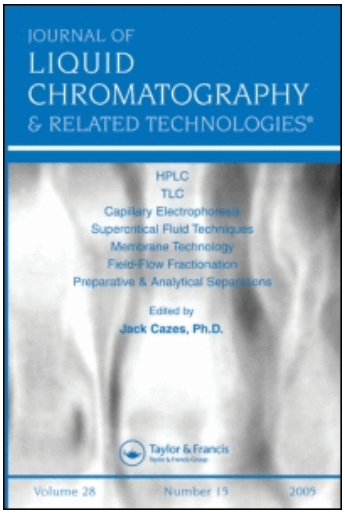

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ANALYSIS OF MOLECULAR SPECIES OF CHLOROPLAST LIPIDS BY HPLC

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ABSTRACT

An improved procedure for the quantitative analysis of fatty acid molecular species of chloroplast thylakoid lipids by HPLC of the p-methoxybenzoyl derivatives of diacylglycerols is described. Data are presented on the chromatographic properties of twenty three commonly occurring molecular species.

INTRODUCTION

The thylakoid membranes of plants and algae are characterised by the presence of four major polar lipids, monogalactosyldiacylglycerol (MGDG), digalactosyldiacyglycerol (DGDG), sulpho-

* Deceased September 14th 1986.

quinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG). A knowledge of the fatty acid molecular species in these lipids is important for understanding their biosynthetic pathway, since the diacylglycerol (DAG) moiety of MGDG, DGDG and SQDG may be synthesised either in the cytoplasm or in the chloroplast and the relative contributions of each pathway vary widely among plant species (1). However, PG appears to be synthesised entirely within the chloroplast in higher plants (1) and its proposed role as a factor in determining chilling sensitivity (2,3,4) is dependent on the presence of specific molecular species.

Detection methods for the analysis of molecular species of chloroplast lipids have depended either on the measurement of UV absorption at about 205nm (5,6,7) or on the preparation of DAG derivatives such as p-methoxybenzoates (5). The former method suffers from a lack of sensitivity and from variations in detector response according to the degree of fatty acid unsaturation. In particular, it fails to detect molecular species containing two saturated fatty acids (5). The latter technique produces an increase in sensitivity and allows a direct quantitative analysis, since the absorption of the aromatic group is measured at about 250nm.

This paper presents data on the analysis of the p-methoxybenzoyl derivatives of DAGs derived from chloroplast lipids using a reversed phase column and a simple isocratic solvent system, which permits excellent separation of molecular species.

MATERIALS AND METHODS

MGDG, DGDG, SQDG and PG were isolated by DEAE-Sepharose chromatography and preparative TLC (2) of lipid extracts from

leaves of silver beet (Beta vulgaris), potato (Solanum tuberosum), passionfruit (Passiflora edulis forma flavicarpa) and banana (Musa sapientum) and from the cyanobacteria, Anacystis nidulans and Synechococcus sp., grown as previously described (8,9,10). DAGs were prepared from purified glycolipids by treatment with periodic acid and 1,1-dimethylhydrazine (11). DAGs were prepared from PG by hydrolysis with phospholipase C. The PG (0.2-2mg) was freed of solvent under a stream of N_2 and the lipid dispersed in 1 ml of 50mM HEPES, pH 7.5, by vortexing for 60 sec at a temperature about ten Centigrade degrees above the phase separation temperature of the lipid. Phospholipase C (12 units, Boehringer), 1M $CaCl_2$ (10 µl) and diethylether (2 ml) were added sequentially and the mixture incubated at 37°C for 2h with occasional shaking. The ether layer was removed and the aqueous fraction re-extracted with a further 2 ml of ether. The combined extracts were evaporated under a stream of N_2. Control experiments showed that a 2 h incubation was sufficient to hydrolyse all the PG present in the sample and that the released DAG had a fatty acid composition identical to that of the PG from which it was derived.

DAGs were purified by TLC and converted to their p-methoxybenzoyl derivatives (5). The derivatives were purified by TLC (5) and after extraction with ether were dissolved in acetonitrile.

Analyses of the p-methoxybenzoyl derivatives of DAGs were performed by HPLC at 24°C in a Waters high performance liquid chromatograph using a model 590 pump and model 490 programmable multiwavelength detector, set at 254 nm. The column was a 150 x 3.9 mm Resolve 5 µm C_{18} reverse-phase (Waters Associates). The solvent used was isopropanol:acetonitrile, 35:65 (12,13) and the flow rate was 0.5 ml/min.

RESULTS AND DISCUSSION

The analyses of the p-methoxybenzoyl derivatives of DAGs prepared from PG and SQDG of P.flavicarpa are shown in Fig. 1. Excellent separation of the major molecular species is obtained under the analytical conditions described and the analysis is complete in less than 20 minutes. PG from leaves of higher plants is characterised by its content of trans-3,-hexadecenoic acid (trans-3,16:1) which only occurs at the sn-2 position and it is possible to resolve molecular species containing this acid from those analogues which contain palmitic acid (16:0), or cis-hexadecenoic acid (cis, 16:1). The technique thus permits accurate estimation of the molecular species 16:0/16:0, 16:0/trans-3,16:1 and 18:0/16:0 which have been proposed to play a major role in determining the relative chilling sensitivity of higher plants (2,3,4).

The relative retention volumes of twenty three molecular species of DAG, together with their Effective Average Chain Length (EACL) (12-13) calculated from the formula:

$$\log (\text{rel RT}) = 0.096\ (\text{EACL}) - 1.54$$

are presented in Table 1. Rel RT is the retention time relative to that of 1,2-dihexadecanoyl-p-methoxybenzoate. The various standard molecular species were obtained from individual chloroplast lipids isolated from specific plants.

The designation of molecular species shows the fatty acid occupying the sn-1 position of the molecule first, and that occupying the sn-2 position second. Such configurations have been determined by positional analysis of the purified lipids using phospholipase A_2 or Rhizopus lipase (ref. 14 and Bishop et al., unpublished results). Those molecular species in which the positional distribution has not been unequivocally determined are marked with an *. All double bonds are of cis configuration, except for those marked^t, which indicates a

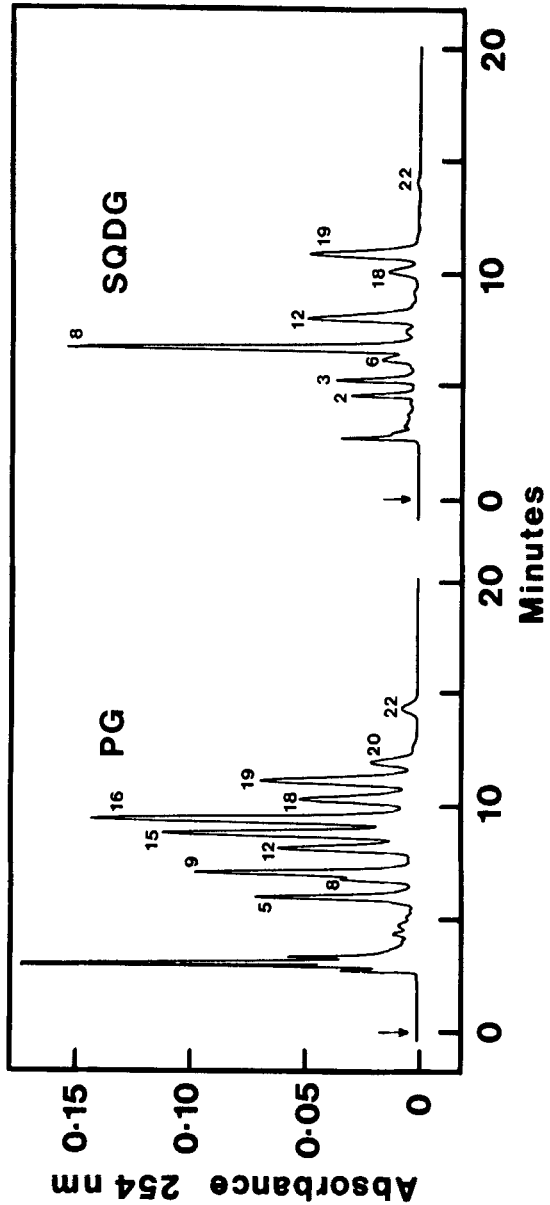

FIGURE 1. Separation of molecular species of p-methoxybenzoyl derivatives of DAGs prepared from PG and SQDG of P. edulis forma flavicarpa. The experimental conditions are described in the Materials and Methods section and the individual peaks are identified in Table 1.

TABLE 1

Relative Retention Times and EACL Values for p-methoxybenzoyl Derivatives of 1,2-Diacylglycerols Prepared from Thylakoid Lipids.

Peak No.	Molecular Species	Rel RT	EACL
1	18:3/16:3	0.36	11.43
2	18:3/18:3	0.40	11.88
3	18:2/18:3*	0.47	12.63
4	18:3/16:1	0.47	12.63
5	18:3/16:1^{t}	0.52	13.08
6	18:2/18:2	0.55	13.33
7	18:2/16:1	0.55	13.33
8	18:3/16:0	0.60	13.73
9	18:2/16:1^{t}	0.61	13.80
10	14:0/14:0	0.63	13.96
11	18:1/16:1	0.69	14.36
12	18:2/16:0	0.71	14.50
13	18:2/18:1*	0.73	14.61
14	16:1/16:0	0.75	14.74
15	18:1/16:1^{t}	0.77	14.85
16	16:0/16:1^{t}	0.83	15.20
17	18:1/18:1	0.87	15.42
18	18:1/16:0	0.93	15.71
19	16:0/16:0	1.00	16.00
20	18:0/16:1^{t}	1.08	16.39
21	18:0/18:1*	1.19	16.83
22	18:0/16:0	1.30	17.23
23	18:0/18:0	1.55	18.02

trans-3 double bond. The terms Rel RT and EACL are defined in the text.

For example, the molecular species 18:3/16:3 is a major component of the MGDG from leaves of potato (9), while 16:1/16:0 is the predominant molecular species in all four lipids of the cyanobacterium, Anacystis nidulans (14). Calculation of the

EACL allows a direct comparison of the effect on the chromatographic properties of specific molecular differences, such as chain length, or the number and configuration of double bonds. For example, the presence of the trans-3 double bond in trans-3,16:1 decreases the EACL by 0.7 to 0.9 units as compared to 16:0, whereas a cis double bond near the centre of the chain decreases the EACL by 1.3 to 1.5 units. At higher levels of unsaturation, the presence of 6 double bonds (e.g. in 18:3/16:3 and 18:3/18:3) reduces the EACL by about 6 units as compared to the fully saturated analogues. The analytical conditions do not discriminate between positional isomers of 1,2-DAGs. For example, the p-methoxybenzoyl-DAG derivatives of 18:3/16:0, 18:2/16:0 and 18:1/16:0 (from Synechococcus MGDG) have respectively the same Rel RT and EACL values as 16:0/18:3, 16:0/18:2 and 16:0/18:1 (from P. edulis forma flavicarpa SQDG).

The present analytical procedure, which is based on the solvent system developed by Batley et al. (12,13) for analyses of E. coli lipids, has a number of advantages over those previously proposed for the analyses of molecular species of chloroplast lipids, in that it uses only a simple isocratic solvent mixture, is more rapid, and provides improved separation. It is also capable of resolving the 1,3-isomers of DAGs from 1,2-isomers (data not shown). The use of the p-methoxybenzoyl derivatives allows accurate quantitative analyses of molecular species, based on the content of one aromatic residue per molecule (13), and permits the detection of molecular species containing two saturated fatty acids, which are not measurable by UV absorption around 200nm. Finally, the procedure is also applicable to analyses of molecular species of natural diacylglycerols or of any other phospholipid which is susceptible to hydrolysis by phospholipase C, such as phosphatidylcholine or phosphatidylethanolamine.

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