Semi-preparative isolation of plant sulfoquinovosyldiacylglycerols by solid phase extraction and HPLC procedures

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Abstract Techniques are described for the semi-preparative isolation of sulfoquinovosyldiacylglycerol from plant leaf tissue lipid extracts and the resolution and analysis of component molecular species. Sulfoquinovosyldiacylglycerol was resolved from phospholipids in a polar lipid fraction by isocratic normal phase HPLC with detection at 208 nm. The mobile phase was composed of heptane–isopropanol–0.001 M KCl 40:52:8 (v/v/v). Yields from spinach leaf lipid extracts were 1.8 mg · 10 g^{-1} fresh wt leaf tissue. Molecular species components of purified sulfoquinovosyldiacylglycerol were separated by reversed-phase C_{18} HPLC, and fatty acid positional distribution was defined.–**Norman, H. A., C. F. Mischke, B. Allen, and J. S. Vincent.** Semi-preparative isolation of plant sulfoquinovosyldiacylglycerols by solid phase extraction and HPLC procedures. *J. Lipid Res.* 1996. **37:** 1372–1376.

Supplementary key words plant sulfolipids

Sulfoquinovosyldiacylglycerol (SQDG), known trivially as plant sulfolipid, is a component of plant photosynthetic membranes. In contrast to the usual sulfate ester, as found in animal sulfatides, SQDG contains a sulfonic acid linkage. SQDG typically constitutes about 5% (wt %) of the total acyl lipid content of higher plant leaves (1). However, in certain algae SQDG is a major lipid component and levels of up to 29% of total lipids have been reported (1). This lipid tends to be more saturated than the major plant chloroplast galactolipids (GL), monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) (1). Various functions have been proposed, including orientation of chlorophyll in the chloroplast membranes and stabilization of chloroplast coupling factors against cold inactivation (2). SQDG is not, however, restricted to chloroplast membranes and a role in the regulation of ATPases has been proposed (2). SQDG functions as a component in the sulfur cycle in plants and is rapidly metabolized for protein production under conditions of sulfur depletion (2). This lipid may also provide a source of cysteine under conditions of oxidative stress (H. A. Norman, unpublished observation), and has been reported to have antiviral properties (3).

With increased interest in SQDG, there is a need for more efficient purification procedures. SQDG is strongly anionic, which has presented some problems in its purification with regard to preventing excessive losses during phase partitioning (4). Milligram amounts were purified from total lipid extracts of alfalfa leaves and *Chlorella* by chromatography on Florisil and DEAE cellulose columns (5), although a final preparative TLC purification step may be required (2). Galactolipid fractions containing SQDG have been typically recovered from plant lipid extracts by silicic acid or acid-treated Florisil column chromatography and further resolved into lipid classes by TLC. However, these procedures may need modification in their application to lipid isolation from pigmented plant tissues on a semi-preparative scale, as found by Allen et al. (6).

Several laboratories have recently described HPLC techniques for the direct isolation of lipid classes. Moreau, Asmann, and Norman (7) were successful in resolving microgram quantities of lipid extracts from different plant tissues by normal phase analytical HPLC with an isooctane–isopropanol–water–ternary gradient system. Rezanka and Podojil (8) separated a crude polar lipid extract of *Chlorella kessleri* by HPLC using a prepa-

Abbreviations: DGDG, digalactosyldiacylglycerol; GL, galactolipid(s); MGDG, monogalactosyldiacylglycerol; PL, phospholipids; SQDG, sulfoquinovosyldiacylglycerol; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; HPLC, high performance 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. Pairs of numbers representing the fatty acids when separated by a slash represent the components in the *sn*-1 and *sn*-2 positions, respectively, of the molecular species.

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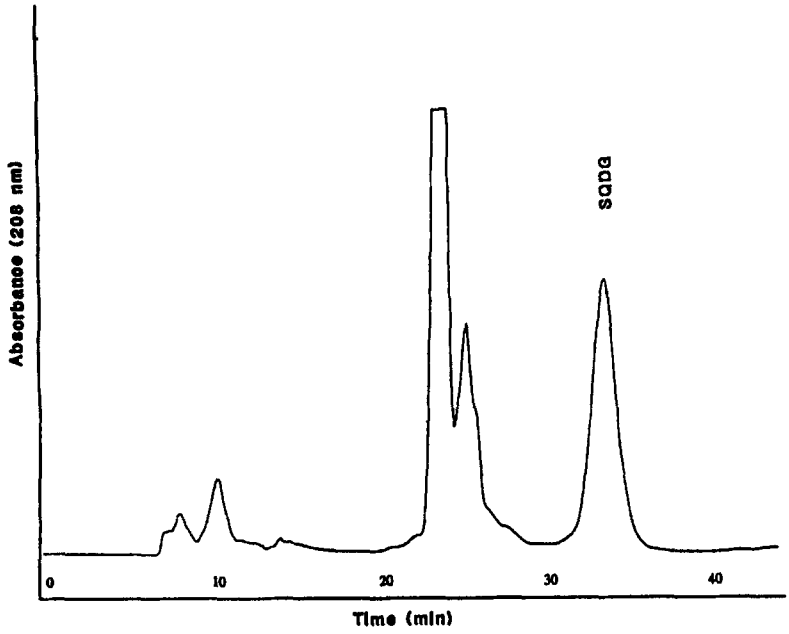

Fig. 1. Normal phase HPLC resolution of SQDG from phospholipids in a polar lipid fraction prepared from spinach leaf tissue. The SQDG peak corresponds to 0.5 mg SQDG with detection at 208 nm.

rative silica gel column. In this case, milligram quantities of lipids, including SL, were recovered. Other systems analyzing plant leaf lipids were unable to omit the use of silicic acid chromatography prior to HPLC because of the large amounts of carotenoids that eluted with polar lipids when milligram quantities of lipids were fractionated (6, 9, 10). In this report we describe chromatographic techniques for the semi-preparative isolation and analysis of SQDG.

MATERIALS AND METHODS

Lipid purification

Spinach was purchased locally. Total lipids were extracted based on the procedure of Bligh and Dyer (11) using sodium chloride solutions in two-phase partitioning (4). Separation into neutral lipid (NL), GL, and PL (phospholipid) fractions was achieved using silica Sep-pak cartridges. Neutral lipids were removed with chloroform, and GL and PL fractions were subsequently eluted with methylene chloride–methanol 93:7 (v/v) and methanol, respectively. Using this solvent system, SQDG eluted in the PL (methanol) fraction. In estimations of purity, lipid phosphorus content was determined spectrophotometrically (12). GLs and SQDG were quantitated based on galactose content (13).

HPLC procedures

Sulfoquinovosyldiacylglycerol was resolved from the PL fraction by normal phase HPLC using a mobile phase composed of heptane–isopropanol–0.001 M KCl 40:52:8 (v/v/v) delivered at 1.0 ml · min^{-1}. The column was a MAXISIL 5 micron SI column (150 × 10 mm) (Phenomenex, Torrance, CA). The column was washed with 100% isopropanol and reequilibrated to 100% heptane at the end of each run. The resolution was achieved using a Hitachi (Model L-6200A) pump and a Linear (UVIS 200) variable wavelength detector operated at 208 nm to detect the peaks. The injection volume was 1 ml. Purity was investigated by TLC on silica gel H using chloroform–acetic acid–methanol–water 75:25:5:2.2 (v/v/v/v) as the developing solvent and detection of lipid bands with I_2 vapor. The purity of SQDG was confirmed by determination of galactose and lipid phosphorous as described above. Recoveries were estimated using ^{14}C-labeled lipids prepared from leaf tissue incubated with radioactive precursors according to Norman and St. John (12). To determine total fatty acid composition, methyl esters were prepared directly with boron trifluoride/methanol (Sigma Chemical Co., St. Louis, MO) and analyzed by GLC (12).

SQDG was resolved into constituent molecular species by reversed-phase HPLC based on previously described analytical methods for MGDG and DGDG (12, 14). The stationary phase was a reversed-phase C_{18} (5 micron) Excello UltraPac ODS (R. E. Gourley, Laurel,

MD) (150 × 10 mm). The mobile phase was methanol–water 96:7 (v/v) delivered at 1.5 ml min, and peaks were detected at 205 nm. SQDG molecular species were collected, dried under N_2, and resuspended in diethyl ether. To determine fatty acid positional distribution, fatty acids were hydrolyzed from the *sn*-1 position by incubating with lipase from *Rhizopus arrhizus* (Boehringer Mannheim) at pH 7.4, according to Norman and St John (12). Analysis of the resultant free fatty acid and 2-acyl SQDG were determined by GLC of methyl esters (12).

RESULTS AND DISCUSSION

In previously developed analytical procedures for plant leaf lipids (15), SQDG was eluted from silicic acid in GL fractions together with MGDG and DGDG. In separations of plant lipids on DEAE-cellulose (15) using chloroform–methanol mixtures, MGDG and DGDG were eluted separately, and SQDG was eluted in a later fraction together with the more acidic PLs. In the current report, solvents were adjusted such that SQDG was eluted in the PL fraction from silica Sep-paks as it was not possible to elute all of the SL in the GL fraction. Using previous elution schemes, by which a GL fraction was eluted with acetone or chloroform–methanol 85:15 (v/v), SQDG was found to be distributed between GL and PL fractions when milligram samples of lipid were analyzed.

Several laboratories have recently reported HPLC techniques for the direct isolation of lipid classes from total plant lipid extractions (16). This is obviously preferable over the need for a preliminary separation of nonpolar, GL, and PL lipid fractions. However, we were unsuccessful in resolving MGDG and DGDG from contaminating pigments when a crude extract from leaf tissue containing milligram amounts of lipid was injected on a semi-preparative normal phase HPLC column (6). Glass (9, 10) also used a solid phase extraction step prior to HPLC in the semi-preparative isolation of phosphatidylcholine from soybean leaf tissue. Rezanka and Podojol (8) resolved milligram quantities of lipids, including SQDG, by normal phase HPLC of a total polar lipid fraction after the removal of pigments on a silica gel column. In the current system, carotenoid pigments consistently contaminated the column when either total lipid or total polar lipid extracts were loaded on the normal phase column. Therefore fractionation was necessary prior to HPLC. An additional problem with HPLC of crude lipid extracts is the tendency for column contamination to build up due to silica-catalyzed chlorophyll breakdown which has been a previously reported problem (7).

Sulfoquinovosyldiacylglycerol was resolved from PLs in the methanol fraction recovered from silica Sep-paks

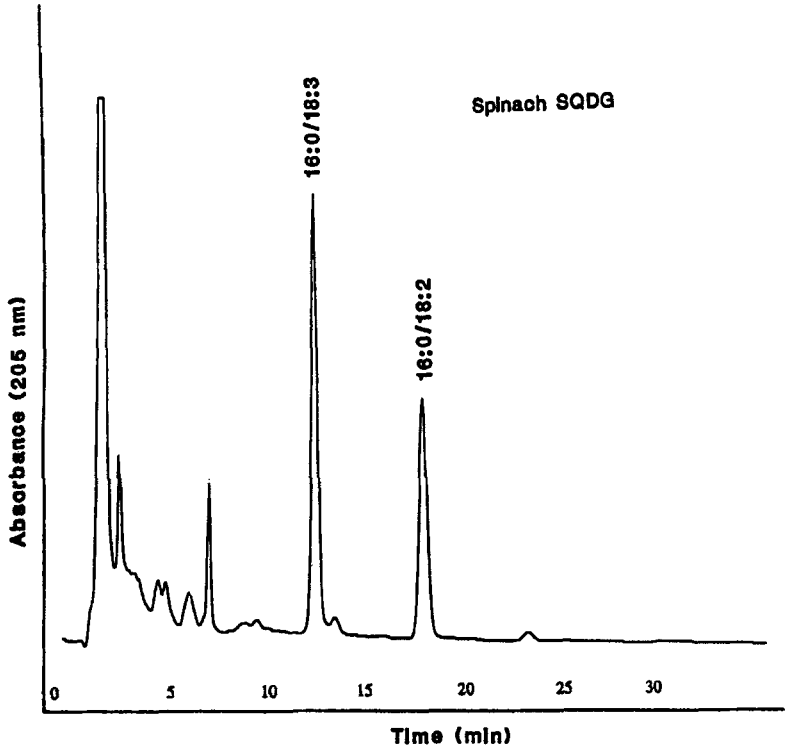

Fig. 2. Reversed phase HPLC resolution of major SQDG molecular species from spinach leaves with detection at 205.nm.

TABLE 1. Molecular species composition of SQDG from spinach leaf

Molecular Species[a]	Wt % of Total Molecular Species	μg/10 g Fresh Wt.
16:0/16:0	3.2	55
16:0/18:1	tr[b]	–
16:0/18:2	38.0	684
16:0/18:3	51.5	927
18:2/18:3	1.5	27
18:3/18:3	5.8	104

[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]Trace, not quantified.

using an isocratic HPLC mobile phase consisting of heptane–isopropanol–0.001 M KCl (40:52:8) (v/v/v) (**Fig. 1**). The PLs were not well resolved in this system, but SQDG had a consistent elution time of about 25 min with a flow rate of 1.5 ml · min^{-1}. Consistent separations were achieved using MAXISIL silica; bonded phases (polar-bonded-phase silica gel) are preferable in many separations. However, SQDG was not eluted from the support tested, as has been found for other acidic lipids (17). Aliquots of the recovered SQDG peak gave a single spot on a TLC plate run in a polar developing solvent, and no lipid phosphorus was detectable. The total recovery of SQDG from the plant extract was determined by spiking a crude lipid extract with SQDG containing ^{14}C-labeled fatty acids that were extracted from pulse-labeled leaves (12). The recovery was found to be 80–82%. Recovery of radioactive SQDG from the HPLC column was 93–95%.

The overall yield of SQDG from spinach leaf tissue was 1.8 mg · 10 g^{-1} fresh wt. This is within the range of levels previously reported that have been between 0.4–8 mg/10 g fresh wt in different leaf species (5, 18). Levels of SQDG vary with plant age and species (2, 18). Previous studies have recorded analytical separations of MGDG and DGDG molecular species (12, 14). We were able to resolve milligram quantities of SQDG from spinach leaf into different components (**Fig. 2**), with 16:0/18:2 and 16:0/18:3 being the major molecular species (**Table 1**). Analysis of fatty acid positional distribution showed that the *sn*-1 position was enriched in 16:0. SQDG from leaf tissue analyzed for total fatty acid composition typically contains at least 30% (of total fatty acids wt) 16:0, with 18:3, or a mixture of 18:3 and 18:2, being the other major component (1). In contrast, spinach MGDG contains 18:3/16:3 and 18:3/16:3 as its major molecular species (19). The situation appears to be very different in algae; Rezanka and Podojil (8) analyzed the molecular species composition of GLs by preparative HPLC from *Chlorella kessleri* and found relatively high levels of 16:3/18:3 and 18:3/18:3 molecular species of both SL and MGDG. Analysis of SQDG in higher plants other than spinach, including *Vicia faba* leaves, also showed that the *sn*-1 position was enriched in 16:0 and the *sn*-2 position in 18:3 or 18:2 (18, 19).

In conclusion, this method should be widely applicable to the isolation of plant SQDGs. This method replaces tedious and less accurate chromatography on Florisil and DEAE cellulose. As MGDG and DGDG are removed in a purification step prior to SQDG resolution, these lipids can be recovered from the same tissue extract and resolved by an HPLC procedure we have previously developed (6).

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Manuscript received 10 October 1995 and in revised form 18 March 1996.

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