for use with the flame ionization detector because they give excessively high background noise.

Another problem encountered with FID is the inadequate electronic noise filtering capability for use with strip chart recorders. High and low filter settings were not sufficient to reduce sharp baseline spikes observed at low attenuation settings when mobile phases for molecular species were employed. This was overcome, however, by utilizing the HP 3390-A integrator at a peak width setting of 0.64 min, which enabled sufficient filtering for relatively flat baselines. There was no observable loss in resolution due to this high filtering or use of FID when separation profiles were compared with profiles of the same lipid mixture obtained using UV detection and no integration (Fig. 1). This comparison also illustrates the major advantage of FID over UV detection, namely the measurement of individual components on a mass basis for quantification. Thus, peaks 4 and 8, which contained approximately the same masses of glycolipids (Table I), displayed this faithfully in the FID profile (Fig. 1A) but not in the UV profile (Fig. 1B) because peak 8 contained relatively saturated lipids. We have previously employed UV detection in conjunction with a quantitative analysis of these same *Dunaliella* glycolipids, but in that study, quantification was achieved through the time consuming GC analysis of fatty acid methyl esters prepared from each eluted peak after the addition of an internal standard[18].

Eluate stream splitting

The development of HPLC separations is ideally accomplished through the

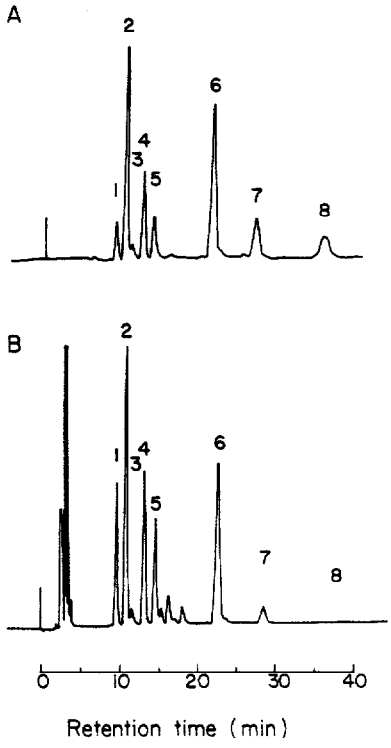

Fig. 1. Reversed-phase HPLC separation of DGDG molecular species utilizing methanol–water (96:4; v/v) as mobile phase. The molecular species resolved were detected by FID (A) and UV (205 nm) (B). Peak identification is given in Table I.

TABLE I

MOLECULAR SPECIES COMPOSITION OF DGDG FROM *DUNALIELLA SALINA*

Peak number	*Peak retention time (min)*	*Fatty acid* composition*	*Percentage of total DGDG molecular species*
1	8.8	18:3/16:4**	5.7 ± 0.4
2	9.8	18:3/16:3 (isomer***)	21.7 ± 0.3
3	11.0	18:3/16:3	1.1 ± 0.2
4	12.5	18:3/16:2; 18:2/16:3 (isomer)	12.9 ± 0.7
5	13.9	18:3/18:3	8.3 ± 0.5
6	21.2	18:3/16:0	28.9 + 1.1
7	27.2	18:2/16:0	11.1 ± 0.2
8	35.5	18:1/16:0	10.3 ± 0.2

* 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.

** The fatty acids separated by a slash represent the components in the *sn*-1 and *sn*-2 positions, respectively, of the molecular species.

*** Believed to be a different isomer from the other 16:3 shown.

§ Trace.

use of pure standards to discern elution order and resolution. The commercial availability of naturally occurring molecular species standards within any given class of lipids, however, is very limited. It will normally be necessary, therefore, to split away a portion of the column effluent prior to flame ionization in order to identify the eluted compounds and evaluate the degree of resolution. A splitting capability also allows the collection of specified portions of each peak for radioactivity analysis or other uses.

In order to accomplish stream splitting prior to FID, we found it necessary to increase the pump flow-rate from the standard to 1.0 ml/min. For example, if only 40% of the column eluate was directed to the detector, and the remaining 60% was split away for collection, then the pump flow-rate had to be increased to 1.9 ml/min in order to maintain the minimum flow-rate to the FID system of 0.7 ml/min required to provide a jet stream of mobile phase for uniform application to the revolving belt. Development of the splitting technique also required delaying the eluate diverted to the fraction collector from emerging into the collection tube until shortly after the equivalent portion directed to the FID system had been dried and passed through the detecting flame. The delay could be varied by changing the length or internal diameter of the collection tubing and measured by connecting a UV detector to its outlet. Once the proper delay was obtained, UV-absorbing glycolipids were injected to evaluate potential band spreading of that portion of eluate diverted through the collection tubing. Satisfactory delays without any detectable band spreading were indicated when a comparison of the FID and UV detector elution profiles showed no differences in peak resolution (data not shown).

HPLC–FID analysis of glycolipid molecular species

The reversed-phase separation and FID of molecular species of chloroplast DGDG from *Dunaliella salina* utilizing methanol–water (96:4; v/v) as the mobile

phase is shown in Fig. 1. The molecular species were identified by stream splitting and collection of each eluted peak followed by a fatty acid GC analysis. These results showed that almost all peaks consisted of one molecular species (Table I) and that this chloroplast lipid was a complex mixture of at least nine molecular species. Approximately 29% of DGDG consisted of the 18:3/16:0 molecular species while the next most abundant component (21.7%) was the 18:3/16:3 (isomer) molecular species. The only peak found to contain more than one molecular species in the chloroplast DGDG analysis was the fourth eluting peak, composed of a combination of the 18:3/16:2 and 18:2/16:3 (isomer) molecular species. The relative amounts (%, w/w) of the major fatty acids of DGDG [16:0 (29%), 16:3 isomer (12%), 18:2 (9%) and 18:3 (39%)] as determined by GC analysis of fatty acid methyl esters[12] were in good agreement with total levels calculated from the weights of the separated molecular species [*i.e.* 16:0 (25%), 16:3 isomer (12%), 18:2 (8%) and 18:3 (40%)]. Levels of minor fatty acid components also correspond well. This confirmed the accuracy of quantitation by HPLC–FID analysis.

Analysis of MGDG molecular species could be accomplished with the same mobile phase. Peak integration during this separation showed that of the four peaks detected by FID, approximately 87% of the total sample was the 18:3/16:4 MGDG molecular species while approximately 10% consisted of a combination of 18:3/16:3 and 18:2/16:4 molecular species (data not shown).

The elution of the individual molecular species of both glycolipids followed the order reported for HPLC separations of other complex lipid molecular species[2,19]. That is, elution time increased with both fatty acyl carbon chain length and the degree of unsaturation.

The relative amounts of each molecular species present in the two glycolipid classes, as determined by FID, was substantiated by total fatty acid analysis of MGDG and DGDG (data not shown) and previous results reported by this lab for these particular *Dunaliella* glycolipids separated by HPLC using a mobile phase containing non-volatile components[18]. Injection of glycolipids at various concentrations showed that reproducible quantitation of the resolved molecular species could be accomplished using a minimum of 75 nmol of chloroplast DGDG of MGDG. Based on this amount, the quantitation limit of minor species contributing only 1–2% of total glycolipid mass (Table I) was approximately 1.2–1.4 nmol. Routine analyses, however, were usually conducted on 0.3–1.4 μmol of each glycolipid class. This working range insured that enough material could be collected by stream splitting for radioactivity counting or GC analysis, while accurate detection could be accomplished on the remaining eluate going to the FID.

HPLC–FID analysis of PG

The reversed-phase separation of *Dunaliella salina* chloroplast PG molecular species was achieved with a mobile phase consisting of 1-ethylpropylamine–acetic acid–methanol–acetonitrile (0.3:0.5:34.7:64.5, v/v/v/v) (Fig. 2). By employing stream splitting and collecting 60% of each peak prior to FID, it was possible to show by a GC analysis of the fatty acid methyl esters that the first and largest peak consisted of 18:3 and t16:1 (*trans*-Δ-3-hexadecenoic acid) in equimolar amounts. The second peak consisted of 18:2 and t16:1 fatty acids, while the third peak contained a mixture of 18:2 isomers paired with 16:0.

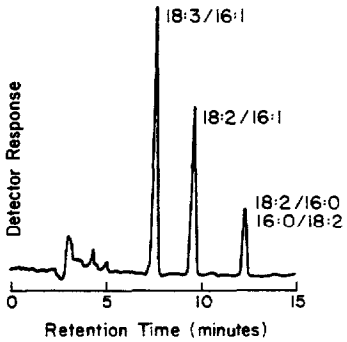

Fig. 2. Reversed-phase HPLC separation of PG molecular species utilizing 1-ethylpropylamine–acetic acid–methanol–acetonitrile (0.3:0.5:34.7:64.5, v/v/v/v) as mobile phase.

When 1-ethylpropylamine was eliminated from the mobile phase, none of the PG molecular species was eluted from the column. While the observed decrease in the column retention properties (capacity factors) after the addition of the 1-ethylpropylamine was clearly due to increased partitioning of PGs into the mobile phase relative to the C_{18} stationary phase, we could not definitely attribute this change to a specific effect such as ion-pairing and sample–C_{18} interactions. The substitution of shorter chain alkylamines, such as triethylamine, did not result in reduced elution times, while the utilization of longer chain amines (*e.g.* hexylamine, octylamine) was precluded since the high boiling point of these compounds resulted in incomplete evaporation from the revolving FID belt. Moreover, studies on separations of neutralized PG species could not be accomplished as the pK of the ionizable lipid phosphate group (pK_a 1–2) is too low to be within a safe pH working range for these columns. Due to these difficulties, no further investigation on this interaction was conducted.

The ability to quantitate molecular species accurately by HPLC–FID detection

TABLE II

MOLECULAR SPECIES COMPOSITION OF PG FROM *DUNALIELLA SALINA* AS DETERMINED BY GC AND BY HPLC

*PG molecular species**	*Percentage of total PG*			
	GC	*HPLC*		
		Total PG injected (nmol)		
		50	*31*	*13*
18:3/t16:1	62.2 ± 1.1	63.4 ± 0.5	63.3 ± 0.9	63.5 ± 1.0
18:2/t16:1	28.2 ± 0.6	26.3 ± 0.2	26.8 ± 0.7	26.5 ± 0.8
18:2/16:0; 16:0/18:2**	9.6 ± 1.2	10.0 ± 0.8	9.9 ± 0.6	10.0 ± 0.6

* The fatty acids separated by a slash represent the components in the *sn*-1 and *sn*-2 positions, respectively, of the molecular species.

** The reverse isomers 18:2/16:0 and 16:0/18:2 are not separated in these conditions. The eluted HPLC peak was a combination of $18{:}2^{\Delta 9,12}$/16:0, 16:0/$18{:}2^{\Delta 6,9}$, and 16:0/$18{:}2^{\Delta 9,12}$.

was investigated in detail using the chloroplast PGs. Relative amounts of component molecular species were determined directly by integration of peaks during HPLC–FID and independently by GC analysis of the TMS derivatives of PG-derived diacylglycerols[17] using the same lipid preparation. Comparison of these results (Table II) indicates that accurate quantitation can be obtained utilizing HPLC–FID. The direct HPLC method offers a major advantage over previous GC analyses in that the lengthy preparation of volatile derivatives is avoided, hence reducing the risk of selective losses due, for example, to oxidation of polyunsaturated fatty acids or incomplete hydrolysis by phospholipase C. Quantitation could be accomplished on a minimum of 13 nmol of total chloroplast PG, indicating that the quantitation limit for one molecular species is approximately 1.3 nmol. Routine analyses, however, were conducted on 30–50 nmol of PG. As shown in Table II, relative peak areas remained essentially the same over this working range.

DISCUSSION

Results of these studies have shown that the revolving belt flame ionization detector can be utilized effectively for the detection of various lipid molecular species separated by HPLC. Quantitative information on the relative amounts of the resolved molecular species present within a lipid class can be readily obtained by direct peak integration without the necessity of first computing response factors for each species. The lower detection limits for quantitation by HPLC–FID were found to be approximately 1.2–1.4 nmol DGDG, enough sample must be injected so that the smallest molecular species-contained peak to be quantitated is present in at least this amount. It was also determined that peak ratios remain the same over a wide range of concentrations (at least one order of magnitude) of glycolipid injected, illustrating that FID can be used effectively within this range.

PG, MGDG, and DGDG are major plant lipids of higher and lower plant chloroplasts[20], and the elucidation of their metabolism, as well as those of additional complex lipid classes, is a primary interest in this laboratory. Preliminary studies have already indicated that a simple modification of the mobile phase employed for the separation of a PG molecular species will permit the separation of phosphatidylcholine and phosphatidylethanolamine molecular species. It should be possible to develop completely volatile mobile phases effective for HPLC–FID separation of most biologically important lipids.

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REFERENCES

1 Y. Nakagawa and L. A. Horrocks, *J. Lipid Res.*, 24 (1983) 1268–1275.
2 M. Batley, N. H. Packer and J. W. Redmond, *Biochim. Biophys. Acta*, 710 (1982) 400–405.
3 G. M. Patton, J. M. Fasulo and S. J. Robins, *J. Lipid Res.*, 23 (1982) 190–196.

4 F. B. Jungalwala, J. E. Evans and R. H. McCluer, *Biochem. J.*, 155 (1976) 55–60.
5 J. M. Charlesworth, *Anal. Chem.*, 50 (1978) 1414–1420.
6 A. Stolyhwo, H. Colin, M. Martin and G. Guiochon, *J. Chromatogr.*, 288 (1984) 253–275.
7 A. Stolyhwo, H. Colin and G. Guiochon, *J. Chromatogr.*, 265 (1983) 1–18.
8 R. Macrae, L. C. Trugo and J. Dick, *Chromatographia*, 15 (1982) 476–478.
9 O. S. Privett and W. L. Erdahl, *Methods Enzymol.*, 72 (1981) 56–108.
10 F. C. Phillips, W. L. Erdahl, J. A. Schmit and O. S. Privett, *Lipids*, 19 (1984) 880–887.
11 J. J. Szakasits and R. E. Robinson, *Anal. Chem.*, 46 (12) (1974) 1648–1653.
12 D. V. Lynch and G. A. Thompson, Jr., *Plant Physiol.*, 69 (1982) 1369–1375.
13 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, 28 (1956) 50–56.
14 G. R. Bartlett, *J. Biol. Chem.*, 234 (1959) 446–468.
15 J. Marinetti, *J. Lipid Res.*, 3 (1962) 1–20.
16 W. R. Morrison and L. M. Smith, *J. Lipid Res.*, 5 (1964) 600–608.
17 D. V. Lynch and G. A. Thompson, Jr., *Plant Physiol.*, 74 (1984) 193–197.
18 D. V. Lynch, R. E. Gundersen and G. A. Thompson, Jr., *Plant Physiol.*, 72 (1983) 903–905.
19 F. B. Jungalwala, V. Hayssen, J. M. Pasquini and R. H. McCluer, *J. Lipid Res.*, 20 (1979) 579–587.
20 J. L. Harwood, in P. K. Stumpf (Editor), *The Biochemistry of Plants, Vol. 4, Lipids: Structure and Function*, Academic Press, New York, 1980, pp. 1–55.