

Estimation of Free Glycolipids in Wheat Flour by HPLC¹

J. B. Ohm^{2,3} and O. K. Chung²⁻⁴

ABSTRACT

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The chloroform-acetone mixture (4:1, v/v) was an effective solvent for eluting the nonpolar lipid fraction, including free fatty acids, from the polar lipid (glycolipid and phospholipid) fractions from free lipids of 21 hard winter wheat flours using a solid-phase extraction system. Amounts of monogalactosyldiglycerides (MGDG) and digalactosyldiglycerides (DGDG) in the glycolipid fraction were determined by normal-phase HPLC with a gradient system using an evaporative light-scattering detector (ELSD) and a diode array detectors (DAD). Unsaturated fatty acids

showed higher UV absorbances from 200 to 213 nm when compared with saturated palmitic acid. However, significant linear correlation coefficients were obtained between the peak areas measured by a DAD and GL contents determined by an ELSD, suggesting that fatty acid composition of flour GL could be fairly constant. Using an ELSD as a reference, equations for determination of MGDG or DGDG quantities were derived from the peak areas of a DAD by multivariate regression methods. Determination of MGDG and DGDG quantities was also possible using only a DAD.

The content of free lipids (FL) in wheat flour has been recognized as an important quality factor in breadmaking (Chung et al 1978). Glycolipid (GL) content has a significant linear correlation with bread loaf volume and a curvilinear relationship with mixing time (Chung et al 1982). FL was extracted from wheat flour with petroleum ether by a Soxhlet system (Chung et al 1980) and fractionated into lipid classes by silicic acid open-column chromatography (Fisher and Broughten 1960, Pomeranz et al 1966, Rouser et al 1967, Bekes et al 1986). Nonpolar lipids (NL), GL, and phospholipids (PL) have been eluted sequentially by chloroform, acetone, and methanol. However, this procedure required a large amount of solvent and time to elute each lipid class solely based upon gravity and on differential solubilities. Unless a great deal of time and chloroform were used for eluting NL, an incomplete separation resulted in free fatty acids being eluted into the GL fraction by acetone.

Recently, solid-phase extraction (SPE) systems with prepacked columns have been applied to separate lipids (Ebeler and Shibamoto 1994). The method has some advantages over open-column chromatography, including less solvent consumption, less hazardous conditions, less use of glassware, shorter sample handling time, and higher analyte recoveries (Wachob 1991). Prieto et al (1992) reported the use of an SPE cartridge for the fractionation of wheat flour lipids extracted by water-saturated butanol.

HPLC also has been used to analyze wheat flour lipids. Because of the significant relationships between GL and the baking characteristics of flours (Chung et al 1980, 1982; Bekes et al 1986), HPLC separation of FL has focused on the characterization and quantification of GL classes (Tweeten 1979, Christie and Morrison 1988, Prieto et al 1992). Tweeten et al (1981) separated GL into molecular species of monogalactosyldiglycerides (MGDG) and digalactosyldiglycerides (DGDG) by reverse-phase HPLC using a

refractive index detector. Marion et al (1988) reported isocratic separation of wheat flour lipids into GL and PL classes using acetonitrile, methanol, and sulfuric acid as mobile phases, and silica gels as stationary phases.

Most lipids display absorbance in the region of 200 to 220 nm, which is determined predominantly by isolated C-C double bonds and partly by other functional groups (Christie 1987). This implies that quantification of a lipid class by a UV detector in the low UV range is difficult when the fatty acid unsaturation of the lipid class is not constant. A refractive index detector also does not allow gradient analysis and shows unstable responses when ambient temperature is fluctuating.

The limitations of a UV or a refractive index detector have been overcome by the development of an evaporative light-scattering detector (ELSD). The advantages of an ELSD include gradient elution and analysis at relatively high temperatures, which decreases the unstable detection caused by the fluctuation of ambient temperature (Shukla 1988). Using an ELSD, Christie (1985) separated lipid classes from animal tissue and Christie and Morrison (1988) separated GL classes from wheat flour lipids. Conforti et al (1993) also separated and quantified the classes of wheat flour lipids using an ELSD.

However, the ELSD also has disadvantages. Moreau (1994) pointed out that it requires large amounts of gas for nebulization, and the response is not linear to the mass of the lipid when the lipid concentration is too low or too high. Also, the detection of a highly volatile solute is difficult. The peak area of ELSD had a linear response with the mass of most lipids in the range of ≈ 10 – $200 \mu\text{g}$ (Moreau 1994).

In addition to fixed- or variable-wavelength UV detectors, a diode array detector (DAD) has been used in HPLC. The DAD allows scanning a range of wavelengths at $\approx 100 \mu\text{sec}$ intervals (Moreau 1994). The DAD appears more useful for the analysis of lipids than ELSD as no gas is required for detection and sensitivity is higher.

The objectives of this research were to 1) develop SPE conditions for the separation of FL of wheat flour into NL, GL, and PL; and 2) quantify the amounts of MGDG and DGDG in GL using a DAD.

MATERIALS AND METHODS

Materials

This experiment used straight-grade flours milled from 21 wheats harvested in Kansas in 1993. Flours were milled on a Brabender Quadrumat Sr. experimental mill. All the organic solvents and reagents used in this experiment were chromatography and analytical grades, respectively. The MGDG and DGDG standards were from Sigma Chemical Co. (St. Louis, MO).

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² Graduate research assistant and professor, respectively, Department of Grain Science and Industry, Kansas State University, Manhattan, KS 66506.

³ Postdoctoral research chemist, and supervisory research chemist, respectively, USDA-ARS, Grain Marketing & Production Research Center, Manhattan, KS 66502.

⁴ Corresponding author. E-mail: okchung@usgmrl.ksu.edu Phone: 785-776-2703. Fax: 785-776-2792.

Extraction and Fractionation of FL

FL were extracted from 10 g of flour with petroleum ether using a Soxhlet system (Chung et al 1980). The SPE system used a pre-packed column (Mega Bond Elut Bonded Phase SI 1GRM Varian) with a vacuum manifold (Visiprep Solid Phase Extraction Vacuum Manifold, Supelco Inc. Bellefonte, PA). After a column was conditioned with 5 mL of chloroform, the FL were applied in 2 mL of chloroform. The NL, GL, and PL fractions were separated by successive elution with 10 mL of a chloroform-acetone mixture (4:1, v/v), followed by 15 mL of an acetone and methanol mixture (9:1, v/v), and 10 mL of methanol, respectively. The flow rate of the SPE was adjusted to 0.7 mL/min by applying a 2.5-in. vacuum (6.35 mm Hg) using a vacuum manifold. The recovery of fractionated lipids was 95–100% of extracted FL. After the solvents were evaporated to \approx 5 mL with a rotary evaporator, the separated lipid fractions were dried under a nitrogen stream.

The purity of fractionated lipids was determined by thin-layer chromatography (TLC). Fractionated lipids (\approx 100 μ g) were applied to a TLC plate (Fisher gel G 20 \times 20). The spotted lipids were developed by a mixture of chloroform, methanol, and water (65:25:4, v/v) and detected by spraying the plate with a 0.2% α -naphthol in ethanol followed by light spray of 95% sulfuric acid. After the plate was heated to 120°C, GL were identified as dark gray purple spots (data not shown).

HPLC of the GL Fraction

A Hewlett-Packard (Palo Alto, CA) HPLC system (HP 1090 liquid chromatograph) was used with a short analytical column (HP Hypersil 5 μ m, 100 \times 4.6 mm i.d.). The GL fraction obtained by SPE separation was diluted in 1 mL of hexane, and 20 μ L of diluted solution was injected. MGDG and DGDG fractions were separated by a gradient of hexane, isopropanol, and water: 0 min (76.2:22.0:1.8), flow rate 2.5 mL/min; 3.5 min (68.0:30.0:2.0), flow rate 2.6 mL/min; 6.5 min (68.0:30.0:2.0), flow rate 2.6 mL/min; 7.0 min (76.2:22.0:1.8), flow rate 2.6 mL/min. The solute was detected successively by a DAD (HP 1040 diode array detector) and an ELSD (Varex MK III, Deerfield, IL). The absorbance was measured at 209, 212, 215, and 218 nm with 4-nm bandwidth by a DAD using absorbance at 597 nm as a reference. Then the flow rate was adjusted to 1.35 mL/min with a solvent flow splitter (Varex) before detection by an ELSD. The drift tube temperature was adjusted to 110°C with an exhaust temperature of 45°C. Nitrogen gas flow rate was set at 2.00 standard L/min. Calibration equations were derived using 30, 50, 70, 90, and 110 μ g of standard MGDG or DGDG based on peak areas obtained from an ELSD and a DAD.

UV Scanning of Fatty Acids

To investigate the effect of unsaturation of fatty acids on UV absorbance, palmitic acid, oleic acid, and linoleic acid (1 mg/mL of hexane) were scanned from 200 to 225 nm in 1-nm intervals using a spectrophotometer (Unicam SP 1750, Pye Unicam Ltd., Cambridge, England).

Derivation of Estimation Equations for MGDG and DGDG

The amounts (mg) of MGDG and DGDG in FL of 21 wheat flours (10 g, db, each) that were estimated by the ELSD were used as references to derive an estimation equation using peak areas

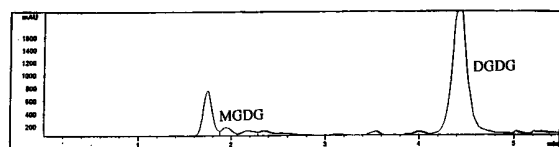


Fig. 1. HPLC results for glycolipid fraction separated by a solid-phase extraction system. Detector was an evaporative light-scattering detector. MGDG = monogalactosyldiglycerides, DGDG = digalactosyldiglycerides.

obtained by the DAD. Peak areas of MGDG and DGDG obtained from the absorbance at 209 nm (A209), 212 nm (A212), 215 nm (A215), and 218 nm (A218), and the ratios of peak areas (A209/A215 and A209/A218) were included in equation development by stepwise multiple regression (SMR). Principal component scores (PCS) also were calculated using A209, A212, A215, and A218, and the ratios of peak areas (A209/A215 and A209/A218). The PCS then were used in stepwise multiple regression (PCSMR) for the derivation of the estimation equations. The SMR and principal component analyses were performed using SAS methods (SAS Institute, Cary, NC). The correlation coefficients (r) and coefficient of determination (R^2) were calculated using the average of two replicates by SAS.

RESULTS AND DISCUSSION

Fractionation of Flour Lipids Using an SPE System

Flour FL was fractionated into three classes (NL, GL, and PL), using the SPE system with a prepacked column. Separation of NL by chloroform alone using silicic acid open-column chromatography has been ineffective. Therefore, separation of NL was attempted with the chloroform-acetone mixture (4:1) using the SPE system. The results were a much clearer separation of NL than by chloroform only, which was confirmed by the TLC chromatogram (data not shown). The clearer separation of NL by chloroform-acetone mixture could be due to the higher polarity than chloroform alone. Our results also demonstrate the advantage of SPE system over open-column chromatography for the fractionation of FL from wheat flour because it requires less solvent consumption, less hazardous conditions, less use of glassware, and shorter analysis time.

HPLC of the GL Fraction

The HPLC of the GL fraction separated by SPE was performed at moderately fast flow rates with a short column to decrease analysis time as much as possible. A gradient of hexane-isopropanol-water was selected as a mobile phase in this experiment because of the compatibility with a DAD and gradient analysis (Jungalwala et al 1976). Two main wheat flour GL classes, MGDG and DGDG, were eluted from the GL fraction in 5 min, and an additional 5 min of elution was suitable to stabilize the column for the next analysis (Fig. 1). Routine analysis assured that retention times were stable and reproducible: the mean peak times and standard deviations ($n = 21$) were 1.75 min and 0.04 for MGDG and 4.42 min and 0.07 for DGDG, respectively. The gradient of flow rate and mobile phase contributed to improving resolution of the MGDG peak from a small peak whose identity was not known, even though the flow rate was changed only from 1.45 to 1.50 mL/min. Other GL classes were not detected significantly by the ELSD in this analysis, except a small peak eluted just after the MGDG peak, possibly because of the small amounts present. Two additional small peaks were detected during column stabilization but were not included in the chromatogram integration due to the minor quantity and unavailability of the corresponding standard lipids.

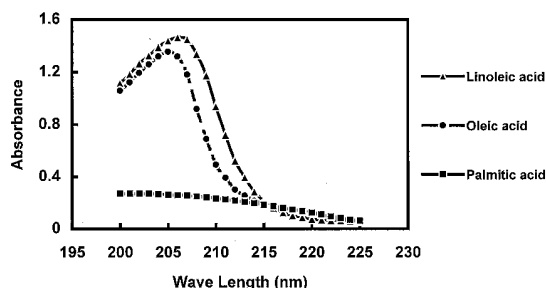


Fig. 2. UV absorbance of fatty acids. Concentration of each standard was 1 mg/mL in hexane. Each lipid was scanned from 200 to 225 nm in 1-nm intervals using a UV spectrophotometer.

The calibration equations were derived using 30, 50, 70, 90, and 110 µg of MGDG and DGDG standards based on the peak areas of an ELSD and a DAD. Those peak areas showed highly linear correlations to the mass of MGDG and DGDG. The linearity of peak areas and lipid mass for the ELSD was confirmed by R^2 values of 0.987 and 0.999 for DGDG and MGDG, respectively. Linearity of the ELSD peak areas over the range of 10–200 µg was observed for most lipids by Moreau (1994) and for flour MGDG and DGDG by Conforti et al (1993). The ranges for GL from 21 wheat flours were 2.7–9.3 (mg/10 g of flour, db) for DGDG and 1.6–4.0 (mg/10 g of flour, db) for MGDG.

Responses of Fatty Acids to UV Detectors

Moreau (1994) reported that the responses of fatty acids to UV detectors are proportional to the degree of unsaturation and observed that the UV signal response of lipids at 205 nm proportionally increased as the degree of unsaturation of lipids increased. Palmitic acid, oleic acid, and linoleic acid were selected to investigate the effect of the double bonds on the UV absorbance of fatty acids, because they are main fatty acids found in flour lipids (Tweeten et al 1981). Palmitic acid is a saturated fatty acid that contains 16 carbons. Oleic and linoleic acids are 18-carbon unsaturated fatty acids, having one and two C-C double bonds, respectively. Linoleic acid and oleic acid, which are unsaturated fatty acids, showed notably higher absorbances in the range of 200–214 nm when compared with saturated palmitic acid (Fig. 2). However, linoleic acid showed higher absorbance than oleic acid over the range of 206–213 nm but showed slightly lower absorbance at >215 nm. The absorbances were similar for all three acids at >214 nm. These results suggest that the effect of double bonds is minimal at >214 nm. Shukla (1988) also investigated this problem using triglycerides that had different fatty acid compositions with different degrees of unsaturation. They found that the double bonds disturbed the quantification of triglycerides at <218 nm, but quantification of triglycerides could be achieved at 220 nm, largely because of the ester C=O functional group. Thus, lipid amount can be determined by a UV detector after selection of proper wavelengths where the unsaturation of fatty acids would not influence the absorbance readings. A UV detector has several advantages over an ELSD, including no need for gas and smaller sample requirement for quantification. Thus, a UV detector seems preferential in HPLC for the routine analysis of lipids whenever possible.

Estimation of DGDG and MGDG by DAD

The estimation of calibration equations for lipid quantification by the DAD was possible by using the ELSD as a reference detector, unaffected by the unsaturation of fatty acids. When the lipid quantifying analysis was performed using the DAD, the HPLC flow could be scanned simultaneously in the various UV ranges. This notable advantage of the DAD was expected to make lipid analysis

as a routine test more convenient in flour quality evaluation. The HPLC peak areas of MGDG or DGDG also were measured at 209, 212, 215, and 218 nm, using the DAD and the ELSD for 21 wheat flours. The peak areas of MGDG and DGDG decreased noticeably as the detecting UV wavelength increased, probably because of saturation of fatty acids. The ratios of A209 to A218 were higher than the ratio of A209 to A215 in both MGDG and DGDG.

Linear correlation coefficients were calculated between peak areas measured by the DAD and the amounts of MGDG and DGDG determined by the ELSD for 21 wheat flours (Table I). The peak areas measured by the DAD were correlated highly with the weights of MGDG and DGDG, respectively, but showed higher r values for DGDG than for MGDG. These results indirectly suggest that the fatty acid compositions of MGDG and DGDG are not complex enough to disturb their quantification using a UV detector. Tweeten (1979) reported that the ratios of two main peaks of MGDG and DGDG separated by reverse-phase HPLC were constant for FL from 20 flours. The present result also indicates fairly constant fatty acid compositions of MGDG and DGDG among 21 samples. The ratios of peak areas (A205/A215 and A209/A218) had significant negative relationships with the amounts of MGDG and DGDG (Table I).

The estimation equations for MGDG and DGDG quantification were derived from the peak areas obtained by the DAD, using the lipid amounts of 21 flours attained by the ELSD. The peak area ratios were used under the assumption that they would be helpful in adjusting the effects of fatty acid double bonds on UV absorbance.

For DGDG, only A218 was included in the estimation equation by SMR (Table II). The PCS1, PCS2, and PCS4 were selected as

TABLE II
Parameters and Coefficient of Determination (R^2 , $n = 21$) of Estimation Equations for Glycolipids Derived from Peak Areas at Various Wavelengths from a Diode Array Detector Using an Evaporative Light-Scattering Detector as a Reference

Regression Method ^a	Parameters ^b	R^2	RMSE ^c
Digalactosyldiglycerides (mg/10 g of flour, db)			
SMR	A218	0.936	0.46
PCSMR	PCS 1,2,4	0.941	0.47
Monogalactosyldiglycerides (mg/10 g of flour, db)			
SMR	A218, A209/A212	0.834	0.29
PCSMR	PCS 1,4	0.845	0.28

^a SMR = stepwise multiple regression, PCSMR = principal component stepwise multiple regression.

^b A209, A212, and A218 = peak areas at 209, 212, and 218 nm, respectively; PCS = principal component score.

^c Root mean square of error.

TABLE III
Coefficient of Determination (R^2 , $n = 21$) Between Glycolipid Contents Determined by Peak Areas at Various Wavelengths from a Diode Array Detector and an Evaporative Light-Scattering Detector

Peak Areas ^a	R^2	RMSE ^b
Digalactosyldiglycerides (mg/10 g of flour, db)		
A209	0.904	0.57
A212	0.850	0.71
A215	0.841	0.73
A218	0.867	0.67
Monogalactosyldiglycerides (mg/10 g of flour, db)		
A209	0.730	0.36
A212	0.755	0.35
A215	0.789	0.32
A218	0.810	0.30

^a A209, A212, A215, and A218 = peak areas at 209, 212, 215, and 218 nm, respectively.

^b Root mean square of error.

TABLE I
Linear Correlation Coefficients Between Peak Areas and Amounts of Digalactosyldiglycerides (DGDG) and Monogalactosyldiglycerides (MGDG) of 21 Wheat Flours^a

Wavelength (nm) ^b	Glycolipids (mg/10 g of flour, db)	
	DGDG	MGDG
209	0.961**	0.813**
212	0.936**	0.843**
215	0.953**	0.866**
218	0.967**	0.864**
209/215	-0.484*	-0.747**
209/218	-0.616**	-0.491*

^a *, ** = Correlation coefficient significant at $P < 0.05$ and $P < 0.01$, respectively.

^b Peak areas of absorbance at each wavelength.

independent variables in the derivation of the estimation equation for DGDG by PCSMR. The standardized scoring coefficients of PCS indicated that PCS1 and PCS4 were mainly responsible for the variation of peak areas, and PCS2 was responsible for the variation of peak area ratios (data not shown).

The A218 and ratio of A209 to A212 were included by SMR in the derivation of estimation equations for MGDG (Table II). The peak area ratio of A209 to A212 was a significant variable at $P < 10\%$. The PCS1 and PCS4 were chosen by PCSMR as the best variables for the derivation of the estimation equation for MGDG. The R^2 values were slightly lower for MGDG than for DGDG, probably because of the narrower range in MGDG than in DGDG contents for the sample set in this study. Though the R^2 value was slightly higher with the PCSMR than with stepwise multiple regression, the SMR has a definite advantage over PCSMR due to its simplicity. These results suggest that the weights of DGDG and MGDG could be determined, using a DAD with the aid of the ELSD as a reference.

The amounts of GL were also determined by calibration equations that were derived from peak areas at various wavelengths using the DAD with known amounts of standards. The R^2 values between GL contents determined by each peak area from a DAD and by an ELSD are shown in Table III. The R^2 values were lower than those estimated from multivariate analysis (Table II). However, the R^2 values were high enough to apply a DAD in free GL analysis for wheat quality evaluation in wheat breeding program and industry. This result suggested that if an ELSD is not available, the amounts of MGDG and DGDG could be quantified by a UV detector because of highly significant correlations with the peak areas at various wavelengths.

SUMMARY

This research was performed to improve the analysis of FL from wheat flour for quality evaluation. FL extracted from 10 g of wheat flour can be separated into NL, GL, and PL by an SPE system. A chloroform and acetone mixture (4:1, v/v) was effective for separating the NL fraction including free fatty acids from the main GL and PL fractions by SPE. The elution of NL with chloroform alone frequently results in incomplete separation of free fatty acids from the GL fraction.

The amounts of MGDG and DGDG in the GL fraction were determined by a normal-phase HPLC in 10 min; the gradient of hexane-isopropanol-water eluted MGDG and DGDG in 5 min; and an additional 5 min of elution was sufficient to stabilize the column for the next analysis. Routine analysis assured that retention times were stable and reproducible day by day.

A higher UV absorbance of unsaturated fatty acids was observed at 200–214 nm when compared with saturated palmitic acid. High linear correlation coefficients occurred between the peak areas obtained by a DAD and GL contents determined by an ELSD, suggesting that fatty acid composition of flour GL is fairly constant. Estimation equations for MGDG or DGDG contents were derived from the peak areas at 209, 212, 215, and 218 nm by SMR and PCSMR. The results of multivariate regression suggested that the weights of MGDG and DGDG could be determined using a DAD with the aid of an ELSD as a reference. When other methodologies, that could determine lipid quantity more precise and accurate than ELSD, are available, they could be more useful as reference methods. Highly significant correlation coefficients

between the peak areas at various wavelengths and GL contents suggested that GL could be quantified using a UV detector even when the ELSD is not available.

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