WHEAT POLAR LIPIDS: SOURCES OF VARIATION AMONG NEAR-ISOGENIC WHEAT LINES WITH DIFFERENT ENDOSPERM HARDNESS

by

SEAN MCILWAIN FINNIE

B.S., University of Idaho. 2004
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Grain Science and Industry
College of Agriculture

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Abstract

Starch granule surface components were studied as a function of puroindoline haplotype, starch isolation method, and processing fraction. Commonly grown cultivars and near-isogenic wheat lines that varied in their wheat endosperm hardness were collected. Wheat whole-meal, flour and starch were evaluated for their polar lipid composition. Water-washed starch was isolated using a modified batter method and a dough method. Direct infusion tandem mass spectrometry was used to identify the lipid species in the extracts.

A total of 155 polar lipid species in wheat meal, flour and starch were quantitatively characterized. The predominant polar lipid classes were digalactosyldiglycerides, monogalactosyldiglycerides, phosphatidylcholine, and lysophosphatidylcholine. Wheat whole-meal, flour and surface-starch contained greater concentrations of total galactolipids while internal-starch lipids contained greater concentrations of monoacyl phospholipids.

Wide ranges in starch surface polar lipid concentrations were observed between the two starch isolation methods. Starch isolation methods provided a greater source of variation than did wheat kernel hardness. When dough is optimally mixed the lipids originally on the surface of wheat starch become incorporated into the gluten phase of the dough, whereas in a batter system the starch-surface lipids stay associated with the starch granule surface.

The greatest quantities of polar lipids on the starch surface occurred when both puroindoline proteins were present on starch in their wild-type form. Starch surface polar lipid content decreased dramatically when one of the puroindoline proteins was null, or if the puroindoline-b (pin-b) was in the mutated form (Tryptophan-44 to Arginine). Within the hard

textured samples, more polar lipids were present on the starch surface when pin-b was in its wild-type form and puroindoline-a (pin-a) was null than when pin-a was in its wild-type form and pin-b was null. The lowest amount of polar lipids were present when pin-b was mutated (Tryptophan-44 to Arginine) and pin-a was in its wild-type form. This indicates the relative importance of pin-b's presence and structure as it relates to lipid association with the starch granule surface.

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Dedication

To my parents, for only expecting me to try my best… regardless of the outcome.

CHAPTER 1 - Introduction

1.1 Background and Initial Goal

Wheat (*Triticum aestivum* L.) flour is a heterogeneous powder comprised of starch granules (70-75%), protein (10-14%), water (14%), non-starch polysaccharides (2-3%) and lipids (1-2%). It is unique in its ability to form a viscoelastic dough capable of retaining gases during fermentation and proofing. The functional properties of wheat flour are mainly influenced by the gliadin and glutenin storage proteins, which together comprise approximately 85% of the total wheat flour proteins. When dough is mixed to optimum (proper hydration and work applied), the gliadin and glutenin proteins interact and form a continuous protein macropolymer (i.e. gluten) surrounding the starch granules (Singh and MacRitchie 2001; Pomeranz 1988) (Figures 1.1). Figure 1.1 presents light microscopic images of dough that has been under mixed (Figure 1.1a) and optimally mixed (Figure 1.1b).

The interactions between and within gliadin and glutenin proteins are important for the functional properties of flour. Gliadins are monomeric proteins responsible for the viscous properties of gluten (Pomeranz 1988; Wrigley and Bietz 1988; Veraverbeke and Delcour 2002), while glutenins are polymeric proteins responsible for the elastic properties of gluten (MacRitchie 1980; Wrigley and Bietz 1988; Veraverbeke and Delcour 2002). A considerable amount of research has been conducted on the interactions between and within gliadin and glutenin proteins as it relates to flour functionality.

One foundational study conducted by Finney and Barmore (1948) found a positive correlation between bread loaf volume and concentration of gluten proteins. Further research has found that besides gluten protein content, gluten protein quality is important in flour

functionality. Gluten quality encompasses the gliadin to glutenin ratio as well as the composition, size distribution and structure of the gliadin and glutenin molecules (Veraverbeke and Delcour 2002).

Even with the considerable amount of research conducted on flour functionality and gluten quality, there are factors that remain unknown. Few conclusive research articles have studied the interaction between the gluten proteins (gliadin and glutenin) and the starch granule surface as it relates to flour functionality (Larsson and Eliasson 1997; Sandsted 1961; Hoseney et al. 1971). Larsson and Eliasson (1997) used *in vitro* modification of the starch granule surface (heat treatment, absorption of wheat protein and absorption of lecithin) and found that these treatments had an effect on the rheological properties of the dough. Those results indicate the importance of the starch granule surface as it relates to dough rheology. However, these results did not provide any indication of the baking performance differences among the treated starch samples. Additionally, the flours were diluted from 15 % to 10 % protein, which may have inadvertently altered the results, since the ratio of starch to protein was increased above a typical flour used for bread. With the large surface area that starch granules have in flour it is logical to hypothesize that the starch granule is not an inert filler and consequently it participates as a functional component of the flour in addition to its gelatinization during baking. Therefore, the initial goal of this research was to determine the functional role of the starch granule components.

1.2 Current Knowledge Regarding Starch Granule Surface Components

The intended end-use of wheat is initially determined by the physical hardness (the amount of force required to crush a kernel of wheat) of the wheat kernel. Hard wheat is milled for use in bread and some noodle flour, while soft wheat is milled for use in cake, cookie,

cracker and noodle flour. Durum wheat (*T. turgidum L. var. durum*), which lacks the D genome found in *Triticum aestivum* L., is the hardest class of wheat and is milled into pasta flour (semolina).

Greenwell and Schofield (1986) determined that grain hardness was caused by the absence of a 15 kDa protein on the surface of water-washed starch granules. These proteins, termed friabilin, were found in greater amounts on water-washed starch from soft wheat than on water-washed starch from hard wheat. Consistent with that trait, the friabilin proteins were absent on water-washed starch from durum wheat. Jolly et al. (1993) and Morris et al. (1994) further discovered the existence of two 15 kDa proteins, puroindoline A (pin-a) and puroindoline B (pin-b), which together comprise friabilin. Morris (2002) found that if one or both of the puroindoline proteins are mutated or absent, then the endosperm texture will become hard.

Similarly to puroindoline proteins, Greenblatt et al. (1995) found that a pattern existed among polar lipids on the surface of starch granules. Galactolipids and phospholipids were found, via thin layer chromatography, to be present in greater amounts on water-washed starch from soft wheat than on water-washed starch from hard wheat (Greenblatt et al. 1995). Konopka et al. (2005) found a negative correlation between starch surface lipids (polar and nonpolar) content and kernel hardness. However, the full profile of the lipid species found on starch granule surfaces has not been reported nor has the relative abundance of each polar lipid class been quantitatively determined.

Furthermore, most of the studies evaluating starch granule surface components have been conducted with starch isolated after dough development. It is unknown if the components on the starch granule surface participate in gluten formation and, thus, are no longer associated with the starch granule isolated after dough development.

1.3 Objectives

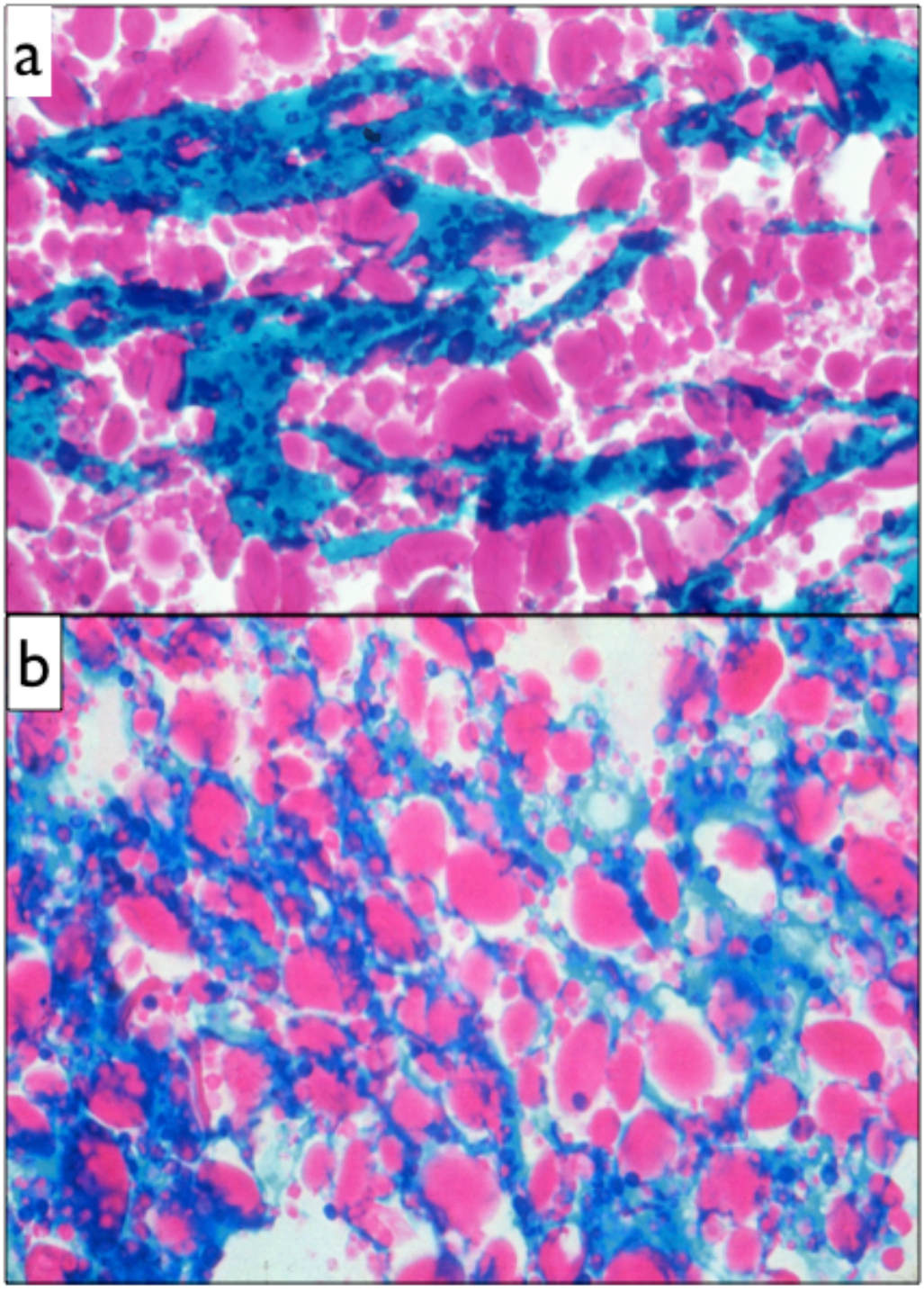
Before the initial goal of determining the functional role of the starch granule surface components can be achieved, further understanding of the starch granule composition is needed. To provide a broader understanding of starch granule surface components, the following objectives were created.

1) To use lipid profiling techniques and quantitatively determine the polar lipid species present in whole-wheat meal, flour, and starch.
2) To quantitatively determine the variation in polar lipids present on the surface of wheat starch granules.
3) To determine the specific polar lipid species present on the surface of wheat starch from near-isogenic wheat lines that contain different puroindoline haplotypes and endosperm hardness.

The following three chapters describe the background, experimental approach, results and discussion of the research implemented for the completion of the above objectives.

1.4 Figures

Figure 1.1: Light micrographs of (a) under-mixed dough and (b) properly mixed dough. Starch granules dyed pink and gluten proteins dyed blue. Images courteously provided by Dr. Finlay MacRitchie, originally taken by R. Moss.



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CHAPTER 2 - Quantitative Characterization of Polar Lipids from Wheat Whole-Meal, Flour and Starch

2.1 Abstract

Even though lipids comprise only a minor proportion of total flour components they have a significant influence on loaf volume and crumb grain of bread. Because the composition and structure of wheat flour polar lipids have an influence on the end-use quality of bread, it is important to determine specifically which lipid class and molecular species are present in wheat. Lipid profiling is a targeted, systematic characterization and analysis of lipids. The use of lipid profiling techniques to analyze grain-based food has the potential to provide new insight into the functional relationships between a specific lipid species and its functionality. The objective of this study was to utilize lipid profiling techniques to quantitatively determine the polar lipid species present in wheat whole-meal, flour, and starch. Two commonly grown wheat cultivars were used in this study, Alpowa and Overley. Direct infusion electrospray ionization tandem mass spectrometry was used to identify and quantitatively determine 155 polar lipid species in wheat whole-meal, flour and starch. The predominant polar lipid classes were digalactosyldiglycerides, monogalactosyldiglycerides, phosphatidylcholine, and lysophosphatidylcholine. ANOVA results concluded that wheat fraction contributed a greater source of variation than did wheat cultivars on total polar, total phospholipid, and total galactolipid contents. Wheat whole-meal, flour and surface starch contained greater concentration of total galactolipids while internal starch lipids contained greater concentrations of monoacyl phospholipids.

2.2 Introduction

Lipids constitute a relatively minor component (2.5-3.3%) of the wheat caryopsis. Between 30-36% of wheat lipids are located in the germ, 25-29% in the aleurone and 35-45% in the endosperm (Morrison 1988; Chung 1986; Chung and Pomeranz 1981; Hargin and Morrison 1980). Germ lipids are predominantly non-polar in character (77-85%) with polar lipids contributing a minor proportion (13-17%) (Morrison 1988). Aleurone lipids are similar in relative composition to germ lipids with non-polar lipids present at 72-83% and polar lipids present at 19-23% of the total (Morrison 1988). Wheat flour (i.e. endosperm) lipids are distinctly different in their relative composition compared to germ and aleurone lipids. Wheat flour lipids provide the only substantial source of galactolipids (predominately monogalactosyldiglycerides (MGDG) and digalactosyldiglycerides (DGDG)), and phospholipids (predominately lysophosphatidylcholine (LPC), phosphatidylcholine (PC), lysophosphatidylethanolamine (LPE), and N-acylphosphatidylethanolamine (APE)) among the various whole-grain components (Carr et al. 1992; Morrison 1988).

Wheat flour lipids are described as either total-flour lipids or non-starch lipids, depending upon their method of extraction. Lipids extracted from wheat flour under conditions that favor starch granule swelling or breakdown are described as total-flour lipids because they include lipids, which are strongly associated with the starch granule matrix (Morrison 1995; Carr et al. 1992; Morrison 1988). Conversely, flour lipid extraction under conditions that do not favor starch granule swelling or breakdown produces non-starch lipids which do not include those lipids strongly associated with the starch granule matrix (Morrison 1995; Carr et al. 1992; Morrison 1988).

As a further differentiation, wheat lipids are categorized as either 'free' or 'bound' depending on their relative solubility in solvents of opposing polarities (Greenblatt et al. 1995; Chung 1986; Zawistowska et al. 1984; Hoseney et al. 1969). Wheat lipids are extracted sequentially; free lipid extraction uses non-polar solvents such as hexane, petroleum ether or diethyl ether, followed by bound lipid extraction with a more polar solvent such as isopropanol-water, chloroform-methanol-water or water-saturated butanol (Greenblatt et al. 1995; Hoseney et al. 1969). This process does not provide a distinct division between the lipid class (non-polar vs. polar) because the composition and abundance of the lipid extracts are dependent on solvent polarity, temperature during extraction, and the sample's particle size (Chung et al. 1980). However, free lipids generally contain primarily the non-polar fractions (tri-, di, mono-acylglycerides and sterol esters) and the bound lipids contain mainly polar fractions (galactolipids and phospholipids) (Mann and Morrison 1974).

Even though flour lipids comprise only a minor proportion of total flour components (1-1.5%) they have a significant influence on loaf volume and crumb grain of bread through the stabilization (and destabilization) of gas cells (Sroan and MacRitchie 2009, MacRitchie and Gras 1973). Sroan and MacRitchie (2009) determined that endogenous wheat flour lipids do not have any influence on the rheological properties (biaxial extension) of dough, indicating the importance of wheat flour lipids as a surface-active component either stabilizing or destabilizing the gas cell structure of dough. Above a critical concentration polar lipids appear to have stabilizing effects on gas cells, resulting in bread with improved volumes; whereas non-polar lipids appear to have destabilizing effects on gas cells resulting in decreased bread volumes (MacRitchie and Gras 1973; Sroan and MacRitchie 2009).

Monolayers at the gas-liquid interface appear to play a major role in the mechanism of gas cell stability (Sroan and MacRitchie 2009). Such monolayers exist in either condensed or expanded orientations (Gains 1966). Condensed monolayers are formed by the tight orientation of the surface-active molecules (i.e. lipids and proteins) and are desirable for gas cell stability, providing resistance to collapse of the liquid lamellae (MacRitchie 1976). Expanded monolayers exist as a loose orientation of the surface-active molecules and are less resistant to collapse of the liquid lamellae (MacRitchie 1976). Long chain free fatty acids and DGDG tend to form condensed monolayers while shorter chain free fatty acid forms an expanded monolayer (Gains 1966 and MacRitchie 1990). Gains (1966) demonstrated that double bonds in fatty acids, especially those in the *cis* conformation, prevent the close packing of monolayers resulting in an expanded orientation. Sroan and MacRitchie (2009) concluded that differences in the gas cell stabilizing characteristics of the various wheat flour lipid classes is most likely due to differences in the adsorption of their monolayers at the gas-liquid interface.

Because the composition and structure of wheat flour polar lipids have an influence on the end-use quality of bread, it is important to determine specifically which class (DGDG, MGDG, PC, LPC, PE, LPE…) and species (combination of acyl groups) are present to influence the stabilization of gas cells. Lipid profiling (i.e. lipidomics) is a targeted, systematic characterization and analysis of lipids. Lipid profiling creates a comprehensive library of targeted lipid species in a sample; providing the lipid class, head group, and acyl group combination of those lipids, e.g. DGDG with an acyl group combination of 36 carbons and 4 double bonds. The profiling approach is based on mass spectrometry methods (typically electrospray ionization tandem mass spectrometry (ESI-MS/MS)) and provides quantitative data with a relatively rapid sample throughput (Devaiah et al. 2006; Welti and Wang 2004).

The use of lipidomic techniques to analyze grain-based food has the potential to provide new insight into the functional relationships between a specific lipid species and end-use quality. By quantitatively determining the specific lipid species present in wheat flours of different baking qualities (loaf volume and gas cell stability) relationships between specific lipid species and their gas cell stabilizing abilities can be determined. Therefore, the objective of this study was to use lipid profiling techniques to quantitatively determine the polar lipid species present in wheat whole-meal, flour, and starch. The results of this study will ultimately provide the ability to determine relationships between specific endogenous wheat flour lipid species and end-use functionality.

2.3 Experimental

2.3.1 Wheat Samples

Two commonly grown wheat cultivars were used in this study; 1) the soft white spring wheat cultivar Alpowa (PI 566596) and 2) the hard red winter wheat cultivar Overley (PI 634974). Alpowa was acquired from the United States Department of Agriculture-Agricultural Research Service, Western Wheat Quality Laboratory and grown in Washington State during the 2007 crop year. Overley was acquired from the Kansas State University, Department of Agronomy and was grown in Kansas during the 2007 crop year.

2.3.2 Milling

Alpowa wheat was milled using a Bühler experimental mill (Uzwil, Switzerland) as per, the American Association of Cereal Chemists International (AACCI) approved method 26-31 (AACCI 2008) tempered to 14 % moisture. Overley wheat was milled using a Quadrumat® Senior mill (Brabender® Instruments, South Hackensack, NJ), after being tempered to 15 % moisture. Wheat samples were milled with a feed rate range of 100 to 130 g/min. Whole-meal samples were milled with a cyclone mill (UDY Corp., Boulder, CO) to pass a 0.5 mm screen.

2.3.3 Starch Isolation

The starch isolation process was adapted from a commercial batter process described by Knight and Olson (1984), with modifications as follows. In this process, 30 grams of flour was hydrated to 140 mL of dH_2O. The samples were vortexed until all the flour was incorporated

into the water (~10 seconds). The samples were rested for 10 minutes, after which they were centrifuged at 100 x g for 5 minutes. After centrifugation the samples were filtered through a series of sieves (425 µm, 180 µm, 106 µm, and 75 µm opening sizes) (Dual MFG. Co., Chicago, IL) to remove gluten particulates from the batter. The thru samples were centrifuged at 4800 x g for 10 mins. After centrifugation the supernatant was decanted out and the different layers of the pellet separated. The pellet contained two main layers; a top layer containing the tailings, and a bottom layer containing the prime-starch. The two layers are separated and the prime-starch was dried for 24 hours at room temperature and then ground into fine powder using an analytical mill, (Tekmar, model A-10, Mason, Ohio). The tailings and gluten fractions were not evaluated further in this study.

2.3.4 Lipid Extraction

Whole-meal, flour and starch surface lipid extraction was conducted as described by Greenblatt et al. (1995) with the sequential extraction of free lipids with hexane and the bound lipids with isopropanol:water (90:10). Free and bound lipid extraction was conducted at room temperature (~21° C). Modifications to the Greenblatt et al. (1995) method were the use of one gram of starch instead of 12 grams.

Internal starch lipids were extracted immediately after bound lipid extraction from prime-starch. Internal starch lipids were extracted based in the method of Morrison and Coventry (1985). Five mL of isopropenol:water (90:10) was added to the starch pellet formed during the final centrifugation step in the starch bound lipid extraction. Prior to vortexing of the starch pellet and solvent, compressed nitrogen gas was flushed into the glass centrifuge tube the tube capped (Kimble HS 15mL, 18 x 102 , screw cap with PTFE liner). The nitrogen flushed samples

were heated to 90° C for 60 mins in a water bath. After heating the samples were centrifuged for 10 minutes at 4800 x g.

All lipid extracts (free, bound, and internal starch) were flushed with nitrogen until dry and dissolved in 1 mL of chloroform.

2.3.5 Lipid Quantification

The polar lipids targeted for analysis were; phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylglycerol (PG), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylglycerol (LPG), digalactosyldiglycerides (DGDG), monogalactosyldiglycerides (MGDG), digalactosylmonoglycerides (DGMG), monogalactosylmonoglycerides (MGMG), and sulfoquinovosyldiacylglycerol (SQDG).

An automated electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) approach was used, and data acquisition and analysis and acyl group identification were carried out as described previously (Devaiah et al. 2006) with modifications. Aliquots of 0.03 mL for wheat whole-meal and flour samples and 0.10 mL for free, bound, and internal starch samples were used. Precise amounts of internal standards, obtained and quantified as previously described (Welti et al. 2002), were added in the following quantities (with some small variation in amounts in different batches of internal standards): 0.60 nmol di12:0-PC, 0.60 nmol di24:1-PC, 0.60 nmol 13:0-lysoPC, 0.60 nmol 19:0-lysoPC, 0.30 nmol di12:0-PE, 0.30 nmol di23:0-PE, 0.30 nmol 14:0-lysoPE, 0.30 nmol 18:0-lysoPE, 0.30 nmol 14:0-lysoPG, 0.30 nmol 18:0-lysoPG, 0.30 nmol di14:0-PA, 0.30 nmol di20:0(phytanoyl)-PA, 0.20 nmol di14:0-PS, 0.20 nmol di20:0(phytanoyl)-PS, 0.23 nmol 16:0-18:0-PI, and 0.16 nmol di18:0-PI,16:0-18:0-MGDG, 0.39

nmol di18:0-MGDG, 0.49 nmol 16:0-18:0-DGDG, and 0.71 nmol di18:0-DGDG. The sample and internal standard mixture was combined with solvents, such that the ratio of chloroform/methanol/300 mM ammonium acetate in water was 300/665/35, and the final volume was 1.4 ml.

Unfractionated lipid extracts were introduced by continuous infusion into the ESI source on a triple quadrupole MS/MS (API 4000, Applied Biosystems, Foster City, CA). Samples were introduced using an autosampler (LC Mini PAL, CTC Analytics AG, Zwingen, Switzerland) fitted with the required injection loop for the acquisition time and presented to the ESI needle at 30 μl/min.

Sequential precursor and neutral loss scans of the extracts produce a series of spectra with each spectrum revealing a set of lipid species containing a common head group fragment. Lipid species were detected with the following scans: PC and lysoPC, $[M + H]^+$ ions in positive ion mode with Precursor of 184.1 (Pre 184.1); PE and lysoPE, $[M + H]^+$ ions in positive ion mode with Neutral Loss of 141.0 (NL 141.0); PG, $[M + NH4]^+$ in positive ion mode with NL 189.0 for PG; lysoPG, $[M – H]^-$ in negative mode with Pre 152.9; PI, $[M + NH4]^+$ in positive ion mode with NL 277.0; PS, $[M + NH4]^+$ in positive ion mode with NL 185.0; PA, $[M + NH4]^+$ in positive ion mode with NL 115.0; MGDG, $[M + NH4]^+$ in positive ion mode with NL179.1; and DGDG, $[M + NH4]^+$ in positive ion mode with NL 341.1. The scan speed was 50 or 100 u per sec. The collision gas pressure was set at 2 (arbitrary units). The collision energies, with nitrogen in the collision cell, were +28 V for PE, +40 V for PC, +25 V for PI, PS and PA, +20 V and PG, +21 V for MGDG, and +24 V for DGDG. Declustering potentials were +100 V for PE, PC, PA, PG, PI, and PS, and +90 V for MGDG and DGDG. Entrance potentials were +15 V for PE, +14 V for PC, PI, PA, PG, and PS, and +10 V for MGDG and DGDG. Exit potentials were

+11 V for PE, +14 V for PC, PI, PA, PG, PS, and +23 V for MGDG and DGDG. The mass analyzers were adjusted to a resolution of 0.7 u full width at half height. For each spectrum, 9 to 150 continuum scans were averaged in multiple channel analyzer (MCA) mode. The source temperature (heated nebulizer) was 100 °C, the interface heater was on, +5.5 kV or -4.5 kV were applied to the electrospray capillary, the curtain gas was set at 20 (arbitrary units), and the two ion source gases were set at 45 (arbitrary units).

The background of each spectrum was subtracted, the data were smoothed, and peak areas integrated using a custom script and Applied Biosystems Analyst software. The lipids in each class were quantified in comparison to the two internal standards of that class. The first and every 11^{th} set of mass spectra were acquired on the internal standard mixture only. Peaks corresponding to the target lipids in these spectra were identified and molar amounts calculated in comparison to the internal standards on the same lipid class. To correct for chemical or instrumental noise in the samples, the molar amount of each lipid metabolite detected in the "internal standards only" spectra was subtracted from the molar amount of each metabolite calculated in each set of sample spectra. The data from each "internal standards only" set of spectra was used to correct the data from the following 10 samples. Finally, the data were corrected for the fraction of the sample analyzed and normalized to the sample "dry weights" to produce data in the units nmol/g.

2.3.6 Statistical Analysis

Five replicated lipid extractions were conducted for each wheat fraction. Sources of variation were determined by conducting a two-way analysis of variation (ANOVA), with wheat cultivar and wheat fraction as the main sources of variation. Multiple mean comparisons were conducted using Scheffé's multiple comparison procedure. The Scheffé's procedure was used to

protect against a type I error, (Dowdy et al. 2004). All statistical analyses were conducted using Statistical Analysis System (SAS) software, General Linear Model (SAS Version 9.0; SAS Institute; Cary, NC). Type III Sums of Squares were reported.

2.4 Results and Discussion

2.4.1 Lipid profiling

The use of direct infusion of lipid samples into an ESI-MS/MS required minimal sample size and relatively simple sample preparation. In this study, phospholipids (PC, PE, PI, PA, PS, PG, LPC, LPE and LPG) and galactolipids (DGDG, MGDG, DGMG, and MGMG) head groups and the acyl carbon atoms amounts and double bonds were quantitatively determined in whole-meal, flour, native-starch, and internal starch fractions from two commercial wheat cultivars. Figure 2.1 illustrates those structures identified for each class with their predominate acyl carbon combination. Acyl chain position was determined based on the typical configuration of the unsaturated fatty acid predominantly located on the sn-2 carbon. Previous wheat lipid research has determined that the most prevalent fatty acids are 18:2 (50-65 %) and 16:0 (19-26%) (Chung et al. 2009 and reference therein). According to the ESI-MS/MS, acyl carbon and double bonds configurations of 36:4 and 34:2 were most prevalent in the wheat fraction extracts and, thus, we can reasonably conclude that the acyl combinations were 18:2/18:2 and 16:0/18:2. SQDG galactolipids were identified in this study. However, obtaining internal standards is difficult, so the relative amounts of SQDG can only be described within its own class. The molecular species distributions of SQDGs are discussed in more detail in a subsequent section.

2.4.2 Polar lipid class variation among wheat kernel fractions

The sources of variation and mean values for total polar lipids, total phospholipids, and total galactolipids are summarized in Table 2.1. Total phospholipids and galactolipids are

additive totals of all the phospholipid species evaluated (PC, PE, PI, PA, PS, PG, LPC, LPE, and LPG) and galactolipid species evaluated (DGDG, MGDG, DGMG, and MGMG) in the free and bound lipid extracts. Total polar lipid amounts are additive values of the total phospholipid and total galacotolipid amounts. Based on the two-way ANOVA *F*-values (Table 2.1), both main effects (wheat fraction and wheat cultivar) were significant sources of variation at $P = 0.0001$ for total polar lipids, total phospholipids and total galactolipids. The interactions between the main effects were also significant at $P = 0.0001$, however, the contributions of the interactions to the overall ANOVA model were minor compared to the main effects. Therefore interactions were considered of practical insignificance. Based on ANOVA *F*-values, wheat fraction (whole-meal, flour, starch surface, and internal starch) were a greater source of variation than were wheat cultivars on total polar, total phospholipid, and total galactolipid contents.

Wheat flour contained the greatest concentration of polar lipids with 5706 nmol/g and 4762 nmol/g for Alpowa and Overley, respectively (Table 2.1). The internal starch lipid samples for Alpowa and Overley contained moderate amounts of total polar lipids, 638 nmol/g and 582 nmol/g, respectively (Table 2.1). However, the starch lipids extracted at room temperature (surface starch) from Alpowa contained five-times the amount of polar lipids extracted from Overley (Table 2.1). These results are consistent with Greenblatt et al. (1995), who demonstrated that starch surfaces from soft wheat starch contained greater amounts of polar lipids than did starch from hard wheat.

Comparisons of the total galactolipids and total phospholipids from various wheat fraction, demonstrated that wheat whole-meal, flour and surface starch contained greater concentrations of total galactolipids while internal starch contained greater concentrations of phospholipids (Table 2.1). This is consistent with previous research, which demonstrated that

internal starch lipids are exclusively monoacyl phospholipids lipids (Morrison 1981; Lee and BeMiller 2008).

The major polar lipid classes for all wheat fractions were DGDG, MGDG, PC and LPC (Table 2.2). Table 2.2 and 2.3 displays mol% of total polar lipid for each lipid fraction. Total polar lipids are defined therein as the sum of the free and bound lipid extracts for each of the wheat fractions. Internal starch free lipids were not extracted because free lipids were extracted from the surface starch and thus no 'free' lipids would be present. The surface starch from Overley contained essentially no free lipids (Table 2.2), while Alpowa surface starch contained free lipids from the DGDG, MGDG, PC, LPC, PG, PA, PI, DGMG and PS polar lipid fractions (albeit in relatively minor mol% of the total polar lipids). In contrast, bound polar lipids from the surface starch were found in the greatest concentration in both the Alpowa and Overley samples in concentrations of 56.40 mol% and 41.56 mol% of total (Table 2.2). The isolated surface starch fraction contains significantly lower quantities of free lipids of all the polar lipid classes indicating that free lipids in the whole-meal and flour fractions are localized in the non-starch components of the grain (Table 2.2). The polar lipids extracted from the starch interior possess a profile different from that of the other wheat fractions. Starch internal lipids contain significantly greater amounts of the mono-acyl polar lipids (LPC, LPE, LPG, DGMG and MGMG) when compared to the other wheat fractions (Table 2.2). LPC contributes 65.36 and 71.90 mol% of the total polar lipids in the internal starch lipid extracted from Alpowa and Overley (Table 2.2).

PI is found in significantly greater concentrations in the bound wheat whole-meal fraction of Alpowa and Overley (4.07 and 4.75 mol% of total) (Table 2.2). In contrast the bound wheat flour fraction for Alpowa and Overley contains only 0.14 and 0.11 mol% of total PI,

respectively. These results indicate that PI is predominately localized in the wheat bran or germ component of the grain.

2.4.3 Polar lipid molecular species of various wheat kernel fractions

Lipid profiling techniques were able to resolve and identify 155 specific wheat polar lipid species; 16 DGDG, 5 DGMG, 16 MGDG, 5 MGMG, 16 SQDG, 20 PC, 6 LPC, 11 PG, 5 LPG, 8 PA, 14 PE, 5 LPE, 11 PI and17 PS (Table 2.3 and 2.4). Each lipid class had one, and sometimes two, major molecular species that were present at considerably greater concentration than any other molecular species from that class. Both wheat cultivars had the same major molecular species from each class. DGDG, MGDG and PE were predominately comprised of molecular species 36:4 (Table 2.3). PC and PA were comprised of the molecular species of 34:2 and 36:4, in relatively similar abundance (Table 2.2). SQDG, PG and PI were predominantly comprised of molecular species of 34:2 (Table 2.3 and 2.4). PS molecular species composition was found to be unique among the wheat polar lipids in that it contained high proportions of very long fatty acids, with predominate molecular species of 40:2, 42:2 and 44:3 (Table 2.3). The lysophospholipids (LPC, LPE and LPG) contained mostly 16:0 and 18:2 species in relatively similar proportions, while the mono-acyl galactolipids (DGMG and MGMG) contained mostly 18:2 species (Table 2.3).

Table 2.4 lists the molecular species present in the SQDG class and their relative abundances. The total bound starch-surface lipid fraction of Alpowa contained the greatest concentration of SQDG among all the wheat fractions within the Alpowa samples, whereas the whole-meal and flour fraction contained the greatest concentration of SQDG in the Overley fractions. These results show that SQDG binds to the surface of starch from soft wheat

(Alpowa) in greater quantity than to the surface of starch of hard wheat (Overley). The abundance of SQDGs on the surface of soft wheat starch may indicate the possible association of SQDGs and the grain softness proteins, puroindoline A and B.

2.4.4 Starch Internal lipids

The images in Figure 2.2 are light micrographs of starch granules from Alpowa after bound surface lipid and starch internal lipid extraction. These images confirm the occurrence of starch granule swelling during the extraction step involving heating of the granules to 90°C in isopropanol:water (90:10). Panels a and b are prime-starch granules before any extraction. Panels c and d are starch granules after bound lipid extraction at room temperature, and panels e and f are starch granules after lipids extraction at 90°C. No visible damage is present in the starch granules that have undergone lipid extraction at room temperature (panel c), indicating that the lipids extracted were located on the surface of the granules, because removal of the internal starch lipids would require starch granule swelling (Morrison and Coventry 1985; Lee and BeMiller 2008). The starch granules that had undergone lipid extraction at 90°C (panel e) show signs of disruption due to swelling of the granules. The dark lines apparent in the granules' center suggest damage to the granule caused by swelling and related to the uptake of solvent during the heating. Even though the starch granules swelled during the 90°C extraction, the granules were not completely gelatinized. The presence of birefringence in the starch granules as observed under polarized light, indicates that the crystalline structure of the starch granules are still intact, thus complete gelatinization of the starch granules did not occur.

Starch internal lipids are predominately monoacyl species (LPC, LPE, LPG, DGMG and MGMG) (Table 2.2 and 2.3). Morrison (1981) indicated that 90 % of starch lipids are

lysophospholipids. In this present study, the internal lipids in Overley were 91.1 % lysophospholipids of the total internal starch lipids, but the internal lipids in Alpowa were 71.1 % lysophospholipids. The difference in phospholipids concentration between the samples might be a result of incomplete extraction of the bound surface lipids in Alpowa during room temperature extraction.

Lee and BeMiller (2008) identified the major lysophospholipids in maize starch as 18:2 LPC and 16:0 LPC. They localized these lipids to the lining of the channels found in maize starch. Thus, it is logical to hypothesize that the monoacyl starch internal polar lipids extracted in the wheat samples from this study also line the channels found in wheat starch.

2.5 Conclusion

In this study, 155 polar lipid species present in wheat whole-meal, flour and starch were quantitatively determined by lipid profiling techniques. The predominant polar lipid classes were DGDG, MGDG, PC, and LPC. Lipid profiling was able to quantitate and differentiate the species present in a variety of processing fractions at a resolution and sensitivity previously unattainable, thus allowing the most complete characterization of wheat polar lipids to date. The ability to profile lipids will help provide new knowledge regarding the functional relationships between specific lipid species and end-use quality. Further research regarding wheat flour lipids functionality is needed, and by using lipid profiling to quantitatively determining the specific lipid species present in wheat flours of different baking qualities (loaf volume and gas cell stability) relationships between specific lipid species and their gas cell stabilizing abilities should be obtainable.

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2.8 Tables and Figures

Table 2.1: Sources of variation and mean values (nmol/g of sample) for total polar lipids, total phospholipids and total galactolipids from the different fractions of cultivars Alpowa and Overley.

Source of Variation/ Sample Fraction	Total Polar Lipids	Total Phospholipids	Total Galactolipids
Wheat Fraction†	8298	1960	11504
Wheat Cultivar†	195	85	164
Interaction†	54	27	48
Alpowa§			
Whole-Meal	4210 b ± 73	1705 a ± 50	2505 b ± 46
Flour	5706 a ± 63	1080 b ± 12	4627 a ± 53
Starch Surface	482 c ± 7	89 d ± 2	392 c ± 5
Internal Starch	638 c ± 15	543 c ± 15	95 d ± 1
Overley§			
Whole-Meal	4073 b ± 39	1585 a ± 28	2488 b ± 14
Flour	4762 a ± 29	713 b ± 4	4049 a ± 26
Starch Surface	89 d ± 3	24 d ± 0	65 c ± 3
Interal Starch	582 c ± 5	535 c ± 4	47 c ± 1

† Values represent *F*-Value with *P<0.0001.*

§ Value represent mean nmol/g of sample ±SE, n=5.

Table 2.2: Mean Values of different polar lipid classes from various fractions of cultivars Alpowa and Overley.

	Alpowa								Overley							
	Whole-Meal		Flour		Starch Surface		Internal Starch		Whole-Meal		Flour		Starch Surface		Internal Starch	
Lipid Class	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound
DGDG	10.11 b ±0.35	27.01 c ±0.44	20.67 a ±0.23	34.75 b ±0.36	0.33 c ±0.14	56.40 a ±0.39	-	8.19 d ±0.20	10.02 b ±0.23	20.81 c ±0.32	20.22 a ±0.23	30.25 b ±0.12	-	41.56 a ±0.64	-	3.54 d ±0.05
MGDG	8.65 b ±0.57	12.85 b ±0.17	12.93 a ±0.16	11.98 c 0.13±	0.42 c ±0.06	24.03 a ±0.25	-	3.39 d ±0.11	10.96 b ±0.18	18.47 b ±0.46	16.04 a ±0.19	16.33 c 0.22±	-	30.35 a ±0.53	-	2.08 d ±0.05
PC	5.58 a ±0.14	20.38 a ±0.38	5.07 b ±0.04	8.32 c ±0.07	0.04 c ±0.03	13.45 b ±0.18	-	2.19 d ±0.02	6.39 a ±0.25	16.53 a ±0.31	4.45 b ±0.04	4.51 c 0.02±	-	7.29 b ±0.29	-	0.50 d ±0.02
LPC	0.76 a ±0.04	2.75 b ±0.06	0.51 b ±0.00	1.45 c ±0.01	0.03 c ±0.02	2.38 b ±0.31	-	65.36 a ±0.44	0.62 b ±0.02	2.31 c ±0.03	1.07 a ±0.02	2.25 c 0.41±	-	17.59 b ±0.75	-	71.90 a ±0.26
PG	0.36 a ±0.02	1.69 a ±0.06	0.26 b ±0.01	0.56 c ±0.01	0.09 c ±0.00	0.79 b ±0.01	-	0.47 c ±0.01	0.17 a ±0.02	0.87 a ±0.01	0.02 b ±0.00	0.06 c ±0.00	0.04 b ±0.01	0.29 b ±0.01	-	0.07 c ±0.00
PA	0.17 b ±0.00	0.53 b ±0.02	0.21 a ±0.01	0.15 c ±0.01	0.01 c ±0.00	0.51 b ±0.01	-	0.72 a ±0.01	0.62 a ±0.04	1.89 a ±0.04	0.28 b ±0.02	0.71 b ±0.34	-	0.27 c ±0.01	-	0.09 d ±0.00
PE	0.24 b ±0.02	2.46 a ±0.09	0.40 a ±0.01	0.96 b ±0.01	-	0.44 c ±0.03	-	0.12 d ±0.00	0.45 a ±0.01	2.18 a ±0.06	0.37 b ±0.01	0.40 b ±0.01	-	0.10 c ±0.01	-	0.02 c ±0.00
LPE	0.04 a ±0.00	0.20 b ±0.00	0.03 b ±0.00	0.09 c ±0.00	-	0.26 b ±0.01	-	9.57 a ±0.40	0.07 b ±0.00	0.27 c ±0.00	0.11 a ±0.00	0.22 c ±0.01	-	0.57 b ±0.04	-	13.48 a ±0.07
PI	0.32 a ±0.04	4.07 a ±0.43	0.32 a ±0.01	0.14 b ±0.01	0.07 b ±0.00	0.19 b ±0.0	-	0.12 b ±0.00	1.15 a ±0.07	4.75 a ±0.24	0.21 b ±0.01	0.11 b ±0.00	-	0.17 b ±0.01	-	0.11 b ±0.00
DGMG	0.12 a ±0.00	0.50 b ±0.01	0.07 b ±0.00	0.36 b ±0.01	0.02 c ±0.00	0.17 c±0.0	-	2.01 a ±0.08	0.12 b ±0.00	0.42 c ±0.01	0.38 a ±0.01	0.88 b ±0.02	-	0.43 c ±0.01	-	1.07 a ±0.01
PS	0.43 a ±0.08	0.27 a ±0.05	0.12 b ±0.01	0.10 c ±0.01	0.04 b ±0.00	0.12 b ±0.01	-	0.18 b ±0.01	0.41 a ±0.14	0.19 a ±0.01	0.05 b ±0.00	0.04 b ±0.00	-	0.05 b ±0.01	-	0.02 c ±0.00
LPG	0.01 b ±0.00	0.24 b ±0.01	0.05 a ±0.00	0.20 b ±0.00	-	0.10 b ±0.01	-	6.28 a ±0.14	-	0.03 c ±0.00	0.02 ±0.00	0.11 c ±0.00	-	0.86 b ±0.09	-	5.74 a ±0.12
MGMG	0.03 b ±0.00	0.23 b ±0.01	0.06 a ±0.00	0.24 b ±0.00	-	0.08 c ±0.00	-	1.39 a ±0.03	0.05 b ±0.00	0.24 c ±0.01	0.26 a ±0.01	0.66 b ±0.04	-	0.31 c ±0.00	-	1.35 a ±0.02

* Each value represents %mol of total polar lipids (free + bound) ±SE, n=5.

Mean values from each lipid extract (free or bound) followed by the same letter are not significantly different at $P = 0.05$. Only comparisons within lipids extracts are depicted.

Table 2.3: Mean values of various polar lipid species from fractions of cultivars Alpowa and Overley

	Alpowa								Overley							
	Whole-Meal		Flour		Starch Surface		Internal Starch		Whole-Meal		Flour		Starch Surface		Internal Starch	
Lipid	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound
DGDG 34:1	0.17 b	0.49 b	0.22 a	0.37 c	0.01 c	2.20 a	-	0.31 c	0.18 b	0.38 b	0.23 a	0.33 b	-	1.13a	-	0.09 c
DGDG 34:2	0.97 b	2.71 c	3.70 a	6.55 b	0.04 c	8.18 a	-	1.24 d	1.08 b	2.37 b	3.55 a	5.39 a	-	5.48 a	-	0.46 c
DGDG 34:3	0.23 b	0.63 c	0.47 a	0.86 b	0.03 c	1.13 a	-	0.18 d	0.31 b	0.63 c	0.71 a	1.07 b	-.	1.16 a	-	0.11 d
DGDG 34:4	-	-	-	0.01 b	0.01	0.03 a	-	0.01 b	-	-	-	-	-	0.01	-	-
DGDG 34:5	-	-	-	-	0.01	0.01	-	-	-	-	-	-	-	-	-	-
DGDG 34:6	-	-	-	-	-	0.02 a	-	0.01 b	-	-	-	-	-	-	-	-
DGDG 36:1	0.01	0.02 c	-	0.01 c	-	0.23 a	-	0.09 b	0.01	0.02 b	-	-	-	0.09 a	-	0.01 b
DGDG 36:2	0.11 b	0.32 c	0.31 a	0.51 b	0.01 c	1.14 a	-	0.18 d	0.11 b	0.24 c	0.26 a	0.39 b	-	0.63 a	-	0.05 d
DGDG 36:3	0.53 b	1.39 c	1.16 a	1.97 b	0.02 c	3.96 a	-	0.54 d	0.48 b	0.97 c	1.02 a	1.48 b	-	2.37 a	-	0.19 d
DGDG 36:4	7.08 b	18.60 c	13.53 a	22.36 b	0.17 c	35.51 a	-	4.98 d	6.65 b	13.68 c	12.57 a	18.77 b	-	26.68 a	-	2.29d
DGDG 36:5	0.93 b	2.56 b	1.17 a	1.98 c	0.05 c	3.25 a	-	0.44 d	1.11 b	2.28 c	1.74 a	2.59 b	-	3.68 a	-	0.33 d
DGDG 36:6	0.02 a	0.11 a	0.02 a	0.03 c	-	0.07 b	-	-	0.03 b	0.08 a	0.05 a	0.07 a	-	0.06 b	-	-
DGDG 38:3	0.03 b	0.08 c	0.04 a	0.06 d	-	0.34 a	-	0.13 b	0.03 b	0.07 b	0.04 a	0.06 b	-	0.19 a	-	0.02 c
DGDG 38:4	0.03 a	0.10 b	0.03 a	0.05 c	-	0.25 a	-	0.07 c	0.04 a	0.08 b	0.04 a	0.07 b	-	0.16 a	-	0.02 c
DGDG 38:5	-	0.02 b	-	0.01 c	-	0.03 a	-	-	-	0.02 a	0.01	0.01 a	-	0.02 a	-	-
DGDG 38:6	-	-	-	-	-	0.06 a	-	0.02 b	-	-	-	-	-	-	-	-
MGDG 34:1	0.03 b	0.05 b	0.06 a	0.04 c	-	0.34 a	-	0.06 b	0.06 b	0.09 b	0.09 a	0.08 b	-	0.28 a	-	0.02 c
MGDG 34:2	0.16 b	0.31 c	0.58 a	0.52 b	0.03 c	1.09 a	-	0.17 d	0.38 b	0.67 c	0.87 a	0.91 b	-	1.50 a	-	0.10 d
MGDG 34:3	0.05 b	0.10 b	0.13 a	0.11 b	0.01 c	0.24 a	-	0.04 c	0.15 b	0.26 b	0.28 a	0.30 b	-	0.43 a	-	0.03 c
MGDG 34:4	-	-	-	-	-	0.07 a	-	0.01 b	-	0.01 b	0.01	0.01 b	-	0.03 a	-	-
MGDG 34:5	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-
MGDG 34:6	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-
MGDG 36:1	-	-	-	-	-	0.02 a	-	0.01 b	-	-	-	-	0.01	0.01	-	-
MGDG 36:2	0.03 b	0.05 c	0.09 a	0.08 b	0.01 c	0.19 a	-	0.03 d	0.04 d	0.07 b	0.09 a	0.08 b	-	0.16 a	-	0.01 c
MGDG 36:3	0.55	0.70 b	0.76 a	0.65 b	0.04 c	1.90 a	-	0.26 c	0.54 b	0.86 b	0.81 a	0.81 b	-	1.87 a	-	0.13 c
MGDG 36:4	7.07 b	10.21 b	10.35 a	9.66 b	0.33 c	18.61 a	-	2.59 c	8.44 b	14.10 b	12.02 a	12.21 c	-	23.24 a	-	1.61 d
MGDG 36:5	0.74 b	1.28 b	0.94 a	0.90 c	0.02 c	1.36 a	-	0.19 d	1.32 b	2.29 b	1.78 a	1.86 c	-	2.76 a	-	0.20 d
MGDG 36:6	0.03 a	0.13 a	0.02 b	0.02 c	-	0.04 b	-	-	0.03 b	0.07 a	0.05 a	0.05 b	-	0.02 c	-	-
MGDG 38:3	-	0.01 c	-	-	-	0.06 a	-	0.02 b	-	0.01 b	0.01	0.01 b	-	0.04 a	-	-
MGDG 38:4	0.01	0.01 c	-	-	-	0.06 a	-	0.02 b	0.01 a	0.02 b	0.01 a	0.01 c	-	0.04 a	-	-
MGDG 38:5	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-

MGDG 38:6	-	-	-	-	-	-	-	-	-	-	-	-	0.01	-	-	-
PC 32:0	0.04 b	0.09 b	0.06 a	0.09 b	-	0.35 a	-	0.06 c	0.06 b	0.15 b	0.08 a	0.10 c	-	0.21 a	-	0.01 d
PC 34:1	0.29 a	1.13 a	0.20 b	0.35 b	-	1.20 a	-	0.19 c	0.36 a	1.00 a	0.22 b	0.21 c	-	0.53 b	-	0.03 d
PC 34:2	2.13 a	6.79 a	2.09 a	2.98 c	0.02 b	5.88 b	-	0.93 d	2.18 a	5.26 a	1.88 b	1.84 c	-	3.38 b	-	0.24 d
PC 34:3	0.09 a	0.48 a	0.10 a	0.16 c	-	0.31 b	-	0.05 b	0.12 a	0.40 a	0.11 a	0.12 c	-	0.17 b	-	0.01 d
PC 34:4	-	0.02 a	-	-	-	0.02 a	-	-	-	0.01	-	-	-	-	-	-
PC 36:1	-	0.01 d	0.01	0.02 c	-	0.15 a	-	0.07 b	0.01 a	0.01 b	0.01 a	0.01 b	-	0.05 a	-	-
PC 36:2	0.20 a	0.71 a	0.15 b	0.26 c	-	0.57 b	-	0.10 d	0.26 a	0.59 a	0.12 b	0.14 c	-	0.28 b	-	0.02 d
PC 36:3	0.67 a	2.63 a	0.42 b	0.87 c	-	1.09 b	-	0.17 d	0.93 a	2.45 a	0.36 b	0.42 b	-	0.56 b	-	0.04 c
PC 36:4	1.94 a	7.37 a	1.87 a	3.27 b	0.01 b	2.74 c	-	0.44 b	2.21 a	5.76 a	1.50 b	1.50 b	-	1.68 b	-	0.11 c
PC 36:5	0.16 a	0.93 a	0.10 b	0.21 b	-	0.21 b	-	0.03 c	0.21 a	0.69 a	0.10 b	0.11 b	-	0.14 b	-	0.01 c
PC 36:6	-	0.03 a	-	-	-	0.03 a	-	0.01 b	-	0.02 a	-	-	-	0.01 b	-	-
PC 38:2	0.01 a	0.04 b	0.01 a	0.01 c	-	0.38 a	-	0.04 b	0.01	0.04 b	-	0.01 c	-	0.11 a	-	-
PC 38:3	0.02 b	0.08 b	0.04 a	0.05 c	-	0.12 a	-	0.04 d	0.02 b	0.08 a	0.03 a	0.03 c	-	0.05 b	-	-
PC 38:4	0.01 a	0.04 a	0.01 a	0.02 b	-	0.02 b	-	0.01 c	0.01 a	0.03 a	0.01 a	0.01 c	-	0.02 b	-	-
PC 38:5	-	0.01 c	-	-	-	0.06 a	-	0.02 b	-	-	-	-	-	0.02	-	-
PC 38:6	-	-	-	-	-	0.22 a	-	0.03 b	-	-	-	-	-	0.05	-	-
PC 40:2	-	0.01 b	0.01	0.01 b	-	0.02 a	-	0.01 b	0.01 a	0.02 a	0.01 a	0.01 b	-	0.01 b	-	-
PC 40:3	-	0.01 b	-	0.01 b	-	0.03 a	-	-	-	0.01	-	-	-	-	-	-
PC 40:4	-	-	-	-	-	0.02	-	-	-	-	-	-	-	-	-	-
PC 40:5	-	-	-	-	-	0.02	-	-	-	-	-	-	-	-	-	-
LPC 16:0	0.34 a	1.13 b	0.24 b	0.67 c	0.02 c	1.27 b	-	27.83 a	0.29 b	0.89 c	0.49 a	1.08 c	-	12.06 b	-	30.24 a
LPC 16:1	-	0.01 b	-	-	-	0.01 b	-	0.34 a	-	0.01 c	-	0.01 c	-	0.08 b	-	0.40 a
LPC 18:0	0.01 a	0.03 c	0.01 a	0.04 bc	-	0.05 b	-	0.50 a	0.01 b	0.03 c	0.02 a	0.05 c	-	0.28 b	-	0.51 a
LPC 18:1	0.04 a	0.18 b	0.02 b	0.07 c	-	0.12 bc	-	2.45 a	0.03 b	0.18 c	0.06 a	0.14 c	-	0.62 b	-	2.81 a
LPC 18:2	0.35 a	1.35 b	0.22 b	0.65 c	0.01 c	0.89 bc	-	32.53 a	0.28 b	1.14 c	0.48 a	0.95 c	-	4.14 b	-	34.98 a
LPC 18:3	0.01	0.05 b	-	0.02 c	-	0.04 bc	-	1.71 a	0.01 a	0.06 c	0.01 a	0.04 c	-	0.40 b	-	2.95 a
PG 32:0	0.02 b	0.06 c	0.02 b	0.04 d	0.03 a	0.15 a	-	0.08 b	0.01 b	0.03 b	-	-	0.03 a	0.07 a	-	0.01 c
PG 32:1	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-
PG 34:0	-	0.01 b	-	0.01 b	-	0.03 a	-	0.01 b	-	-	-	-	-	0.01	-	-
PG 34:1	0.03 a	0.14 a	0.02 ab	0.05 c	0.01 b	0.09 b	-	0.05 c	0.02 a	0.10 a	-	-	0.01 b	0.03 b	-	-
PG 34:2	0.22 a	1.11 a	0.14 b	0.32 b	0.02 c	0.35 b	-	0.22 c	0.10 a	0.52 a	0.01 b	0.04 c	0.01 b	0.11 b	-	0.04 c
PG 34:3	0.01 a	0.08 a	0.01 a	0.01 b	-	0.01 b	-	0.01 b	-	0.04 a	-	-	-	0.01 b	-	-
PG 34:4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PG 36:1	-	-	-	-	-	0.01 a	-	0.01 a	-	-	-	-	-	0.01	-	-
PG 36:2	-	0.01 b	-	0.01 b	-	0.02 a	-	0.01 b	-	0.01 a	-	-	-	0.01 a	-	-
PG 36:3	0.01 a	0.05 a	0.01 a	0.02 bc	0.01 a	0.03 b	-	0.01 c	0.01	0.03 a	-	-	-	0.01 b	-	-
PG 36:4	0.06 a	0.21 a	0.05 b	0.10 b	0.01 c	0.10 b	-	0.06 c	0.03	0.14 a	-	0.01 c	-	0.04 b	-	0.01 c
PG 36:5	-	0.02	-	-	-	-	-	-	-	0.02	-	-	-	-	-	-
PG 36:6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

PA 32:0	-	-	-	-	-	-	-	0.01	-	0.01	-	-	-	-	-	-
PA 34:1	0.01 a	0.03 c	0.01 a	0.01 d	-	0.05 b	-	0.08 a	0.04 a	0.11 a	0.01 b	0.03 b	-	0.03 b	-	0.01 c
PA 34:2	0.07 b	0.15 b	0.09 a	0.07 c	-	0.16 b	-	0.25 a	0.16 a	0.45 a	0.09 b	0.20 b	-	0.09 c	-	0.03 d
PA 34:3	-	0.01 a	-	-	-	0.01 a	-	0.01 a	0.01	0.04 a	-	0.01 b	-	-	-	-
PA 34:4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PA 34:5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PA 34:6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PA 36:2	-	0.01 c	0.01	-	-	0.02 b	-	0.03 a	0.02 a	0.05 a	0.01 b	0.03 b	-	0.01 c	-	-
PA 36:3	0.02 a	0.08 b	0.02 a	0.01	-	0.09 b	-	0.11 a	0.11 a	0.35 a	0.05 b	0.12 b	-	0.05 c	-	0.02 d
PA 36:4	0.06 b	0.24 a	0.08 a	0.06 d	-	0.17 c	-	0.22 b	0.25 a	0.78 a	0.11 b	0.30 b	-	0.09 c	-	0.03 d
PA 36:5	-	0.02 a	-	-	-	0.01 b	-	0.01 b	0.03 a	0.09 a	0.01 b	0.02 b	-	-	-	-
PA 36:6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PE 34:1	-	0.03 a	0.01	0.02 b	-	0.02 b	-	-	0.01 a	0.05 a	0.01 a	0.01 b	-	-	-	-
PE 34:2	0.06 b	0.55 a	0.10 a	0.24 b	-	0.13 c	-	0.04 d	0.11 a	0.47 a	0.10 b	0.11 b	-	0.04 c	-	0.01 d
PE 34:3	-	0.04 a	-	0.01 c	-	0.02 b	-	-	0.01	0.03	-	-	-	-	-	-
PE 34:4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PE 36:1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PE 36:2	-	0.03 a	0.01	0.02 b	-	0.01 c	-	-	0.01 a	0.04 a	0.01 a	0.01 b	-	-	-	-
PE 36:3	0.02 b	0.13 a	0.03 a	0.09 b	-	0.03 c	-	0.01 d	0.04 a	0.18 a	0.03 b	0.04 b	-	-	-	-
PE 36:4	0.13 b	1.45 a	0.17 a	0.47 b	-	0.15 c	-	0.04 c	0.23 a	1.24 a	0.13 b	0.16 b	-	0.04 c	-	0.01 c
PE 36:5	0.01 a	0.17 a	0.01 a	0.05 b	-	0.03 c	-	-	0.02 a	0.13 a	0.01 b	0.01 b	-	-	-	-
PE 36:6	-	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PE 38:2	-	-	0.01	0.01	-	-	-	-	-	-	0.01	0.01	-	-	-	-
PE 38:3	-	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PE 38:4	-	0.01	-	-	-	-	-	-	-	0.01	-	-	-	-	-	-
PE 38:5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PE 38:6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PE 40:2	0.01 b	0.01 c	0.04 a	0.04 a	-	0.03 b	-	0.01 c	0.01 b	0.01 b	0.04 a	0.03 a	-	0.01 b	-	-
PE 40:3	-	-	-	-	-	-	-	-	-	-	0.01	-	-	-	-	-
PE 42:2	0.01 a	0.01 a	0.01 a	0.01 a	-	0.01 a	-	-	-	-	0.01	0.01	-	-	-	-
PE 42:3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PE 42:4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LPE 16:0	0.01 a	0.05 b	0.01 a	0.02 b	-	0.09 b	-	3.18 a	0.02 b	0.07 c	0.03 a	0.06 c	-	0.28 b	-	5.09 a
LPE 16:1	-	-	-	-	-	-	-	0.04	-	-	-	-	-	-	-	0.06
LPE 18:1	-	0.02 b	-	-	-	0.02 b	-	0.29 a	-	0.02 b	0.01	0.01 b	-	0.02 b	-	0.32 a
LPE 18:2	0.02 a	0.12 bc	0.02 a	0.06 c	-	0.13 b	-	5.84 a	0.04 b	0.17 bc	0.07 a	0.14 c	-	0.25 b	-	7.58 a
LPE 18:3	-	0.01 b	-	-	-	0.01 b	-	0.22 a	-	0.01 b	-	-	-	0.02 b	-	0.43 a
PI 32:0	-	-	0.01	-	0.01	0.02	-	-	0.01	0.01 a	-	-	-	0.01 a	-	-
PI 32:1	-	-	-	-	-	-	-	-	-	0.01	-	-	-	-	-	-
PI 32:2	-	-	-	-	-	-	-	-	-	0.01	-	-	-	-	-	-

PI 32:3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PI 34:1	0.02 a	0.28 a	0.02 a	0.01 b	0.01 b	0.02 b	-	0.01 b	0.10 a	0.44 a	0.01 b	0.01 b	-	0.01 b	-	-
PI 34:2	0.17 a	2.66 a	0.13 a	0.07 b	0.01 b	0.05 b	-	0.07 b	0.62 a	2.60 a	0.04 b	0.05 b	-	0.07 b	-	0.09 b
PI 34:3	0.02 a	0.18 a	0.01 b	-	0.01 b	0.01 b	-	0.01 b	0.05	0.16 a	-	-	-	0.01 b	-	0.01 b
PI 34:4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PI 36:1	-	-	-	-	-	0.01	-	-	-	0.01 a	-	-	-	0.01 a	-	-
PI 36:2	0.02 a	0.07 a	0.01 b	-	-	0.01 b	-	-	0.03 a	0.11 a	0.01 b	-	-	0.01 b	-	-
PI 36:3	0.03 a	0.21 a	0.04 a	0.01 b	0.01 b	0.01 b	-	-	0.08 a	0.33 a	0.04 b	0.01 b	-	0.01 b	-	-
PI 36:4	0.04 b	0.59 a	0.09 a	0.03 b	0.01 c	0.03 b	-	0.01 b	0.23 a	0.97 a	0.08 b	0.03 b	-	0.03 b	-	-
PI 36:5	0.01 a	0.07 a	0.01 a	-	0.01 a	0.01 b	-	-	0.03 a	0.11 a	0.01 b	-	-	0.01 b	-	-
PI 36:6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DGMG 16:1	-	-	-	-	-	-	-	0.01	-	-	-	-	-	-	-	0.01
DGMG 16:2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DGMG 16:3	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-
DGMG 18:1	0.01 a	0.05 b	0.01 a	0.03 c	-	0.02 c	-	0.15 a	0.01 b	0.04 c	0.03 a	0.07 b	-	0.06 b	-	0.09 a
DGMG 18:2	0.10 a	0.42 b	0.07 b	0.31 b	-	0.12 c	-	1.73 a	0.10 b	0.35 c	0.32 a	0.75 b	-	0.33 c	-	0.88 a
DGMG 18:3	0.01	0.03 b	-	0.02 c	-	0.01 c	-	0.11 a	0.01 b	0.03 c	0.03 a	0.06 b	-	0.02 c	-	0.10 a
PS 34:1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PS 34:2	-	0.03 a	0.02	0.02 b	-	0.01 c	-	0.01 c	0.01 a	0.04 a	0.01 a	0.01 b	-	-	-	-
PS 34:3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PS 34:4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PS 36:1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PS 36:2	-	-	0.01 a	0.01	-	-	-	0.01 a	-	-	-	-	-	-	-	-
PS 36:3	-	0.01	-	-	-	-	-	-	-	0.01	-	-	-	-	-	-
PS 36:4	-	0.01	0.01	0.01 a	-	-	-	-	-	0.03	-	-	-	-	-	-
PS 36:5	-	-	-	-	-	-	-	-	-	0.01	-	-	-	-	-	-
PS 36:6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PS 38:1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PS 38:2	-	0.01 a	0.01	0.01 a	-	-	-	0.01 a	-	0.01	-	-	-	-	-	-
PS 38:3	-	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PS 38:4	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-
PS 38:5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PS 38:6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PS 40:1	-	0.01 a	-	-	-	0.01	-	0.01 a	-	-	-	-	-	-	-	-
PS 40:2	0.01 b	0.08 a	0.04 a	0.03 b	-	0.01	-	0.05 b	0.01 a	0.04 a	0.01 a	0.01 b	-	0.01 b	-	0.01 b
PS 40:3	-	0.02	-	-	-	-	-	0.01	-	0.01	-	-	-	-	-	-
PS 40:4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PS 42:1	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PS 42:2	0.04 a	0.04 a	0.01 b	0.01 b	-	0.01 b	-	0.04 a	0.05 a	0.02 a	0.01 b	-	-	0.01 b	-	-
PS 42:3	0.01	-	-	-	-	0.02 a	-	0.01 a	-	-	-	-	-	-	-	-

PS 42:4	-	-	-	-	0.01	0.02 a	-	0.01 b	-	-	-	-	-	-	-	-
PS 44:2	0.06 a	-	-	-	0.01 a	-	-	-	0.06	-	-	-	-	-	-	-
PS 44:3	0.30 a	0.04 a	0.01 b	-	0.02 b	0.01 a	-	-	0.28 b	-	0.01 b	-	-	0.01 b	-	-
LPG 16:0	-	0.13 b	0.02	0.08 b	-	0.05 b	-	3.02 a	-	0.01 c	0.01	0.04 c	-	0.73 b	-	3.62 a
LPG 16:1	-	-	-	-	-	-	-	0.08	-	-	-	-	-	0.04 b	-	0.15 a
LPG 18:1	-	-	-	0.01 b	-	-	-	0.19 a	-	-	-	0.01 b	-	0.01 b	-	0.15 a
LPG 18:2	0.01 b	0.10 b	0.02 b	0.10 b	-	0.05 b	-	2.73 a	-	0.01 b	0.01	0.05 b	-	0.08 b	-	1.52 a
LPG 18:3	-	-	-	0.01 b	-	-	-	0.26 a	-	-	-	0.01 b	-	0.01 b	-	0.30 a
MGMG 16:1	-	-	-	-	-	-	-	0.01	-	-	-	-	-	-	-	0.01
MGMG 16:2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MGMG 16:3	-	-	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-
MGMG 18:1	0.01	0.03 b	-	0.01 c	-	0.01 c	-	0.15 a	0.01 b	0.03 d	0.02 a	0.05 c	-	0.06 b	-	0.16 a
MGMG 18:2	0.02 b	0.18 b	0.05 a	0.22 b	-	0.06 c	-	1.11 a	0.04 b	0.19 c	0.22 a	0.55 b	-	0.22 c	-	1.00 a
MGMG 18:3	-	0.02 b	-	0.01 c	-	-	-	0.13 a	-	0.02 c	0.02	0.06 b	-	0.03 c	-	0.19 a

Each value represents %mol of total polar lipids (free + bound), n=5. Mean values from each lipid extract (free or bound) followed by the same letter are not significantly different at $P = 0.05$. Only comparisons within lipids extracts are depicted.

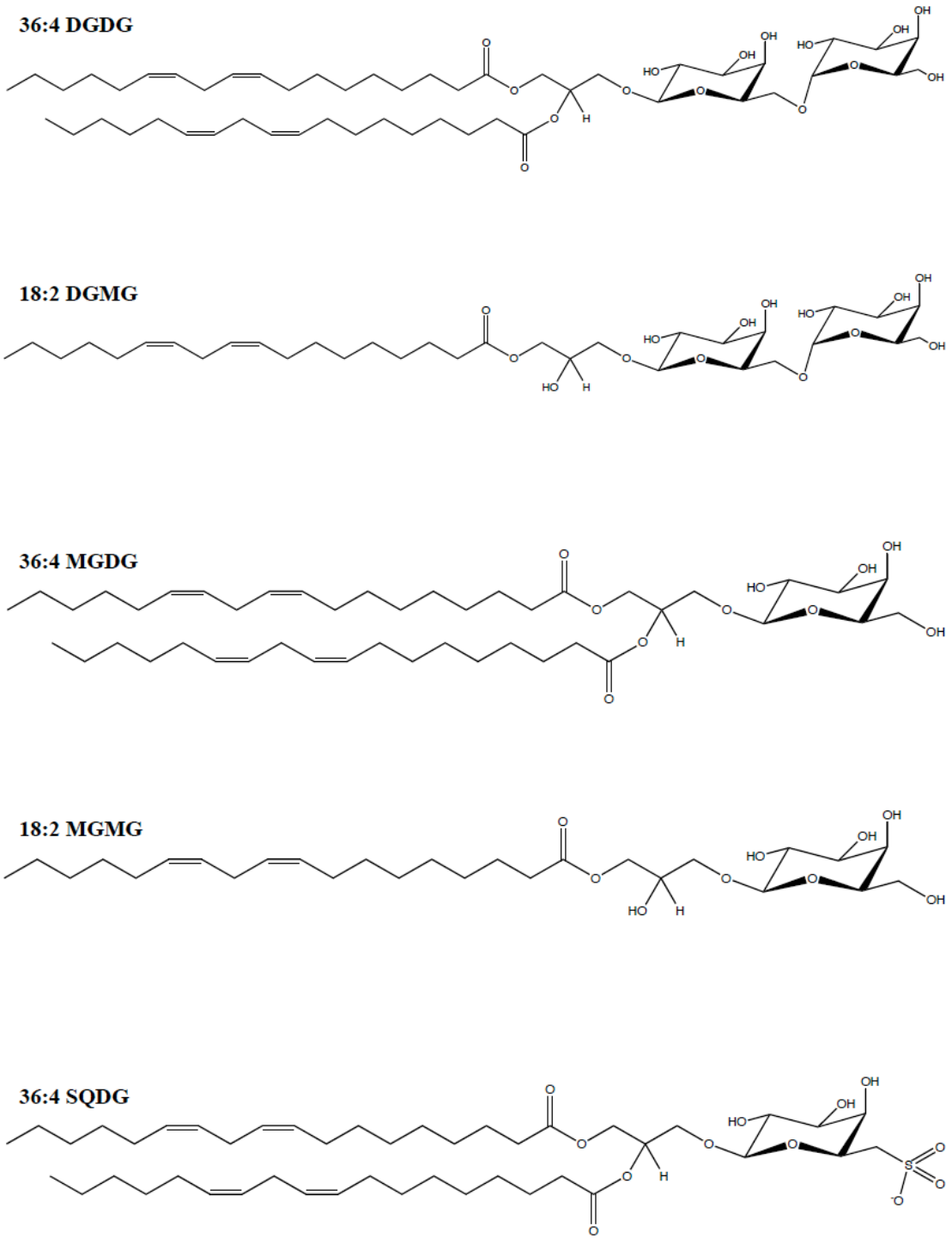
Table 2.4: Mean values of SQDG Lipids from various fractions of cultivars Alpowa and Overley.

	Alpowa						Overley					
	Whole-Meal		Flour		Starch Surface		Whole-Meal		Flour		Starch Surface	
SQDG	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound
32:0		0.17 a	0.02 b	0.13 b	0.57 a	0.62 a	0.01 c	0.07 b	0.01 b	0.03 b	0.15 a	0.20 a
	-	±0.03	±0.00	±0.01	±0.06	±0.03	±0.00	±0.00	±0.00	±0.01	±0.04	±0.02
32:1					0.09	0.20						0.03 a
	-	-	-	-	±0.02	±0.03	-	-	-	-	-	±0.01
32:2		0.20		0.26 a	0.18 a	0.18 b		0.03 a		0.03 a		0.01 a
	-	±0.03	-	±0.02	±0.02	±0.01	-	±0.01	-	±0.00	-	±0.00
32:3		0.01		0.02 b	0.28 a	0.17 a		0.01 b		0.01 b		0.03 a
	-	±0.00	-	±0.00	±0.01	±0.01	-	±0.00	-	±0.00	-	±0.00
34:1			0.01 b	0.02 b	0.46 a	0.44 a	0.03 b	0.06 a		0.03 a	0.10	0.10 a
	-	-	±0.00	±0.01	±0.07	±0.06	±0.01	±0.02	-	±0.00	±0.03	±0.02
34:2	0.21 b	0.44 a	0.58 a	1.64 a	0.59 a	0.79 b	0.24 c	0.42 b	0.42 a	0.60 a		0.19 c
	±0.02	±0.04	±0.02	±0.04	±0.04	±0.06	±0.04	±0.02	±0.02	±0.03	-	±0.01
34:3		0.14 a	0.03 b	0.13 b	0.15 a	0.22 a	0.01 c	0.03 b	0.03 b	0.09 a		0.03 b
	-	±0.01	±0.01	±0.01	±0.01	±0.03	±0.00	±0.01	±0.01	±0.01	-	±0.01
34:4					0.01	0.09						0.02
	-	-	-	-	±0.00	±0.02	-	-	-	-	-	±0.01
34:5		0.01		0.05 b	0.01	0.35 a						0.02
	-	±0.00	-	±0.01	±0.00	±0.02	-	-	-	-	-	±0.00
34:6				0.01 b	0.04	0.23 a						0.04
	-	-	-	±0.00	±0.01	±0.04	-	-	-	-	-	±0.01
36:1		0.05		0.02 b	0.05	0.19 a		0.04 a		0.03 a		0.02 a
	-	±0.01	-	±0.00	±0.01	±0.03	-	±0.01	-	±0.01	-	±0.01
36:2		0.07	0.01 b	0.10 b	0.14 a	0.18 a		0.05 a		0.04 a		0.04 a
	-	±0.01	±0.00	±0.01	±0.01	±0.01	-	±0.01	-	±0.01	-	±0.01
36:3	0.03 b	0.09 a	0.05 b	0.13 b	0.14 a	0.27 a	0.03 c	0.15 a	0.03 b	0.04 b		0.03 b
	±0.01	±0.01	±0.01	±0.03	±0.00	±0.02	±0.01	±0.01	±0.01	±0.01	-	±0.01
36:4	0.11 b	0.45 a	0.31 a	0.89 a	0.13 b	0.41 b	0.18 c	0.46 a	0.18 b	0.27 b		0.08 c
	±0.01	±0.05	±0.01	±0.02	±0.02	±0.02	±0.01	±0.06	±0.01	±0.01	-	±0.01
36:5		0.05 a	0.01 b	0.08 a	0.03 a	0.11 a	0.01 b	0.04 a	0.01 b	0.04 a		0.01 a
	-	±0.01	±0.00	±0.01	±0.01	±0.02	±0.00	±0.02	±0.00	±0.01	-	±0.00
36:6	-	0.01	-	-	0.03	0.25	-	0.03 a	-	-	-	0.02 ab

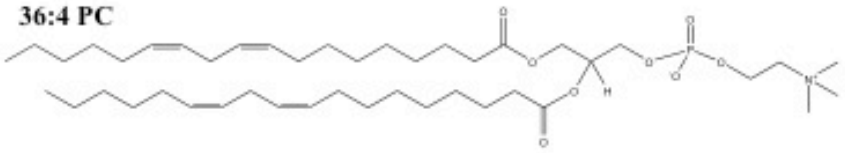
		±0.00			±0.01	±0.03		±0.01				±0.01
Total	0.42 c	1.70 a	1.02 b	3.49 b	2.92 a	4.86 a	0.52 c	1.40 a	0.70 b	1.22 a	0.28 c	0.52 b
	±0.05	±0.09	±0.03	±0.06	±0.20	±0.25	±0.04	±0.05	±0.04	±0.05	±0.07	±0.19

* Each value represents the mean nmol analyzed of SQDG lipids ±SE, n=5. No internal standards were used in the analysis, only comparisons within the SQDG lipid class can be obtained.

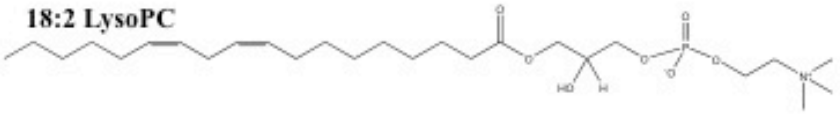
Figure 2.1: Structural representations of each polar lipid class found in wheat. Labels represent total acyl carbons : total double bonds and specific class of lipid, respectively.



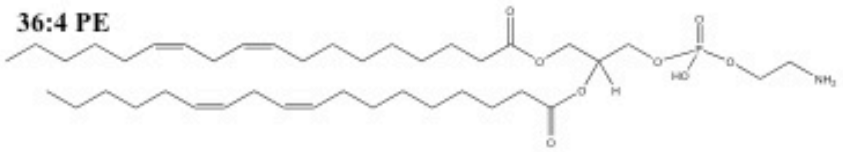
36:4 PC



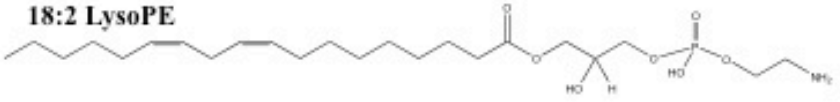
18:2 LysoPC



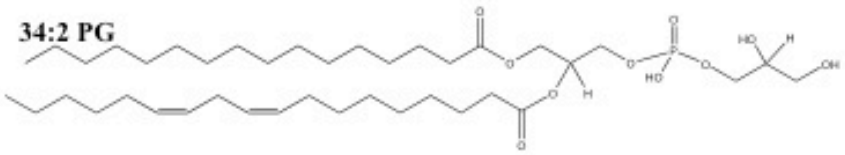
36:4 PE



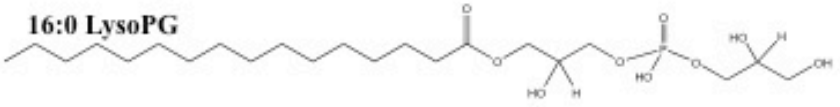
18:2 LysoPE



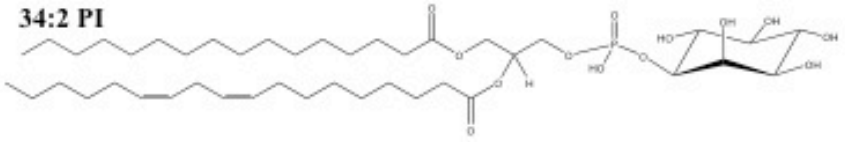
34:2 PG



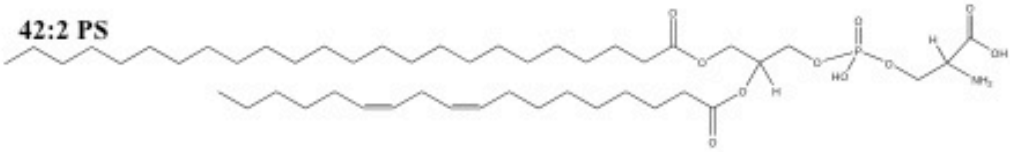
16:0 LysoPG



34:2 PI



42:2 PS



36:4 PA

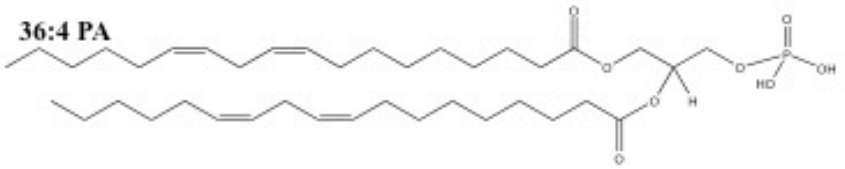
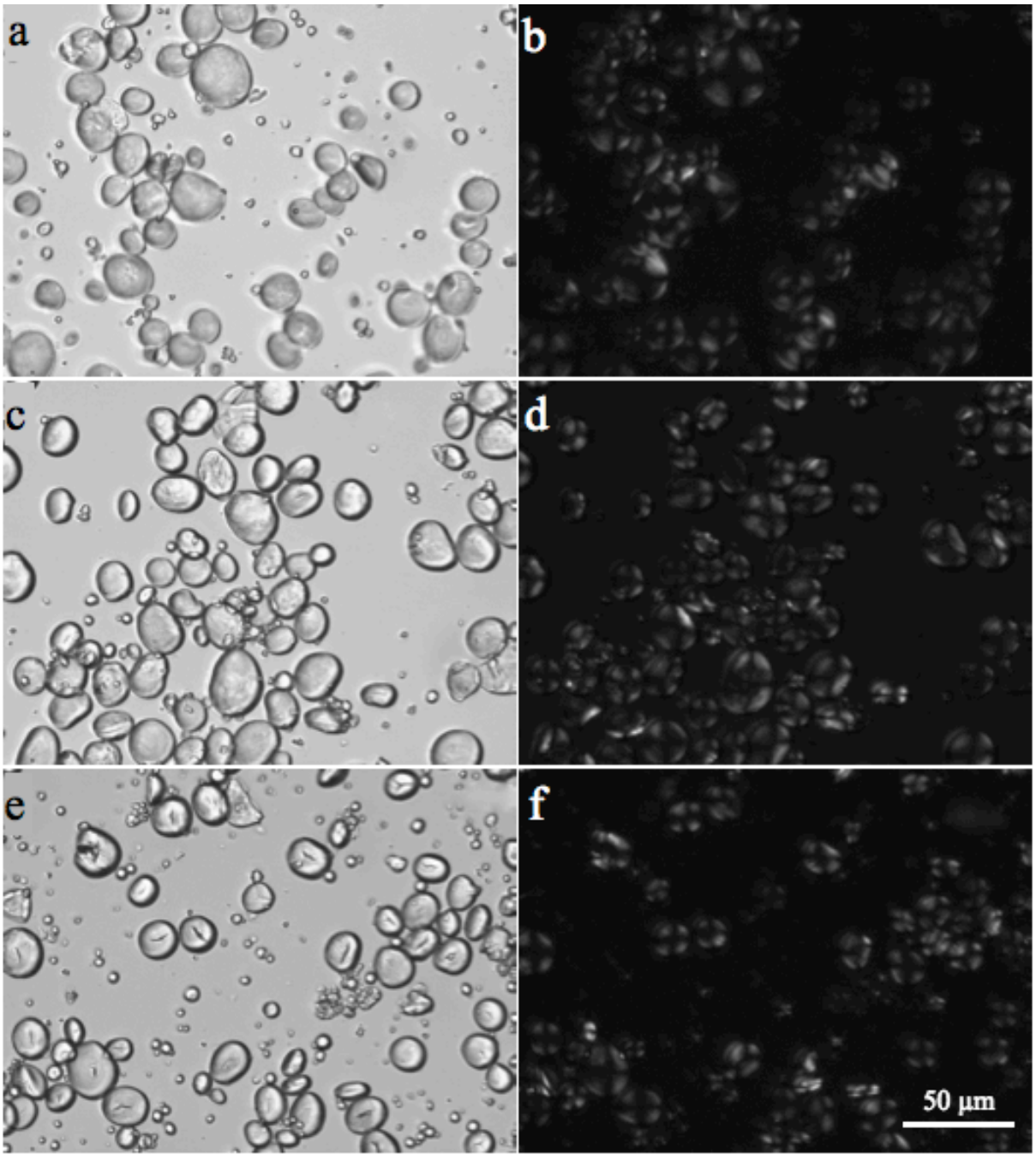


Figure 2.2: Light micrographs of wheat starch using brightfield (a, c, and e) and polarized (b, d, and f) illumination. Panels a and b, native starch; panels c and d, polar lipid-extracted starch at room temperature; panels e and f polar lipid-extracted starch at room temperature proceeded by 90ºC extraction.



CHAPTER 3 - Variation in Polar Lipids Located on the Surface of Wheat Starch

3.1 Abstract

The starch granule surface contributes the greatest proportion of surface area in a dough and it is unknown if starch isolated before dough development would have the same surface lipid composition as starch isolated after dough development. The compositional structure of the surface of wheat starch granules plays a crucial role in wheat endosperm hardness. The abundance of starch granule surface polar lipids is related to the physical hardness of the endosperm, but the variation in specific lipid class and molecular species is unknown. The objective of this study was to determine quantitatively the variation in polar lipids present on the surface of wheat starch granules. Experimental wheat lines were used, which within each set, are near-isogenic to each other but, vary in their grain endosperm hardness and therefore their puroindoline protein content and polar lipid content on the starch granule surface. Starch was isolated using two different processes; a dough and batter method. Direct infusion electrospray ionization tandem mass spectrometry was used to identify and quantitatively determine the polar lipid species in wheat flour and starch. Wide ranges in starch surface polar lipid concentrations were observed between the two starch isolation methods. Starch isolation method provided a greater source of variation than did wheat kernel hardness. When dough is optimally mixed the lipids originally on the surface of wheat starch become incorporated into the gluten phase of the dough, whereas in a batter system the starch surface lipids stay associated with the starch granule surface. The predominant starch surface polar lipids were di-acyl lipids of DGDG, MGDG and PC polar lipid classes and the predominant internal starch polar lipids were LPC lipids.

3.2 Introduction

Wheat (*Triticum aestivum* L.) starch constitutes 70-75% of the flour and is comprised of three distinctive types of granules, A-type, B-type and C-type. The A-type granules are lenticular in shape with a diameter greater than 16 μm, the B-type granules are spherical in shape with a smaller diameter of 5-16 μm and the C-type are spherical with the smallest diameter of less than 5 μm (Bechtel et al. 1990). During the development of the wheat endosperm, A-type granules are synthesized first, beginning at four days after flowering. B-type granules begin developing at 10 days after flowering and C-type granules begin developing 21 days after flowering (Bechtel 1990).

The internal structures of normal starch granules consist of the high molecular weight polymers, amylose and amylopectin. Amylose, the minor component, is a linear polymer of ∝-(1,4)-linked D-glucopyranosyl units and amylopectin is a highly branched polymer of ∝-(1,4)-linked D-glucopyranosyl units with ∝-(1,6)-branch points. The non-reducing ends of amylopectin and amylose are orientated toward the granule's center and the reducing ends are towards the surface of the starch granule. Wheat starch granules contain channels, which allows some molecules to enter the granule matrix (Kim and Huber 2007). A-type granules contain two types of channels; larger channels located on the equatorial groove and smaller channels located throughout the granule. B-type starch granules contain only one type of channel, which are large void-like channels that are less defined than the channels on the A-type granules (Kim and Huber 2007). Han et al. (2005) and Lee and BeMiller (2008) found that starch channels are lined with proteins and polar lipids. Han et al. (2005) identified the proteins as 38 kDa - 40 kDa brittle-1 proteins. Lee and BeMiller (2008) identified the channel lipids in maize starch as

lysophosphatidylcholine polar lipids with either palmitic acid (16:0) or linoleic acid (18:2) fatty acid moieties.

The compositional structure of the surface of wheat starch granules plays a crucial role in the wheat endosperm hardness. Greenwell and Schofield (1986) determined that kernel hardness is caused by the absence of 15 kDa proteins on the surface of water-washed starch granules. These proteins (called friabilin) are found in greater amounts on water-washed starch from soft wheat than on water-washed starch from hard wheat. The friabilin proteins are absent on water-washed starch from durum wheat (the hardest class of wheat). Jolly et al. (1993) and Morris et al. (1994) further discovered, through N-terminal sequencing, the existence of two proteins, puroindoline A (pin-a) and puroindoline B (pin-b), which together comprise friabilin. Besides puroindoline proteins, 30 kDa glycoproteins and 60 kDa starch granule-bound starch synthase enzymes are located on the surface of starch granules (Baldwin 2001).

As is the case for puroindoline proteins, Greenblatt et al. (1995) found that a pattern existed among polar lipids on the surface of starch granules. Both galactolipids and phospholipids were found, via thin layer chromatography, to be present in greater amounts on water-washed starch from soft wheat than on water-washed starch from hard wheat (Greenblatt et al. 1995). Konopka et al. (2005) further found a negative correlation between starch surface lipids (polar and nonpolar) and kernel hardness. In the previous chapter 155 different lipid species are present on the surface of wheat starch granules, in mean amounts ranging from 89 nmol/g to 482 nmol/g. The major polar lipid species identified were digalactosyldigylceride (DGDG) (36:4), monogalactosyldigylceride (MGDG) (36:4), phosphatidylcholine (PC) (36:4 and34:2), and lysophosphatidylcholine (LysoPC) (18:2 and 16:0).

Addition of water and energy to wheat flour produces unique viscoelastic dough, capable

of retaining gases during fermentation and proofing. When dough is mixed to optimum (i.e. with sufficient hydration and work applied), the gliadin and glutenin proteins interact and form a continuous protein macropolymer (gluten) surrounding the starch granules (Singh and MacRitchie 2001; Pomeranz 1988). Few research articles have studied the interaction between the gluten proteins (gliadin and glutenin) and the starch granule surface as it relates to flour functionality (Larsson and Eliasson 1997; Sandsted 1961; Hoseney et al. 1971). Larsson and Eliasson (1997) used *in vitro* modification of the starch granule surface (heat treatment, absorption of wheat protein and absorption of phosphatidylcholine) and found that these treatments had an effect on the rheological properties of the dough, thereby indicating the importance of the starch granule surface components as it relates to dough functionality. Because the starch granules surface components appear to interact with the other components in dough, their abundance on the starch granule surface and or composition could vary depending on whether the starch was isolated before or after dough development (i.e. before or after interactions).

The results presented in the previous chapter were derived from starch isolated using a batter method, thus representing the starch granule surface polar lipids absent of dough development. If the starch granule-gluten interactions are mediated or affected by the starch granule's surface composition, it may be that the polar lipids on the surface of the starch granules will not be present in the same abundance or ratios after dough development. Additionally, the results of Greenblatt et al. (1995) indicate that the abundance of the starch granule surface polar lipids is related to the physical hardness of the endosperms (implying a relationship between puroindoline proteins and polar lipids). Greenblatt et al. (1995) results indicated a genetic source of variation in the relative abundance of starch granule polar lipids, but does not indicate how the

specific classes (DGDG, MGDG, PC and LysoPC) and species (class and fatty acids esterified to the glycerol backbone) of polar lipids are affected depending on their wheats' puroindoline protein expression. Therefore, the objective of this study was to quantitatively determine the variation in polar lipids present on the surface of wheat starch granules. Variation in the starch granule surface polar lipids was evaluated as resulting from either native variation; from differences in wheat endosperm hardness or induced; by the starch isolation processes. Native variation studies was conducted using three pairs of near-isogenic wheat lines, which varied in their puroindoline expression and kernel endosperm hardness. Artificial variation studies were conducted using two distinctly different starch isolation processes; a dough-ball method (Wolf 1964), representing starch after experiencing dough development, and a batter method, representing starch, which has not experienced dough development.

3.3 Experimental

3.3.1 Wheat Samples

A series of unique experimental wheat lines (Table 3.1) were collected that vary in their starch granules surface components. Experimental wheat lines were used that, within each set are near-isogenic to each other but, varied in their grain endosperm hardness and therefore their puroindoline protein and lipid contents on the starch granule surface. One set was derived from the hard cultivar Hi-line (PI 549275), the second set derived from the hard cultivar Bobwhite and the final set derived from the soft cultivar Alpowa (PI 566596). (Hogg et al. 2005 and Morris and King 2008).

The Alpowa-derived samples were grown near Pullman, WA by the USDA-ARS Western Wheat Quality Laboratory. The wheat samples derived from Hi-line and Bobwhite were grown near Bozeman, MT by the Department of Plant Sciences and Plant Pathology at Montana State University. The wheat was grown in the 2007 season with two field replications for each sample. Once the wheat lines were harvested and cleaned, single kernel hardness was determined using the Single Kernel Characterization System 4100 (Perten Instruments North America, Inc., Springfield, IL). To provide enough wheat for these proposed experiments, the two field replicates were bulked into one sample (hardness values were compared to ensure no combinations of multiple wheat lines).

3.3.2 Milling

The wheat lines were milled using a Bühler experimental mill as per, the American Association of Cereal Chemists International (AACC) approved method 26-31 (AACC_2008). Modifications included a short time tempering (20 minutes) instead of the 16-24 hours for the Hi-line and Bobwhite derived samples. The 20 minutes tempering was short enough to insure

that the water did not penetrate into the endosperm, but provided enough time for the bran to become plastic and not break into small particles, potentially contaminating the flour. The Alpowa derived samples were tempered (24 hours) as per AACC 26-31. All wheat samples were tempered to 14 % moisture content and the wheat was milled at a reduced feed rate of 100 g/min.

3.3.3 Starch Isolation

Prime-starch was isolated using two different processes; a dough-ball method (Wolf 1964), and a batter method (see previous chapter, 2.3.3). In the dough-ball method dough was mixed to optimum (3-4 minutes) by a 100 g pin mixer (National Manufacturing Co., Division of TMCO, Lincoln, NE). After dough washing the starch slurry was filtered through a sieve with 75 μm opening sizes (Dual MFG. Co., Chicago, IL). After the filtration step the two starch isolation processes were identical to each other as described in the previous methods section.

3.3.4 Lipid Extraction

Based on the results from the previous chapter, which found that free lipids (hexane extractable) are extracted in relatively minor proportions when compared to the bound lipid extracts (mol% basis) only bound lipids were analyzed in this study. For a detailed description of the lipid extraction see (chapter section 2.3.4).

3.3.5 Lipid Quantification

The polar lipids targeted for analysis were; phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylglycerol (PG), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylglycerol (LPG), digalactosyldiglycerides (DGDG), monogalactosyldiglycerides (MGDG), digalactosylmonoglycerides (DGMG), and monogalactosylmonoglycerides (MGMG).

An automated electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) approach was used, and data acquisition and analysis and acyl group identification were carried out as described previously (Devaiah et al. 2006 and see chapter section 2.3.5).

3.3.6 Puroindoline Isolation and Determination

SDS-PAGE precast gels with a tris-HCL gradient of 10-20% were used (Bio-Rad, Hercules, CA). Puroindoline proteins were extracted using the modified method described by Giroux et al. (2003), which uses a Triton X-114 non-ionic detergent partitioning step. Modification includes, the use of SYPRO® Tangerine protein stain (Invitrogen, Carlsbab, CA) instead of Coomassie blue to visualize the protein bands. Gel images were obtained with a UVP (Upland, CA) Multidoc-It Imaging System equipped with the Doc-it LS Analysis software.

3.3.7 Statistical Analysis

For wheat flour polar lipid determination, five replicated lipid extractions were conducted on each wheat flour sample. Differences in flour means were assessed using Scheffé's multiple comparison procedure, $P = 0.05$. Wheat starch polar lipid determination was conducted using a completely randomized block experimental design. 'Day of starch isolation' was the blocking factor (three blocks with two duplicated lipid extractions each, $n = 6$). To determine significant sources of variation in lipid content found on the surface of wheat starch a two-way ANOVA was conducted using starch isolation method and wheat endosperm hardness classification as the sources of variation. Data for starch surface lipids were non-normally distributed. To fulfill the assumption of normality the data were *log* transformed. Multiple mean comparisons of starch isolation and genotype were conducted using the Scheffé's test, $P = 0.05$. All statistical analyses were conducted using Statistical Analysis System (SAS) software, General Linear Model (SAS version 9.1; SAS Institute; Cary, NC). Type III Sums of Squares were reported.

3.4 Results and Discussion

3.4.1 Wheat flour bound polar lipids

Wheat flour bound polar lipid composition and content were found to be similar to the results in the previous chapter. The four major lipid classes were DGDG, MGDG, PC, and LPC for all the wheat flour samples. DGDG was the most prevalent lipid class, representing 42 - 59 mol% of the total bound polar lipids. In general, wheat lines within the cultivar/experimental line pair were more similar than different. Mean comparisons for each class within the cultivar/experimental line pairs showed only slight differences. The overall polar lipid totals had no significant difference between the cultivar/experimental line pairs for all the samples (Table 3.2). The Bobwhite/BW2 pair had the most significantly different lipid class means, with BW2 containing greater amounts of DGDG and PA lipids and Bobwhite containing greater amounts of MGDG, PC, LPC, PE, MGMG, PI and LPE (Table 3.2). Within the HI-Line and HGAB18 pair, Hi-Line contained greater amounts of PC, and PI and HGAB18 contained greater amounts of DGDG, DGMG, MGMG, PA and PS (Table 3.2). The Alpowa/Alpowa/Canadian Red//7*Alpowa pair had the least difference between the lipid classes, with Alpowa containing greater amounts of DGDG, LPG and PS lipids and Alpowa/CanadianRed containing greater amounts of PI lipids (Table 3.2). Only DGDG and PI lipid classes were found to be significantly different between all the cultivar/experimental line pairs. It is worth noting that, the soft textured wheat lines (Alpowa, BW2 and HGAB18) contained significantly greater concentrations of DGDG lipids and the hard texture wheat lines contained significantly greater concentrations of PI lipids (Table 3.2).

Mean differences among the cultivar/experimental line pairs were significantly different. The Hi_line derived samples contained the greatest concentration of total polar lipids, 3638 nmol/g followed by Alpowa derived samples, at 3288 nmol/g and Bobwhite derived samples with 3055 nmol/g. Overall, these results indicate that there was more variability in total polar lipid content among the cultivar/experimental line pairs than within the pairs.

3.4.2 Variation in starch polar lipids

In this study two starch isolation methods were evaluated for differences in their effects on starch granule surface lipids. The two isolation methods represent starch isolated before dough development (batter method) and after dough development (dough method). In the randomized complete design where block represented the day of starch isolation and treatment was the starch isolation method, the blocking effect had no significant influence while the treatment was found to be a significant source of variation in polar lipid concentration (data not shown).

Wide ranges in starch surface polar lipid concentrations were observed between the two starch isolation methods. Included in this study were three near-isogenic wheat line pairs that varied in their puroindoline expression levels and thus were expected to vary in starch granule surface polar lipid concentrations. The combined polar lipid class means showed a broad range in polar lipid concentrations among the samples studied (Table 3.3). Minimum mean values for the four major starch surface polar lipids (DGDG, MGDG, PC and LPC) were 0.01, 0.04, 0.01 and 0.94 nmol/g, respectively, while their maximum values were 551, 222, 195 and 39 nmol/g. This broad range in lipid concentrations indicates that the starch granule surface is considerably different in its capacity to interact and bind lipids.

ANOVA results identifying the sources of variation within the samples are presented in Table 3.3. Data were non-normally distributed as determined by residual plots. Consequently, the data were *log* transformed to satisfy the normality assumption. Interactions between the main effects (starch isolation method and wheat endosperm hardness) were not significant for all the lipid classes except LPC and LPE (Table 3.3). The interactions between the main effects in the LPC and LPE lipids were caused by mean crossovers, where the starch isolated using the dough method contained greater amounts of lipids in the soft textured samples, and the starch isolated using the batter method contained greater amounts of lipids in the hard textured samples (Table 3.3 and Figures 3.1 and 3.2). Both main effects where found to be significant sources of variation in DGDG, MGDG, PC, PE, PG, DGMG, MGMG, PI, PS and PA lipid classes (Table 3.3). Based on ANOVA *F*-values for the polar lipid classes (Table 3.3) starch isolation method constituted a greater source of variation than did wheat kernel hardness.

All the model R^2 values for the di-acyl lipid classes (DGDG, MGDG, PC, PE, PG, PA, PI and PS) were relatively high, 0.69 to 0.89 (data not shown). The high R^2 value indicates a good model fit in which 69 to 89 % of the variation in the data can be explained by this 2-way ANOVA. The mono-acyl lipid classes (LPC, DGMG, MGMG, LPG) exhibited lower R^2 values, 0.14 to 0.35 (data not shown), indicating that the 2-way ANOVA only explains 14 to 35 % of the total variation in those lipid classes and that other sources of variation may exist for the mono-acyl lipids. Possible sources of variation other then isolation method and endosperm hardness could be differences in the extractability of the lyso-phospolipids from the channels of the wheat granule between the samples. Additionally, differences in lipid acyl-hydrolase activities or specificities of the various wheat flour samples could exist.

Figures 3.1 – 3.7 present the differences in polar lipids between starch isolation method and wheat endosperm hardness on a nmol/g basis. Figures 3.1 and 3.2 provide information regarding the polar lipid class differences, while Figure 3.3 – 3.7 provide information regarding molecular species differences within classes. For the molecular species figures, the y-axes within a lipid class were kept at a constant scale, providing the ability to visually observe the lipid contents in a 'mol% of total' basis as well as the labeled nmol/g. It is evident that starch isolation method had a significant influence on the abundance of the polar lipids, where starch isolated using the batter method contained greater amounts of polar lipids than did starch isolated using the dough method. The only non-significant difference between isolation methods was observed in the mono-acyl lipid classes (Figures 3.1 – 3.7). All the di-acyl lipids were in greater concentration on the batter isolated starches (Figures 3.1 – 3.7). The predominant lipid classes (DGDG, MGDG, PC and LPC) were different between the batter and dough isolated starch samples. For the dough isolated starch samples, LPC was the most predominant lipid class, but in the batter isolated samples DGDG, MGDG and PC lipid classes were found in greater quantities (Figures 3.1, 3.4, 3.8 and 3.9).

The molecular species present in this study were similar in composition to those identified previously. The most prevalent molecular species were those that contained 16:0 fatty acids and 18:2 fatty acids (36:4, 34:2 and 16:0). In general, the effect of starch isolation method was non-discriminant to the molecular species within a class, where the differences between the dough and batter starch lipids where relatively constant among all the molecular species (Figures 3.3 – 3.7).

The difference in starch surface lipids provides insight into the role starch surface lipids play in the development of gluten. During gluten formation the majority of flour polar lipids

become incorporated into the gluten phase of dough (Chung and Tsen 1975). It appears that as gluten is being developed polar lipids located originally on (or near) the surface of the starch granules become incorporated into the gluten phase of the dough. Starch granule surfaces are thus, dynamic and depending on their surface surrounding environment, their composition can be significantly altered.

Wheat endosperm hardness also had a significant effect on the composition and concentrations of the polar lipids located on the surface of starch (Table 3.3 and Figures 3.1-3.7). Endosperm hardness was a significant source of variation in the 2-way ANOVA for all the lipid classes except LPG and PA. Overall, the starch from soft textured wheat contained greater amounts of polar lipids than did starch from hard textured wheat (Figures 3.1- 3.7). An exception to this pattern was the PA and PI lipids from the Hi-line derived samples. The starch isolated from Hi-line flour (hard textured wheat) contained greater amounts of PA and PI lipids than did the starch isolated from HGAB18 flour (soft textured wheat). One possible explanation for this finding is that the activity of phospholipase D might be greater in the Hi-line wheat sample than the other wheat samples. Products of phospholipase D reactions are PA lipids. The molecular species difference between the starches isolated from soft and hard textured wheat also appears to be non-discriminating, with relatively similar differences between the near-isogenic pairs (Figures 3.3 – 3.7).

For some batter food systems (some cakes and pancakes) soft wheat flour is treated with chlorine gas to improve its color and baking quality (Montzheimer 1931 and Finnie et al. 2006). The starch granule properties are considerably altered due to chlorination (Sollars 1958 and Hoseney et al. 1988, Johnson et al. 1980). Seguchi (1987) attributed the starch granule property change to be caused by an increase in hydrophobicity of the starch granule surface. Since the

results of the current study show that starch from the soft textured wheat samples contained significantly greater amounts of polar lipids, the increase in starch granule surface hydrophobicity in chlorinated flour might be caused by a change in the polar lipids. The change in hydrophobicity could be the result of a change in polar lipid structure (e.g. physical removal of the polar components) or by an indirect change were the non-polar components (acyl groups) of the polar lipid become more accessible for association. Even though the results of the current study provide possible insight into the effects of chlorination on the starch granule surface lipids, more research is needed to fully determine how chlorination of wheat flour alters the properties of the starch granule surface. Especially since, other research has shown that the improving effect of chorine gas on starch granules is due to the oxidative depolymerization of glucose residues in the starch molecules (Johnson et al. 1980).

3.4.3 Resting time effect on starch polar lipids between the two isolation methods

During starch isolation, the time in which the dough or batter were allowed to rest after initial hydration were different between the two methods. The samples using the dough method were allowed to rest for 60 minutes after mixing, and the batter method samples were hydrated and rested for 10 minutes prior to starch isolation. To determine if the difference in resting time affected the starch granule surface lipids, a study was conducted using Alpowa flour, with two starch isolation methods, and three resting times (0, 30 and 60 minutes).

Varying resting time produced different results, depending on the isolation method (Figures 3.8 and 3.9). The four major classes in the batter isolated starch exhibited increases in their class totals over time. When evaluating the result on a nmol/g basis, DGDG, MGDG and PC class totals increased significantly between 0 minutes to 30 minutes after which no further increase was observed. The LPC class totals increased over the 0, 30 and 60 minute rests, but

were not found to be statistically different from each other. When analyzing the same data on the mol% of total basis no difference was observed between the resting times (Figure 3.8). The lack of differences in the mol% of total data show that even though the actual amount of lipids increased from 0 to 30 minutes in the major polar lipid classes, each class increased in relatively equal proportions.

The four major lipid classes from the dough-isolated starch exhibited a different response to time than did those from the batter isolated starch. On the nmol/g basis, LPC, the most predominant polar lipid in the dough isolated starch, increased between 0, 30 and 60 minutes (Figure 3.9). DGDG, MGDG and PC had an initial significant decrease between 0 and 30 minutes followed by a significant increase between 30 to 60 minutes, (Figure 3.9). The mol% of total value shows an initial increase between 0 and 30 minutes for the LPC lipids and an initial decrease in DGDG, MGDG and PC. After this point the mol% of total LPC decreased and the mol% of total for DGDG, MGDG and PC increased. These results provide evidence that the starch surface lipids (DGDG, MGDG and PC) are removed during dough development and that the internal starch lipids are not affected (LPC).

Even though the results for the resting time study show differences caused by rest time, the differences were minor when compared to the variation between the starch isolation methods. For future work evaluating starch surface lipids, the use of the batter method with a 30 minute resting period is recommended.

3.4.4 Effect of starch isolation method on starch surface proteins

A preliminary study was conducted to determine if the starch granule surface proteins (puroindoline a and b) exhibited the same response between the two starch isolation methods. The same starch samples used in the lipid extractions for Bobwhite and BW2 were used to

extract puroindoline proteins and measure their relative abundance via SDS-PAGE gels (Figure 3.10). The preliminary results show that starch isolated using the batter method contained more puroindoline proteins than did starch isolated using the dough method. The results between the soft and hard textured samples were expected, the starch from the soft textured wheat (BW2) contained greater puroindoline proteins than did the Bobwhite starch. However, to confirm and extend these results analysis of starch isolated from the other flour samples using the different isolation methods will need to be conducted.

3.5 Conclusion

This research provides evidence that as dough is mixed to optimum the lipids on the surface of wheat starch become incorporated into the gluten phase of the dough, whereas in a batter the starch surface lipids stay associated with the starch granule surface. The puroindoline proteins located on the surface of starch granules also are removed during the mixing of dough. This research also indicates that the predominant starch surface polar lipids are di-acyl lipids of DGDG (36:4), MGDG (36:4) and PC (34:2 and 36:4) polar lipid classes and the predominant internal starch polar lipids of are LPC (16:0 and 18:2) lipids. Based on the results of this study, we recommend that future studies evaluating starch surface components use a starch isolation method that involves no (or slight) gluten formation. We recommend the use of the batter method containing a 30 minute resting period as described in the methods section.

3.6 Acknowledgements

The lipid analyses described in this work were performed at the Kansas Lipidomics Research Center Analytical Laboratory. Instrument acquisition and method development at the Kansas Lipidomics Research Center was supported by National Science Foundation (EPS 0236913, MCB 0455318, DBI 0521587), Kansas Technology Enterprise Corporation, K-IDeA Networks of Biomedical Research Excellence (INBRE) of National Institute of Health (P20RR16475), and Kansas State University.

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3.8 Tables and Figures

Table 3.1: Cultivar and corresponding experimental line, puroindoline haplotype, molecular change and SKCS hardness value of the wheat samples obtained form Dr. Craig Morris and Dr. Mike Giroux.

Cultivar / Experimental Line	Puroindoline Haplotype	Molecular change from 'wild-type'†	Hardness (SKCS)§
Alpowa	*Pina-D1a/Pinb-D1a*	-	31
Alpowa/Canadian Red//7*Alpowa	*Pina-D1a/Pinb-D1e**	Pinb null (Trp-39 to stop)	68
Hi-Line	*Pina-D1a/Pinb-D1b*	Pinb Gly46 to Ser	82
HGAB18	*Pina-D1a**/Pinb-D1a**	-	9
Bobwhite	*Pina-D1b/Pinb-D1a*	Pina null	84
BW2	*Pina-D1a**/Pinb-D1a*	-	24

† 'Wild-type' defined as *Pina-D1a/Pinb-D1a* puroindoline haplotype
* Indicates puroindoline gene modified through backcross breeding method
** Indicates puroindoline gene modified through transgenic method
§ SKCS; single kernel characterization system hardness index value

Table 3.2: Mean values (nmol/g of sample) of different bound polar lipid classes from wheat flour cultivars and corresponding experimental wheat lines.

	Cultivar	Experimental line	Cultivar	Experimental line	Cultivar	Experimental line
Lipid Class	**Alpowa**	**Alpowa/Canadian Red**	**Bobwhite**	**BW2**	**Hi-line**	**HGAB18**
DGDG	1984 a ± 41	1764 b ± 34	1334 b ± 41	1509 a ± 37	1549 b ± 17	1762 a ± 27
MGDG	684 a ± 14	691 a ± 15	530 a ± 14	482 b ± 10	700 a ± 18	726 a ± 11
PC	475 a ± 7	489 a ± 19	743 a ± 16	626 b ± 17	887 a ± 45	719 b ± 6
LPC	82 a ± 2	80 a ± 4	165 a ± 4	147 b ± 3	176 a ± 2	165 a ± 4
PE	55 a ± 1	58 a ± 2	133 a ± 2	107 b ± 2	93 a ± 24	84 a ± 2
PG	32 a ± 1	31 a ± 2	33 a ± 1	34 a ± 1	54 a ± 1	58 a ± 1
DGMG	20 a ± 1	19 a ± 1	18 a ± 1	17 a ± 1	16 b ± 1	18 a ± 0
MGMG	14 a ± 0	15 a ± 1	15 a ± 1	13 b ± 1	14 b ± 0	16 a ± 0
LPG	11 a ± 0	10 b ± 1	13 a ± 0	14 a ± 0	19 a ± 0	20 a ± 0
PA	9 a ± 0	9 a ± 1	23 b ± 1	26 a ± 0	31 b ± 1	44 a ± 1
PI	8 b ± 0	16 a ± 1	58 a ± 2	29 b ± 1	49 a ± 1	24 b ± 1
PS	6 a ± 0	4 b ± 0	11 a ± 0	11 a ± 0	13 b ± 0	17 a ± 1
LPE	5 a ± 0	5 a ± 0	10 a ± 0	9 b ± 0	10 a ± 3	12 a ± 1
Total Polar	3385 a ± 61	3192 a ± 60	3086 a ± 77	3023 a ± 67	3664 a ± 54	3611 a ± 47

Value represent mean nmol of polar lipids per gram of sample ±SE, n=5. Letter difference represents significant difference between cultivar and corresponding experimental line, $P < 0.05$.

Table 3.3: Sample statistics and *F*-values from two-way ANOVA for starch isolation method and wheat endosperm hardness sources of variation.

Statistic/ Source	DGDG	MGDG	PC	LPC	PE	PG	DGMG	MGMG	LPG	PA	PI	PS	LPE
Minimum	0.01	0.04	0.01	0.94	0.00	0.04	0.01	0.08	0.13	0.00	0.02	0.00	0.00
Mean	92	35	29	17	2	1	1	1	2	2	0.5	0.5	1
Maximum	551	222	195	39	11	7	3	2	4	12	1	2	4
Isolation Method	258	399	396	ns	209	439	11	4	ns	140	220	122	9
Hardness	61	77	80	5	33	94	24	19	ns	ns	4	29	7
Interaction	ns	ns	ns	5	ns	ns	ns	ns	14	ns	ns	ns	ns

Sample statistics are expressed as nmol/g of sample. *F*-values are significant at $P = 0.05$; ns, not significant. Data used for 2-way ANOVA were transformed to satisfy normality assumption using log. *F*-values derived from Type III sums of squares.

Figure 3.1: Polar lipid class means (nmol/g) from starch isolated using batter (solid bars) and dough (stripped bars) methods. Values are means and ± SE (*n* = 6). * indicates significantly greater lipid content between the starch isolation method, for the specific wheat sample, § indicates significantly greater lipid content between cultivar and corresponding experimental line within starch isolation method, both at $P < 0.05$.

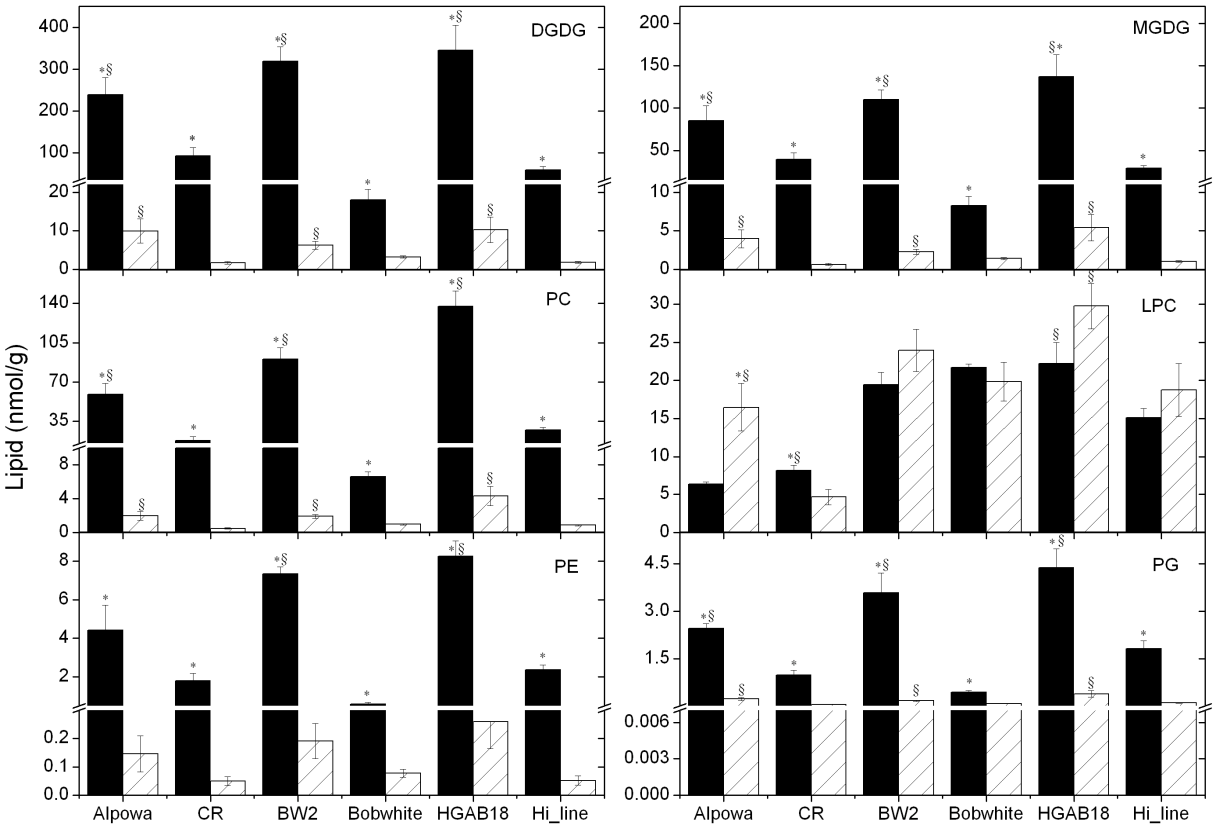


Figure 3.2: Polar lipid class means (nmol/g) from starch isolated using batter (solid bars) and dough (stripped bars) methods. Values are means and ± SE (*n* = 6). * indicates significantly greater lipid content between the starch isolation method, for the specific wheat sample, § indicates significantly greater lipid content between cultivar and corresponding experimental line within starch isolation method, both at *P* < 0.05.

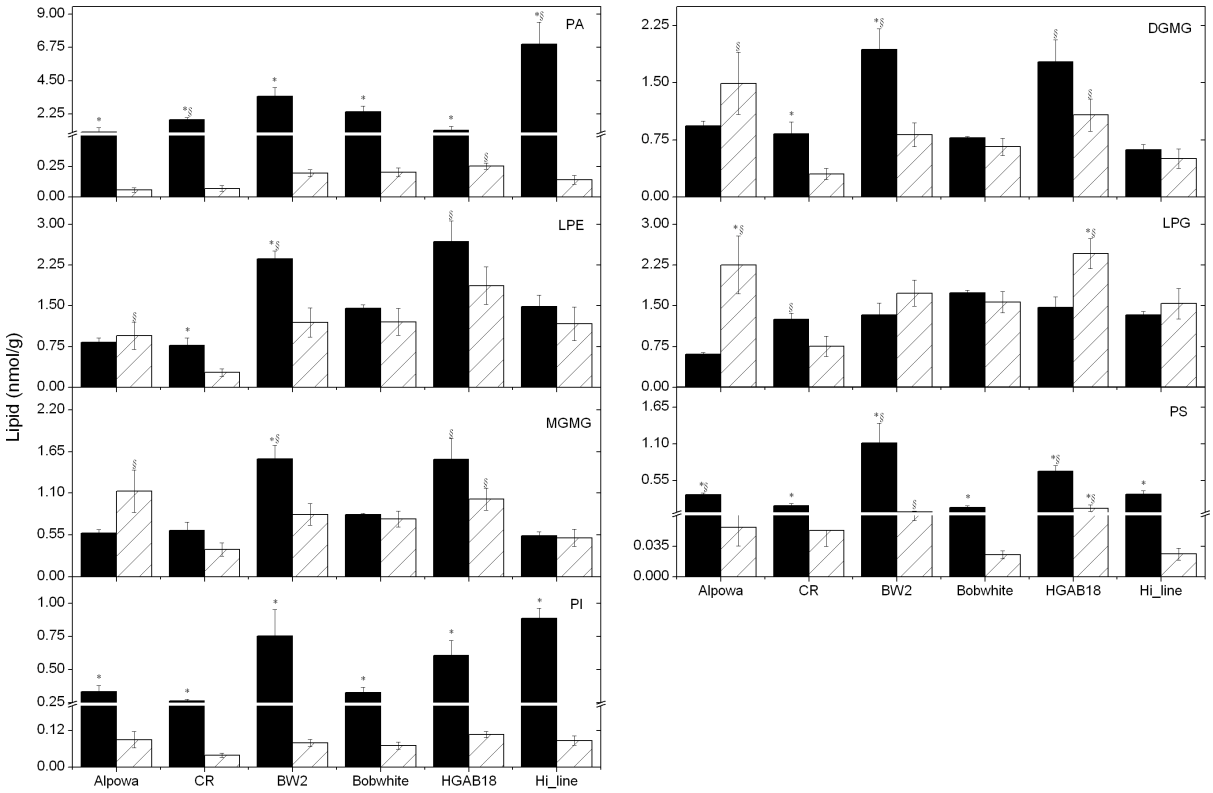


Figure 3.3: DGDG and MGDG molecular species means in starch isolated using batter (solid bars) and dough (stripped bars) methods. Values are means and ± SE (nmol/g) (*n* = 6).

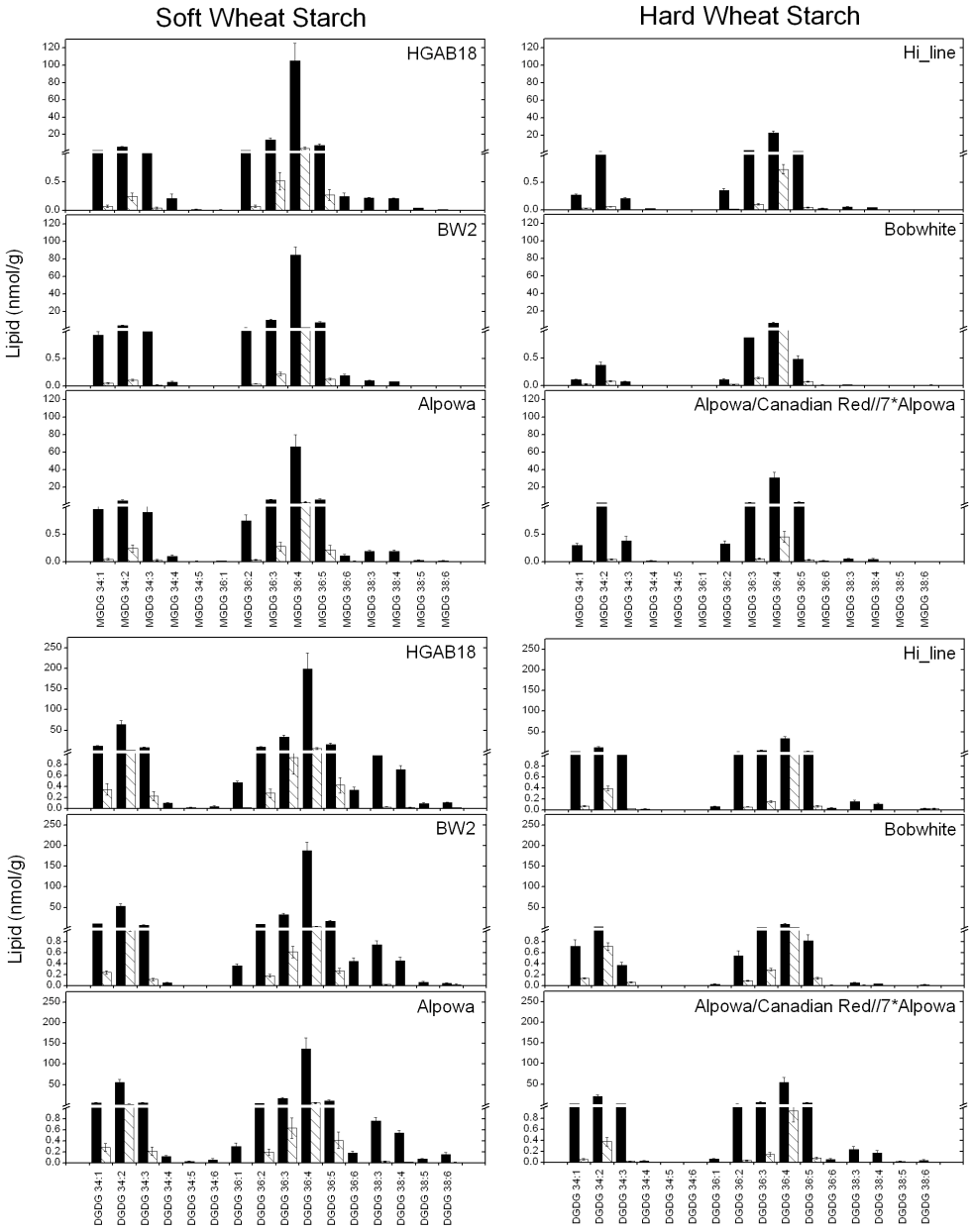


Figure 3.4: PC and PE molecular species means in starch isolated using batter (solid bars) and dough (stripped bars) methods. Values are means and ± SE (nmol/g) (*n* = 6).

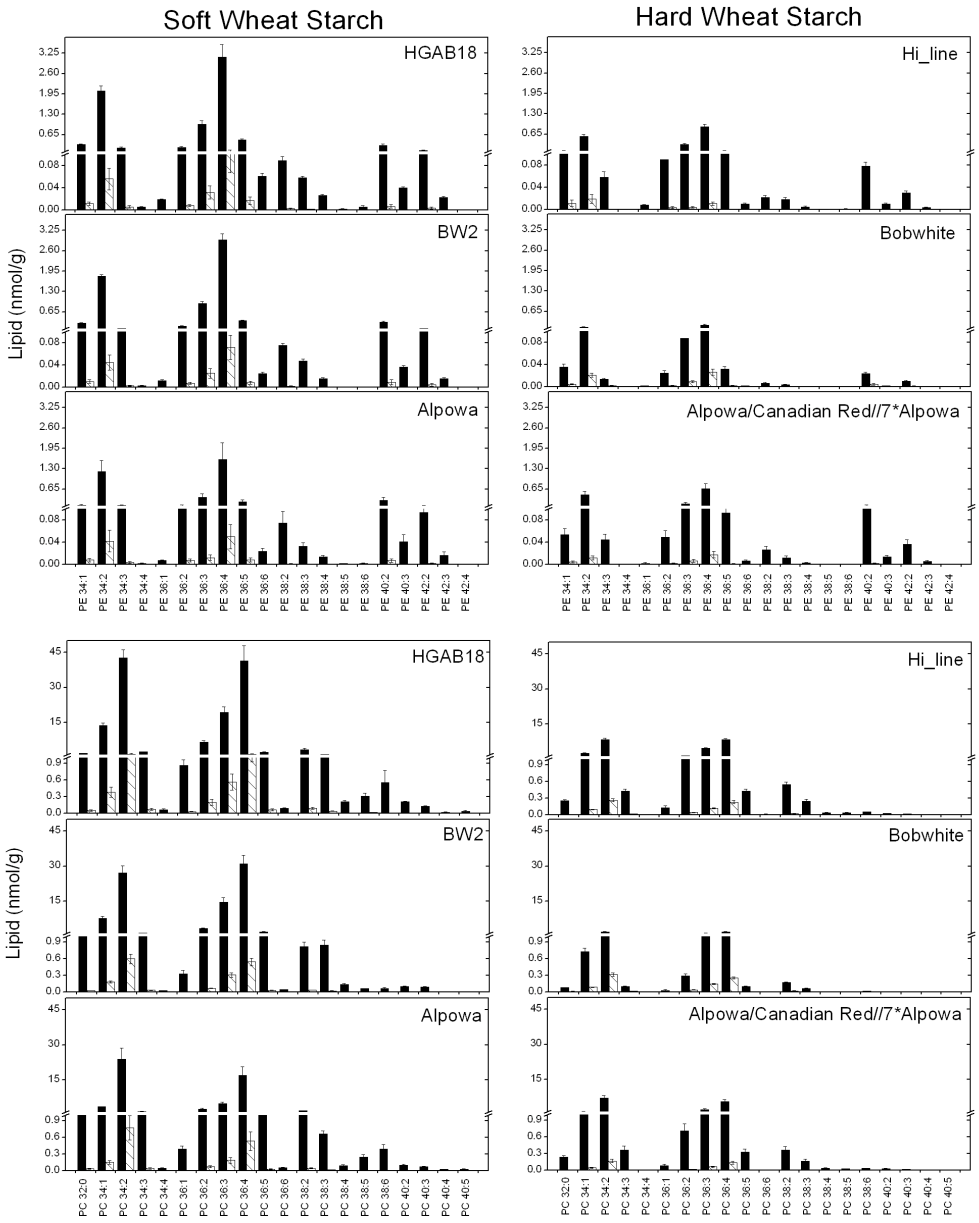


Figure 3.5: PA and PG molecular species means in starch isolated using batter (solid bars) and dough (stripped bars) methods. Values are means and ± SE (nmol/g) (*n* = 6).

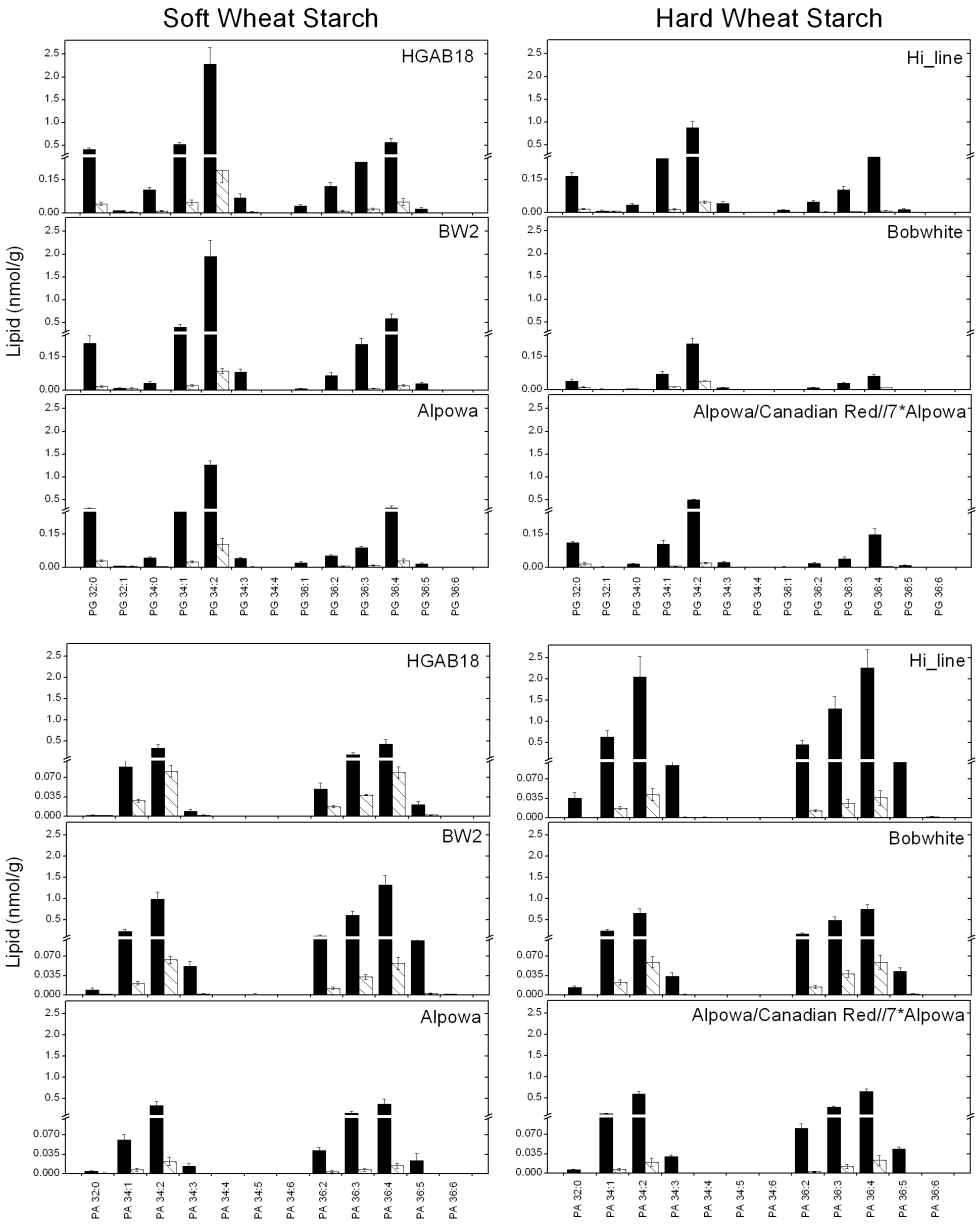


Figure 3.6: PI and PS molecular species means in starch isolated using batter (solid bars) and dough (stripped bars) methods. Values are means and ± SE (nmol/g) (*n* = 6).

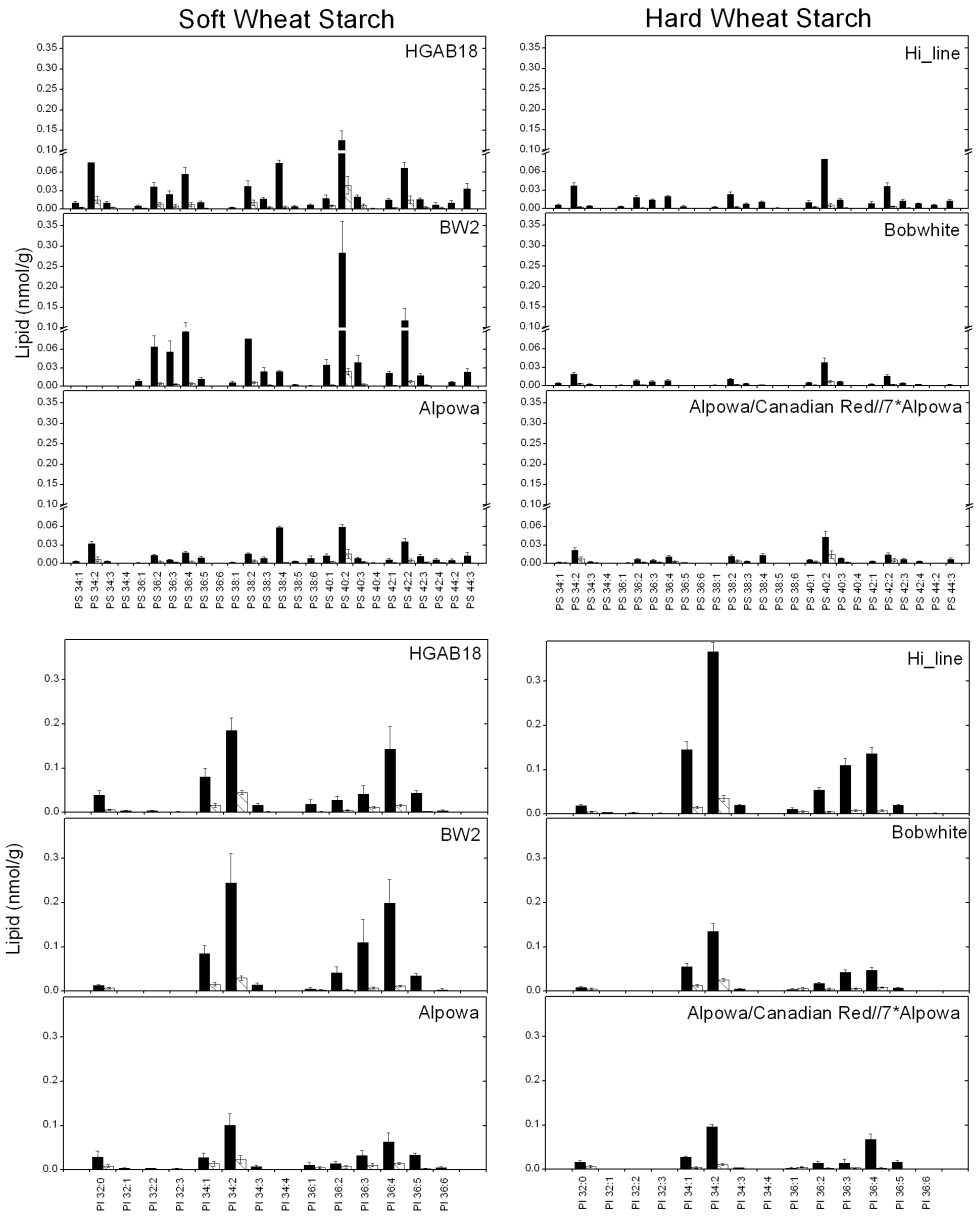


Figure 3.7: DGDG, MGDG, LPC, LPE and LPG molecular species means in starch isolated using batter (solid bars) and dough (stripped bars) methods. Values are means and ± SE (nmol/g) (*n* = 6).

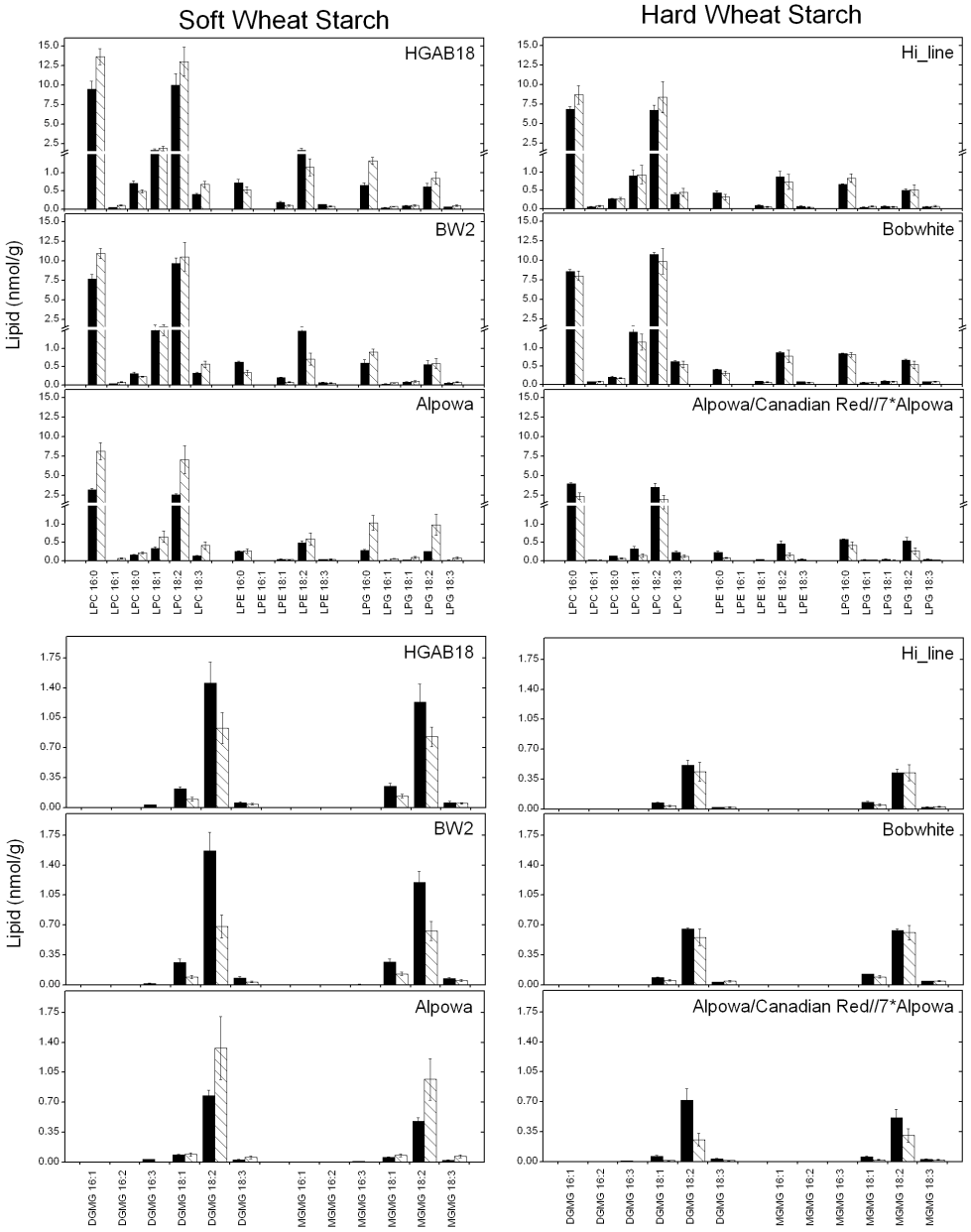


Figure 3.8: Major polar lipid classes (DGDG, MGDG, PC and LPC) in starch isolated from Alpowa flour using the batter method with three different resting periods, (0, 30 and 60 minutes) prior to fractionation. Values are means and ± SE of two starch isolation replicates with five lipid extractions each (*n* = 10). The Top graph represents data in nmol/g while bottom graph represents data in mol% of total

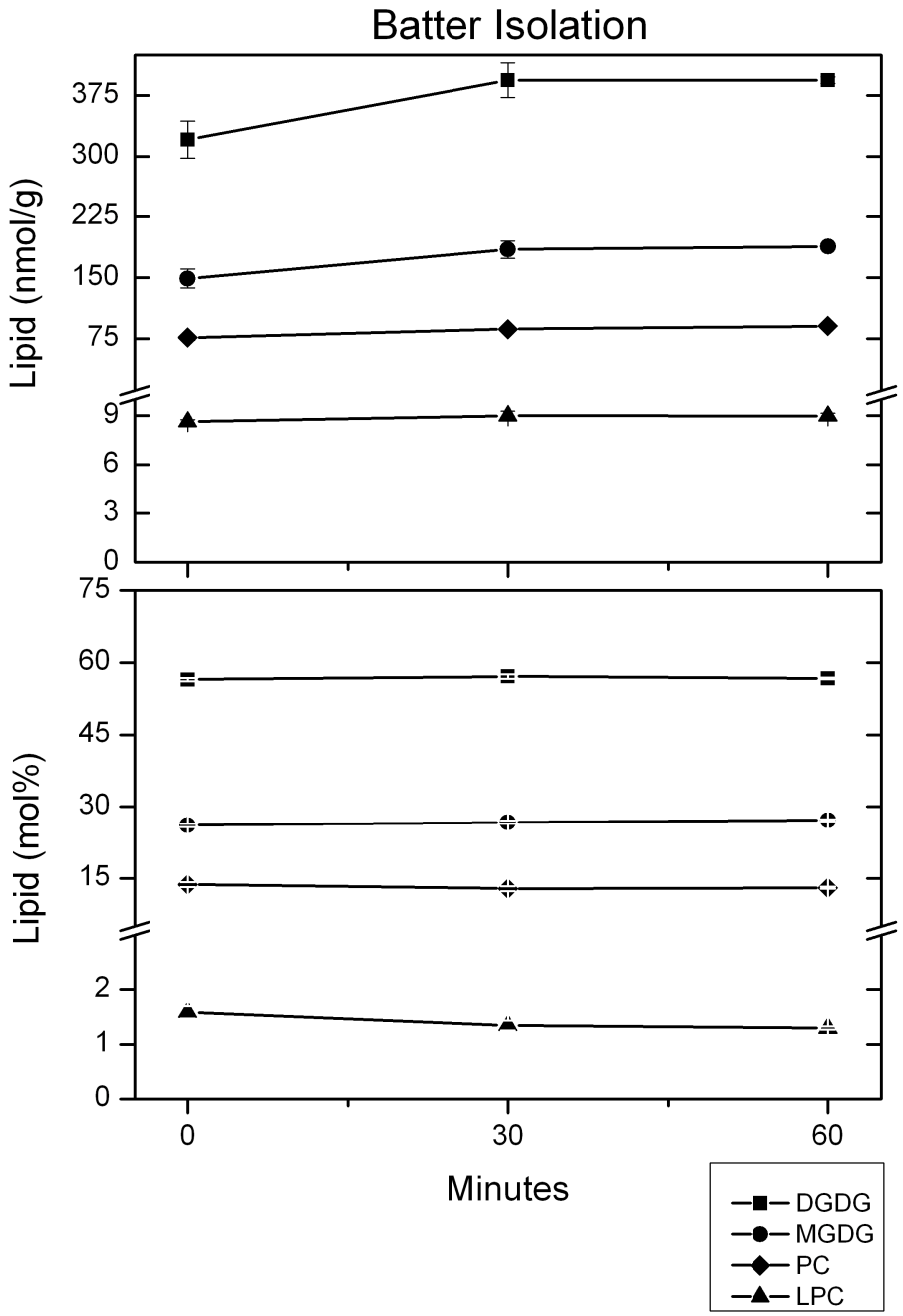


Figure 3.9: Major polar lipid classes (DGDG, MGDG, PC and LPC) in starch isolated from Alpowa flour using the dough method with three different resting periods (0, 30 and 60 minutes) prior to fractionation. Values are means and ± SE of two starch isolation replicates with five lipid extractions each (*n* = 10). The Top graph represents data in nmol/g while bottom graph represents data in mol% of total

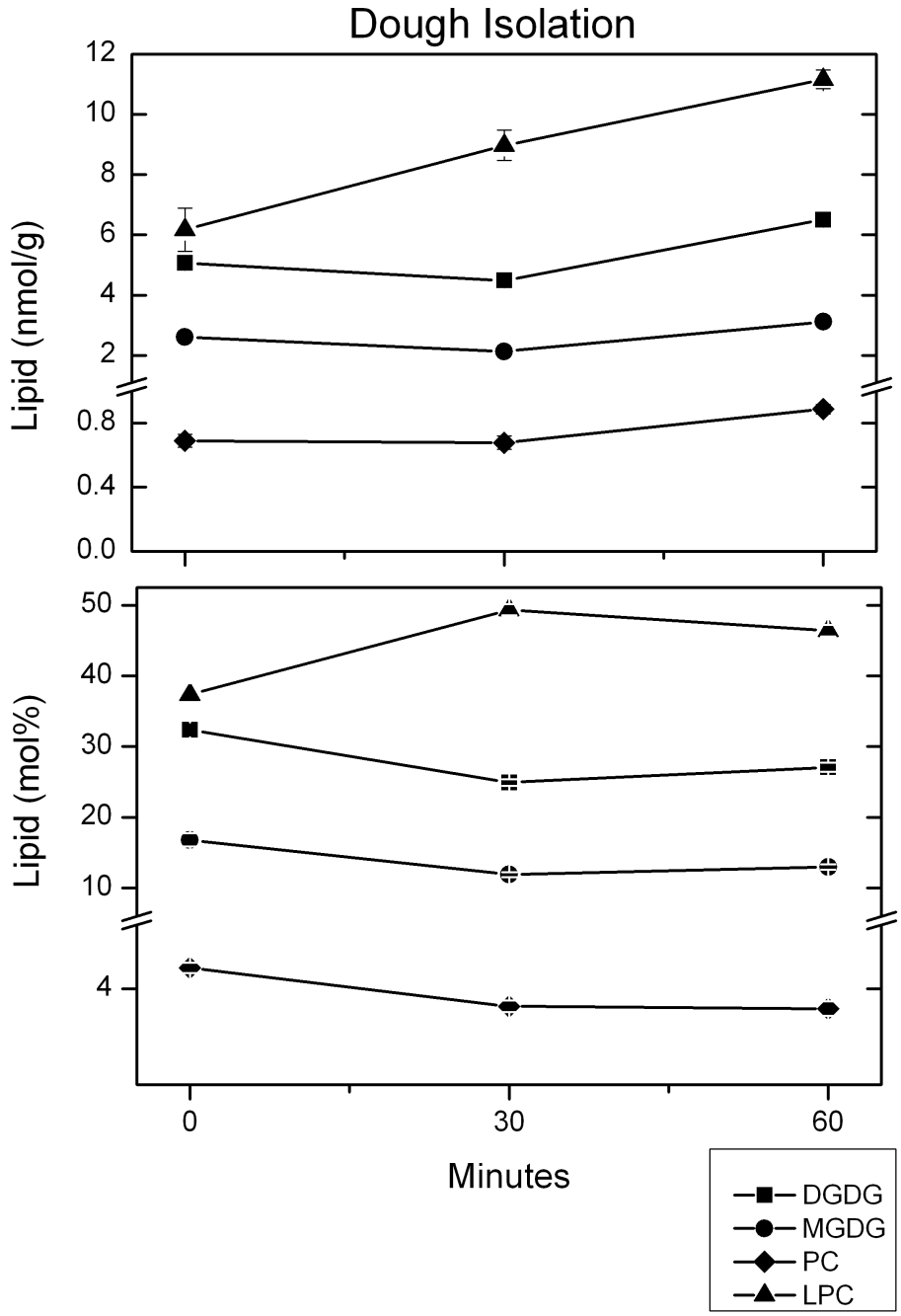
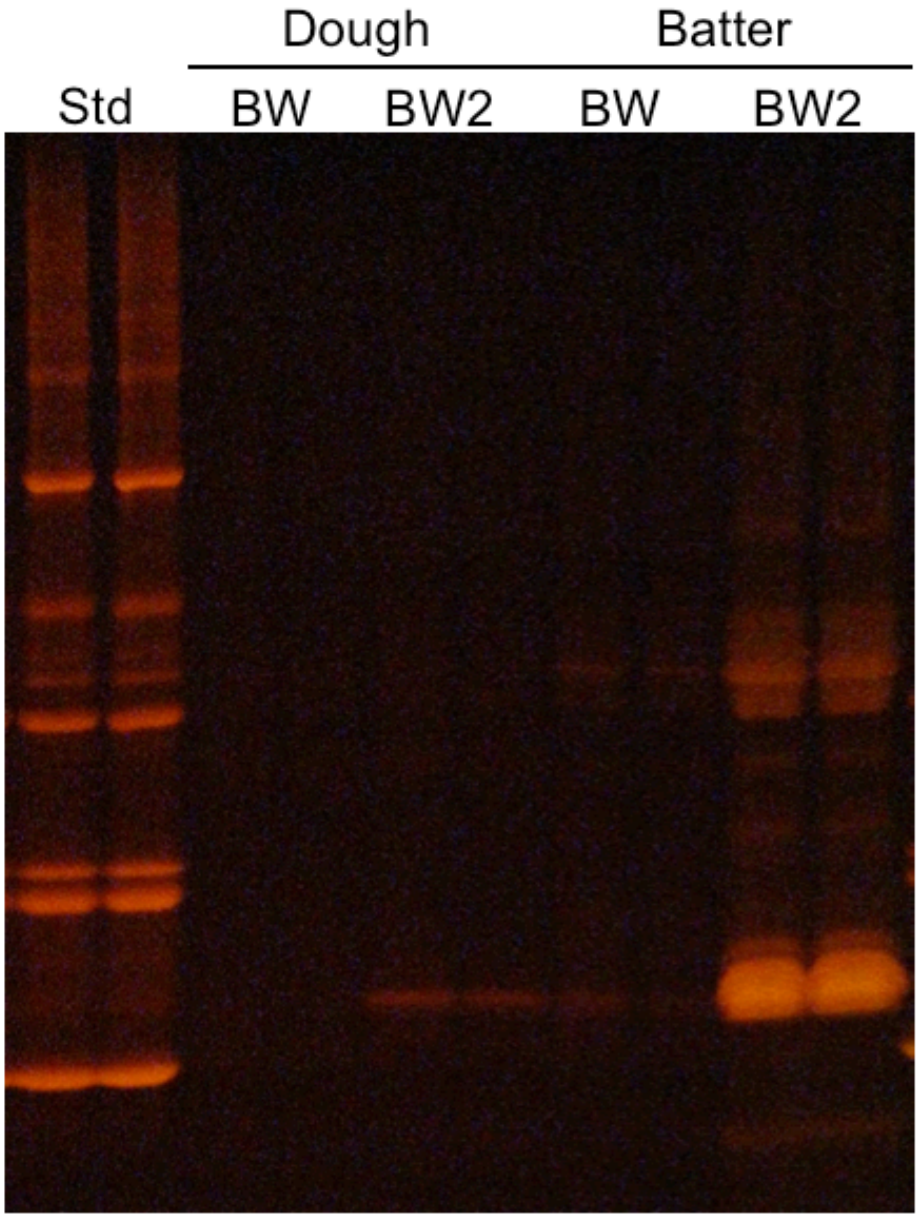


Figure 3.10: SDS-PAGE image of puroindoline proteins extracted from starch isolated from Bobwhite (BW) and BW2 wheat lines using the batter and dough method. Standard molecular markers (Std) with molecular mass range of 14 to 66 kDa.



CHAPTER 4 - Variation in Polar Lipid Composition within Near-Isogenic Wheat Lines Containing Different Puroindoline Haplotypes

4.1 Abstract

An extensive amount of research has investigated kernel hardness. However, the exact mechanism underlying this phenomenon is unknown. Puroindoline-a and puroindoline-b proteins must be present in their wild-type form to create soft textured wheat. Similar to puroindoline proteins, polar lipids are present on the surface of starch granules. The objective of this research was to determine the specific polar lipid species present on the surface of wheat starch from near-isogenic wheat lines that contain different puroindoline haplotypes and endosperm hardness. Five near-isogenic wheat lines were used in this analysis, all derived from the soft cultivar Alpowa. Water-washed starch was isolated using a modified batter method. Direct infusion tandem mass spectrometry was used to identify the lipid species in both the flour and starch samples. Endosperm hardness had no significant effect on the polar lipid contents in wheat flour, had a slight influence on the polar lipid contents of the whole-meal and had a significant influence on the polar lipid composition of the polar lipids located on the surface of wheat starch. The greatest quantities of polar lipids on the starch surface occurred when both puroindoline proteins where present in their wild-type form. Starch surface polar lipid content dramatically decreased when one of the puroindoline proteins were null, or if the pin-b was in the mutated form (Trp-44 to Arg). Within the hard textured samples, more polar lipids were present on the starch surface when pin-b was in its wild-type form and pin-a was null than when

pin-a was in its wild-type form and pin-b was null; the least amount of polar lipids where present when pin-b was in its mutated form (Trp-44 to Arg) and pin-a as in its wild-type form.

4.2 Introduction

Wheat kernel physical hardness, often referred to as texture, is the most important trait used for the classification of its intended end-use. An extensive amount of research has investigated wheat kernel hardness, however, the exact mechanism underlying this phenomenon remains unknown. Despite the lack of understanding regarding the specific mechanism of kernel hardness, the molecular source has been located at the interface between the starch granule surface and storage proteins of the wheat endosperm. Using a micropenetrometer, Barlow et al. (1973), measured the hardness of starch granules and storage protein within wheat kernels from different hardness classes and found no significant difference between the hardness of the starch granules and that of the surrounding storage protein. Barlow et al. (1973) concluded that differences between soft and hard textured wheat varieties must be in the adhesive characteristics between the starch granule surface and the storage proteins.

By evaluating the surface components of water-washed starch granules, Greenwell and Schofield (1986) discovered an un-broken molecular pattern between soft and hard textured wheat. A group of ~15 kDa proteins (friabilin) were found in greater quantities on water-washed starch from soft wheat than on equivalently treated starch from hard wheat. These proteins (friabilins) were absent from water-washed starch from durum wheat, the hardest wheat class. These results established a foundation for the molecular basis of wheat endosperm hardness.

Further investigation of the friabilin proteins revealed the existence of two protein isomers, puroindoline A (pin-a) and puroindoline B (pin-b), which together comprise friabilin (Jolly et al. 1993 and Morris et al. 1994). The name puroindoline is derived from their unique tryptophan-rich domain (indoline) and the Greek word for wheat (Puro). Pin-a contains five tryptophan residues in the sequence WRWWKWWK, while in pin-b that region is truncated to

three tryptophan residues in the sequence WPTKWWK (Gautier et al. 1994). Kooijman et al. (1997) suggest the tryptophan rich domains in puroindolines form loop structures at the exterior of the protein.

Puroindolines in their wild-type state (*Pina-D1a/Pinb-D1a*) expressed a soft-wheat phenotype (Morris 2002 and references therein). Pin-a and pin-b act complementary to each other to form friabilin and both must be present in their wild-type state for the expression of soft textured wheat (Martin et al. 2006). When either of the puroindoline proteins are mutated or absent, the resulting phenotype will be hard in texture. Several puroindoline mutations are known which result in hard endosperm texture. They include; pin-a null and pin-b wild-type (*Pina-D1b/Pinb-D1a*) (Morris et al. 2001), pin-a wild-type and pin-b Gly46 to Ser (*Pina-D1a/Pinb-D1b*) (Martin et al. 2001), pin-a wild-type and pin-b Lue60 to Pro (*Pina-D1a*/ *Pinb-D1c*) (Morris et al. 2001), pin-a wild-type and pin-b Trp44 to Arg (*Pina-D1a*/*Pinb-D1d*) (Lillemo and Morris 2000.), pin-a wild-type and pin-b Trp39 to stop codon (*Pina-D1a/Pinb-D1e*) (Morris et al. 2001) pin-a wild-type and pin-b Trp44 to stop codon (*Pina-D1a*/*Pinb-D1f*) (Morris et al. 2001) and pin-a wild-type and pin-b Cys56 to stop codon (*Pina-D1a*/*Pinb-D1g*) (Morris 2001, Morris and Bhave 2008, Bhave and Morris 2008(a) and Bhave and Morris 2008(b)).

Morris and King (2008) developed a series of unique puroindoline allele near-isogenic hexaploid wheat experimental lines. The soft white spring cultivar, Alpowa (PI 566595), was used as the recurrent parent to which donor parents containing specific puroindoline haplotypes (pin-a null, pin-b Gly46 to Ser, pin-b Lue60 to Pro, pin-b Trp44 to Arg, pin-b Trp39 to stop codon, pin-b Trp44 to stop codon and pin-b Cys56 to stop codon) were crossed as the male donor plant. These near-isogenic wheat lines provide the unique ability to study the molecular basis of

endosperm hardness and have the potential to help solve the enigma of the mechanism of endosperm hardness.

Similar to puroindoline proteins, Greenblatt et al. (1995) found that a pattern existed among polar lipids present on the surface of starch granules. Galactolipids and phospholipids were found, via thin layer chromatography, to be in greater amounts on water-washed starch from soft wheat than from water-washed starch from hard wheat (Greenblatt et al. 1995). Konopka et al. (2005) further found a negative correlation to exist between starch surface lipids (polar and nonpolar) and kernel hardness. However, a full profile of the lipid species found on starch granule surface and the relationship of these molecules to endosperm hardness has not been reported.

The interaction between the puroindoline proteins and polar lipids has been intensively researched for their roles in endosperm hardness and gas cell stabilization effects (Wilde et al. 1993, Dubreil et al. 1997, Bottier et al. 2008, Clifton et al. 2007, and Clifton et al. 2008). The unique tryptophan-rich loop of puroindoline proteins plays a role in the proteins' interactions with lipids (Clifton et al. 2007). Wilde et al. (1993) demonstrated the ability of a single puroindoline molecule to bind ~5 lysophosphatidylcholine molecules. Dubreil et al. (1997) determined that pin-a associates tightly to phospholipids and galactolipids while pin-b is loosely associated with galactolipids and preferentially binds to negatively charged phospholipids. These studies have been conducted *in vitro*, where the puroindoline proteins have been extracted from the wheat and combined with either natural or synthetic polar lipids. It is unknown if the results of the *in vitro* studies above are valid *in vivo*. By studying the relationship between the polar lipids and puroindoline proteins located on the surface of wheat starch *in vivo* and implementing the relationships demonstrated with the *in vitro* studies, a clearer understanding of the potential

mechanism of endosperm hardness may be established. Therefore the objective of this research was to determine the specific polar lipid species present on the surface of wheat starch from near-isogenic wheat lines that contain different puroindoline haplotypes and endosperm hardness. The near-isogenic wheat lines used in this study were developed and characterized by Morris and King (2008). Three of the hard-textured experimental lines were used in this study, each with different puroindoline expressions. In a different backcrossing series, Dr. Craig Morris introduced a 'supersoft' phenotype into the Alpowa lines. It has been observed that some soft wheat varieties genetically possess a softer texture endosperm than other soft wheat varieties (Bettge et al. 2000). One of these 'supersoft' varieties (SS163) were backcrossed into the Alpowa cultivar using a similar procedure as described by Morris and King (2008). By using these near-isogenic wheat lines we were able to establish relationships between the polar lipid compositions of different puroindoline haplotypes as they relate to endosperm hardness.

4.3 Experimental

4.3.1 Wheat Samples

A series of near-isogenic wheat lines (NILs) were collected that varied in their puroindoline haplotypes (Table 4.1). The near-isogenic wheat lines used in this study were developed and characterized by Morris and King (2008). The use of these NILs, that contain different hardness phenotypes but are nearly genetically identical, provide the unique ability to analyze starch granule surface lipids from wheat lines that vary in grain texture while all other genetically controlled wheat components are constant.

The wheat samples were grown near Pullman, WA with the assistance of the USDA-ARS Western Wheat Quality Laboratory. The wheat was grown in the 2007 and 2008 season with two field replications for each sample. Alpowa, Alpowa/ID377s//7*Alpowa, Alpowa/Mjølner//7*Alpowa, Alpowa/Canadian Red//7*Alpowa were all grown in 2007 and Alpowa/SS163//7*Alpowa was grown in 2008. Once the wheat lines were harvested and cleaned, single kernel hardness was determined using the Single Kernel Characterization System 4100 (Perten Instruments North America, Inc., Springfield, IL). To provide enough wheat for these proposed experiments, the two field replicates were bulked into one sample (hardness values were compared to ensure no combinations of multiple wheat lines).

4.3.2 Milling

The wheat lines were milled using a Bühler experimental mill as per, the American Association of Cereal Chemists International (AACC) approved method 26-31. All wheat samples were tempered to 14 % moisture content for 24 hours and the wheat was milled with a

reduced feed rate of 100 g/min. Whole-meal samples were milled with a cyclone mill (UDY Corp., Boulder, CO) through a 0.5 mm screen.

4.3.3 Starch Isolation

Prime-starch was isolated using the batter method as described earlier in this document (See chapter section 2.3.3).

4.3.4 Lipid Extraction

Lipids were extracted from whole-meal, flour, and starch fractions. Based on the first chapter's results indicating that starch surface free lipids (hexane extractable) are extracted in minor proportions when compared to the bound lipid extracts, only bound lipids were analyzed in this study. For a detailed description of the lipid extraction see (See chapter section 2.3.4)

4.3.5 Lipid Quantification

The polar lipids targeted for analysis were; phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylglycerol (PG), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylglycerol (LPG), digalactosyldiglycerides (DGDG), monogalactosyldiglycerides (MGDG), digalactosylmonoglycerides (DGMG), and monogalactosylmonoglycerides (MGMG).

An automated electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) approach was used, and data acquisition and analysis and acyl group identification were carried out as described previously (Devaiah et al. 2006 and see chapter section 2.3.5).

4.3.6 Statistical Analysis

For all polar lipid determinations (meal, flour and starch extracts), five replicated lipid extractions were conducted on each sample. Differences in means were assessed using the Scheffé multiple comparison procedure, $P = 0.05$. To determine if wheat kernel hardness was a significant source of variation in polar lipids extracted in whole-meal, flour and starch surface samples, a one-way ANOVA was conducted. Data for each fraction were determined to be normally distributed by observations of residual plots and normal probability plots. All statistical analyses were conducted using Statistical Analysis System (SAS) software, Proc GLM and Proc Univariate (SAS version 9.1; SAS Institute; Cary, NC). Type III Sums of Squares were reported.

4.4 Results and Discussion

4.4.1 Total polar lipid class variation among wheat fractions

The sample statistics and wheat endosperm hardness *F*-values for total polar lipids are summarized in Table 4.2. Total polar lipids are additive values of all the bound polar lipid classes analyzed (DGDG, MGDG, PC, PE, PI, PA, PS, PG, LPC, LPG, LPE, DGMG and MGMG). The sample statistics for the bound polar lipids from both the whole-meal and flour fractions contained similar ranges with 2750 to 3600 nmol/g and 2550 to 3600 nmol/g, respectively (Table 4.2). The similarities between the whole-meal and flour fractions were expected, since the majority of polar lipids are located in the endosperm of wheat (Carr et al. 1992 and Morrison 1988).

The range in total bound polar lipids in the whole-meal and flour samples are relatively minor (0.3 and 0.4 fold increase, respectively) when compared to the range in the starch-surface lipids (10 fold increase, 50 to 500 nmol/g) (Table 4.2). These results are expected since the samples analyzed are nearly genetically identical to each other, but with different endosperm hardness phenotypes. The one-way ANOVA for endosperm hardness source of variation further emphasizes the genetic similarities of the samples. Based on *F*-values, wheat endosperm hardness had no significant effect on total bound lipids from the flour fractions and a relatively minor effect on the whole-meal samples (*F*-value of six with a significance level of $P=0.05$) (Table 4.2). Whereas the effect of endosperm hardness on the starch-surface bound polar lipids were significant to a $P<0.0001$ level and an *F*-value of 69 (Table 4.2). These results were expected since it has been shown that the surface of wheat starch isolated from soft wheat flour contains greater amounts of polar lipids than wheat starch isolated from hard wheat flour

(Chapters 2 and 3 from this document and Greenblatt et al. 1995). Overall, the results from Table 4.2 indicate that whole-meal and flour from soft and hard wheat will have relatively similar quantities of bound polar lipids, but the starch-surface from those same flours will have dramatically different amounts of bound polar lipids.

4.4.2 Whole-meal polar lipid class differences among wheat genotypes

The genotype class totals were more similar to each other than different with no apparent pattern for the polar lipid class totals among the wheat genotypes. The hard textured genotypes contained the greatest quantities of DGDG and MGDG lipids while the soft textured genotypes contained the greatest quantities of PC, LPC, PG, DGMG, MGMG, PA and LPE (Table 4.3). Alpowa/SS163//7*Alpowa had significantly greater amounts of PE and PA lipids while the other four genotype class totals were statistically the same (Table 4.3). PI and PS class totals were statistically similar for all the genotypes (Table 4.3). The predominant lipid classes for all the genotypes were the same with the order of decreasing abundance for all the class totals greater then 100 nmol/g being DGDG, MGDG, PC, LPC, PE and PI. Interestingly, PI class totals were relatively large when compared to the flour and starch-surface lipids (Tables 4.3, 4.4 and 4.5). The PI results are similar to results from Chapter 2, where PI lipids were localized predominantly in the bound lipids from the whole-meal fractions.

4.4.3 Flour polar lipid class differences among wheat genotypes

Similar to the whole-meal polar lipid class totals, the flour totals appeared more similar to each other than different (when compared to the class totals from the starch-surface fractions) Tables 4.4 and 4.5. MGMG lipids were statistically similar between all the genotypes in the flour fractions (Table 4.4). Interestingly, Alpowa/SS163//7*Alpowa contained the greatest quantity of PC, LPC, PE, PG, DGMG, LPG, PA, and PS, but the least amount of DGDG and

MGDG (Table 4.4). The Alpowa/SS163//7*Alpowa plants were grown in the 2008 crop year whereas all other genotypes were grown in the 2007 crop year. The observed differences between Alpowa/SS163//7*Alpowa and the other genotypes could be a result of the introduction of the 'supersoft' phenotype into Alpowa or could be a result of differences contributed by growing the plants in different environments ('years'). Chung et al. (2009) and references therein state that genotype, environment and their interactions have a significant influence on the lipid content and composition in wheat flour and depending in the growing environment a two-fold difference in DGDG, MGDG and PC can be observed.

4.4.4 Variation within starch-surface polar lipid classes

Bound polar lipid class totals varied significantly between the NILs with Alpowa cultivar containing the greatest concentrations for all the class totals and the hard textured genotypes containing considerably fewer bound polar lipids (Table 4.5). LPG was the only polar lipid class that was not significantly different among the genotypes (Table 4.5). Similar to results presented in Chapters 2 and 3, the most predominant lipids located on the surface of wheat starch were DGDG, MGDG, PC and LPC (Table V).

Because the endosperm texture of Alpowa/SS163//7*Alpowa was softer than the endosperm texture of Alpowa, it was unexpected that the Alpowa/SS163//7*Alpowa genotype contained fewer bound polar lipids than the Alpowa cultivar (Table 4.5). These results suggested that starch surface bound lipids contribute to variation among hardness classes, but within (at least within the soft texture class) other factors may contribute to differences in endosperm hardness. Additionally, the effect of growing the two soft texture genotypes in different years make it difficult to directly compare between the bound polar lipids in Alpowa and Alpowa/SS163//7*Alpowa. Even though the soft textured wheat lines are nearly genetically

identical to each other and the wheat lines contain the same puroindoline haplotype (*Pina-D1a/Pinb-D1a*), differences in the quantity of bound polar lipids located on the surface of the starch granules exist. These results indicate that a possible threshold exists in terms of the necessary quantity of starch surface components (puroindoline proteins and polar lipids) needed to produce soft textured endosperm and that any additional starch surface components do not necessarily result in a softer textured endosperm.

The results from the hard textured wheat lines provide interesting insight into the effect of lipid binding on the starch surface between the different puroindoline haplotypes. Unlike the soft textured wheat lines, the hard textured wheat lines were grown in the same year and the same location, affectively removing any environmental source of variation. All the bound polar lipid class totals were significantly different within the hard texture sample except LPC, PE, DGMG, LPG and PS (Table 4.5). The Alpowa/ID377s//7*Alpowa (pin-a null) sample contained the greatest quantity of DGDG, MGDG, PC, PG, MGMG, PA, PI, and LPE class totals (Table 4.5). Alpowa/Canadian Red//7*Alpowa (pin-b null) contained intermediate quantities and Alpowa/Mjølner//7*Alpowa (pin-a wild-type and pin-b Trp-44 to Arg) containing the least of those same bound polar lipids (Table 4.5). These results indicate that starch granule surfaces that contain pin-b in its wild-type state are able to associate with approximately twice as many polar lipids than starch granule surfaces that contain pin-a in its wild-type state and approximately three times as much as starch granules surfaces that contain pin-a wild-type and pin-b Trp-44 to Arg (Table 4.5). It appears that by themselves pin-b (when associated with the surface of starch granules) binds more polar lipids than when pin-a is associated with the surface of starch granules (Table 4.5).

Based on previous work done *in vitro,* which demonstrated that pin-a associates tightly to phospholipids and galactolipids while pin-b is loosely associated with galactolipids and preferentially binds to negatively charged phospholipids (Dubreil et al. 1997), it would be expected that the genotype with pin-a wild-type (Alpowa/Canadian Red//7*Alpowa and Alpowa/Mjølner//7*Alpowa) would contain greater amounts of bound polar lipids than would the pin-b wild-type (Alpowa/ID377s//7*Alpowa) genotype. However, this was not the case when analyzing the samples *in vivo*. In this study, the pin-b wild-type sample contained the greatest quantity of bound polar lipids. One important difference between the *in vivo* and *in vitro* studies is that the *in vivo* study involved a third component, the starch granule surface.

The interactions between the puroindoline proteins, the polar lipids and the starch granule surface were such that when both puroindoline proteins were present in their wild-type state on the starch granule surface, the greatest concentrations of polar lipids were present (476.6 and 233.8 nmol/g of total polar lipids) (Table 4.5). When only pin-b wild-type was present on the surface of the starch granules 110.8 nmol/g of total polar lipids were present and when only pin-a wild-type was present on the surface of the starch granule 62.9 nmol/g of total polar lipids were present (Table 4.5). The least amount of polar lipids were present on the surface of the starch granule when pin-a wild-type and pin-b Try-44 to Arg was present (38.8 nmol/g).

4.4.5 Variation of specific polar lipid molecular species within hardness classes

Figures 4.1-4.3 represent specific molecular species of the bound polar lipids on a mol% of total lipid basis. Sample means were compared within hardness class using Scheffé's procedure. Overall, 138 specific molecular species were identified with a range from 0.002 to 35.890 mol% of total polar lipids. The predominant molecular species were similar to previous results where the acyl-carbon and double bond configurations were of 18:2 and 16:0 fatty acids

(34:2 and 36:4). PI and PG were the lipid classes that contained the only relatively substantial amounts of 32:0 acyl-carbons, while PS lipids were unique in their abundance of larger fatty acid molecules of 40:0, 42:2 and 42:3 molecular species.

Even though Alpowa starch contained the greatest overall quantity of polar lipids on a nmol/g basis, it was only significantly greater on a mol% of total basis for the DGDG molecular species, (34:2, 36:4, 38:3, and 38:4) MGDG molecular species (34:2, 36:3 and 36:4) and PE molecular species (34:1, 34:2, 34:3, 36:1, 36:2 36:3, 38:3, 40:2 and 40:3). For all other molecular species, Alpowa/SS163//7*Alpowa contained the greatest mol% of total lipids (Figures 4.1-4.3). These results indicate that the overall greater amount of polar lipids in the Alpowa starch was not equally distributed across all the lipid classes and was specific to greater proportions of DGDG, MGDG and PE lipids (Figures 4.1-4.3). It is unclear whether the differences observed in mol% of total polar lipids on the surface of starch granules between the Alpowa and Alpowa/SS163//7*Alpowa samples are due to the introduction of the 'supersoft' phenotype or whether it is caused by the different growing environments. It is possible that the greater proportions of PC, PG, PA, PI, PS, DGMG, MGMG, LPC, LPE and LPE lipid classes on the surface of Alpowa/SS163//7*Alpowa starch could result in the 'supersoft' endosperm texture, however, further evaluation of the sources of variation in starch surface polar lipids within soft texture wheat is needed.

The mean comparisons between the lipid molecular species from the starch isolated from hard textured wheat samples revealed that differences in bound polar lipids were present. In general, the starch from Alpowa/ID377s//7*Alpowa (pin-a null) contained greater proportions of the various molecular species than did the starch from Alpowa/Canadian Red//7*Alpowa (pin-b null) and Alpowa/Mjølner//7*Alpowa (pin-a null pin-b Trp-44 to Arg). Comparisons between

the class total data (nmol/g of sample) (Table 4.5) and molecular species data (Figures 4.1-4.3) reveal that an increase in the total amount of lipid class results in a general uniform increase in the proportion of the molecular species of the lipid class, for the hard textured samples. Figure 4.3 demonstrates differences in the mono-acyl molecular species mol% of total. The significant decrease in the proportion of starch bound mono-acyl lipid species associated with the Alpowa/ID377s//7*Alpowa samples is due no significant quantity difference (nmol/g of sample) in the mono-acyl lipids on the nmol/g basis (Table 4.5). No difference in the actual quantity of the mono-acyl lipids occurred, but because other lipids classes increase in quantity, the overall effect on the mol% of total was a decrease in the proportion of the mono-acyl lipids.

4.5 Conclusion

By using the near-isogenic wheat lines, we were able to establish relationships between the polar lipid compositions of samples containing different puroindolines. We found endosperm hardness had no significant effect on the polar lipid contents in wheat flour, had a slight influence on the polar lipid contents of the whole-meal fractions and had a significant influence on the polar lipid composition of the polar lipids located on the surface of wheat starch. The greatest quantities of polar lipids on the starch surface occurred when both puroindoline proteins where present in their wild-type form. Starch surface polar lipid content dramatically decreased when one of the puroindoline proteins where null, or if the pin-b was in the mutated form (Trp-44 to Arg). Within the hard textured samples, more polar lipids where present on the starch surface when pin-b was in its wild-type form and pin-a was null than when pin-a was in its wild-type form and pin-b was null; the least amount of polar lipids where present when pin-b was in its mutated form (Trp-44 to Arg) and pin-a as in its wild-type form. These results demonstrate that the mechanism of endosperm hardness is, at a minimum, a three-way interaction between the starch granule surface, puroindoline proteins and polar lipids. It is unknown exactly how these components are configured, but it is evident that when the puroindoline proteins are in their wild-type state, dramatically more polar lipids are associated with the puroindoline proteins and strongly implies that the result is a structure that provides an anti-adhesion characteristic between the starch granule surface and the storage proteins in the endosperm of the soft textured wheat kernels.

4.6 Acknowledgments

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4.8 Tables and Figures

Table 4.1: Sample identification and corresponding source, puroindoline haplotype, molecular change and SKCS hardness value of the wheat samples obtained from Dr. Craig Morris.

Sample Identification	Puroindoline Haplotype	Molecular change from 'wild-type'†	Hardness (SKCS)§
Alpowa	*Pina-D1a/Pinb-D1a*	-	31
Alpowa/SS163//7*Alpowa	*Pina-D1a/Pinb-D1a*	-	17
Alpowa/ID377s//7*Alpowa	*Pina-D1b/Pinb-D1a*	Pina null	72
Alpowa/Mjølner//7*Alpowa	*Pina-D1a/Pinb-D1d*	Pinb Trp-44 to Arg	59
Alpowa/Canadian Red//7*Alpowa	*Pina-D1a/Pinb-D1e*	Pinb null (Trp-39 to stop)	68

† 'Wild-type' defined as *Pina-D1a/Pinb-D1a* puroindoline haplotype
§ SKCS; single kernel characterization system hardness index value

Table 4.2: Sample statistics[a] and *F*-values from one-way ANOVA[b] of total bound polar lipids in one-way ANOVA for wheat endosperm hardness.

Statistic / Source	Whole-Meal	Flour	Starch-Surface
Minimum	2750	2550	50
Mean	3150	3150	200
Maximum	3600	3600	500
Hardness	6*	ns	69**

[a] Sample statistics are expressed as nmol/g of sample.
[b] ns = not significant, * and ** represent *F*-values significant at $P = 0.05$ and 0.0001, respectively. *F*-values derived from Type III sums of squares.

Table 4.3: Mean content (nmol/g) of different bound polar lipid classes from wheat whole-meal of the six near-isogenic wheat lines.

Lipid Class	Alpowa	Alpowa/SS163//7*Alpowa	Alpowa/Canadian Red//7*Alpowa	Alpowa/ID377s//7*Alpowa	Alpowa/Mjølner//7*Alpowa
DGDG	1138 b ±33	1086 b ±6	1348 a ±34	1278 a ±24	1347 a ±62
MGDG	541 c ±14	533 c ±16	774 a ±31	692 a ±5	675 b ±21
PC	859 a ±25	855 ab ±11	786 b ±28	749 bc ±20	727 c ±24
LPC	116 a ±3	83 b ±2	64 c ±2	67 bc ±1	73 c ±5
PE	103 b ±3	127 a ±3	108 b ±2	105 b ±2	92 b ±4
PG	71 a ±3	61 b ±1	52 c ±1	50 c ±2	52 c ±2
DGMG	21 a ±1	9 c ±0	14 b ±0	12 b ±1	15 b ±2
MGMG	10 a ±0	4 c ±0	7 b ±0	6 b ±0	7 b ±1
LPG	10 a ±1	6 b ±1	6 b ±1	5 b ±1	6 b ±0
PA	22 b ±1	30 a ±0	16 cd ±1	13 d ±1	18 bc ±2
PI	172 a ±20	189 a ±12	222 a ±6	145 a ±36	151 a ±20
PS	11 a ±2	15 a ±1	14 a ±1	9 a ±2	11 a ±2
LPE	8 a ±0	7 b ±0	6 c ±0	6 b ±0	7 b ±1
Total	3083 ab ±87	3005 b ±24	3416 a ±73	3138 ab ±57	3181 ab ±121

Each value represents nmol/g of sample ±SE, n=5. Mean values from each lipid extract followed by the same letter are not significantly different between genotypes at $P = 0.05$ (based on Scheffé multiple-comparison procedure).

Table 4.4: Mean content (nmol/g) of different bound polar lipid classes from wheat flour of the six near-isogenic wheat lines.

Lipid Class	Alpowa	Alpowa/SS163//7*Alpowa	Alpowa/Canadian Red//7*Alpowa	Alpowa/ID377s//7*Alpowa	Alpowa/Mjølner//7*Alpowa
DGDG	1984 a ±40	1464 c ±24	1764 b ±34	1808 b ±17	1851 ab ±16
MGDG	684 a ±13	428 c ±9	691 a ±15	649 ab ±4	628 b ±5
PC	475 b ±7	542 a ±18	489 abc ±18	534 ab ±7	470 c ±5
LPC	82 c ±2	96 a ±3	80 b ±4	90 a ±2	78 c ±1
PE	55 d ±1	78 a ±3	58 c ±2	67 b ±1	49 d ±1
PG	32 b ±1	44 a ±2	31 b ±2	32 b ±1	30 b ±0
DGMG	20 bc ±1	26 a ±1	19 bc ±1	21 b ±1	18 c ±0
MGMG	14 a ±0	13 a ±0	15 a ±1	14 a ±1	14 a ±1
LPG	11 b ±0	15 a ±1	10 c ±1	13 b ±0	12 b ±0
PA	9 c ±0	22 a ±1	9 c ±1	12 b ±0	9 c ±0
PI	8 d ±0	43 a ±6	16 c ±1	26 b ±1	15 c ±0
PS	6 b ±0	9 a ±0	4 c ±0	7 b ±0	7 b ±0
LPE	5 c ±0	6 b ±0	5 c ±0	7 a ±0	5 c ±0
Total	3385 a ±61	2784 b ±60	3192 a ±60	3281 a ±23	3185 a ±11

Each value represents nmol/g of sample ±SE, n=5. Mean values from each lipid extract followed by the same letter are not significantly different between genotypes at $P = 0.05$ (based on Scheffé multiple-comparison procedure).

Table 4.5: Mean content (nmol/g) of different bound polar lipid classes from wheat starch of the six near-isogenic wheat lines.

Lipid Class	Alpowa	Alpowa/SS163//7*Alpowa	Alpowa/Canadian Red//7*Alpowa	Alpowa/ID377s//7*Alpowa	Alpowa/Mjølner//7*Alpowa
DGDG	271.6 a ±3.9	125.7 b ±1.9	31.6 d ±3.4	58.4 c ±0.4	18.6 e ±0.5
MGDG	115.7 a ±2.4	48.3 b ±0.8	16.2 d ±1.6	27.8 c ±0.3	8.9 e ±0.2
PC	64.8 a ±1.6	38.8 b ±0.7	6.1 d ±0.5	14.4 c ±0.3	3.4 d ±0.2
LPC	11.5 a ±0.3	10.6 a ±0.1	6.7 b ±0.6	6.3 b ±0.10	6.2 b ±0.2
PE	2.1 a ±0.1	0.9 b ±0.0	0.1 c ±0.0	0.3 c ±0.0	0.0 c ±0.0
PG	3.8 a ±0.1	3.5 b ±0.0	0.5 d ±0.0	1.0 c ±0.0	0.3 d ±0.0
DGMG	0.8 a ±0.0	0.9 a ±0.0	0.3 b ±0.0	0.2 b ±0.0	0.2 b ±0.0
MGMG	0.4 a ±0.0	0.4 a ±0.0	0.2 b ±0.0	0.1 c ±0.0	0.2 b ±0.0
LPG	0.5 a ±0.1	0.5 a ±0.1	0.4 a ±0.1	0.4 a ±0.0	0.5 a ±0.1
PA	2.5 a ±0.1	2.1 b ±0.1	0.6 d ±0.0	1.2 c ±0.0	0.1 e ±0.0
PI	0.9 a ±0.0	0.8 b ±0.0	0.2 d ±0.0	0.3 c ±0.0	0.1 e ±0.0
PS	0.6 a ±0.1	0.5 a ±0.0	0.0 c ±0.0	0.1 c ±0.0	0.0 c ±0.0
LPE	1.2 a ±0.1	0.9 b ±0.0	0.0 d ±0.0	0.3 c ±0.0	0.1 d ±0.0
Total	476.5 a ±7.7	233.8 b ±3.3	62.9 d ±5.9	110.8 c ±0.9	38.8 e ±0.9

Each value represents nmol/g of sample ±SE, n=5. Mean values from each lipid extract followed by the same letter are not significantly different between genotypes at $P = 0.05$ (based on Scheffé multiple-comparison procedure).

Figure 4.1: DGDG, MGDG, PC and PE bound polar lipids molecular species means from starch isolated from the NIL wheat lines. Values represent means and ± SE in mol% of total lipids, (*n* =5). To determine the lipid molecular species variation within hardness class, samples were analyzed within each hardness classes (hard and soft). Different letters above column bars indicates significantly different means between the NILs within the hardness class (based on Scheffé multiple-comparison procedure)

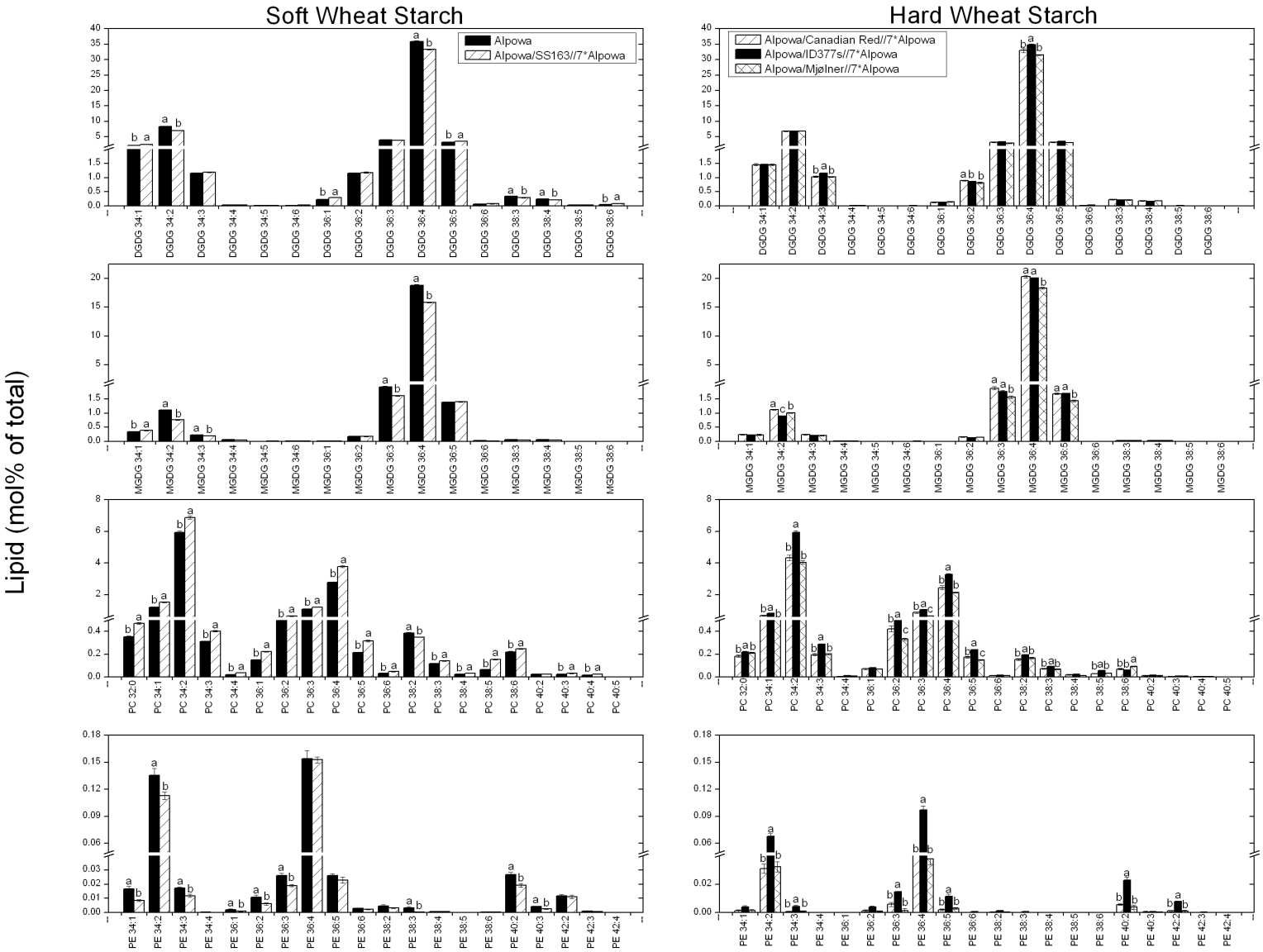

Soft Wheat Starch
Hard Wheat Starch
Lipid (mol% of total)
Alpowa
Alpowa/SS163//7*Alpowa
Alpowa/Canadian Red//7*Alpowa
Alpowa/ID377s//7*Alpowa
Alpowa/Mjølner//7*Alpowa
DGDG 34:1
DGDG 34:2
DGDG 34:3
DGDG 34:4
DGDG 34:5
DGDG 34:6
DGDG 36:1
DGDG 36:2
DGDG 36:3
DGDG 36:4
DGDG 36:5
DGDG 36:6
DGDG 38:3
DGDG 38:4
DGDG 38:5
DGDG 38:6
MGDG 34:1
MGDG 34:2
MGDG 34:3
MGDG 34:4
MGDG 34:5
MGDG 34:6
MGDG 36:1
MGDG 36:2
MGDG 36:3
MGDG 36:4
MGDG 36:5
MGDG 36:6
MGDG 38:3
MGDG 38:4
MGDG 38:5
MGDG 38:6
PC 32:0
PC 34:1
PC 34:2
PC 34:3
PC 34:4
PC 36:1
PC 36:2
PC 36:3
PC 36:4
PC 36:5
PC 36:6
PC 38:2
PC 38:3
PC 38:4
PC 38:5
PC 38:6
PC 40:2
PC 40:3
PC 40:4
PC 40:5
PE 34:1
PE 34:2
PE 34:3
PE 34:4
PE 36:1
PE 36:2
PE 36:3
PE 36:4
PE 36:5
PE 36:6
PE 38:2
PE 38:3
PE 38:4
PE 38:5
PE 38:6
PE 40:2
PE 40:3
PE 42:2
PE 42:3
PE 42:4

Figure 4.2: PG, PA, PI and PS bound polar lipid molecular species means from starch isolated from the NIL wheat lines. Values represent means and ± SE in mol% of total lipids, (*n* =5). To determine the lipid molecular species variation within hardness class, samples were analyzed within each hardness classes (hard and soft). Different letters above column bars indicates significantly different means between the NILs within the hardness class (based on Scheffé multiple-comparison procedure).

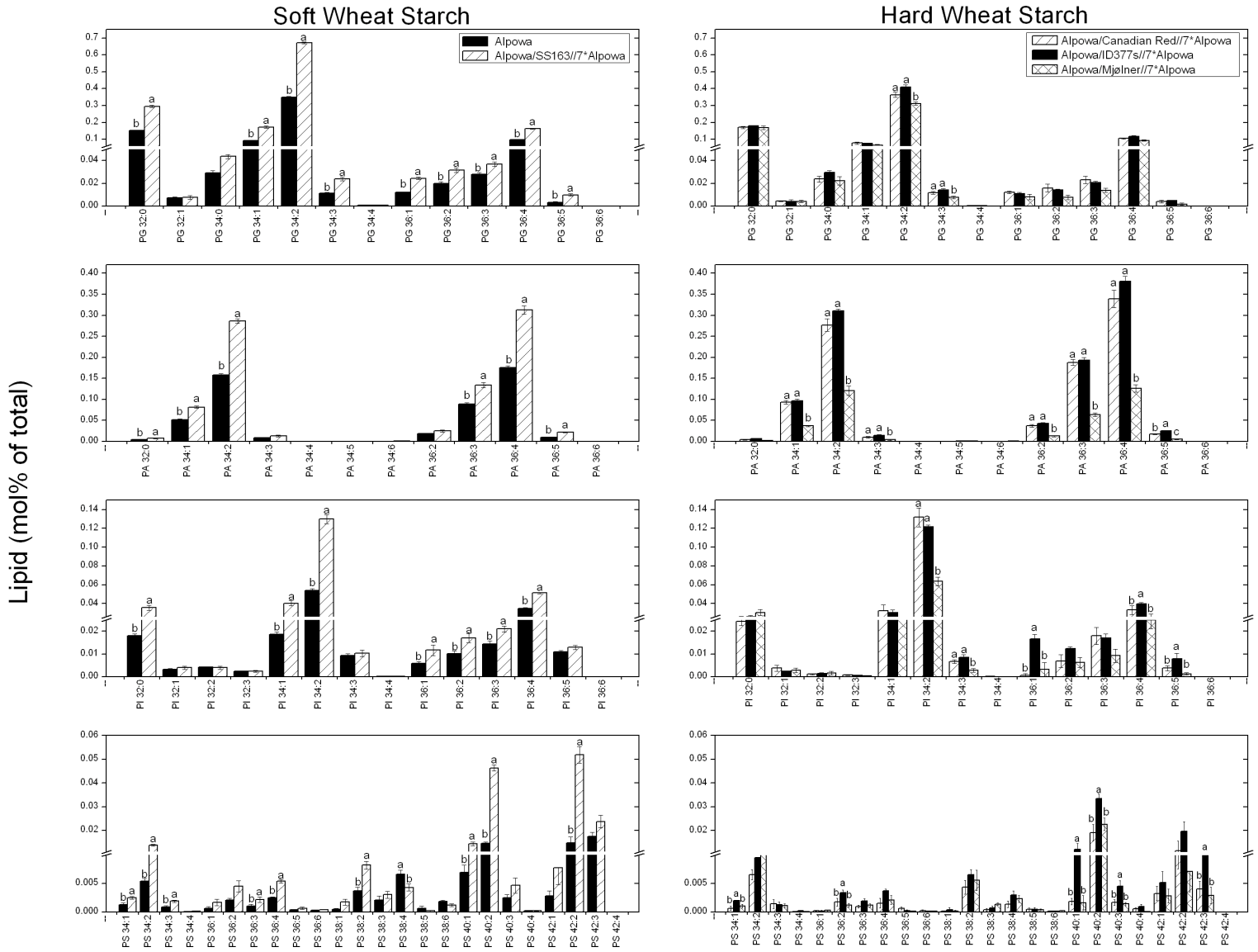
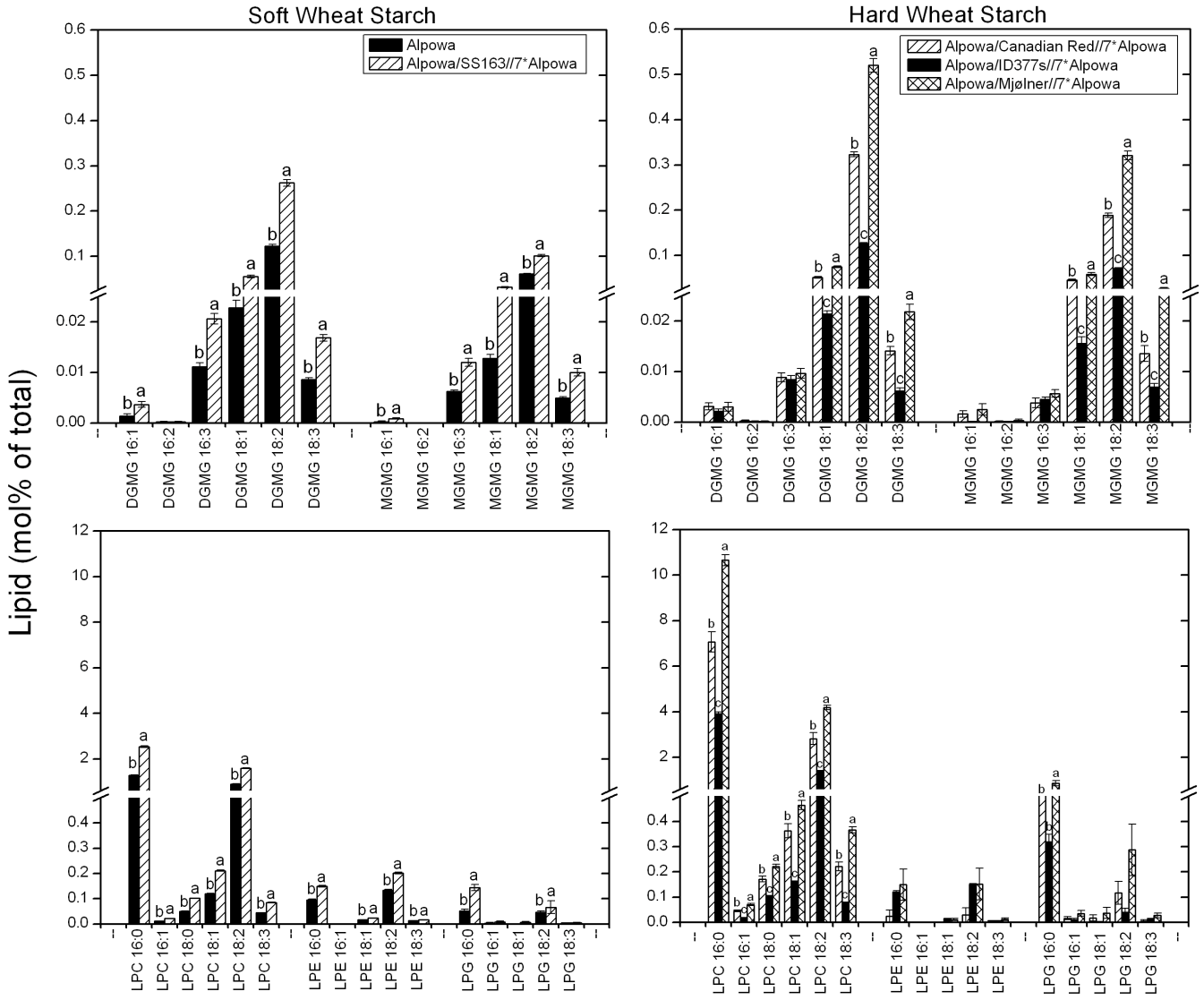

Soft Wheat Starch
Hard Wheat Starch
Lipid (mol% of total)
Alpowa
Alpowa/SS163//7*Alpowa
Alpowa/Canadian Red//7*Alpowa
Alpowa/ID377s//7*Alpowa
Alpowa/Mjølner//7*Alpowa
PG 32:0
PG 32:1
PG 34:0
PG 34:1
PG 34:2
PG 34:3
PG 34:4
PG 36:1
PG 36:2
PG 36:3
PG 36:4
PG 36:5
PG 36:6
PA 32:0
PA 34:1
PA 34:2
PA 34:3
PA 34:4
PA 34:5
PA 34:6
PA 36:2
PA 36:3
PA 36:4
PA 36:5
PA 36:6
PI 32:0
PI 32:1
PI 32:2
PI 32:3
PI 34:1
PI 34:2
PI 34:3
PI 34:4
PI 36:1
PI 36:2
PI 36:3
PI 36:4
PI 36:5
PI 36:6
PS 34:1
PS 34:2
PS 34:3
PS 34:4
PS 36:1
PS 36:2
PS 36:3
PS 36:4
PS 36:5
PS 36:6
PS 38:1
PS 38:2
PS 38:3
PS 38:4
PS 38:5
PS 38:6
PS 40:1
PS 40:2
PS 40:3
PS 40:4
PS 42:1
PS 42:2
PS 42:3
PS 42:4

Figure 4.3: DGMG, MGMG, LPC, LPE and LPG bound polar lipids molecular species means from starch isolated from the NIL wheat lines. Values represent means and ± SE in mol% of total lipids, (*n* =5). To determine the lipid molecular species variation within hardness class, samples were analyzed within each hardness classes (hard and soft). Different letters above column bars indicates significantly different means between the NILs within the hardness class (based on Scheffé multiple-comparison procedure).


Soft Wheat Starch
Hard Wheat Starch
Lipid (mol% of total)
Alpowa
Alpowa/SS163//7*Alpowa
Alpowa/Canadian Red//7*Alpowa
Alpowa/ID377s//7*Alpowa
Alpowa/Mjølner//7*Alpowa
DGMG 16:1
DGMG 16:2
DGMG 16:3
DGMG 18:1
DGMG 18:2
DGMG 18:3
MGMG 16:1
MGMG 16:2
MGMG 16:3
MGMG 18:1
MGMG 18:2
MGMG 18:3
LPC 16:0
LPC 16:1
LPC 18:0
LPC 18:1
LPC 18:2
LPC 18:3
LPE 16:0
LPE 16:1
LPE 18:1
LPE 18:2
LPE 18:3
LPG 16:0
LPG 16:1
LPG 18:1
LPG 18:2
LPG 18:3

CHAPTER 5 - Conclusion and Further Studies

5.1 Conclusion and Significance

In this study the three objectives were completed.

Objective 1) To establish lipid profiling techniques to quantitatively determine the polar lipid species present in whole-wheat meal, flour, and starch.

Using lipid profiling techniques we identified and determined the quantity of 155 polar lipid species present in wheat meal, flour and starch. The predominant polar lipid classes were DGDG, MGDG, PC, and LPC. Starch internal lipids were monoacyl species (LPC, LPE, LPG, DGMG and MGMG) and starch surface lipids were primarily diacyl species (Table 2.2 and 2.3). Lipid profiling was able to quantify and differentiate the species present in a variety of processing fractions at a resolution and sensitivity previously unattainable, thus allowing the most complete characterization of wheat polar lipids to date. The ability to profile lipids will help provide new knowledge regarding the functional relationships between specific lipid species and end-use quality. By using lipid profiling to quantitatively determining the specific lipid species present in wheat flours of different baking qualities, relationships between specific lipid species and their gas cell stabilizing abilities should be obtainable.

Objective 2) To quantitatively determine the variation in polar lipids present on the surface of wheat starch granules.

This research provided evidence that as dough is mixed to optimum development the lipids on the surface of wheat starch become incorporated into the gluten phase of the dough, whereas in a batter the starch surface lipids stay associated with the starch granule surface. The difference in starch surface lipids provides insight into the role starch surface lipids play in the development of gluten. It appears that as gluten is being developed polar lipids located originally on (or near) the surface of the starch granules become incorporated into the gluten phase of the dough. Starch granule surfaces are thus, dynamic and depending on the their surface surrounding environment, their composition can be significantly altered. Future studies evaluating starch surface components will have to use starch isolation methods that do not involve gluten formation.

3) To determine the specific polar lipid species present on the surface of wheat starch from near-isogenic wheat lines that contain different puroindoline haplotypes and endosperm hardness.

By using the near-isogenic wheat lines, we were able to establish relationships between the polar lipid compositions of samples containing different puroindoline haplotypes. The greatest quantities of polar lipids on the starch surface occurred when both puroindoline proteins where present in their wild-type form. It is possible that threshold exists in terms of the necessary quantity of starch surface components (puroindoline proteins and polar lipids) needed to produce soft textured endosperm and that any additional starch surface components do not necessarily result in a softer textured endosperm. Starch surface polar lipid content was dramatically reduced when one of the puroindoline proteins was null, or if the pin-b was in the

mutated form (Trp-44 to Arg). Within the hard textured samples, more polar lipids were present on the starch surface when pin-b was in its wild-type form and pin-a was null than when pin-a was in its wild-type form and pin-b was null; the least amount of polar lipids were present when pin-b was in its mutated form (Trp-44 to Arg) and pin-a as in its wild-type form. These results demonstrate that the mechanism of endosperm hardness is, at a minimum, a three-way interaction between the starch granule surface, puroindoline proteins and polar lipids. It is as of yet unknown exactly how these components are configured, but it is evident that when the puroindoline proteins are in their wild-type state, dramatically more polar lipids are associated with the puroindoline proteins and strongly implies that the result is a structure that provides an anti-adhesion characteristic between the starch granule surface and the storage proteins in the endosperm of the soft textured wheat kernels.

5.2 Further Studies

There are two separate areas for future studies; 1) continuation on the determination of the functionality of the starch granule surface components, and 2) further evaluation of wheat flour lipids.

Studies on the determination of the functionality of starch granule surface components include, but are not limited to; their involvement in starch pasting and gelatinization, dough development, dough rheology, and breakmaking functionality. The near-isogenic wheat lines used in this dissertation research would provide an excellent template for the future studies on the functional role of starch granule surface components. Additionally, those near-isogenic lines would be useful in determining the exact mechanism involved in wheat endosperm hardness. More research is needed for the determination of mechanistic basis for endosperm hardness.

Only one of the pin-b mutated lines were evaluate in this study. It would be useful to determine if the other mutated pin-b lines from the Alpowa derived lines contained similarly reduced amounts of polar lipids when compared to the non-mutated pin-b lines.

There are numerous possible further studies evaluating wheat lipids. This dissertation only briefly studied SQDG lipids. It would be valuable to determine the exact quantities of SQDG present in wheat, as well as, determine the sources of variation contributed to SQDG amounts in wheat. There is value in further understanding of the endogenous wheat lipid degrading enzymes and how those enzymes affect flour functionality. There are also other lipid classes in wheat flour that could to be researched in more detail, such as sphingolipids and estolides. Wheat lipid research is currently limited and I am confident that with the new technologies of lipid profiling through the use of mass spectrometry, new discoveries and findings will be possible.