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ORIGINAL ARTICLE

Simultaneous Quantification of Plant Glyceroglycolipids Including Sulfoquinovosyldiacylglycerol by HPLC–ELSD with Binary Gradient Elution

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Abstract Membrane lipids of photosynthetic organisms consist of glycerophospholipids and glyceroglycolipids. We investigated a method for the simultaneous quantitative analysis of neutral and acidic lipids using HPLC–ELSD, and quantified monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG). Ten complex lipid classes were separated with a binary gradient system consisting of chloroform and methanol–acetone–water–acetic acid (30:60:9:1, v/v/v/v) with 0.3% triethylamine (pH 4), and were eluted within 16 min. The contents of SQDG in ten edible plants ranged from 3 to 101 mg/100 g, and were positively correlated to the neutral glyceroglycolipids contents.

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Introduction

Glyceroglycolipids are major membrane lipids in photosynthetic organisms such as higher plants and algae. MGDG, DGDG and SQDG are all polar lipids particularly found in chloroplasts, and account for 90% of the total lipids in the chloroplast thylakoid membrane [1]. It has been established that SQDG, which is a negatively charged (acidic) sulfolipid present in the thylakoid membrane with phosphatidylglycerol, is important for preservation of structure and function of membranes, despite its slight amount [2, 3]. Moreover, these glycolipids have been evaluated for the functionality of their bioactive substances and are known to have various biological activities, including improving the intestinal environment [4], antitumor activity [5–7], anti-inflammatory activity [8, 9] and protection against cell death [7, 10]. Among them, SQDG has been identified as a new functional component because it has specific biological inhibitory activities against DNA polymerase [11–15], certain types of viruses [16–19], P-selectin receptor [20], telomerase [21], angiogenesis [22] and inflammation/proliferation [23].

People consume these glycolipids daily from plant foodstuffs. Sugawara et al. [24] analyzed the contents of neutral glycolipids in various plants using HPLC. In this way, the main neutral glycolipids in plants present no particular difficulties for analysis and are easily separated

from phospholipids by HPLC. However, since SQDG presents greater analytical problems because of its highly polar anionic (acidic) nature, the only methods that have been devised for their analysis is the TLC/densitometry method [25] and the complicated triadic gradient elution by HPLC [26]. Therefore, publications reporting SQDG contents in plants are rare. In the present study, we developed optimal HPLC conditions with a binary gradient system to separate and quantify neutral and acidic glyceroglycolipids in edible plants.

Materials and Methods

Materials

Chromatographically pure working standards of SQDG, MGDG and DGDG were prepared from spinach total lipids. Firstly, spinach TL (prepared as described below) was subjected to silicic acid column chromatography. After removing the non-polar lipids from the cartridge with chloroform, the glycolipid fraction was eluted with acetone [27]. The crude glycolipid fraction obtained was further fractionated with stepwise elution of chloroform–acetone [27]. The DGDG fraction, including SQDG, was applied to a DEAE column and separated to neutral DGDG and acidic SQDG lipid fractions as described below. Finally, each glycolipid was subjected to preparative TLC with a solvent system of chloroform–methanol–water (70:35:7, v/v/v) to give one spot. The glycolipids were dissolved in chloroform–methanol (2:1, v/v) and 0.001% butylhydroxytoluene (Wako, Japan) was added. Standard samples of cholesterol, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG) from egg yolk, and phosphatidylinositol (PI) from soybeans were purchased from Sigma (St Louis, USA), and cerebroside (CE, lower spot) from bovine brain was purchased from Doosan Serdary Research Laboratories (Toronto, Canada). Commercial standards of MGDG, DGDG, acylsterylglucoside (ASG, from soybeans) and sterylglucoside (SG, from soybeans) were purchased from Funakoshi Co. (Tokyo, Japan). SQDG was purchased from Kanto Chemical Co. (Tokyo, Japan). They were used for comparing LC retention times.

Spinach (leaf, *Spinacia oleracea* L.), parsley (leaf, *Petroselinum crispum*), perilla (leaf, *Perilla frutescens*), Chinese chive (leaf, *Allium tuberosum*), green onion (leaf, *Allium cepa*), crown daisy (leaf and stem, *Chrysanthemum coronarium*), broccoli (flower and stem, *Brassica oleracea* var. *italica*), green pepper (fruit, *Capsicum annuum* cv. *grossum*), cucumber (fruit, *Cucumis sativus*) and pumpkin (fruit, *Cucurbita maxima*) were purchased at a local supermarket in Obihiro, Japan. The pumpkin fruit was cut into two pieces to remove the loose stringy pulp with seeds, and the outer green epidermis was removed to obtain the isolated pumpkin flesh.

Extraction, Separation and Detection of Lipids

Fresh plant samples (5–50 g) were cut into pieces and heated in a microwave oven for 1 min to deactivate lipolytic enzymes and then extracted three times with chloroform–methanol (2:1, v/v) for 1 h, respectively, after homogenization (Polytron, Kinematica, Switzerland). After filtration using filter paper (Advantec No. 2, Toyo Roshi Kaisha, Japan), the extracts were combined and washed once with water in a mixture of chloroform–methanol–water (with a ratio of 8:4:3, v/v/v) [28]. After partition, the lower phase was concentrated to dryness using a rotary evaporator, and the lipids were weighed to yield the total lipids (TL).

Then, green pepper TL was separated into neutral and acidic lipid fractions by anion-exchange column chromatography. Briefly, 50 mg of TL was inserted into a diethylaminoethyl (DEAE) column (Toyopak DEAE M, Tosoh, Tokyo, Japan), and stepwise eluted with chloroform–methanol (neutral lipid fraction), followed by elution with chloroform–methanol–0.1 M ammonium acetate (20:80:0.2, v/v/v) [29]. The latter fraction was washed twice in a mixture of chloroform–methanol–water (with a ratio of 8:4:3, v/v/v), then the lower phase was concentrated to dryness to yield the acidic lipid fraction.

To detect glyceroglycolipids, TL was analyzed by silicic acid TLC (Silica gel 60, 0.25 mm, Merck, Germany) with a chloroform–acetone–methanol–acetic acid–water mixture (50:20:10:10:5, v/v/v/v/v). Glyceroglycolipid species were visualized after spraying with anthrone/sulfuric acid, followed by brief heating.

HPLC and GC Analyses

A portion of the total lipids was dissolved in chloroform–methanol (2:1, v/v) solution (1 mg/ml) and filtered (GL Chromatodisc, 0.45 μm, GL science, Japan) for HPLC analysis. Plant TL was analyzed by a normal-phase HPLC equipped with an evaporative light scattering detector. Instrument: Shimadzu LC-10AD (Kyoto, Japan); mobile phase: solvent A, chloroform and solvent B, methanol–acetone–water–acetic acid (30:60:9:1, v/v/v/v) with 0.3% triethylamine (pH 4); gradient: Table 1; column: LiChrospher 100 Diol (column size 250 × 4 mm i.d.; particle size 5 μm, Merck, Germany) with an LiChroCART 4–4 guard column; flow rate: 0.9 ml/min; injection volume 10–20 μl; column temperature 35 °C; detector ELSD (70 °C, 350 kpa, Sedex 75, Sedere, France). The amounts of SQDG, MGDG and DGDG in plant samples were calculated from the calibration curve of each standard

Table 1 Elution program for the binary gradient system

Time (min)	A (%)	B (%)
1	100	0
2	70	30
6	70	30
8	50	50
13	50	50
15	0	100
17	0	100
20	100	0

A Chloroform

B Methanol–acetone–water–acetic acid (30:60:9:1) with 0.3% triethylamine (pH 4)

component described above, since ELSD provided a curvilinear response to mass of standard lipids. All data are averages ± SD of independent experiments from three samples.

MGDG, DGDG and SQDG from spinach leaves were methanolyzed with 5% HCl/methanol at 95 °C for 2 h, and fatty acid methyl esters were analyzed by GC according to our previous report [30].

Results

HPLC Analysis of Standard Lipids and Component Fatty Acids of Glyceroglycolipids

Chromatographically pure working standards were analyzed by HPLC (Fig. 1). MGDG, DGDG and SQDG prepared from spinach leaves each exhibited a single peak (Fig. 1a) and were identified by co-chromatography with commercial standards, and were used as standard compounds for their calibration curves. The correlation coefficients were from 0.9980 to 0.9998. The detection limit of SQDG was 0.1 μg. The predominant fatty acids were α-linolenic acid (68 mol%) and oleic acid (30 mol%) for MGDG, α-linolenic acid (86 mol%) for DGDG, and palmitic acid (46 mol%) and α-linolenic acid (46 mol%) for SQDG. The glycolipids (MGDG, SQDG, DGDG, ASG, SG and CMH) and phospholipids (PC, PE, PG and PI) were completely separated from each other (Fig. 1b–e).

TLC of Plant Total Lipids

Total lipids obtained from spinach leaf, broccoli flower and stem, green pepper fruit and pumpkin fruit were analyzed by TLC (Fig. 2). Spinach TL (lane A) contained not only a significant amount of neutral glyceroglycolipids such as MGDG and DGDG but also SQDG, which was located below the DGDG spot (identified by co-chromatography with commercial MGDG, DGDG and SQDG standards). In this TLC system, SQDG could be clearly separated, but DGDG overlapped with PC. Broccoli TL (lane B) contained a significant amount of glycerophospholipids and a small amount of glyceroglycolipids. In green pepper TL (lane C), glycolipids such as sterylglycoside (SG) and ceramide monohexoside (CMH), were found in relatively significant amounts. In pumpkin TL (lane D), two large spots of Rf values 0.01 and 0.04 with positive coloring reaction to anthrone/sulfuric acid reagent were assumed to be triglycosyldiacylglycerol (TGDG) and tetraglycosyldiacylglycerol (TeGDG), respectively [31].

HPLC Profiles of Plant Total Lipids

Lipid extracts from four plant tissues shown in Fig. 2 were analyzed by HPLC (Fig. 3). Non-polar lipids and pigments including chlorophyll were eluted within 4 min after the start of analysis. Ten complex lipid species were detected from green pepper TL (injection amount 20 μg), and all were eluted within 16 min. These peaks were identified by analyzing the neutral and acidic lipid fractions of green pepper, and comparing them with retention times of standard components (Fig. 1). It was confirmed that peaks 4, 6 and 11 shown in the acidic lipid fraction were PG, SQDG and PI, respectively. The peak at about 4 min in acidic lipid fraction was tentatively identified as free fatty acids. Although all glyceroglycolipids and glycerophospholipids were detected in spinach TL, SG and CMH, of which the contents are low, were not detected with an injection amount of 10 μg (within the maximum detection limit of MGDG). When 20 μg of broccoli TL was injected the SQDG peak was very small and the PC peak exceeded the detection limit of the ELSD. In pumpkin TL, peaks 12 and 13 around 18–19 min were assumed to be TGDG and TeGDG, respectively, because the preparative isolates obtained from HPLC analysis corresponded to Rf value and color reaction by anthrone/sulfuric acid of their spots on TLC shown in Fig. 2.

Determination of Glyceroglycolipids in Plant Foodstuff

Contents of MGDG, DGDG and SQDG in ten fresh edible plants were determined (Table 2). The glyceroglycolipid profile was significantly different among the plants. Contents of SQDG ranged from 3 mg/100 g for green onion to 101 mg/100 g for parsley. Total glycolipid contents were generally low in green onion, green pepper and cucumber with low TL contents (0.17/100 g). Although the TL content (1.1/100 g) of perilla was close to that of broccoli, the glycolipid content (397 mg/100 g) of perilla was three times higher than broccoli. Green leafy vegetables, such as spinach, parsley, perilla and chive, were rich in MGDG, whereas crown daisy, green pepper and pumpkin were

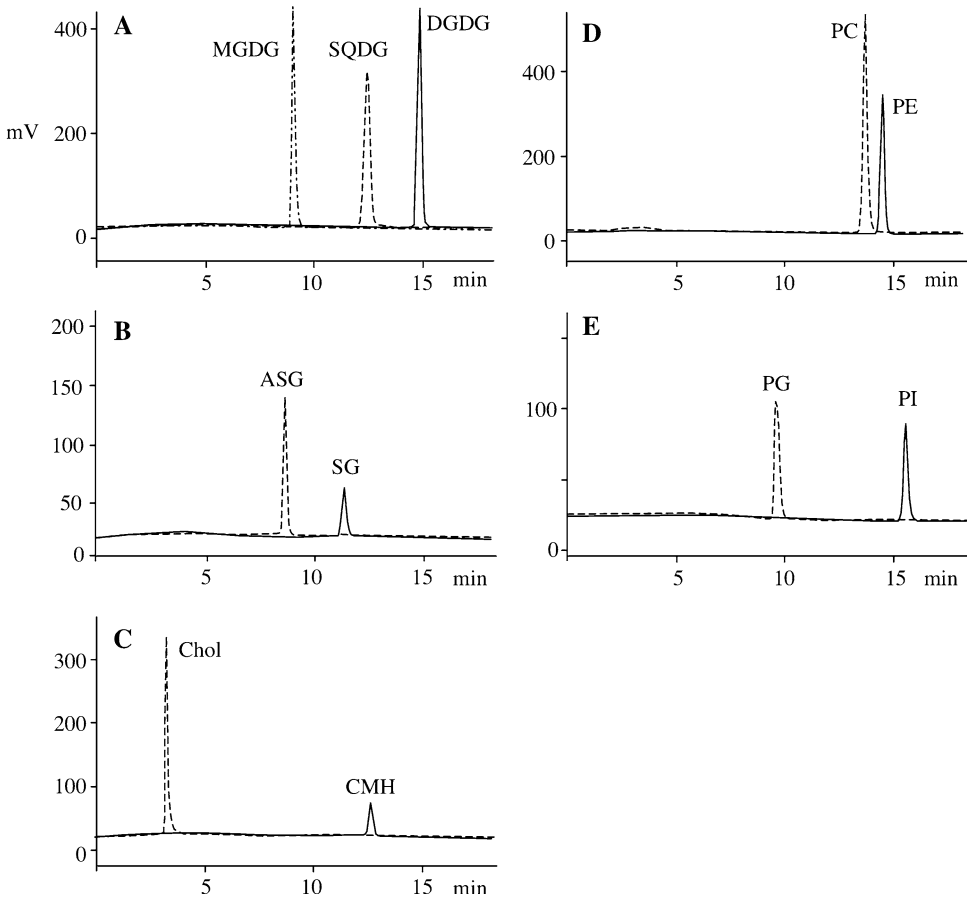

Fig. 1 HPLC profiles of standard compounds. Injection: *A* 2 μg, *B* 0.6 μg, *C* 1 μg, *D* 1 μg, *E* 3 μg. *MGDG* monogalactosyldiacylglycerol, *DGDG* digalactosyldiacylglycerol, *SQDG* sulfoquinovosyldiacylglycerol, *ASG* acylsterylglycoside, *SG* sterylglycoside, *Chol* cholesterol, *CMH* ceramide monohexoside, *PE* phosphatidylethanolamine, *PC* phosphatidylcholine, *PG* phosphatidylglycerol, *PI* phosphatidylinositol

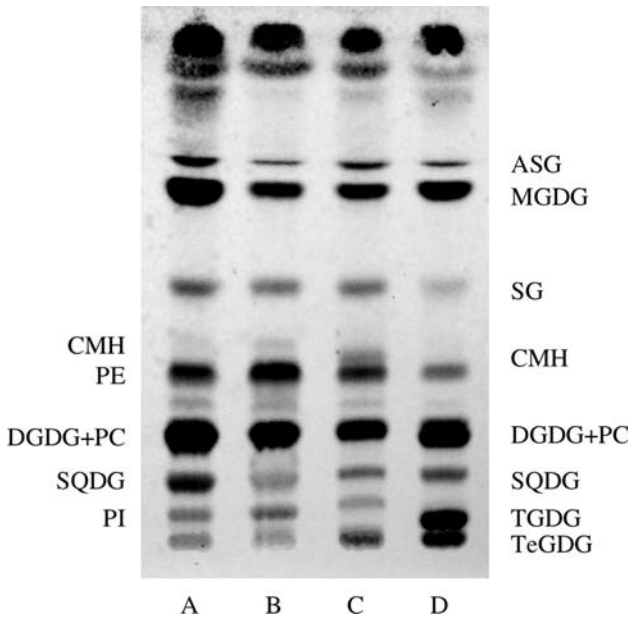

Fig. 2 TLC of total lipids prepared from plant foodstuff. *A* spinach, *B* broccoli, *C* green pepper, *D* pumpkin. Solvent chloroform–acetone–methanol–acetic acid–water (50:20:10:10:5, v/v/v/v/v), Detection anthrone-sulfuric acid followed by heating. See Fig. 1 for abbreviations. *TGDG* triglycosyldiacylglycerol, *TeGDG* tetraglycosyldiacylglycerol

relatively high in DGDG. SQDG was highest in parsley (101 mg), followed by crown daisy (48 mg) and spinach (45 mg). Also, SQDG was comparatively rich even in pumpkin (26 mg), a fruit vegetable. Relative amounts of SQDG in glyceroglycolipids were high in cucumber, pumpkin and crown daisy.

Discussion

Glyceroglycolipids are the main lipid class present in photosynthetic plant tissues and are accompanied by glycerophospholipids. Acidic glycerophospholipids and glyceroglycolipids including PG, PI and SQDG, as well as neutral glycolipids including acylsterylglycoside, CMH, MGDG and DGDG, and neutral phospholipids including PC and PE, were detected in a single HPLC run without any pretreatment such as anion-exchange column chromatography. This may have resulted from not only addition of acid (acetic acid), but also a basic substance (triethylamine), which may have improved the dissolubility of acidic lipid groups.

Since green pepper TL contained almost all of the glycolipid and phospholipid classes detected by TLC analysis

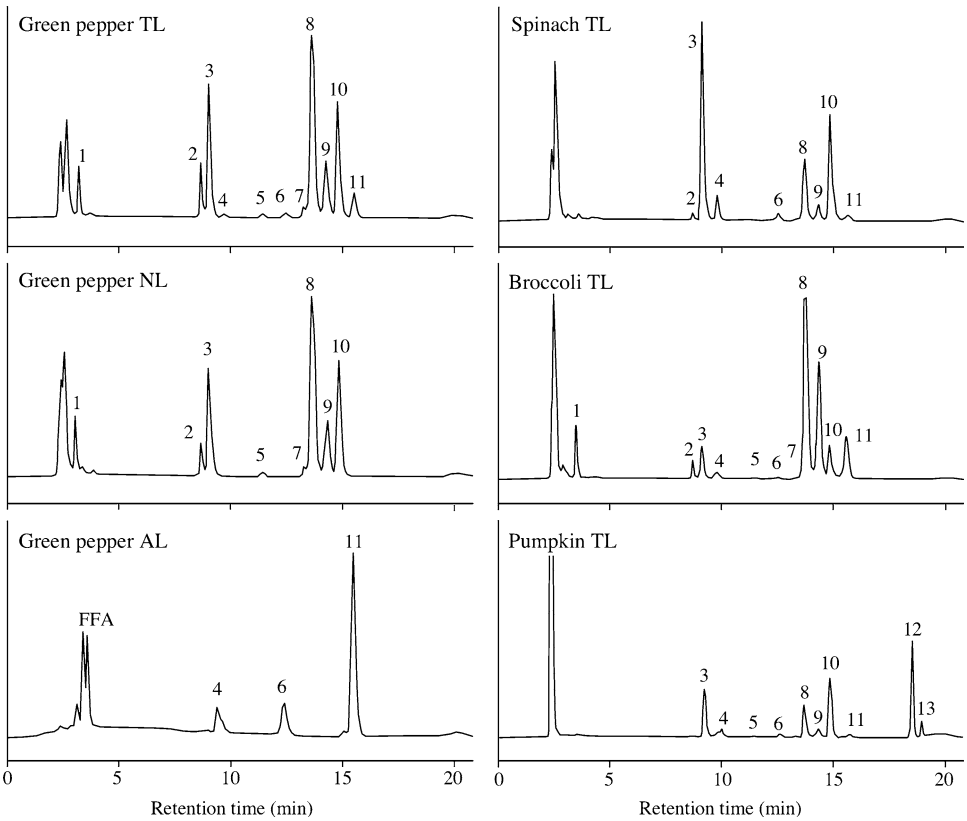

Fig. 3 Typical HPLC profiles of lipid fractions prepared from plant foodstuffs. *TL* total lipid, *NL* neutral lipid fraction, *AL* acidic lipid fraction. *1* Chol, *2* ASG, *3* MGDG, *4* PG, *5* SG, *6* SQDG, *7* CMH, *8* PC, *9* PE, *10* DGDG, *11* PI, *12* TGDG, *13* TeGDG. See Fig. 1 for abbreviations. *FFA* free fatty acids

Table 2 Glyceroglycolipid contents of edible plants determined by HPLC–ELSD

Vegetables	Organ	Total lipids (g/100 g)	MGDG[a]	DGDG[a]	SQDG[a]	Total[a]	Ratio M:D:S
Spinach	Leaf	0.7 ± 0.01	243 ± 26	136 ± 10	45 ± 4	424 ± 38	57:32:11
Parsley	Leaf	1.6 ± 0.05	697 ± 51	147 ± 15	101 ± 7	945 ± 74	74:15:11
Perilla	Leaf	1.1 ± 0.08	222 ± 21	139 ± 15	36 ± 8	397 ± 36	56:35:9
Chive	Leaf	0.6 ± 0.06	158 ± 46	57 ± 19	18 ± 6	233 ± 72	68:24:8
Green onion	Leaf	0.2 ± 0.01	12 ± 3	8 ± 1	3 ± 0	23 ± 4	52:35:13
Crown daisy	Leaf and stem	0.7 ± 0.13	123 ± 22	132 ± 24	48 ± 9	303 ± 56	40:44:16
Broccoli	Flower and stem	1.0 ± 0.06	50 ± 1	39 ± 1	14 ± 1	103 ± 2	48:38:14
Green pepper	Fruit	0.2 ± 0.01	21 ± 5	23 ± 5	6 ± 1	50 ± 10	42:46:12
Cucumber	Fruit	0.2 ± 0.01	11 ± 3	8 ± 2	4 ± 0	23 ± 5	48:35:17
Pumpkin	Fruit	0.7 ± 0.01	66 ± 11	77 ± 5	26 ± 1	169 ± 12	39:46:15

M:D:S indicate the ratio of MGDG/DGDG/SQDG

Data indicate lipid content per fresh weight

[a] Values are mg/100 g

(Fig. 2), it was possible to simultaneously detect both glycolipids and phospholipids from a single analytical HPLC. However, since broccoli TL was rich in glycerophospholipids, SQDG could not be detected within the detection limit of PC (Fig. 3). Moreover, since spinach TL was rich in glyceroglycolipids, SG and CMH could not be detected within the detection limit of MGDG. In these cases, in order to quantify all lipids including phospholipids, it may be necessary to analyze them repeatedly by changing the injection volume, or to measure them separately by separating the neutral and acidic lipids using an anion-exchange column like DEAE (as shown in Fig. 3). Despite large quantitative differences in SQDG, this study revealed that plant glyceroglycolipids, including SQDG, could be simultaneously detected in all samples. Some research into the occurrence of TGDG has previously been reported in pumpkin [24, 31], but not research into TeGDG. Since TeGDG has also been identified in oat kernels

[32], further verification of these oligoglycosyl-DGs by determining the molar ratio of fatty acid/sugar or by using LC–MS will be needed.

The glycerolglycolipid levels in the various tissues (leaf, fruit, flower, and stem) of each common species were unique. The levels of MGDG were especially high in dark green leafy vegetables such as parsley, chive, and spinach. The levels of DGDG were especially high in crown daisy leaf and stem, pumpkin fruit, and were relatively high in green pepper. It was assumed that this is because the thylakoid membrane with high amount of MGDG in the chloroplasts develops in tissues with active photosynthesis, whereas non-photosynthetic tissues such as fruit, flower and stem were high in DGDG derived from the outer envelope of chloroplasts [1]. It was found that SQDG contents in vegetables were positively correlated ($R = 0.970$, $P < 0.01$) to neutral glyceroglycolipid contents (sum of MGDG and DGDG). Moreover, although sulfur compounds are present in allium including chive and green onion as allyl sulfide, and in broccoli as isothiocyanate, this was not related to the content of SQDG, which has the same sulfur component.

In the future, we plan to use this new HPLC method to compare the levels of SQDG in various agricultural by products and identify one or more of them as potential commercial sources of SQDG.

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