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Distribution of Lipids in the Grain of Wheat (cv. Hereward) Determined by Lipidomic Analysis of Milling and Pearling Fractions

Irene González-Thuillier,[†] Louise Salt,[§] Gemma Chope,[⊗] Simon Penson,[⊗] Peter Skeggs,[⊥] Paola Tosi,[Δ] Stephen J. Powers,[#] Jane L. Ward,[†] Peter Wilde,[§] Peter R. Shewry,[*,†,Δ] and Richard P. Haslam[‡]

[†]Plant Biology and Crop Science, Rothamsted Research, Harpenden AL5 2JQ, United Kingdom

[‡]Biological Science and Crop Protection, Rothamsted Research, Harpenden AL5 2JQ, United Kingdom

[§]Food & Health Programme, Institute of Food Research, Norwich Research Park, Norwich NR4 7UA, United Kingdom

[⊗]Campden BRI, Station Road, Chipping Campden, Gloucestershire GL55 6LD, United Kingdom

[⊥]Hovis Limited, Lord Rank Centre, Lincoln Road, High Wycombe HP12 3QS, United Kingdom

[Δ]School of Agriculture, Policy and Development, University of Reading, Whiteknights, P.O. Box 237, Reading RG6 6AR, United Kingdom

[#]Computational and Systems Biology, Rothamsted Research, Harpenden AL5 2JQ, United Kingdom

Supporting Information

ABSTRACT: Lipidomic analyses of milling and pearling fractions from wheat grain were carried out to determine differences in composition that could relate to the spatial distribution of lipids in the grain. Free fatty acids and triacylglycerols were major components in all fractions, but the relative contents of polar lipids varied, particularly those of lysophosphatidylcholine and digalactosyldiglyceride, which were enriched in flour fractions. By contrast, minor phospholipids were enriched in bran and offal fractions. The most abundant fatty acids in the analyzed acyl lipids were C16:0 and C18:2 and their combinations, including C36:4 and C34:2. Phospholipids and galactolipids have been reported to have beneficial properties for breadmaking, whereas free fatty acids and triacylglycerols are considered detrimental. The subtle differences in the compositions of fractions determined in the present study could therefore underpin the production of flour fractions with optimized compositions for different end uses.

KEYWORDS: *wheat grain, lipids, breadmaking, lipidomics, milling*

INTRODUCTION

Cereal grains are the main source of food for humankind, with total global yields of over 2780 million tonnes in 2013 and with the three major cereals (maize, rice, and wheat) accounting for almost 90% of this.[1] Most bread wheat (*Triticum aestivum* L.) is milled into flour and bran for human consumption, but substantial quantities are also used to feed livestock and poultry. The wheat "grain" is actually a single-seeded fruit, called a caryopsis, in which maternal fruit and seed coats (pericarp and testa, respectively) surround the embryo and the endosperm. The endosperm itself comprises two tissues. The outer part is the aleurone layer, which comprises a single layer of cells in wheat. The aleurone cells have thick walls (and hence high dietary fiber), contain storage lipids (i.e., triacylglycerol) and globulin storage proteins, and are rich in minerals, vitamins, and phytochemicals (micronutrients). By contrast, the central starchy endosperm is the major grain tissue and is rich in starch and storage proteins, but has lower contents of dietary fiber and micronutrients. Whereas the aleurone cells remain alive in the mature grain, the starchy endosperm cells die and their contents become disordered. In the mature grain, the outer layers account for 7−8% of the dry weight, the aleurone for about 6.5%, the starchy endosperm for about 83%, and the embryo (germ) for 6.0%.[2] When the grain is milled, the starchy endosperm forms the white flour fraction, whereas the outer layers, aleurone, and germ are together recovered in the bran. Pearling is a treatment in which the kernel outer layers are removed by friction and abrasion. The resulting kernels can then be processed into flour by roller milling.

Although the starchy endosperm is often regarded as a single homogeneous tissue, it actually comprises several types of cells, which differ in their size and composition. In wheat these include two to three layers of small subaleurone cells directly below the aleurone layer, elongated prismatic cells that radiate from the subaleurone cells toward the center of the grain, and large central cells in the centers of the cheeks. Bradbury et al.[3] reported approximate sizes of 60 μm diameter for the subaleurone cells, 128−200 μm × 40−60 μm for prismatic cells, and 72−144 μm × 69−120 μm for central cells. Differences in composition between these cell types have been known for some time, with the subaleurone cells being richer in protein with fewer and less regular starch granules[4,5] Differences in the distributions of gluten proteins and structural variation in cell wall arabinoxylan have also been determined in thin sections of developing wheat grain using immunomicroscopy[6] and FT-IR microspectroscopy,[7] respectively. Recently, De Brier et al.[8] reported that the total lipid content varied between 2.1 and 3.3% dry weight in pearling fractions produced by removal of between 3 and 12% of the grain

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Table 1. Weights and Contents of Protein and Ash of Milling and Pearling Fractions Prepared from Wheat cv. Hereward[a]

sample	% grain wt	% grain wt cumulative	total lipid (nmol/g)	total lipid cumulative (nmol/g)	ash (% dry wt)
wholemeal	100	100	8971	8971	1.3
milling fractions					
break 1 (B1)	8.1	8.1	6379	6379	0.3
reduction 1 (R1)	33.1	41.2	6528	6499	0.3
break 2 (B2)	9.3	50.5	8006	6776	0.4
reduction 2 (R2)	21.1	71.6	12041	8328	0.4
break 3 (B3)	1.9	73.5	13396	8459	0.8
reduction 3 (R3)	4.2	77.7	19845	9074	0.6
bran flour	1.4	79.1	10799	9105	1.6
offal flour	2.4	81.5	25263	9581	2.3
offal overtail (O-OT)	5.7	87.2	23920	10518	3.8
bran overtail (B-OT)	12.8	100	6639	10021	4.
pearling fractions					
PF1	7	7	11831	11831	3.4
PF2	6	13	19053	15164	2.8
PF3	7	20	18543	16347	2.4
PF4	10	30	16603	16432	2
PF5	10	40	18910	17051	1.6
PF6	10	50	13485	16338	1.1
core (C)	50	100	5957	11147	0.7

[a]Lipid analyses were carried out on one set of fractions from Buhler milling and two biological replicate sets of fractions from pearling. Four technical replicate samples of each fraction were extracted for lipids and single replicates for N and ash determination.

dry weight. Differences in the contents and compositions of proteins, cell wall polysaccharides and other components (amino acids, minerals, and phytochemicals) may also occur in white flour fractions produced by conventional milling of wheat (mill streams).[9−12] It is likely that these differences result from intrinsic differences between the cell types that comprise the wheat starchy endosperm, as well as the degree of contamination with bran tissues.

Lipids in wheat grains display large structural diversity and comprise neutral (acylglycerols and free fatty acids) and polar (glycolipids and phospholipids) components. As in most seed tissues, triacylglycerols are the main storage lipids and are contained in subcellular organelles called oil bodies. Although lipids are minor components of wheat flour (about 2−2.5% dry weight), they are considered to have significant impacts on flour and dough functionality, by interacting with gluten proteins and starch and by stabilizing gas cells in breadmaking (reviewed by Pareyt et al.[13]). Furthermore, they have also been shown to vary in amount and composition between cultivars and mill-streams.[14−16]

Traditional methods of lipid analysis such as thin layer chromatography are limited in their ability to identify and quantify specific lipid molecular species. Recently, newer techniques using sensitive mass spectrometry-based high-throughput methods have allowed the detailed and systematic characterization of lipids. Such lipid profiling, or lipidomics, creates a comprehensive library of lipid species in each sample with quantitative information on lipid class, headgroup, and acyl group combination. It is therefore possible through the application of these methods to gain new insights into the distribution of grain lipids during processing and the relationship to end-use quality. However, no systematic studies of wheat lipids have been carried out using modern high-resolution "lipidomic" approaches, with the exception of analyses of wholemeal, flour, and starch from two U.S. cultivars.[17] In flour, the term "non-starch" lipids is used to refer to all lipids including those present on the outer surface of starch granules, but excluding lipids entrapped within starch granules, which are termed "internal starch" lipids and can only be extracted when the starch granules are broken down or disrupted. "Non-starch" lipids have a significant role in determining the characteristics of the final baked product. We have therefore carried out a detailed study of the composition and distribution of "non-starch" lipids in the U.K. breadmaking cultivar Hereward, comparing mill streams and sequential pearling fractions from the same grain sample.

MATERIALS AND METHODS

Samples. Wheat grain (cv. Hereward) grown at Rothamsted Research in 2011 was milled in a Buhler−MLU-202 mill to give four bran and six flour fractions. The milling scheme is shown graphically in Supporting Information Figure S1. The fractions were three break flours (B1, B2, B3), three reduction flours (R1, R2, R3), offal overtail (O-OT), offal flour (OF), bran overtail (B-OT), and bran flour (BF). Samples of Hereward grown under similar agronomic conditions in 2012 and 2014 were also milled using the same mill setup for comparative analyses. Two replicate 50 g samples from the 2011 harvest were also abraded in a Streckel and Schrader (Hamburg, Germany) pearling mill as described by Tosi et al.[6] This gave six sequential fractions (PF1−PF6), which together accounted for about 50% of the grain weight, with PF1−PF3 each accounting for about 6−7% and PF4−PF6 for about 10% of the weight (Table 1). PF1−PF3 are enriched in the pericarp tissue (bran), aleurone layer, and subaleurone cells, respectively, whereas PF4−PF6 correspond to progressively more central areas of the starchy endosperm.[6] The grain remaining after pearling, called the core (C), was milled using a ball mill (Glen Creston, Stanmore, UK) and corresponded to about 50% of the original weight. Three replicates of 10 g of grain were milled using a freezer/mill 6770 (SPEX SamplePrep, Metuchen, NJ, USA) to generate wholemeal samples.

Chemical Analysis. Nitrogen content was determined on 300 mg samples of all fractions and the wholemeal flours using a LECO TruMac Combustion Analyzer (St. Joseph, MI, USA) based on the DUMAS digestion method. Ash content was determined by Sciantec Analytical Services (Cawood, UK).

Lipid Extraction. Non-starch lipids were extracted from flour samples as described by Finnie, Jeannotte, and Faubion[17] with some modifications. The flour (150 mg) was heated in boiling water (100 °C)

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for 12 min to inactivate any hydrolytic enzymes.[18] Three sequential extractions were then carried out with petroleum ether (PEt), water-saturated butan-1-ol (1:10) (WSB), and propan-2-ol/water (90:10) (IW), with sample to solvent ratios of 1:10, 1:14, and 1:10, respectively. The PEt and WSB extracts were washed by shaking with 1:1 (v/v) 0.88% KCl, centrifugation for 2 min at 650*g*, and recovery of the upper layer to a new tube, in which all three lipid phases were combined. The combined extracts were evaporated under nitrogen at 40 °C, resuspended in an equal volume of chloroform, and washed again with 0.88% KCl, retaining the lower phase. The solutions were then filtered (0.45 μm Millex-FH filters, Merck Millipore, Germany), dried under a stream of nitrogen, resuspended in 2 mL of chloroform, flushed with nitrogen, and stored at −80 °C.

Quantitative Lipid Analysis. Quantitative analyses of neutral lipids (NL) (free fatty acids (FFA), diacylglycerols (DAG), and triacylglycerols (TAG)) and polar lipids, which comprise phospholipids (PL) (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG), lysophosphatidylcoline (LPC)) and galactolipids (GL) (digalactosyldiglycerol (DGDG) and monogalactosyldiglycerol (MGDG)), were carried out using electrospray ionization tandem triple-quadrupole mass spectrometry (API 4000 QTRAP; Applied Biosystems; ESI-MS/MS). Monoacylglycerols (MAG) were not targeted for analysis given the low levels present in the flour samples. The lipid extracts were infused at 15 μL/min with an autosampler (HTS-xt PAL, CTC-PAL Analytics AG, Switzerland). Data acquisition and acyl group identification of the polar lipids was as described in Ruiz-Lopez et al.[19] with modifications. The internal standards for polar lipids were supplied by Avanti (Alabaster, AL, USA), incorporated as 0.857 nmol of 13:0-LPC, 0.086 nmol of di24:1-PC, 0.080 nmol of di14:0-PE, 0.800 nmol of di18:0-PI, and 0.080 nmol of di14:0-PG. The standards dissolved in chloroform and 25 μL of the samples in chloroform were combined with chloroform/methanol/300 mM ammonium acetate (300:665:3.5 v/v) to make a final volume of 1 mL.

To quantify FFA in the Q1 ESI-MS negative mode, 0.607 nmol of 15:0-FFA (Sigma-Aldrich, St. Louis, MO, USA) and 25 μL of sample were combined with propan-2-ol/methanol/50 mM ammonium acetate/dichloromethane (4:3:2:1; v/v) to a final volume of 1 mL. The ESI-MS/MS method described by Li et al.[20] was modified to quantify TAG and DAG contents. For quantifying TAG, 15 μL of lipid extract and 0.857 nmol of tri15:0-TAG (Nu-Chek Prep, Elysian, MN, USA) were combined with chloroform/methanol/300 mM ammonium acetate (24:24:1.75: v/v), and for DAG, 25 μL of sample and 0.857 nmol of 18:0−20:4-DAG (Sigma-Aldrich) were combined with propan-2-ol/methanol/50 mM ammonium acetate/dichloromethane (4:3:2:1; v/v), to final volumes of 1 mL for direct infusion into the mass spectrometer. TAG and DAG were detected as $[M + NH_4]^+$ ions by a series of different neutral loss scans, targeting losses of fatty acids. The scans as well as the parameters used for the three neutral lipids are shown in Table S1 of the Supporting Information. The data were processed using the program Lipid View Software (AB-Sciex, Framingham, MA, USA) where isotope corrections are applied. The peak area of each lipid was normalized to the internal standard and further normalized to the weight of the initial sample. There is variation in ionization efficiency among acyl glycerol species with different fatty acyl groups, and no response factors for individual species were determined in this study; therefore, the values are not directly proportional to the TAG/DAG contents of each species. However, the approach does allow a realistic comparison of TAG/DAG species across samples in this study.

Determination of Total Fatty Acid Methyl Esters (FAMEs). Total fatty acids were methylated by heating the samples at 80 °C for 2 h with 2 mL of a solution containing methanol/toluene/dimethoxypropane/H_2SO_4 (66:28:2:1 by volume). Methyl heptadecanoate (C17:0) was added to samples as an internal standard. After cooling, 1.5 mL of hexane was added, and FAMEs were recovered from the upper phase. Methyl ester derivatives of total fatty acids extracted were analyzed by GC-FID (flame ionization detection) using an Agilent 6890 gas chromatography system (Palo Alto, CA, USA) with an AT-225 capillary column of fused silica (30 m length, 0.25 mm i.d., 0.20 μm film thickness). The oven temperature cycle was set as follows: a start temperature of 50 °C was held for 1 min to allow vaporized samples and the solvent (hexane) to condense at the front of the column. Oven temperature was then increased rapidly to 190 °C at a rate of 40 °C/min followed by a slower increase to 220 °C at a rate of 1.5 °C/min. The final temperature of 220 °C was held for 1 min, giving a total run time of 25 min and 50 s per sample. Hydrogen was used as the carrier gas. FAMEs were identified by comparison with known standards (Sigma, St. Louis, MO), and they were confirmed by GC-MS. Values presented are representative numbers derived from replicated analyses.

Determination of Calibration Factors for Lipid Groups. It is necessary to correct the quantitative data obtained by MS for differences in sensitivity for the different groups of lipids, as polar components are more efficiently detected and hence overestimated in comparison with other components. Correction factors were therefore determined for FFA and TAG, as these groups are overestimated and underestimated, respectively, compared with PL and GL. Equal volumes of lipid extracts from 25 flour samples containing different concentrations of TAG and FFA were quantified using two different systems: ESI-MS/MS as described above and thin layer chromatography−gas chromatography (TLC-GC-FID). For the latter analysis, 50 μg of 45:0 TAG/15:0 FFA standard was added to each sample, and the neutral lipid classes were separated by silica gel TLC (plate thickness = 0.25 mm) using the solvent hexane/diethyl ether/acetic acid (150:50:2 by volume). The individual lipid classes were identified under UV light after spraying with primuline (0.05% w/v in acetone/water, 80:20 v/v), and TAG and FFA were scraped from the plate and used directly for methylation to give FAMEs as described above. The quantity of each fatty acid was calculated in comparison to the internal standards and then normalized for the sample weight (g of flour). The values (nmol/g flour) from the two analyses were compared in a scatter plot (Supporting Information Figure S2). For FFA, the data from the two methods were directly compared (Supporting Information Figure S2A), whereas for TAG the data for molecular species determined by ESI-MS/MS system were compared with the sum of FAMEs determined by GC-FID (Supporting Information Figure S2B). Both exponential and logistic curves were fitted using the method of nonlinear least-squares to estimate the three (exponential) and four (logistic) parameters with standard errors for FFA and TAG data, respectively, using the GenStat statistical package (2014, 17th ed., © VSN International Ltd., Hemel Hempstead, UK). The equation predicted from each fitted curve was used to correct the values for TAG and FFA obtained from ESI-MS/MS. The curves were fitted using quantitative data of lipid species expressed as nanomoles of lipid per gram of flour.

Multivariate Statistical Analyses. Principal component analysis (PCA) was conducted on the full data sets for molecular species of the major lipid groups in the milling and baking fractions using SIMCA-P software (version 13, Umetrics, Umea, Sweden) with unit variance scaling to compensate for differential concentrations of each lipid species.

Canonical variates analysis (CVA)[21] was used to assess the differences between combinations of years and milling fractions with respect to proportions of FAMEs. This analysis obtains linear combinations (CVs) of the FAMEs that maximize the ratio of the between treatment combinations variance to the within-treatments variance and thereby performing a discrimination between combinations. The first few CVs are those that maximize the discrimination, and so the results are presented in these new dimensions as CV scores. Assuming a multivariate N distribution for the data, 95% confidence circles can be placed around the means of CV scores, with radius $\sqrt{\chi^2_{2,0.05}}/\sqrt{n}$, where n is the replication and $\chi^2_{2,0.05} = 5.99$, is the upper 5% point of a chi-squared distribution on 2 degrees of freedom. Non-overlapping confidence circles give evidence of significant differences between treatment combinations at the 5% level of significance, although in the present case they are approximate due to the replication being technical rather than biological in nature. The magnitude of CV loadings (coefficients in the linear combinations) on the FAMEs can be inspected to identify which are important in the discrimination. The GenStat statistical package was used for this analysis.

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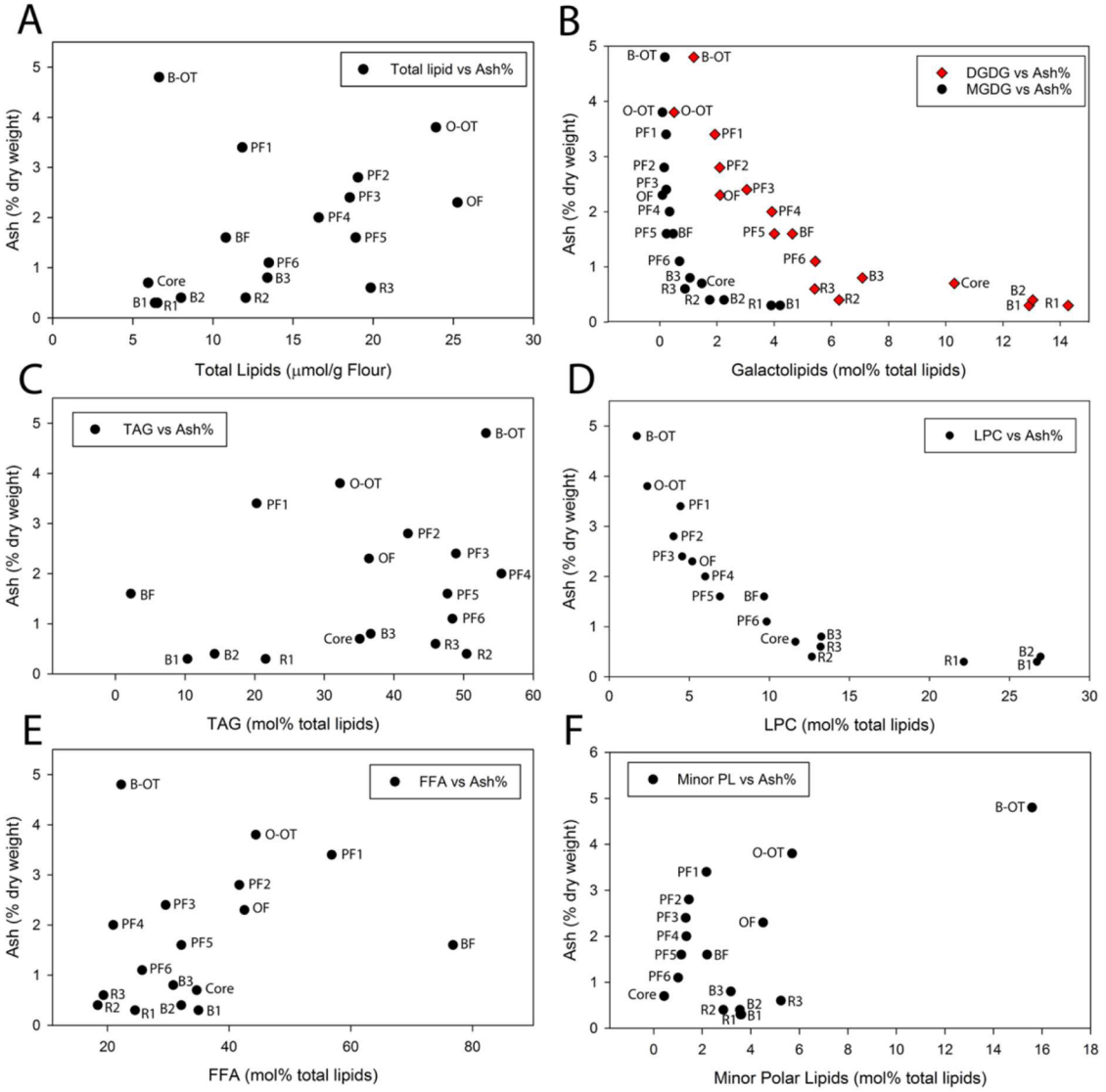

Figure 1. Relationship between ash (% dry weight) and lipid contained in 7 and 10 flour pearling and milling fractions, respectively, representing ash contained against (A) total lipid accumulation and proportions of (B) galactolipid, including MGDG and DGDG; (C) TAG; (D) LPC; (E) FFA; and (F) minor polar lipids, such as PI, PG, and PE. Seventeen fractions are indicated. Pearling fractions: PF1, pearling fraction 1; PF2, pearling fraction 2; PF3, pearling fraction 3; PF4, pearling fraction 4; PF5, pearling fraction 5; PF6, pearling fraction 6 and core. Milling fractions: B1, break 1; R1, reduction 1; B2, break 2; R2, reduction 2; B3, break 3; R3, break 3; OF, offal fraction; O-OT, offal overtail; BF, bran fraction; B-OT, bran overtail.

RESULTS AND DISCUSSION

Characterization of Fractions by Ash Content. To characterize the distribution of lipids within the wheat grain, two complementary approaches were used. First, 5 kg of grain was milled in a Buhler−MLU-202 mill to give 10 fractions consisting of 6 flours (3 breaks and 3 reductions) and 4 bran/offal fractions. Second, two 50 g aliquots of the same grain sample were fractionated using a pearling mill, removing about half of the grain dry weight in six sequential fractions, with the remaining "cores" being milled in a ball mill. The proportions of starchy endosperm cells and other grain tissues in these fractions were estimated by determination of the ash content, as this is largely derived from minerals present in the grain outer layers (Figure 1A; Table 1). The ash contents were low, <1% of total dry weight, in the three break and reduction fractions, but increased from B1/R1 to B3/R3, 0.3/0.3% to 0.8/0.6%, respectively, and increased substantially in both the break and offal flours and in the bran and offal overtail fractions (from 1.6 to 4.8%). The core (remaining after pearling) had a similar ash content (0.7%) to the third break and reduction fractions (0.8 and 0.6%, respectively), which then increased from PF6 to PF1, from 1.1 to 3.4% (i.e., from the inside to the outside of the grain, see Table 1).

Lipid Extraction and Profiling. Total non-starch lipids were extracted from the milling and pearling fractions using an optimized procedure, and ESI-MS-MS was used to identify and quantify individual lipid molecular species. Combining the values for the individual lipids allowed the total lipid contents of the fractions to be calculated, and these are plotted against ash content in Figure 1A. The total lipid content increased from the pure endosperm flour fractions to the outer layers, for example, increasing from 6.379 nmol/g flour in B1 or 5.957 nmol/g flour in the core to 25.263 and 19.053 nmol/g flour for OF and PF2, respectively. The lipid content then decreased in the B-OT (6.639 nmol/g flour) and PF1 (11.831 nmol/g flour) fractions (Table 1). The decline in lipid content observed in the PF1 fraction probably results from the high content of fibrous tissue

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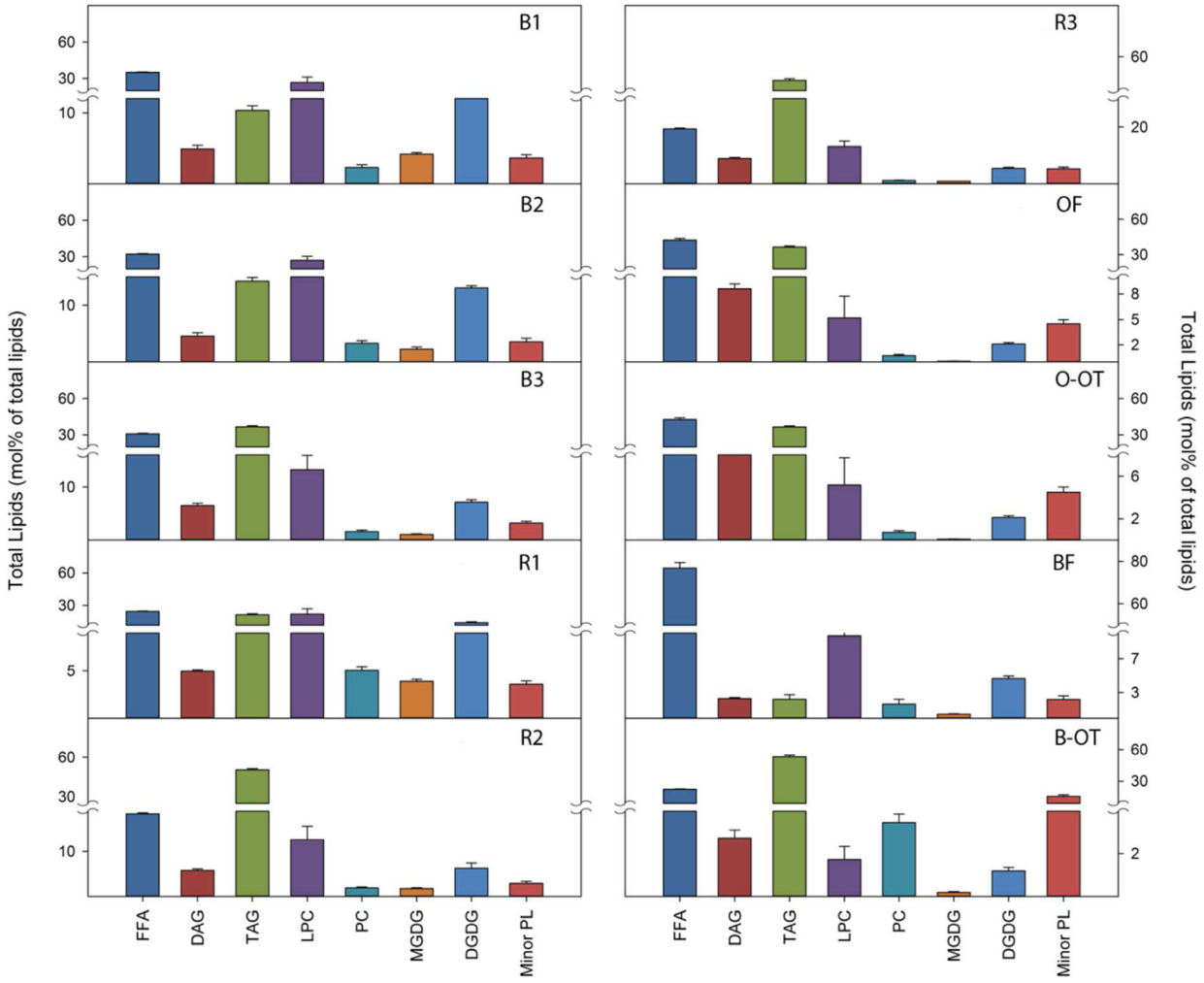

Figure 2. Total lipid profile (mol % of total lipids) in 10 flour milling fractions. Major classes of lipids are represented, including neutral lipids, free fatty acids (FFA), diacylglycerol (DAG), and triacylglycerol (TAG), the polar lipids lysophosphatidylcholine (LPC), phosphatidylcholine (PC), monogalactosyl diglyceride (MGDG), and digalactosyl diglyceride (DGDG), and the sum of different minor polar lipids, including phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylinositol. Milling fractions: B1, break 1; R1, reduction 1; B2, break 2; R2, reduction 2; B3, break 3; R3, break 3; OF, offal fraction; O-OT, offal overtail; BF, bran fraction; B-OT, bran overtail. Data are mean values ± SE of three independent samples analyzed via ESI-MS/MS.

in the outer pericarp, and it is probable that the B-OT fraction is similarly enriched.

Lipid profiling was able to resolve and identify a total of 72 specific lipid molecular species. Although a large number of lipid groups and species were determined for the individual fractions (Supporting Information Table S2), we will focus on groups and species that made significant contributions to the total lipid content. These groups were free fatty acids (FFA), the neutral lipids diacylglycerol (DAG) and triacylglycerol (TAG), the phospholipids phosphatidylcholine (PC) and lysophosphatiyl-choline (LPC), and the glycolipids monogalactosyl diglycerol (MGDG) and digalactosyl diglycerol (DGDG). Within each class one or two species predominated. Among the 12 detected FFA molecular species, 18:2 and 16:0 were predominant in the different flour fractions, accounting for 76 and 42% of total FFA in the inner and outer layers of the grain, respectively, followed by 18:1, 18:3, and 18:0. Totals of 11 and 10 molecular species were identified for DAG and TAG, respectively; specifically DAG 36:4, TAG 52:3, TAG 52:4, TAG 54:5, and TAG 54:6 were enriched in most of the fractions (Figures 4 and 5 and Supporting Information Table S2). Of the phospholipids, PC 34:2 and PC 36:4 were predominant among the eight PC species detected, representing 60−70% of the total. Notably, PC36:3 was enriched in outer layers of the grain, representing 18.9% of total PC. Only four molecular species of lysophosphatidylcholine were found, with LPC16:0 and LPC18:2 being the major species collectively representing up to 92% of total LPC in most of the fractions. The galactolipids represented 0.5−18% of the total lipids in the fractions, being higher in those derived from the inner part of the grain (Figures 2 and 3). The predominant species were DGDG 36:4, DGDG 34:2, DGDG 36:5, and MGDG 36:4, accounting for 45−61, 12−15, 9−15, and 3−18% of total galactolipids, respectively, with five other minor molecular species (Supporting Information Figures S4 and S5 and Table S2). The total "minor" phospholipids (PL) (comprising PI, PE, and PG) represented between 0.4 and 15% of the total lipids across the fractions studied, with the lowest contents in the core fraction and the highest in the B-OT. The major molecular species of minor phospholipids contained either 36:4 or 34:2 acyl groups. The proportions of the groups of minor phospholipids in the individual milling and pearling fractions are summarized in

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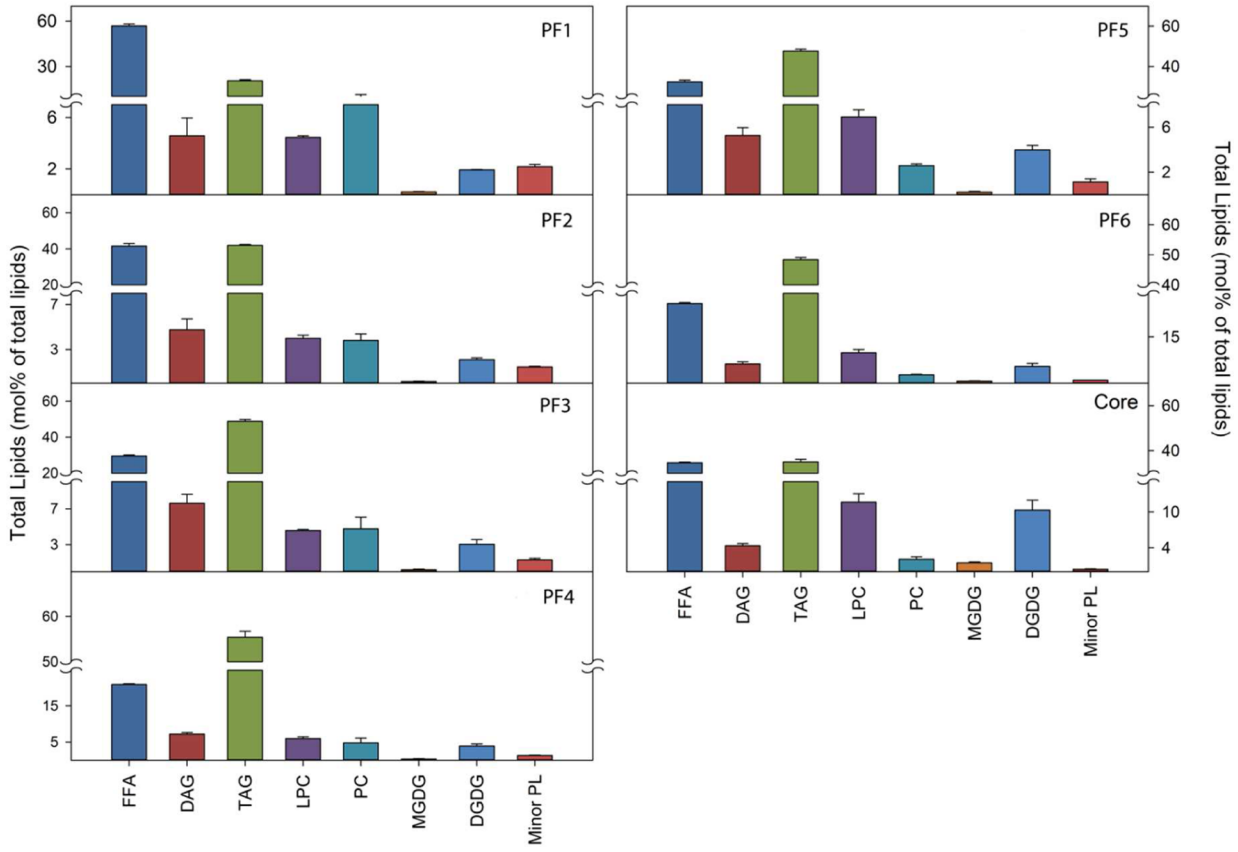

Figure 3. Total lipid profile (mol % of total lipids) in seven flour pearling fractions. Major classes of lipids are represented, including neutral lipids, free fatty acids (FFA), diacylglycerol (DAG), and triacylglycerol (TAG), the polar lipids lysophosphatidylcholine (LPC), phosphatidylcholine (PC), monogalactosyl diglyceride (MGDG), and digalactosyl diglyceride (DGDG) and the sum of different minor polar lipids, including phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylinositol. Pearling fractions: PF1, pearling fraction 1; PF2, pearling fraction 2; PF3, pearling fraction 3; PF4, pearling fraction 4; PF5, pearling fraction 5; PF6, pearling fraction 6 and core. Data are mean values ± SE of three independent samples analyzed via ESI-MS/MS.

Figures 2 and 3 and the individual species in Supporting Information Figures S5 and S6.

Milling Fractions. Clear trends and differences were observed in the distributions of the major lipid groups between milling fractions (Figure 2). FFA was consistently the major NL component in all fractions, ranging up to 76% of the total lipids in the bran fraction and from 24 to 19% of total lipids in the three reduction fractions (R1−R3). By contrast TAG, which is the major form of storage lipid in wheat, increased from B1 to B3 (from 10 to 36% of total lipids) and was similarly high in all three reduction fractions and in the bran/offal fractions, except for the bran flour, where it was very low, representing only 2.2% of total lipids. The ratio FFA/TAG varied across the different fractions, being approximately 1 in some, but substantially higher in the B1 (3.4) and bran flour (34) and lower in the R2 (0.4) fractions. In general, DAG was present at lower levels than TAG and FFA in all of the samples, between 2- and 8-fold less in most fractions, with the concentration of FFA being >30-fold greater in bran flour. DAG was particularly high in the offal fractions (up to 14% of total lipids) and minor PL in the bran OT fraction, where they represented 15% of total lipids. The most abundant minor PL was PI, which accounted for 52−88% of the total minor PL in bran flour and R3, respectively. The proportion of LPC decreased from B1 (26% of total lipids) to B3 (13% of total lipids) and from R1 (22% of total lipids) to R3 (13% of total lipids) and was low (<5%) in the offal/bran fractions (except for bran flour, 9.6% of total lipids). Glycolipids (MGDG/DGDG) were higher in the break/reduction (up to 18% of total lipids) fractions than in the bran/offal fractions (up to 5% of total lipids) and were particularly high in B1 and R1.

Of the FFA, C18:2 was the major species in all fractions except the offal fractions, where C16:0, C18:1, and C18:3 were higher (Figure 4). TAG 54:5 and 54:6 were the major TAG species in all fractions with TAG 52:3 and TAG 52:4 also being particularly high in B3 and R1-R3. The TAG notations reflect the total numbers of carbon atoms and double bonds in the three fatty acid moieties, and these "species" could therefore be mixtures of forms with similar masses (which cannot be discriminated by the MS method).

Pearling Fractions. Some trends were observed in the proportions of major lipid groups (Figure 3), moving from the outer part of the grain (PF1) to the center (core). The PF1 fraction was particularly high in minor PL (2.2% of total lipids), where PI 34:2 accounted for almost 36% of the total minor PL (see Figure 3 and Supporting Information Figure S6). The results showed that TAG was highest in PF4, accounting for 55% of total lipids. The core was particularly rich in DGDG (10% of total lipids; especially in DGDG 36:4 and 34:2) and in LPC (11% of total lipids), with LPC 18:2 and 16:0 the major molecular species (Figure 3 and Supporting Information Figure S4). The proportion of PC was highest in PF1, representing >9% of total lipids, and then decreased from the outer to the inner layers of the grain, representing only 2% of total lipids in the core. A clear gradient in FFA was observed, with C16:0 being the major

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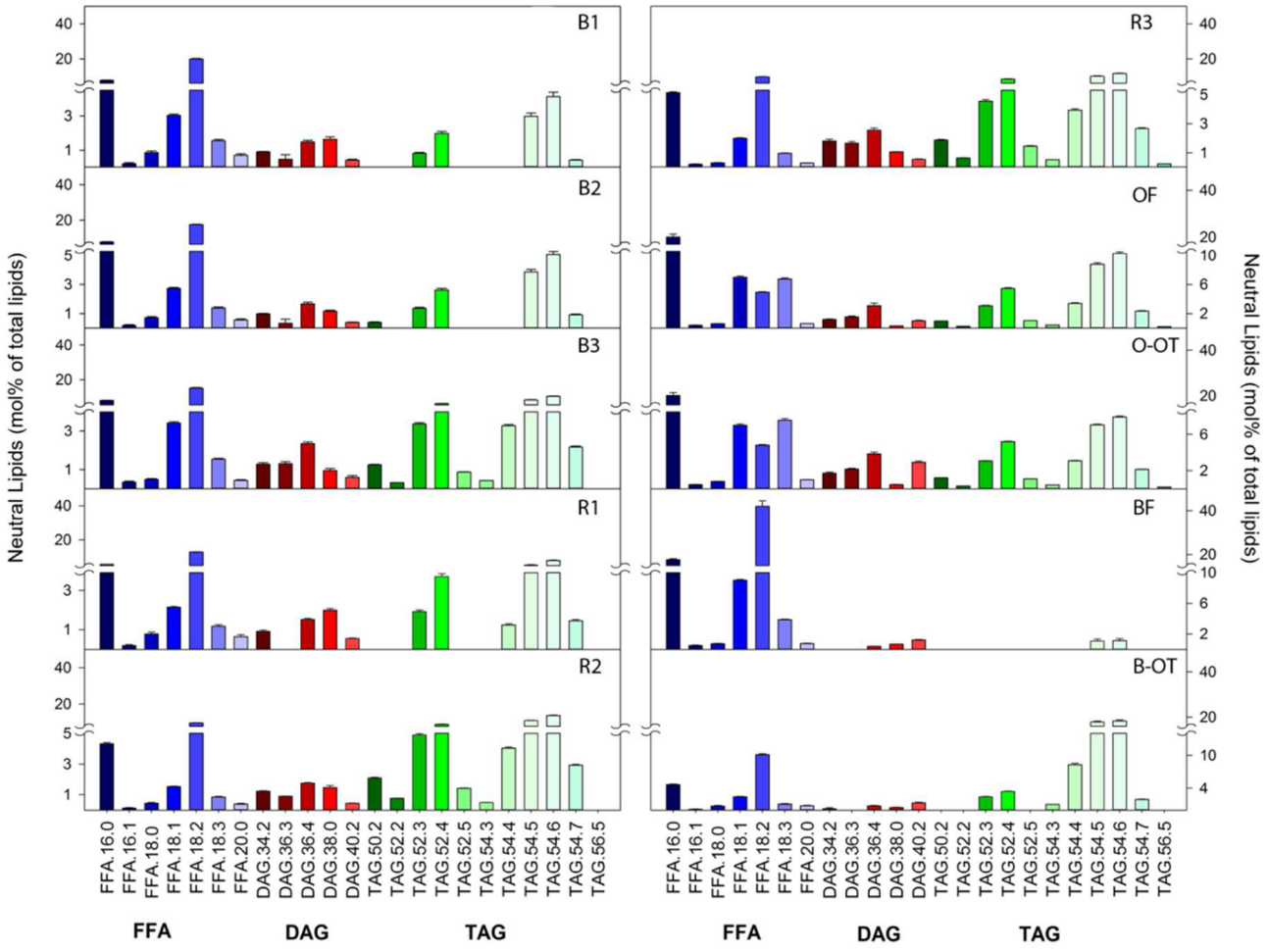

Figure 4. Molecular species of neutral lipids classes, free fatty acids (FFA), diacylglycerol (DAG), and triacylglycerol (TAG) (mol % of total lipids) in 10 flour milling fractions. Milling fractions: B1, break 1; R1, reduction 1; B2, break 2; R2, reduction 2; B3, break 3; R3, break 3; OF, offal fraction; O-OT, offal overtail; BF, bran fraction; B-OT, bran overtail. Data are mean values ± SE of three independent samples analyzed via ESI-MS/MS.

component in the outer fractions (PF1−PF4, up to 46% of total FFA) and C18:2 the major component in the central fractions (PF5−PF6 and core, up to 52% of total FFA). Total FFAs were highest in PF1 (>56% of total lipids) and decreased from PF1 to PF4 (to 20%). However, the proportions of FFA in the inner parts of the kernel (PF5−PF6 and core) were higher, between 25 and 35%. The major DAG species in all fractions was DAG36:4, representing up to 35% of total DAG, and the major TAG species were TAG52:3, TAG52:4, TAG54:5, and TAG54:6, accounting for 9.6−11.8, 16−19, 19−22, and 23−26% of total TAG, respectively.

Multivariate Analyses. The full data sets for molecular species of the major lipid groups in the milling and pearling fractions were compared by PCA (Figure 6), including data for the replicate samples used for lipid extraction and analysis. The first two principal components explained 58% of the total variance. From the PCA scores plot (Figure 6A) it is possible to say that five of the "purest" flour samples (based on their ash contents, Figure 1A) are explained by a negative score in principal component 1 (PC1) and form a group together in the left-hand part of the plot (B1, B2, B3, R1, R2, and core). The B-OT and PF1 fractions have a high positive score along the PC2 axis and group together in the upper central part of the scores plot: as discussed above, these samples have low lipid contents and probably both contain the outer pericarp of the grain. The offal fractions have the highest PC1 score and occur together on the right-hand side of the PCA scores plot. Other samples having a positive score along the PC1 axis include PF2, PF3, and PF4. Analysis of the PCA loadings plot for PC1 versus PC2 (Figure 6B) shows that these samples are rich in FFA and additionally also enriched in TAG and may contain the oil-rich aleurone layer. The PF5 and PF6 fractions form a group in the center of the plot, reflecting their intermediate purity and composition between the purest fractions (B1, B2, B3, R1, R2, core) and the offal/PF2/PF3/PF4 fractions. Only two fractions separate as completely clear clusters: the bran four (BF), which has a distinctive lipid composition (with low TAG and high PE), and the reduction R3, which differs from all other fractions by having a low PC2 score as a consequence of being lower in FFA, but rich in TAG.

Comparative Analysis of Milling Fractions from Grain Grown in 2011, 2012, and 2014. To determine whether the distribution of lipids between milling fractions was similar for grain grown under different conditions, we analyzed mill streams from samples of Hereward wheat grown under similar agronomic conditions over three seasons, 2011, 2012, and 2014. These three years differed significantly in weather conditions, particularly in total precipitation during the period from June to August, which was 208.8 mm in 2011, 349.6 mm in 2012, and 180.7 mm in 2014.

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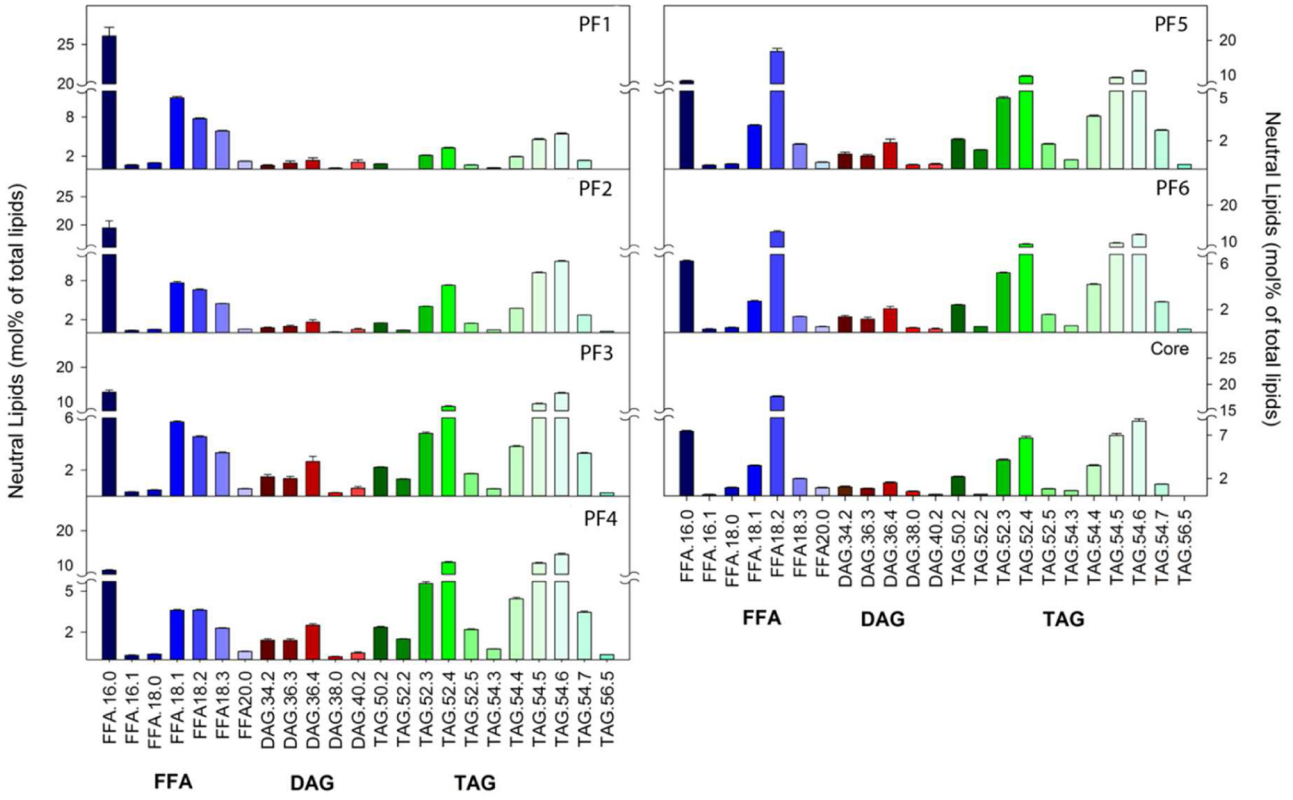

Figure 5. Molecular species of neutral lipids classes, free fatty acids (FFA), diacylglycerol (DAG), and triacylglycerol (TAG) (mol % of total lipids) in seven flour pearling fractions. Pearling fractions: PF1, pearling fraction 1; PF2, pearling fraction 2; PF3, pearling fraction 3; PF4, pearling fraction 4; PF5, pearling fraction 5; PF6, pearling fraction 6 and core. Data are mean values ± SE of three independent samples analyzed via ESI-MS/MS.

To provide a broad overview of lipid distribution, total lipids were transmethylated and the FAMEs determined by gas chromatography. CVA was applied to the proportions of C16:0, C18:0, C18:1, C18:2, and C18:3 to consider the differences between the combinations of years by milling fractions. The first two CVs accounted for 98.36% of the variation and possible discrimination, with CV1 (94.04%) largely separating the fractions and CV2 (4.32%) the years (Figure 7). Hence, the very small percentage accounted for by CV2 relates to lesser overall importance of differences between years compared to differences between fractions. The loadings for CV1, 1.155 (C16:0), −4.357 (C18:0), −4.777 (C18:1), 0.831 (C18:2), and −8.763 (C18:3), suggest that C18:3 and to a lesser extent C18:0 and C18:1 were mainly responsible for the separation between fractions. The loadings for CV2, 2.054 (C16:0), 0.893 (C18:0), −2.263 (C18:1), −1.356 (C18:2), and 8.951 (C18:3), suggest that C18:3 was mainly responsible for separation of the years. The CVA plot shows that 2011 was somewhat different from the other two years for R1 and BF milling fractions in particular, and the means show that 2011 had the lowest proportion of 18:3 for all of the fractions. This comparison demonstrates that fractions from different grain samples show similar distributions of lipid components, although some differences in detailed compositions may occur between years. This is to be expected as environmental factors are expected to affect grain development and composition. For example, it is possible that the unusually high precipitation during the summer of 2012 resulted in effects on grain composition.

Relationship to Ash Content. As discussed above, the ash content can be used as a measure of the purity of the flour samples. This is because the outer starchy endosperm cells are more likely to become mixed with the aleurone and other outer tissues during milling/pearling; the ash content also indicates whether the flour fractions correspond to the outer or inner parts of the grain. Figure 1B–F and Supporting Information Figure S3 therefore show the proportions of lipid groups and molecular species in relation to ash content.

The two types of glycolipid, MGDG and DGDG, both show inverse relationships with ash content (Figure 1B), which reflects the fact that they are characteristic of the membranes of plastids.[22] In wheat grain the plastids, termed amyloplasts, are the sites of starch synthesis and storage and are largely restricted to the starchy endosperm cells. However, these two groups of glycolipids differ in that higher proportions of DGDG are present in the fractions that are high in ash. It has been previously reported that DGDG is transported from plastids to other membranes, including the plasma membrane,[23,24] tonoplast,[24] and mitochondria[25] under some conditions such as phosphate starvation.[26] By contrast, there is no evidence that MGDG is present in membranes outside the plastid. Our results may therefore reflect the fact that DGDG is present in amyloplast and non-amyloplast membranes in the outer layers of the grain.

The polar lipid LPC shows a distribution similar to that of DGDG (Figure 1D), which is consistent with the fact that LPC and other lysophosholipids (LPL) are concentrated in starch granules, but not restricted to these structures.[17,27] LPC and other LPL require polar solvents and harsh conditions for complete extraction from starch granules.[17,28] Although these conditions were not used in the present study, it is probable that some of the LPC was extracted from the starch granules present in the flour samples. The proportion of minor PL increased with ash content, with the B-OT fraction being particularly rich (as

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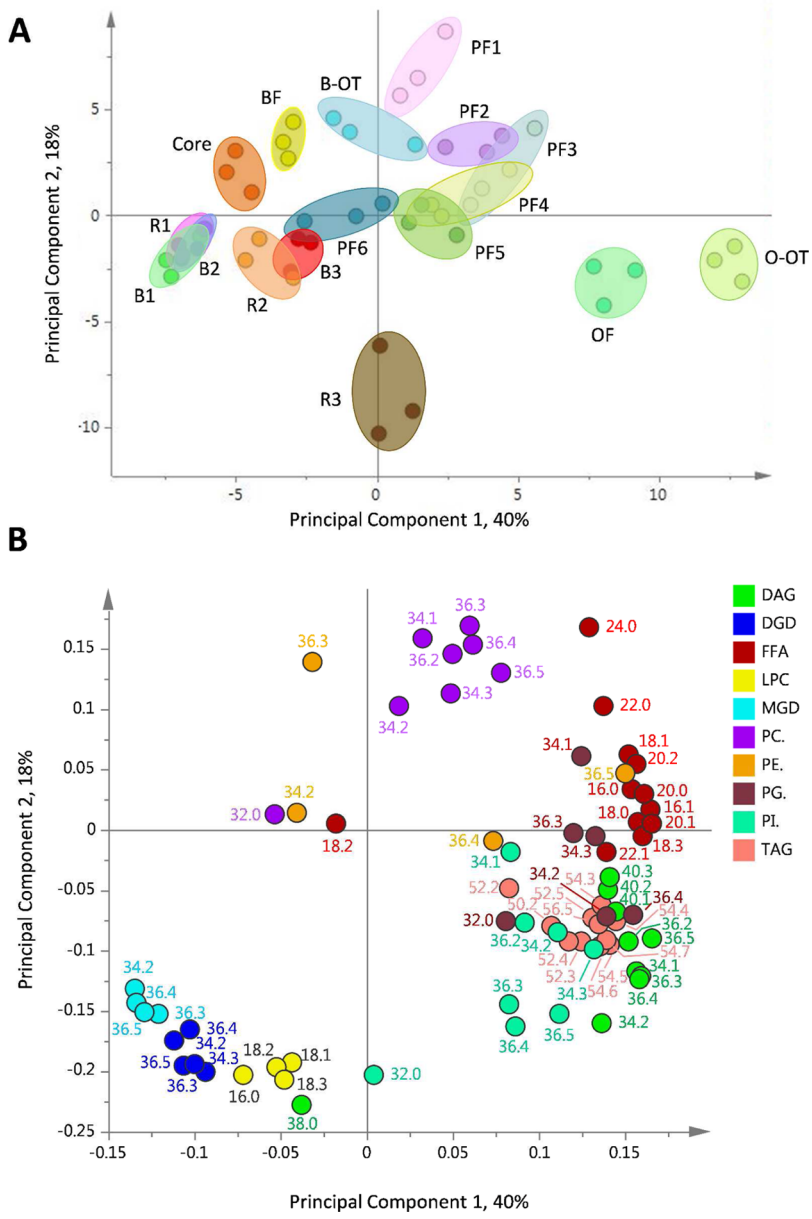

Figure 6. Principal component analysis (PCA) of 17 flour fractions. (A) PCA scores plot showing PC1 (40%) versus PC2 (18%). Pearling fractions: PF1, pearling fraction 1; PF2, pearling fraction 2; PF3, pearling fraction 3; PF4, pearling fraction 4; PF5, pearling fraction 5; PF6, pearling fraction 6 and core. Milling fractions: B1, break 1; R1, reduction 1; B2, break 2; R2, reduction 2; B3, break 3; R3, break 3; OF, offal fraction; O-OT, offal overtail; BF, bran fraction; B-OT, bran overtail. (B) PCA loadings plot of PC1 vs PC2. Variables are colored according to their lipid class and are labeled according to chain length and double-bond number. Lipid classes are free fatty acids (FFA), diacylglycerol (DAG), triacylglycerol (TAG), monogalactosyl diglyceride (MDG), digalactosyl diglyceride (DGD), lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylinositol (PI).

above) (Figure 1F). This is an interesting observation, but the biological significance is not known.

The relationships between ash content and the proportions of TAG (Figure 1C) and FFA (Figure 1E) are less clear-cut. Although both TAG and FFA tend to increase with increasing ash content, the TAG content also increases significantly from B1 to B3 and from R1 to R2/R3 with little increase in ash content. This presumably reflects a gradient in the distribution of storage lipids within the starchy endosperm. The two bran fractions form outliers in the FFA plot, with B-OT having high ash, but low FFA and BF the highest content of FFA.

Comparison with Previous Studies. It has been reported that nonstarch polar lipids have beneficial effects on breadmaking by stabilizing gas bubbles in dough.[13,29,30] We have therefore focused on total nonstarch lipids from different milling fractions to identify differences in composition that may be related to the spatial distribution of lipids in the grain and allow the lipid compositions of flours to be optimized for different end uses. Although historically flour lipids have been divided into free and bound groups according to their extractability in solvents,[18,29−32] this distinction has little biological validity or functional significance and has not been retained in the present study.

Lipidomic analyses showed that neutral lipids were the major components of the non-starch total lipids from all flour fractions (Figures 2 and 3). This agrees with the review of Chung et al.,[33] which concluded that fractions that were rich in non-starch lipids

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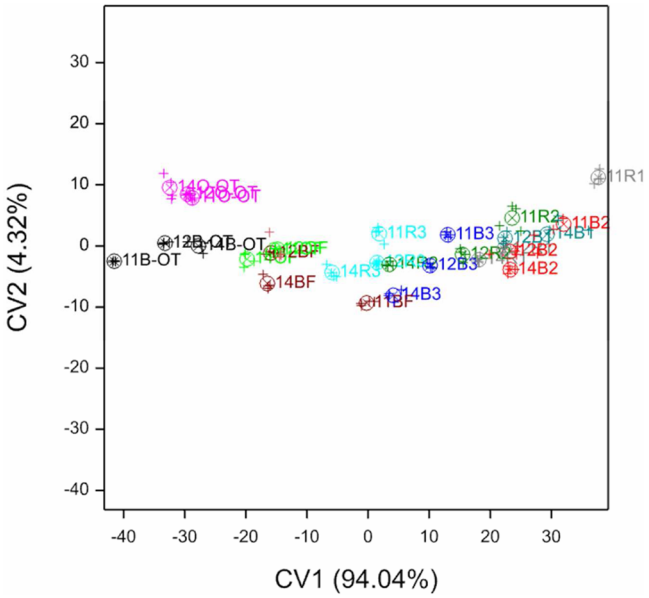

Figure 7. Canonical variates analysis (CVA) plot of variation in the proportions of fatty acid methyl esters in milling fractions from wheat cv. Hereward grown at Rothamsted Research in 2011, 2012, and 2014. Milling fractions: B1, break 1; R1, reduction 1; B2, break 2; R2, reduction 2; B3, break 3; R3, break 3; OF, offal fraction; O-OT, offal overtail; BF, bran fraction; B-OT, bran overtail; 11, 12, and 14 refer to years. CV scores (+) and means of scores (×) in the two dimensions are plotted. In the present case the 95% confidence circles around the means may be used only as a rough guide due to the replication being technical rather than biological in nature.

also had high levels of neutral lipids, especially the germ and aleurone. However, previous studies reported much lower proportions of FFA compared to TAG in flour fractions of four cultivars (Atou, Flinor, Waldron, and Edmore)[33,34] than in the flour fractions from cv. Hereward, which had more similar levels of FFA and TAG, with FFA being higher than TAG in several fractions (PF1, B1, B2, and BF). However, more recent analyses of the soft wheat cultivar Claire showed lower TAG/FFA ratios, which were more similar to those reported here.[27] This suggests that variation in grain composition between cultivars and growth conditions may affect the composition of non-starch lipids in flour.[15,16,33−36]

The major polar lipid classes detected here were, from highest to lowest, LPC, DGDG, PC, and MGDG (Figures 2 and 3 and Supporting Information Figures S3 and S4). Finnie et al.[17,37] reported the same major polar lipids in two U.S. varieties, but their proportions differed. By contrast, the most abundant fatty acids in the acyl lipids were C16:0 and C18:2 and their combinations, including 36:4 and 34:2 in DAG and diacyl polar lipids, which agrees with Finnie et al.[17,37] (Figures 4 and 5; Supporting Information Figures S4−S7). The most abundant acyl carbon and double-bond configurations in TAG, such as 52:3, 52:4, 54:4, 54:5, and 54:6, were probably combinations of these two major fatty acids with lower proportions of C18:3, C18:1, and C18:0.

Relationship between Fractions and Grain Tissues. The differences reported here between the lipid compositions of milling and pearling fractions reflect differences between the compositions of the grain tissues and the efficiency with which these are separated by milling. The fractions produced by pearling do not represent individual grain tissues, as the elongated nature of the grain and the presence of a crease means that the removal of tissue does not occur evenly, with the cores being more rounded in shape with some evidence of a crease still being present. Hence, pearling results in a series of overlapping fractions with gradients in composition rather than fractions corresponding to distinct layers. By contrast, clear differences are observed between the milling fractions, which demonstrates the efficiency of modern roller milling in separating tissues that are highly enriched in the outer pericarp (B-OT), aleurone layer (offal fractions), and central starchy endosperm (breaks and reductions). Furthermore, differences are also observed between the individual breaks and reductions, which may indicate that they correspond to different regions of the starchy endosperm tissue.

Exploitation of Differences in Lipid Composition. The Buhler laboratory mill used in the present study generated only 10 fractions compared to up to 30 fractions produced in the more complex commercial roller mills. Hence, it may be predicted that streams from commercial mills would also show differences in composition, although perhaps more subtle differences than those described here. Selective modification of the flour lipid composition by lipase activity affects loaf volume,[32] and it has been shown that polar lipids (PL and GL) have beneficial properties with NL (TAG and FFA) being detrimental.[38−41] It has been suggested that most of the endogenously supplied lipids bind to the gluten fraction during the first stages of dough mixing, especially free polar lipids,[30,42,43] with galactolipids promoting the formation of glutenin protein/lipid complexes, whereas phospholipids interact with the gliadins or lipid binding protein of gluten.[44] These interactions result in increased dough strength and gas-retaining capacity and, therefore, in a greater loaf volume and better crumb structure.

However, in addition to their impact on the gluten network, flour polar lipids also have direct effects on gas cell stability, possibly through the formation of monolayers at the gas−liquid interface that prevent coalescence of neighboring gas cells.[45,46] For example, the large polar headgroup of DGDG would be expected to become oriented as a condensed monolayer that would promote gas bubble stability in the dough. A role for glycolipids in breadmaking was initially suggested by Pomeranz et al.[47,48] on the basis of their structural similarity with bread softeners and surfactants used to improve bubble stability. Lipolytic enzymes, which hydrolyze the endogenous wheat lipids to release surface active lipids, are also used in commercial breadmaking[32,49] Gerits and collaborators[32] recently reported that lipases that hydrolyze polar lipids, particularly galactolipids, release lyso-forms of galactolipids, which stabilize bubbles structures in the dough.[49]

The differences in lipid compositions of mill streams reported here therefore suggest that it may be possible to produce flours with specific lipid compositions for different end uses, by optimizing the operation of the mill and combining selected individual mill streams. Furthermore, these effects may be enhanced by selective modification by lipases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b05289.

Table S1, ESI-MS/MS methods and parameters for TAG, DAG, and FFA molecular species identification; Figure S1, scheme for milling using a Buhler-MLU-202 mill; Figure S2, comparison of quantitative values obtained via GC vs

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ESI-MS/MS; Figure S3, relationship between ash (% dry weight) and most relevant lipid molecular species in 7 and 10 flour pearling and milling fractions, respectively, showing patterns across the fractions; Figure S4, molecular species (mol % of total lipids) of most relevant polar lipids in 10 milling fractions; Figure S5, molecular species (mol % of total lipids) of most relevant polar lipids in 7 pearling fractions; Figure S6, molecular species (mol % of total lipids) of minor polar lipids in 10 milling fractions; Figure S7, molecular species (mol % of total lipids) of minor polar lipids in 7 pearling fractions(DOCX)

Table S2, lipid molecular species in milling and pearling fractions (XLS)

AUTHOR INFORMATION

Corresponding Author

*(P.R.S.) E-mail: peter.shewry@rothamsted.ac.uk.

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Notes

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ABBREVIATIONS USED

DAG, diacylglycerols; DGDG, digalactosyldiglycerol; ESI-MS/MS, electrospray ionization tandem triple-quadrupole mass spectrometry; FAME, fatty acid methyl ester; FFA, free fatty acids; LPL, lysophospholipids; LPC, lysophosphatidylcoline; MGDG, monogalactosyldiglycerol; PC, phosphatidylcholine; PCA, principal component analysis; PE, phosphatidylethanolamine; Pet, petroleum ether; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, phospholipids; TAG, triacylglycerol; TLC-GC-FID, thin layer chromatography−gas chromatography; WSB, water-saturated butan-1-ol

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