

# Isolation of Polar Lipid Classes from Wheat Flour Extracts by Preparative High-Performance Liquid Chromatography

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## ABSTRACT

Cereal Chem. 78(6):663–665

A preparative high-performance liquid chromatographic method was developed for the isolation of polar lipid classes of mono- and digalactosyldiacylglycerols (MGDG and DGDG), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylcholine (PC) from wheat flour lipid extracts in relatively large quantities. The method allowed the

separation of  $\leq 100$  mg/injection of lipid mixture. MGDG, DGDG, and PC were isolated as pure lipid classes, whereas PE was isolated in a mixture with PG, PC, and lysophosphatidylcholine (LPC). The availability of this methodology will facilitate research aimed at investigating the functional role of such lipids in foods.

Lipids are quantitatively minor components of wheat flour occurring at levels of 1.5–2.0% (w/w), although they can have important functional effects in baked products (Chung et al 1978). These lipids occur as a complex mixture, comprising a number of lipid classes ranging in polarity from nonpolar classes, such as triacylglycerols and steryl esters, to very polar classes, such as galactolipids and phospholipids (Morisson 1983). Baking studies aimed at assessing the functional role of the different flour lipid classes in determining the quality of baked products require relatively large amounts of individual lipid classes for addition to defatted flour. Separation of these lipid classes is usually achieved by methods such as thin-layer chromatography (TLC), column chromatography, and countercurrent extraction. While these procedures are powerful means of separating lipid mixtures for characterization, they suffer from several disadvantages for preparative separations including one or more of the following: limited capacity, poor resolution, and long separation times with the attendant hazard of degradation of labile lipids if the separation is too slow. The first-mentioned disadvantage is particularly troublesome when the separated lipids are required for studies of functionality.

The literature describes an efficient high-performance liquid chromatography (HPLC) method for separation of wheat flour lipids on an analytical scale (Conforti et al 1993). However, good preparative methods have not been described for the full range of wheat flour lipids. Preparative HPLC was used in production of phospholipids from soybean lecithin (Hanras and Perrin 1991) and from egg lecithin (Geurts van Kessel et al 1981, Amari et al 1990). Also, Bergqvist and Herslof (1995) employed semipreparative HPLC to purify digalactosyldiacylglycerols (DGDG) previously isolated by means of solid-phase extraction from oat kernel lipid extracts. In the past, crude fractions of DGDG and monogalactosyldiacylglycerols (MGDG) were isolated from wheat flour using a simple batch adsorption technique with silica gel (DeStefanis and Ponte 1969). This method was able to separate relatively large amounts of galactolipids, but the fractions obtained were very crude and phospholipid classes were not fractionated.

The objective of this research was the development of a preparative HPLC method for the rapid isolation of relatively large quantities of very pure galactolipids and phospholipids from wheat flour lipid extracts without incorporating any preliminary fractionation step. The availability of such lipids will facilitate studies aimed at determining their functional roles in baked products.

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## MATERIALS AND METHODS

### Extraction of Flour Lipids

The extraction procedure was based on that of MacRitchie and Gras (1973). Flour cultivar Consort, commonly used for cookie (biscuit) making (1 kg, milled at Heygates Mills, Carlisle, UK) was used. This was mixed with chloroform (1.5 L) in a stainless steel bucket and stirred with a glass rod for 3 min. The resulting slurry was filtered through a sintered glass filter under vacuum. This procedure was repeated twice with fresh chloroform each time. Lipids dissolved in chloroform were filtered through Whatman No. 1 filter paper under vacuum to remove residual flour. The chloroform was evaporated under vacuum on a rotary evaporator at 30°C and the last traces of chloroform were removed by flushing nitrogen through the sample. The extraction produced 14.5 g of flour lipids (1.45%). These lipids were then redissolved in chloroform under nitrogen and stored at –20°C until required. Chloroform extracts virtually all the nonstarch lipid, which comprises three quarters of flour lipids, and which is known to have technological importance, but not the starch lipids, which are associated with starch granules. The latter can only be extracted using more polar solvents such as hot water-saturated butanol. However, this solvent brings about deleterious effects on other flour components such as proteins that are undesirable when the flour is to be used for baking studies (MacRitchie and Gras 1973).

### Development of Preparative HPLC Method

All natural lipid extracts (dissolved in chloroform) were filtered through Acrodisc PTFE CR disposable filters (0.45  $\mu$ m pore size, Whatman, Kent, UK), and evaporated with a stream of nitrogen.

TABLE I<sup>a</sup>  
Optimized Gradient Elution System Used for Separation  
of Major Polar Lipid Classes

Step	Time (min)	% A	% B	% C	Gradient Form <sup>b</sup>
0	0.4	100	0	0	0
1	1	95	5	0	1
2	5	30	68	2	2
3	10	40	51	9	3
4	15	40	50	10	2
5	15	40	60	0	0
6	15	100	0	0	0

<sup>a</sup> A = hexane: tetrahydrofuran (99:1); B = isopropanol; C = water; the flow rate was 6.0 mL/min throughout the chromatography run.

<sup>b</sup> Numbers describing the gradient form relate to the way the gradient elution of solvents between steps takes place (Perkin-Elmer Operator's Manual). Zero indicates an instant change from one step to the next, whereas 1 refers to a linear gradient between the steps, and 2 indicates that the gradient from one step to the next is a slightly concave curve following the equation:  $y = 0.16e^{0.006x}$  in the first half, but then it is almost linear in the second. Parameter  $y$  is 100 $\times$  the solvent ratio, whereas  $x$  is time (min). Finally, 3 indicates a concave gradient with a steeper curvature:  $y = 0.474e^{0.0089x}$ .

They were then redissolved in toluene before analysis at a concentration of 200 mg/mL. The injection consisted of 500  $\mu$ L of each sample.

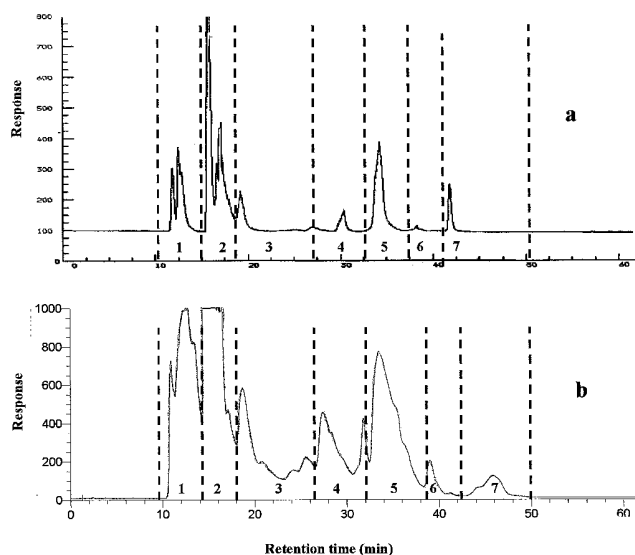
The preparative HPLC method was developed from the analytical HPLC method for the characterization of flour lipid classes (Conforti et al 1993). Lipid extracts were separated on a Supelcosil Si-60 silica column (12  $\mu$ m; 250  $\times$  21.2 mm; Sigma, Poole, UK). The optimized gradient elution system used for the separation of the major polar lipid classes is given in Table I. The solvents used were A, hexane-tetrahydrofuran (99:1); B, isopropanol; and C, water. All solvents were degassed before analysis.

The HPLC system consisted of a Perkin-Elmer 410 series controller with dual pumps, Perkin-Elmer ISS-200 autosampler with a 500- $\mu$ L injection loop and a data acquisition controller with analysis software (Atlas). The detector used was an evaporative light scattering detector from Perkin Elmer, model 750/14. The drift tube temperature was set at 45°C. A stream splitter was connected at the end of the column to allow the separation of the mobile phase into two streams passing through the detector (15%) and the fraction collector (85%). The capacity of the preparative column was checked by injecting increasing amounts of flour lipid mixture in the range of 5–200 mg.

The timings corresponding to the collected fractions (one collection tube/min) were compared with the retention times of the peaks in the chromatogram and the tubes were combined accordingly. After the individual lipid fractions were collected, the solvents were removed under vacuum and the yields were calculated on a weight basis. Analytical-scale HPLC using gradient elution blending A, hexane-tetrahydrofuran (99:1); B, isopropanol; and C, water as described by Conforti et al (1993) was performed to characterize and assess the purity of the collected fractions.

## RESULTS AND DISCUSSION

HPLC chromatograms for the separation of flour lipid extract are shown in Fig. 1. Low loading of lipids on the column gave good resolution but injection of 100 mg of lipid mixture per injection onto the column represented a balance between loss of resolution and speed of isolation of sufficient material. A total of seven fractions were collected after injection of 100 mg per run. All the neutral lipid classes including triacylglycerols (TG), diacylglycerols (DG), and monoacylglycerols (MG) were eluted as a mixture during



**Fig. 1.** HPLC chromatograms of a wheat flour lipid extract analyzed with a Supelcosil Si-60 silica column (12  $\mu$ m; 250  $\times$  21.2 mm) with gradient elution at 6 mL/min as described in Table I. Numeric indices are fraction numbers. Injections of 10 mg of lipid (a), 100 mg of lipid (b).

the first 27 min of the chromatography run (fractions 1–3). This was confirmed by both TLC (result not shown) and analytical HPLC. After this period, the polar components of interest, galactolipids (fractions 4 and 5) and phospholipids (fractions 6 and 7) were eluted.

Addition of increasing amounts of isopropanol and water in the early stages (steps 1 and 2, Table I) of the elution was necessary to elute the nonpolar lipid classes rapidly. Steps 3, 4, and 5 were crucial for the separation of the polar lipid classes, whereas step 6 was included to restore the initial mobile phase. A flow rate of 6.0 mL/min for the eluting solvent was optimum for the resolution of the peaks of the polar lipid classes for this particular column.

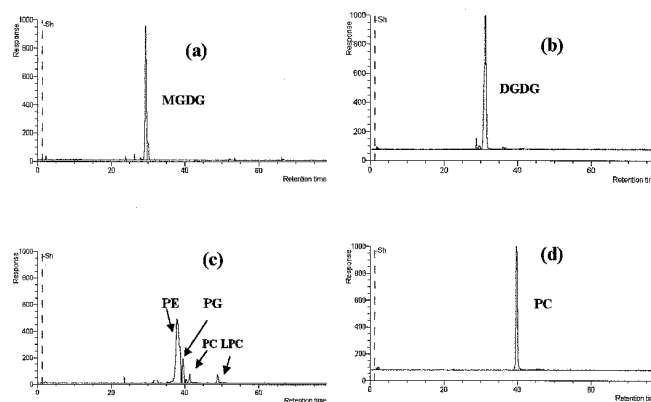
The analytical HPLC chromatograms for each of the polar fractions are shown in Fig. 2. Fraction 4 contained 95% MGDG and fraction 5 contained 94% DGDG. Fraction 6 mainly contained phosphatidylethanolamine (PE) (78%) and phosphatidylglycerol (PG) (14%), but small amounts of phosphatidylcholine (PC) (3.5%) and lysophosphatidylcholine (LPC) (4.5%) were also present. Finally, fraction 7 contained 97% PC. The degree of purity of these fractions was considered very satisfactory because the starting material was a complex lipid mixture and the separation of the polar lipid classes was achieved without any preliminary sample fractionation. The relatively short chromatographic run (61 min) allowed the separation to be repeated several times per day, thus providing a rapid means of production of several milligrams of very pure wheat flour galactolipid and phospholipid classes for baking studies. On average, 32.5 mg of MGDG, 55 mg of DGDG, 16 mg of PE and PG mixture, and 13 mg of PC were produced each day using this method.

However, the yield of isolated lipids was  $\approx$ 65% of the initial injected mass, suggesting that some material was retained on the column. Therefore, the column was washed with a blank run at the start of each day to remove any remaining material that may reduce the efficiency of the column. The solvent consumption per chromatographic run was 203 mL of hexane, 150 mL of isopropanol, and 15 mL of water. This is quite low compared with the volumes of solvents required for the separation of smaller amounts of lipid classes using traditional fractionation methods, such as column chromatography.

In conclusion, this preparative HPLC method enables researchers to produce relatively large amounts of highly pure wheat flour polar lipid classes to assess their functionality in baking studies.

## ACKNOWLEDGMENTS

We thank United Biscuits Ltd. Group Research and Development, UK, for providing facilities and some financial support for this research, and the State Scholarships Foundation of Greece for financial support.



**Fig. 2.** Analytical HPLC chromatograms of the four main polar flour lipid fractions isolated using the preparative technique in this study. Fractions a–d: 4, 5, 6, and 7. MGDG = monogalactosyldiglyceride; DGDG = digalactosyldiglyceride; PE = phosphatidylethanolamine; PG = phosphatidylglycerol; PC = phosphatidylcholine; and LPC = lysophosphatidylcholine.

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[Received October 27, 2000. Accepted May 18, 2001.]