Composition and Distribution of Individual Molecular Species of Major Glycolipids in Wheat Flour

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The distribution of individual molecular species of the main wheat flour glycolipids, digalactosyldiglyceride (DGDG), monogalactosyldiglyceride (MGDG), digalactosylmonoglyceride (DGMG) and monogalactosylmonoglyceride (MGMG) has been investigated by reversed phase high-performance liquid chromatography of their benzoate derivatives after the respective galactosylglyceride classes were obtained by semi-preparative high-performance liquid chromatography. Combinations of linoleic acid at the *sn*-2 position with linoleic, oleic and palmitic acids at the *sn*-1 position predominated as major common molecular species of MGDG and DGDG. The pairs 16:0/20:4, 18:3/20:1, 18:0/18:3, 18:0/18:1 and 20:0/18:2 were determined only among MGDG molecular species. Five common molecular species containing 16:0, 18:0, 18:1, 18:2 and 18:3 fatty acids, respectively, were determined in MGMG and DGMG, with 18:2 being the most predominant form, and 18:1 (MGMG) and 16:0 (DGMG) as the next major fatty acids.

KEY WORDS: Benzoate derivatives, diglyceride classes, glycolipids, individual molecular species, monoglyceride classes, RP-HPLC, semi-preparative HPLC, wheat flour.

Polar lipids, especially glycolipids, make important contributions to dough properties, baking performance and resistance to bread staleness (1–8). Although discrepancies concerning the effects of glycolipids on the breadmaking potential and prediction of loaf volume of wheats grown in different countries have been reported (9–14), there is a general agreement on the improving effects of glycolipids. Significant correlations between loaf volume and galactose contents of wheat or flour free lipids have been found (15,16). Among glycolipids classes, monogalactosyldiglyceride (MGDG) has been described as a weak improver (17–19), but digalactosyldiglyceride (DGDG), which is the main glycolipid in flour (19), is an outstanding improver (18) that enhances baking quality (20,21).

Several attempts have been made to define the functionality of lipid classes in dough development and breadmaking quality, but progress undoubtedly has been hampered by the lack of an appropriate methodology for analyzing a wide spectrum of chemically similar lipids functioning in a complex environment, such as flour and dough.

Traditional methods for quantitation of glycolipid classes are based on the analysis of carbohydrates (22) or fatty acids (23) in the hydrolyzed lipid extract after column chromatography, thin-layer chromatography (TLC) or TLC followed by densitometry (3,4), as well as on the combination of two or more chromatographic procedures [column, TLC, gas chromatography (GC)], and chemical and enzymatic analysis.

More recently, high-performance liquid chromatography (HPLC) on normal (5) and reversed-phase (RP) (21,24,25) columns with UV, RI or mass spectrometric detection of intact glycolipids (21,24,25) and their derivatives (5) has been successfully used in addition to GC for separation and quantitation of glycolipid classes (5) and molecular species (21,24,25) from rye and wheat flour and from spinach.

Fatty acid composition and positional distribution of major individual molecular species of MGDG and DGDG have been reported in the literature (21,25,26), but no data have been found concerning monogalactosylmonoglyceride (MGMG) and digalactosylmonoglyceride (DGMG), minor glycolipids in flour (27), in spite of their potential functional role in breadmaking.

In this paper, the positional distribution and molecular association of fatty acids from individual molecular species of mono- and digalactosylglycerides of wheat flour are determined to provide preliminary structural information in the investigation of glycolipid breadmaking functionality.

EXPERIMENTAL PROCEDURES

Reagents and solvents. MGDG and DGDG from wheat flour, benzoic anhydride, 4-dimethylaminopyridine and free fatty acid standards were purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile, methanol and propan-2-ol, all HPLC-grade, were obtained from SDS (Peypin, France). Chromatography-quality water and organic solvents (analytical grade) were from Merck (Darmstadt, Germany).

Apparatus. A Hewlett-Packard (Palo Alto, CA) HPLC system composed of a 1050 gradient pumping system, a 1040A diode array detector and a 9000 Pascal Chem Station was used. The analytical column was an ODS (5 μm, 150 × 4.6-mm i.d.) from Supelco, Inc. (Bellefonte, PA), in line with a low-dispersion column-inlet filter (Hewlett-Packard). The separation among glycolipid classes, mono- and digalactosylglycerides, was performed with a Hypersil (Hewlett-Packard) column (3 μm, 60 × 4.6-mm i.d.). Fractionation of digalactosylglycerides required two columns coupled in series.

HPLC. Molecular species of benzoate derivatives of 1-monoacyl and 1,2-diacylgalactosylglycerides were separated with a linear gradient of 20% propan-2-ol (0.5%/min) in acetonitrile. Galactosylglyceride classes were fractionated by a gradient system from an initial mobile phase of hexane/propan-2-ol/water (60:38.5:1.5, vol/vol/vol to 52.6/42/5.4 in 9 min). The columns were operated at a flow rate of 1 mL/min for all experiments, except for the preparative run of digalactosylglycerides (1.5 mL/min), and at room temperature. Detection was at 230 nm and 206 nm for benzoate derivatives and native glycolipids, respectively.

GLC. The GLC analyses were performed with a Perkin-Elmer (Norwalk, CT) gas-chromatography system composed of a Sigma-2 gas chromatograph coupled to an LCI-100 automated integrator (Perkin-Elmer). Samples were derivatized by acid methanolysis. The fatty acid methyl esters were resolved by means of a capillary Supelcowax-10 fused-silica column (Supelco; 30 m, 0.53 mm i.d., 1.0 μm film thickness). The elution program was initially adjusted at 190°C for 5 min, then up to 240°C at 4°C/min, and finally isothermal for 14 min. The car-

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rier gas was helium at a linear flow of 20 cm/s and a split ratio of 13.5. The temperature for both the oven and injector was programmed at 270°C. Peaks were identified by reference standards and by retention times relative to the hepatadecanoic acid methyl ester.

Extraction and purification of glycolipids. Extraction and initial purification of glycolipids were carried out as described previously (28). Briefly, lipid separation was performed by solid-phase extraction (SPE) on Bond-Elut (500 mg) prepacked columns (stainless-steel frits). Neutral lipids were eluted with chloroform, and monogalactosyl and digalactosylglycerides were resolved with chloroform/acetone (50:50, vol/vol) mixture and acetone, respectively. Glycolipid fractions were freed of nonlipid contaminants by solvent partitioning (29), evaporation to dryness under nitrogen and solution in the initial mobile phase for HPLC fractionation of glycolipid classes. Samples containing 1.5–3 mg of glycolipids (20 μL) were injected into the chromatograph, and individual peaks were collected separately. Several runs were made for each sample to collect enough material for derivatization and analysis. Purity of each peak was checked by TLCL on silica gel 60 plates (28,30).

Preparation of benzoate derivatives. Galactosylglycerides (less than 2 mg) were dissolved in dry pyridine (0.3 mL) containing dimethylaminopyridine (4 mg) and benzoic anhydride (10 mg), and the mixture was kept overnight at room temperature. Sodium carbonate-saturated methanol/water (80:20, vol/vol; 2 mL) was added, and the derivatives were extracted with 3 × 2 mL of isooctane. Evaporation of the organic solvent led to the benzoate derivatives ready for HPLC analyses without further purification.

Lipase treatment. RP-HPLC fractions from diacylgalactosylglycerol benzoates were treated with lipase for 15 min according to the method of Batley *et al.* (31), modified by using methyltirbutirylether (MTBE) (3 × 2 mL) as organic solvent for extraction. The reaction products, 2-acyl-3-galactosylbenzoate-*sn*-glycerols, were isomerized to the more stable 1-acyl-3-galactosylbenzoate-*sn*-glycerol by incubation in 0.2M Tris-HCl buffer, pH 8.0 (32).

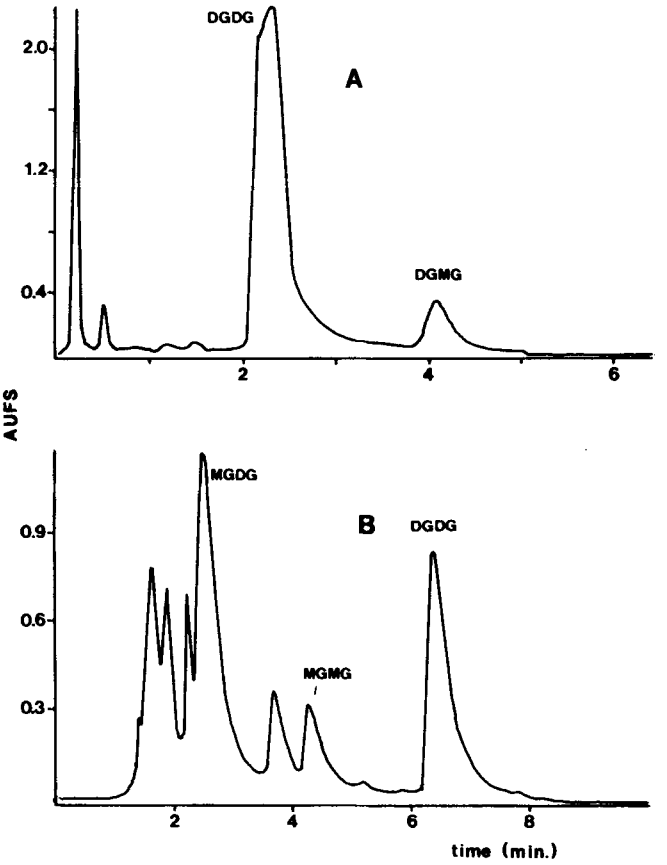

FIG. 1. High-performance liquid chromatography fractionation of digalactosylglycerides (A) and monogalactosylglycerides (B) of wheat flour. Chromatographic conditions are described in the Experimental Procedures section.

RESULTS AND DISCUSSION

Main glycolipids of wheat flour, monogalactosyl- and digalactosylglycerides, were previously obtained from water-saturated butanol extracts by optimized SPE (28) and purified from nonlipidic contaminants by solvent partition (29) before chromatographic separation of lipid classes.

HPLC separation of glycolipid classes. Normal-phase HPLC on hypersil columns properly separated mono- and digalactosylglyceride classes (Fig. 1) in high homogeneity. Digalactosylglyceride chromatograms performed on one column accounted for two main peaks (Fig. 1A), corresponding to DGDG (Rt = 2.292 min) and DGMG (Rt = 4.087 min), respectively. Monogalactosylglycerides separated on two coupled columns (Fig. 1B) gave two peaks, identified as MGDG (Rt = 2.489 min) and MGMG (Rt = 3.463 min), in addition to a few unidentified peaks and to a contaminant DGDG (Rt = 6.048 min), which did not interfere with chromatographic separation and identification. No previous attempt on semi-preparative HPLC separation of intact glycolipid classes has been found in the literature.

Molecular species determination of galactosyldiglycerides classes. The determination of MGDG and DGDG molecular species was performed in SPE extracts purified by semi-preparative normal-phase HPLC after RP-HPLC of their respective benzoate derivatives was optimized (30).

Chromatographic separation of molecular species of MGDG and DGDG is plotted in Figure 2. Eight common peaks were separated for both MGDG and DGDG, in addition to five peaks corresponding to particular molecular species of MGDG. Fractions assigned to each individual chromatographic peak were analyzed by capillary GLC after transmethylation to know the *sn*-1 and *sn*-2 fatty acid composition of each molecular species. Determination of the fatty acid at the second position by RP-HPLC of the respective benzoate derivatives after lipase treatment and isomerization failed. The enzyme attack was ineffective, even after 15 min reaction. This was probably due to sterical hindrance. Conversion of absolute retention times to retention times relative to phosphatidylcholine (PC) (30) permitted determination of the positional distribution of fatty acids (Table 1) because relative retention time (RRT) is only dependent on the nature of the fatty acids.

Main chromatographic peaks corresponded to individual components, except for peak No. 10, which includes

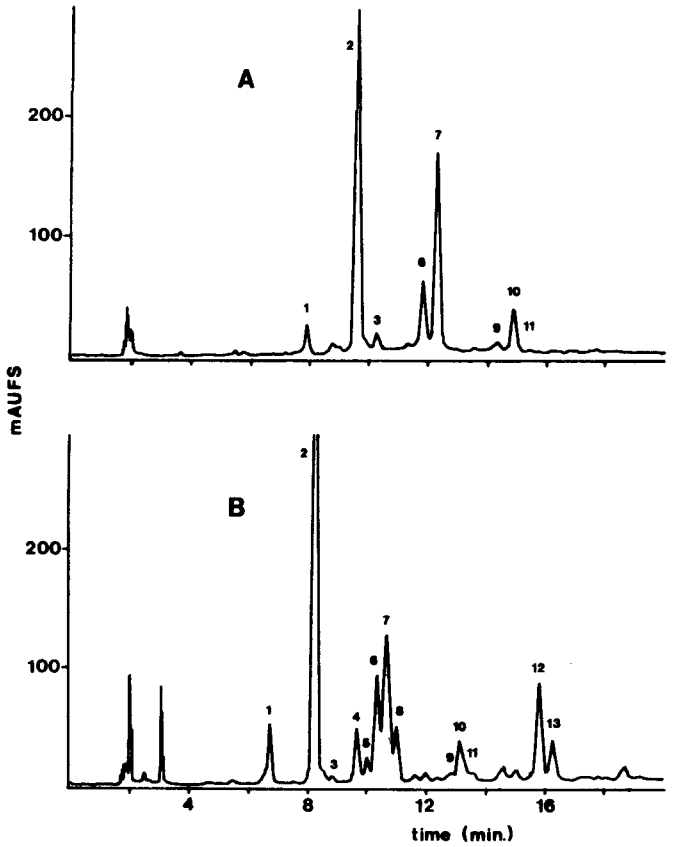

FIG. 2. Reversed-phase high-performance liquid chromatography separation of benzoate derivatives of molecular species from digalactosyldiglyceride (A) and monogalactosyldiglyceride (B). Chromatographic conditions are described in the Experimental Procedures section. Peak number identification is shown in Table 1.

TABLE 1

Relative Retention Time (RRT) and Identification of Molecular Species of MGDG and DGDG from Wheat Flour[a]

Peak number	RRT[b] MGDG	RRT[b] DGDG	Molecular species[c]
1	0.595	0.614	18:2/18:3
2	0.750	0.758	18:2/18:2
3	0.815	0.824	16:0/18:3
4	0.897	—	16:0/20:4[d]
5	0.934	—	18:3/20:1[d]
6	0.967	0.961	18:1/18:2
7	1.000	1.000	16:0/18:2
8	1.037	—	18:0/18:3
9	1.218	1.186	18:1/18:1
10	1.252	1.232	16:0/18:1 18:0/18:2
11	1.289	1.278	20:0/18:3[d]
12	1.523	—	18:0/18:1
13	1.569	—	20:0/18:2

[a]MGDG, monogalactosyldiglyceride; DGDG, digalactosyldiglyceride.
[b]16:0/18:2 Used as reference standard; absolute retention times corrected in respect to dead volume.
[c]Fatty acid at *sn*-1 (left) and *sn*-2 (right) position, respectively. For analog diacyl-unsaturated, the *sn*-1 position is assumed for the less unsaturated fatty acid.
[d]Identification estimated from log RRT/equivalent chain-length, described (ref. 30).

TABLE 2

Distribution of Molecular Species of MGDG and DGDG from Wheat Flour[a]

Molecular species	% (± σ) (n = 3) MGDG	DGDG
18:2/18:3	4.46 (± 0.13)	7.17 (± 0.17)
18:2/18:2	41.51 (± 0.84)	51.97 (± 0.17)
16:0/18:3	0.45 (± 0.06)	2.32 (± 0.05)
16:0/20:4	4.14 (± 0.07)	—
18:3/20:1	1.57 (± 0.04)	—
18:1/18:2	9.05 (± 0.08)	9.33 (± 0.07)
16:0/18:2	15.41 (± 0.18)	23.81 (± 0.29)
18:0/18:3	3.70 (± 0.04)	—
18:1/18:1	0.74 (± 0.05)	1.16 (± 0.02)
16:0/18:1	4.57 (± 0.04)	4.00 (± 0.02)
18:0/18:2		
20:0/18:3	0.64 (± 0.03)	0.25 (± 0.03)
18:0/18:1	9.51 (± 0.07)	—
20:0/18:2	4.12 (± 0.10)	—

[a]Quantitation was performed by reversed-phase high-performance liquid chromatography as described in the Experimental Procedures section. Abbreviations as in Table 1.

two molecular species, 16:0/18:1 and 18:0/18:2. Assignment of minor molecular species was carried out through extrapolation of the graphic plot of log RRT *vs.* ECL (equivalent chain length), as described previously (30).

The quantitative distribution of molecular species of MGDG and DGDG is given in Table 2. Among common fatty acids of diglycerides, linoleic acid at the second position, combined with linoleic, oleic and palmitic acids at the *sn*-1 position, accounted for more than 65% (MGDG) and 75% (DGDG) of the total respective molecular species, with pairs 18:2/18:2 and 16:0/18:2 the most predominant (Table 2), in good agreement with previously reported results for wheat (21,26) and rye (24) flours. The pairs 16:0/20:4, 18:3/20:1, 18:0/18:3, 18:0/18:1 and 20:0/18:2 were quantitated only among MGDG molecular species (Table 2). Minor common diglyceride molecular species included 16:0/18:3, 18:1/18:1, 16:0/18:1, 18:0/18:2 and 20:0/18:3 as fatty acid distribution. When data are compared with previous findings, a similarity in fatty acid distribution between PC and DGDG, and *N*-acyl-phosphatidylethanolamine (NAPE) and MGDG, respectively, is noticed among prominent molecular species (30) separated from the same wheat flour.

Molecular species determination of galactosylmonoglycerides classes. Molecular species determination of MGMG and DGMG was carried out by RP-HPLC of their respective 1-acyl-2-benzoyl-3-galactosylbenzoate derivatives, as illustrated in Figure 3. Capillary GC of the respective methylated fatty acid at the *sn*-1 position after transesterification of the collected RP-HPLC peaks made the identification of MGMG and DGMG species of wheat flour possible.

Five common molecular species with 16:0, 18:0, 18:1, 18:2 and 18:3 fatty acids, respectively, were determined (Table 3). The most prominent species include 18:2 and accounts for more than 74% of the total molecular forms in both MGMG and DGMG, which is in good agreement with the main fatty acid of lysoPC species from the same wheat flour (30). The next major fatty acids were 18:1 (MGMG) and 16:0 (DGMG), respectively. No previous

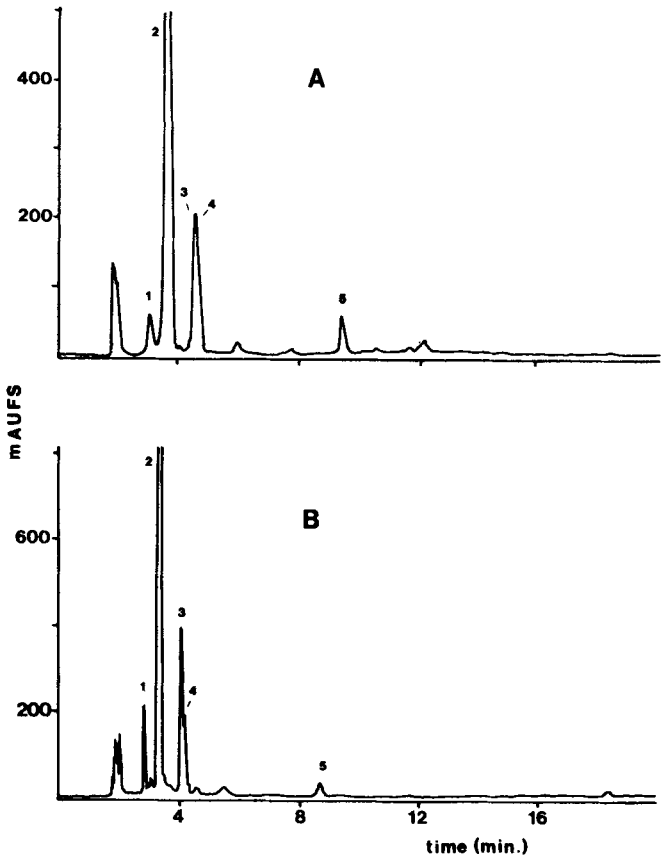

FIG. 3. Reversed-phase high-performance liquid chromatography of benzoate derivatives of molecular species of digalactosylmonoglyceride (A) and monogalactosylmonoglyceride (B), following the elution program described in the Experimental Procedures section. Peak number identification is shown in Table 3.

TABLE 3

Molecular Species Composition of MGMG and DGMG from Wheat Flour[a]

	% (± σ) (n = 3)	
Fatty acid/code	MGMG	DGMG
16:0/4	4.62 (± 0.11)	12.20 (± 0.50)
18:0/5	1.63 (± 0.11)	1.00 (± 0.59)
18:1/3	11.29 (± 0.23)	8.30 (± 0.34)
18:2/2	77.60 (± 0.48)	74.69 (± 0.16)
18:3/1	4.50 (± 0.14)	3.81 (± 0.31)

[a]Abbreviations as in Table 1.

data have been found in the literature concerning research of molecular species of monoglyceride classes.

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