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Wheat (*Triticum aestivum* L.) lipid species distribution in the different stages of straight dough bread making

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ABSTRACT

Although wheat endogenous lipids strongly impact bread quality, knowledge on their detailed distribution throughout the different stages of straight dough bread making is lacking. We here compared the lipid populations in hexane [containing free lipids (FLs)] and water-saturated butanol extracts [containing bound lipids (BLs)] of wheat flour, freshly mixed and fermented doughs, and bread crumb using high-performance liquid-chromatography [for nonpolar lipids, *i.e.* mainly free fatty acids (FFA) and triacylglycerols] and electrospray ionization tandem mass spectrometry (for polar lipids). Freshly mixed doughs had lower FL and higher BL levels than flour, a phenomenon referred to as lipid-binding. Furthermore, probably due to the disintegration of flour particles, the overall extractability of nonpolar lipids was higher in freshly mixed dough than in flour. Dough fermentation decreased the extractability of glycolipids, but increased that of nonpolar lipids and phospholipids. We hypothesize that these phenomena result from stretching of the gluten network due to gas cell expansion, which leads to the replacement of some lipids associated with gluten proteins by others. Baking increased the extractability of bound lysophospatidylcholine (LPC) levels, but decreased that of free FFA. This is probably due to *in situ* dissociation of amylose-LPC inclusion complexes and formation of amylose-FFA inclusion complexes during bread baking and cooling, respectively.

The approach and ESI-MS/MS methodology we developed provided valuable insights regarding the distribution of lipids at the different stages of bread making. Hence, it opens perspectives for future efforts to relate differences in lipid composition between wheat cultivars to their bread making quality.

1. Introduction

Although lipids only make up 1.5 to 2.5% *w*/w of wheat flour, they strongly impact its bread making quality (Chung, Ohm, Ram, Park, & Howitt, 2009; Hargin & Morrison, 1980; Pareyt, Finnie, Putseys, & Delcour, 2011). Wheat flour lipid structure, classification, and functionality has been a topic of debate over the past century. The first reports on wheat lipids were by Sullivan, Near, and Foley (1936) and Olcott and Mecham (1947). Later, several research groups studied the extraction and separation of wheat lipids (Christie, 1985; Christie, 1986; Christie & Morrison, 1988; Morrison & Coventry, 1985; Prieto, Ebri, & Collar, 1992) and their role in bread making (Chung, Pomeranz, & Finney, 1982; Chung & Tsen, 1975; Fisher, Broughton, Peel, & Bennett, 1964; Hargin & Morrison, 1980; Hoseney, Finney, Pomeranz, & Shogren, 1969; MacRitchie & Gras, 1973; Pomeranz, Chung, & Robinson, 1966). More recently, additional lipid extraction procedures (Hubbard, Downing, Ram, & Chung, 2004; Moreau, Powell, & Singh, 2003), analytical techniques (Finnie, Jeannotte, & Faubion, 2009;

Abbreviations: AM-L, Amylose-lipid; AU, Arbitrary units; BL(s), Bound lipid(s); DGDG, Digalactosyldiacylglycerol; DGMG, Digalactosylmonoacylglycerol; Dm, Dry matter; ELSD, Evaporative light scattering detector; ESI, Electrospray ionization; FA(s), Fatty acid(s); FFA(s), Free fatty acid(s); FL(s), Free lipid(s); HPLC, High-performance liquid-chromatography; LPC, Lysophosphatidylcholine; LPE, Lysophosphatidylethanolamine; LPI, Lysophosphatidylinositol; LPS, Lysophosphatidylserine; MGDG, Monogalactosyldiacylglycerol; MGMG, Monogalactosylmonoacylglycerol; MRM, Multiple reaction monitoring; MS, Mass spectrometry; MS/MS, Tandem mass spectrometry; NALPE, N-acyl lysophosphatidylethanolamine; NAPE, N-acyl phosphatidylethanolamine; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PI, Phosphatidylinositol; PS, Phosphatidylserine; TAG, Triacylglycerol; TL(s), Total lipid(s); WSB, Water-saturated butanol

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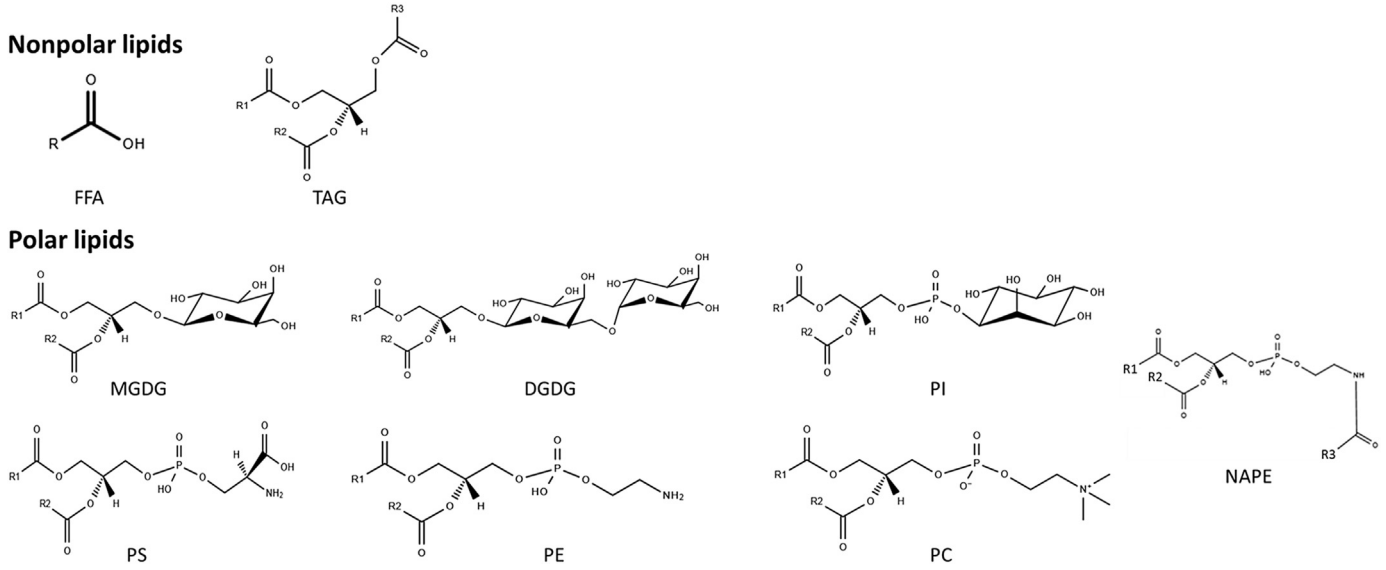

Fig. 1. Chemical structures of nonpolar [free fatty acids (FFA) and triacylglycerols (TAG)], and polar [monogalactosyldiacylglycerols (MGDG), digalactosyldiacylglycerols (DGDG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and N-acyl phosphatidylethanolamine (NAPE)] lipids commonly present in wheat flour. R_X indicates the alkane/alkene chain of a fatty acid (FA). Lipid maps, lipidomics gateway [online] available from: www.lipidmaps.org. 15/01/2018.

Finnie, Jeannotte, Morris, & Faubion, 2010; Finnie, Jeannotte, Morris, Giroux, & Faubion, 2010), or innovative approaches, such as those based on synthetic lipid-like compounds (Selmair & Koehler, 2008; Selmair & Koehler, 2009) or lipases (Gerits, Pareyt, & Delcour, 2014; Gerits, Pareyt, Masure, & Delcour, 2015; Schaffarczyk, Østdal, Matheis, & Koehler, 2016), have allowed further elaborating on wheat lipid structure, classification, and functionality.

Today, wheat lipids are typically classified either as starch lipids, which - as their name implies - occur inside starch granules, or as non-starch lipids (Morrison, 1981). The latter are further subdivided in free lipids (FLs) and bound lipids (BLs) based on their sequential extractability with nonpolar (*e.g.* hexane) and polar [*e.g.* water saturated butanol (WSB)] solvents, respectively (Chung et al., 2009; Morrison, 1988; Pareyt et al., 2011). In addition, flour lipids are also classified as either nonpolar or polar. Fig. 1 shows the chemical structures of the most common wheat flour lipid classes. The most abundant flour nonpolar lipids are triacylglycerols (TAGs) and free fatty acids (FFA). Glyceroglycolipids and glycerophospholipids (further referred to as glycolipids and phospholipids, respectively) make up most wheat flour polar lipids. Mono- (MGDG) and digalactosyldiacylglycerols (DGDG) are the main glycolipids, whereas N-acyl phosphatidylethanolamine (NAPE), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and their respective lysoforms lyso-NAPE (NALPE), lyso-PE (LPE), and lyso-PC (LPC) represent the most abundant phospholipid classes (Finnie et al., 2009; Hargin & Morrison, 1980; Pareyt et al., 2011).

Evidently, wheat flour contains a complex mixture of lipids with varying polarities. Some of its lipids play a prominent role in each stage of the bread making process which typically starts by mixing water, flour, yeast, and salt and some nonessential ingredients into viscoelastic dough. During mixing, air is incorporated in the dough (Baker & Mize, 1941; Delcour & Hoseney, 2010). In this process also, gluten proteins interact with one another and form a viscoelastic network while, at the same time, the majority of native flour lipids is redistributed from the surface of starch granules to this gluten network (Chung & Tsen, 1975; Gerits, Pareyt, & Delcour, 2013; Olcott & Mecham, 1947). This phenomenon is known as lipid-binding (Carr, Daniels, & Frazier, 1992; Gerits et al., 2013; Olcott & Mecham, 1947; Ponte, Titcomb, & Cerning, 1964). In the early stages of fermentation, gas cells are embedded in and physically stabilized by the gluten-starch matrix (Gan, Ellis, & Schofield, 1995; Sroan, Bean, & MacRitchie, 2009). However, during late fermentation and early baking, stretching of the gluten network results in discontinuities in the matrix that leave neighboring gas cells only separated by a thin liquid film. From that moment onwards, the stabilization of these gas cells at the air/water interface (Gan et al., 1995; Sroan & MacRitchie, 2009) is taken over by protein and lipid surface-active constituents. During the initial phases of baking, gas cells continue to expand until the liquid films fail to withstand the increase in interfacial area and the gas cells rupture which more or less coincides with the setting of the crumb as a result of starch gelatinization and gluten polymerization. Starch gelatinization provokes (i) migration of water from the gluten to the starch phase which then leads to release of polar lipids from the gluten phase (Eliasson, 1985; Köhler, 2001) and (ii) the *in situ* dissociation and reformation of amylose-lipid (AM-L) inclusion complexes during bread baking and cooling, respectively (Goderis, Putseys, Gommes, Bosmans, & Delcour, 2014; Kugimiya, Donovan, & Wong, 1980; Putseys, Lamberts, & Delcour, 2010).

It follows from the above that lipids play a decisive role in all stages of bread making. However, because the role of certain lipid classes or even lipid species in the process remains unclear at present, there is a need for a systematic approach for characterizing and analyzing lipids, especially those present in complex matrices (Wenk, 2005) such as wheat flour, dough, or bread crumb. This area of study in general is referred to as lipidomics or lipid profiling (Finnie et al., 2009; Finnie, Jeannotte, Morris, & Faubion, 2010; Finnie, Jeannotte, Morris, Giroux, & Faubion, 2010). Over the last decade, several advanced proteomic techniques [*e.g.* high-resolution chromatography, mass spectrometry (MS), and nuclear magnetic resonance] have become available (German, Gillies, Smilowitz, Zivkovic, & Watkins, 2007). MS-based lipidomics allows simultaneous identification and quantification of (hundreds of) lipid species in crude lipid extracts (*i.e.* shotgun lipidomics) [as reviewed by Wenk, 2005, 2010 and Dehairs, Derua, Rueda-Rincon, & Swinnen, 2015]. Lipid profiling of wheat whole meal, flour, and starch (Finnie et al., 2009; Finnie, Jeannotte, Morris, & Faubion, 2010; Finnie, Jeannotte, Morris, Giroux, & Faubion, 2010), milling and pearling fractions (González-Thuillier et al., 2015), and flour, dough liquor, and dough liquor foam (Salt et al., 2018) with electrospray ionization tandem MS (ESI-MS/MS) already allowed identification and quantification of different wheat glycolipid and phospholipid classes and the analysis of their distinctive acyl groups. However, detailed knowledge on the distribution of glycolipids and phospholipids during

the different phases of bread making is lacking. Furthermore, none of the above studies have included the quantification of NAPE and NALPE, even if they have a unique molecular structure amongst wheat phospholipids because they contain a fatty acid (FA) attached to the *sn*-3 position.

Recently, a method based on single-run high-performance liquid-chromatography (HPLC) with evaporative light scattering detection (ELSD) was developed at our research group to study wheat lipids (Gerits et al., 2013). Although this method is valuable for studying the role of wheat lipids in bread making (Gerits et al., 2013; Gerits et al., 2014; Gerits et al., 2015), shortcomings include that it is not quantitative and cannot differentiate between individual lipid species within a lipid class. Therefore, we here implemented an MRM-based ESI-MS/MS method for polar lipid profiling, including NAPE and NALPE, thus complementing the HPLC-ELSD lipid analyses, which are still used to assess the distribution of nonpolar lipids. Using this approach, we here set out to study the distribution of polar and nonpolar lipids throughout the entire bread making process by analyzing and comparing lipid extracts from wheat flour, fresh and fermented dough, and bread crumb.

2. Materials and methods

2.1. Materials

Wheat flour has a much lower lipase activity than its whole grain counterpart (Almeida, Pareyt, Gerits, & Delcour, 2014). Hence, to minimize the impact of endogenous lipases, wheat flour instead of whole grain flour was used for bread making. Kernels from soft wheat cultivar Claire were from Limagrain (Rilland, The Netherlands) and were conditioned to 16.0% moisture before milling with a Bühler (Uzwil, Switzerland) MLU-202 laboratory mill as in Delcour, Vanhamel, and De Geest (1989). Wheat flour contained 12.6% moisture, 2.1% damaged starch [on dry matter (dm) basis], 0.54% ash, and 9.6% protein (N × 5.70) [on dry matter (dm) basis], as determined with AACCI Approved Methods 44–19.01 (AACCI, 2000a), 76.31.01 (AACCI, 2000b), 08–01.01 (AACCI, 2000c), and an adaptation of the AOAC Official Method (AOAC, 1995) to an automated Dumas protein analysis system (EAS Vario Max CN, Elt, Gouda, The Netherlands), respectively. Sugar, salt, and fresh compressed yeast (AB Mauri, Dordrecht, Nederland) were bought in a local supermarket. Cholesterol (purity > 99%) was from Larodan (Solna, Sweden). Glycolipid and phospholipid standards for ESI-MS/MS analyses were from Matreya LLC (State College, PA, USA) and Avanti Polar Lipids (Alabaster, AL, USA), respectively, and are described in detail in Section 2.2.4. Commercial wheat starch was from Tereos Syral (Aalst, Belgium). Palmitic acid (purity > 99%) was from Sigma-Aldrich (Steinheim, Germany). All solvents used were from VWR (Haasrode, Belgium) unless specified otherwise and of at least analytical grade.

2.2. Methods

2.2.1. Bread making

Wheat dough optimal water level and mixing time were determined by mixograph (National Manufacturing, Lincoln, NE, USA) analysis as in AACCI method 54–40.02 (AACCI, 2000d). The optimum water absorption and mixing time were 45% (*w*/w on 14% moisture flour basis) and 150 s, respectively. As in Shogren and Finney (1984), Flour (10.0 g), deionized water (45% w/w on 14% moisture flour basis), sugar (6.0% w/w on flour basis), compressed fresh yeast (5.3% w/w on flour basis), and salt (1.5% w/w on flour basis) were mixed in a pin mixer (National Manufacturing) for 150 s. The speed of mixing applied during dough making was 90 rpm. No shortening was used. Dough samples were placed in a fermentation cabinet (National Manufacturing) at 30.0 °C and 90% relative humidity for 126 min and sheeted (2.5 mm gap) after 52, 77, and 90 min after the start of fermentation. Finally, fermented dough samples were baked in a rotary oven (National Manufacturing) for 13 min at 232 °C.

2.2.2. Starch gelatinization in the presence of palmitic acid

To mimic the formation (and reformation) of AM-L inclusion complexes during baking (see section 1), commercial wheat starch samples were gelatinized in the absence and presence of added palmitic acid (16:0). Palmitic acid (0.034 g) dissolved in 0.50 ml chloroform was added to 1.50 g of wheat starch in a test tube. The chloroform was allowed to evaporate under a fume hood. Likewise, control samples were prepared by adding 0.50 ml chloroform to 1.50 g wheat starch. Deionized water (4.5 ml) was added to both sample types to obtain a dm/water ratio of 1/3. The samples were heated in a water bath at 95 °C for 10 min to gelatinize the starch, immediately frozen with liquid nitrogen, and freeze-dried prior to lipid extraction (see Section 2.2.3.3) and HPLC-ELSD analyses (see Section 2.2.5).

2.2.3. Lipid extraction

2.2.3.1. Lipids in flour, fresh and fermented doughs, and bread crumb. Lipids were extracted in triplicate from flour, fresh and fermented dough, and bread crumb (all on same dm basis) as in Gerits et al. (2013, 2014). In short, total lipid (TL) content was determined gravimetrically as the sum of FLs and BLs (Gerits et al., 2013). FLs were extracted with hexane, the solvent was evaporated, and the FL weight was determined. Next, BLs were extracted using WSB, the solvent was evaporated, and the extract was treated according to Bligh and Dyer (1959) to remove non-lipid material (primarily proteins). The latter is based on extraction with solvents of opposing polarity. In short, chloroform, methanol, and milli-Q water were sequentially added to the samples in a 1.0/1.0/0.9 ratio. After centrifugation (540 *g*, 20 min, 21 °C), the upper methanol/water phase, which contained non-lipid material, was removed. The lower chloroform phase, which contained lipid material, was collected, the solvent was evaporated, and BLs were gravimetrically quantified. All lipid extracts were then stored at −80 °C prior to ESI-MS/MS (see Section 2.2.4) and HPLC-ELSD (see Section 2.2.5) analyses.

2.2.3.2. Lipids in compressed fresh yeast. To gain insight in the potential importance of the yeast lipid population, yeast lipids were extracted and purified in triplicate as in Section 2.2.3.1. After centrifugation (540 *g*, 20 min, 21 °C), the lower chloroform phase contained lipids, the middle phase yeast residue, and the upper methanol-water phase non-lipid material (mainly protein). The upper phase was discarded, the lower phase collected and the yeast residue extracted twice more. The chloroform phases were then combined, the solvent was evaporated, and lipid levels were gravimetrically quantified. These lipid extracts were then stored at −80 °C until analyzed by HPLC-ELSD (see Section 2.2.5).

2.2.3.3. Lipids in gelatinized starch. Lipids were extracted in triplicate from freeze-dried gelatinized starch samples (see Section 2.2.2) as in Section 2.2.3.1. FLs were extracted with hexane. Next, BLs were extracted using WSB and purified as in Section 2.2.3.1. After centrifugation (540 *g*, 20 min, 21 °C), the lower chloroform phase was collected and the solvent evaporated. These lipid extracts were then stored at −80 °C until analyzed by HPLC-ELSD (see Section 2.2.5).

2.2.4. Lipid profiling with electrospray ionization tandem mass spectrometry

2.2.4.1. Sample preparation. ESI-MS/MS analyses of glycolipids [*i.e.* MGDG and DGDG and their respective lysolipid forms mono- (MGMG) and digalactosylmonoacylglycerols (DGMG)] and phospholipids [*i.e.* PC, PE, NAPE, phosphatidylinositol (PI), phosphatidylserine (PS), and their respective lysolipid forms LPC, LPE, NALPE, lyso-PI (LPI), and lyso-PS (LPS)] were carried out with a hybrid triple quadrupole/linear ion trap mass spectrometer (4000 QTRAP system, AB SCIEX, Framingham, MA, USA) equipped with a

Table 1
Amounts (μg), limits of detection (pmol), and limits of quantification (pmol/μl assay) of free (FL) and bound lipid (BL) extracts analyzed with electrospray ionization (ESI) tandem mass spectrometry (MS/MS) for a given lipid class.

Lipid class	Amount of lipid extract analyzed (μg)		Limit of detection (pmol)	Limit of quantification (pmol/μl assay)
	Free	Bound	Free/Bound	Free/bound
Glycolipids	2.70	2.70	5.00	0.125
LPC	26.70	0.35	0.50	0.007
PC	26.70	17.70	0.50	0.007
LPE	26.70	17.70	0.50	0.017
PE	26.70	17.70	0.50	0.017
LPI	80.00	53.30	0.50	0.017
PI	80.00	53.30	0.50	0.017
LPS	80.00	53.30	0.50	0.017
PS	80.00	53.30	0.50	0.017
NALPE	32.00	8.00	1.00	0.025
NAPE	32.00	8.00	1.00	0.025

Abbreviations: LPC, lysophosphatidylcholine; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; LPI, lysophosphatidylinositol; PI, phosphatidylinositol; LPS, lysophosphatidylserine; PS, phosphatidylserine; NALPE, N-acyl lysophosphatidylethanolamine; NAPE, N-acyl phosphatidylethanolamine.

TriVersa NanoMate system (Advion Biosciences, Ithaca, NY, USA) for chip-based infusion, which eliminated sample-to-sample carry-over effects. Prior to MS analyses, lipid extracts (see Section 2.2.3.1) were diluted to a concentration of 20.0 mg lipid extract/ml with appropriate running solutions [running solution A, *i.e.* chloroform/methanol/143.0 mM ammonium acetate, 300/665/35 (*v*/v/v), for the analysis of glycolipids, NAPE, and NALPE or running solution B, *i.e.* chloroform/methanol/ammonium hydroxide (28% NH_3 in water) 10/90/1.25 (v/v/v), for analyzing PC, LPC, PE, LPE, PI, LPI, PS, and LPS]. Table 1 lists the amounts (μg), limits of detection (pmol), and limits of quantification (pmol/μl assay) of FL and BL extracts analyzed with ESI-MS/MS for a given lipid class. These amounts were selected based on the linear dynamic range of the assays (data not shown) and equal suppression of the respective internal lipid standard(s) signals due to matrix effects (see below). Samples were infused in the ESI source with a spray voltage of 1.5 kV and a nitrogen gas pressure of 0.25 psi.

2.2.4.2. Lipid detection. Identification of MGDG/MGMG $[M + NH_4]^+$ and DGDG/DGMG $[M + NH_4]^+$ was based on the neutral loss of masses 179 and 341, respectively. Applied collision energies/CXP values were 21 eV/9 eV and 24 eV/12 eV for monogalactosyl and digalactosyl species, respectively (Xiao et al., 2010). Identification of phospholipids was based on the generation of *m/z* 184 for PC/LPC $[M + H]^+$, on the neutral loss of mass 141 for PE/LPE $[M + H]^+$, on the neutral loss of mass 87 for PS/LPS $[M-H]^-$, and on the generation of m/z 241 for PI/LPI $[M-H]^-$ (Marien et al., 2015). Applied collision energies/CXP values were 50 eV, 35 eV, −35 eV, and − 60 eV, respectively. Identification of NAPE and NALPE species was based on the neutral loss of the following ammoniated *N*-fatty amide head group fragments $[M + NH_4]^+$: 396.3 (N-16:0), 394.3 (N-16:1), 392.3 (N-16:2), 390.3 (N-16:3), 424.3 (N-18:0), 422.3 (N-18:1), 420.3 (N-18:2), 418.3 (N-18:3), 416.3 (N-18:4), 452.3 (N-20:0), 450.3 (N-20:1), 448.3 (N-20:2), 446.3 (N-20:3), and 444.3 (N-20:4) (Kilaru et al., 2012). Glycolipid internal standards were MGDG 36:0 (0.61 nmol) and DGDG 36:0 (0.61 nmol). Phospholipid internal standards were PC 25:0 (0.062 nmol), PC 43:6 (0.047 nmol), PE 25:0 (0.068 nmol), PE 43:6 (0.048 nmol), PI 25:0 (0.043 nmol), PI 43:6 (0.032 nmol), PS 25:0 (0.048 nmol), PS 31:1 (0.041 nmol), PS 37:4 (0.036 nmol), PS 43:6 (0.034 nmol), LPC 13:0 (0.043 nmol), LPC 17:1 (0.039 nmol), LPE 13:0 (0.049 nmol), LPI 13:0 (0.027 nmol), LPI 17:1 (0.025 nmol), LPS 13:0 (0.031 nmol), LPS 17:1 (0.028 nmol), and NAPE 19:0 [36:2] (0.08 nmol). Part of the standards and lipidomics consumables were kindly provided by prof. J. Swinnen (KU Leuven).

2.2.4.3. Data processing. Prior to (semi)quantification of specific lipid species, raw data were subjected to background subtraction, isotope correction and FA chain length correction (for phospholipids). As background, we considered the intensities of species detected in "internal standards only" spectra after being divided by the ion suppression factor of each sample. The ion suppression factor was calculated for each sample separately by dividing the intensity of the standards in the "internal standards only" spectrum by the intensity of the standards in the sample spectrum.

Background corrected intensities were subjected to MRM isotope correction, which takes into account the isotopic contribution of the (di)acylglycerol portion of glycolipids and phospholipids. For NAPE and NALPE, an additional correction for isotopic overlap between the N-acyl head groups was executed as described in Kilaru et al. (2012). FA chain length correction was only executed for phospholipids (not for their lysoforms) because it requires at least two internal standards per phospholipid class. (see above). Chain length correction was executed because the intensity of signals in MS/MS scans is the sum of variation in ionization and fragmentation efficiency (Koivusalo, Haimi, Heikinheimo, Kostiainen, & Somerharju, 2001). (Semi)quantification was executed by ratio comparison of (background, isotope, and FA chain length corrected) intensities of the species to the average of the intensities of the corresponding internal standards. For MGMG, DGMG and NALPE quantification, the MGDG, DGDG and NAPE standards were used, respectively, as no standards of the former were commercially available. Finally, data were corrected for the absolute amount of lipid extract analyzed (see above) and expressed as nmol/g of free, bound, or total polar lipids. Polar lipid levels within FL, BL, or TL fractions (expressed as mg/g) were calculated as the sum of the absolute amounts of all different lipid species within a given fraction, as determined with ESI-MS/MS. ESI-MS/MS analyses were single measurements on each of two lipid extracts obtained as in section 2.2.3.1.

2.2.5. Lipid profiling with high-performance liquid-chromatography

HPLC-ELSD analyses were as in Gerits et al. (2013, 2014). To each of the flour lipid extracts, fresh and fermented dough, bread crumb, and yeast, 1.0 ml isooctane was added. Injection volumes were 2.0 and 5.0 μl for the elution and detection of FLs and BLs, respectively. For gelatinized starch samples, the injection volume was 15 μl for the elution and detection of BLs. Lipid levels were expressed as the peak area relative to that of the internal standard cholesterol [in arbitrary units (AU)]. It is of note here that absolute lipid quantification was not possible because of variation in detector response between different lipid classes [see also Gerits et al., 2013]. Thus, one can only compare lipid extracts of different samples for a given lipid class. HPLC-ELSD analyses were single measurements on each of three lipid extracts obtained as in section 2.2.3.1.

2.2.6. Statistical analyses

Data were analyzed with statistical software JMP pro 12 (SAS Institute, Cary, NC, USA). Significant differences were identified using one-way analysis of variance combined with a post-hoc test such as the Tukey's honest significant difference (HSD) test. Tukey's HSD test assumes that the data are (i) normally distributed and (ii) homoscedastic (*i.e.* that there is homogeneity of variance). The former assumption was tested using the Shapiro-Wilk's W test ($\alpha = 0.05$) and was met for the distributions of TAG and FFA in the FL fractions and for TAG in the BL ones. *P*-values were 0.1647, 0.0643, and 0.1077, respectively. The assumption of homoscedasticity was tested using the Bartlett's Test ($\alpha = 0.05$) and was met for each of these distributions with respective P-values of 0.8505, 0.2019, and 0.2585. Thus, Tukey's HSD test was a valid post-hoc analysis for detecting significant differences ($\alpha = 0.05$) in the distributions of free TAG, free FFA, and bound TAG. The

Table 2
Free lipids (FLs), bound lipids (BL), and total lipids (TLs, sum of FLs and BLs), expressed in mg/g dry matter (dm) of flour, freshly mixed dough, fermentation dough, and bread crumb.

	Flour	Freshly mixed dough	Fermented dough	Bread crumb
FLs (mg/g)[1]	5.8 (100)	3.9 (100)	4.6 (100)	2.5 (100)
Polar (mg/g FL)[2]	1.26 (22)	0.58 (15)	0.06 (1)	0.07 (3)
Glycolipids (mg/g polar FL)	1.17 (93)	0.57 (98)	0.04 (73)	0.02 (29)
Phospholipids (mg/g polar FL)	0.09 (7)	0.01 (2)	0.02 (27)	0.05 (71)
Nonpolar (mg/g FL)[3]	4.54 (78)	3.32 (85)	4.54 (99)	2.43 (97)
BLs (mg/g)[1]	6.5 (100)	9.6 (100)	9.7 (100)	8.5 (100)
Polar (mg/g BL)[2]	6.81 (105)	7.33 (76)	6.21 (64)	6.52 (77)
Glycolipids (mg/g polar BL)	6.48 (95)	6.79 (93)	5.41 (87)	5.83 (89)
Phospholipids (mg/g polar BL)	0.33 (5)	0.54 (7)	0.80 (13)	0.69 (11)
`Nonpolar (mg/g BL)[3]	0.00[A] (0)	2.27 (24)	3.49 (36)	1.98 (23)
TLs (mg/g)[1]	12.3 (100)	13.5 (100)	14.3 (100)	11.0 (100)
Polar (mg/g TL)[2]	8.07 (66)	7.91 (59)	6.27 (44)	6.59 (60)
Glycolipids (mg/g polar TL)	7.66 (95)	7.36 (93)	5.45 (87)	5.85 (89)
Phospholipids (mg/g polar TL)	0.42 (5)	0.55 (7)	0.81 (13)	0.74 (11)
Nonpolar (mg/g TL)[3]	4.23 (34)	5.59 (41)	8.03 (56)	4.41 (40)

Values between parentheses are the percentages they represent within a given fraction.
[1] Determined gravimetrically (see section 2.2.3.1).
[2] Calculated based on electrospray ionization (ESI) tandem mass spectrometry (MS/MS) data (see section 2.2.4).
[3] Calculated by subtracting the polar lipid content from the TL content.
[A] The calculated value was negative (−0.33), which is probably due to slight experimental error in the gravimetric measurements. Since lipid levels cannot be negative, it was changed to 0.00.

distribution of FFA in the BL fractions did not meet the assumption of normality. Here, one-way analysis of variance was combined with the non-parametric Wilcoxon signed-rank test as post-hoc analysis for detecting significant differences ($\alpha = 0.05$) in the distribution of bound FFA.

3. Results & discussion

Table 2 lists gravimetrically determined (see section 2.2.3.1) FL, BL, and TL levels of flour, freshly mixed dough, fermented dough, and bread crumb. Polar lipid levels within FL, BL, or TL fractions were calculated by summing up the amounts (expressed in mg/g) of all different lipid species within a given fraction such as determined with ESI-MS/MS. As it was not possible to ionize nonpolar lipids with the ESI-MS/MS set-up used here, their levels were obtained by subtracting the polar lipid level from the TL level within a given fraction. Table 3 presents the levels (expressed as nmol/g) of the different polar lipid classes in FL, BL, and TL extracts of the different samples, quantified with ESI-MS/MS and Table 4 lists an overview of the most abundant acyl groups (for lysolipids) or acyl group combinations of the different polar lipids in these extracts. Finally, Fig. 2 shows nonpolar lipid levels (expressed in AU), calculated as peak areas relative to that of the internal standard cholesterol, of FL and BL extracts of the different samples such as determined with HPLC-ELSD. In what follows, we evaluate and discuss the distribution of polar and nonpolar lipids throughout the process from flour to bread.

Table 3
Free lipids (FLs), bound lipids (BL), and total lipids (TLs, sum of FLs and BLs), expressed in nmol/g dry matter (dm) of flour, freshly mixed dough, fermented dough, and bread crumb.

Lipid class	Flour			Freshly mixed dough			Fermented dough			Bread crumb		
	Free	Bound	Total	Free	Bound	Total	Free	Bound	Total	Free	Bound	Total
MGMG	36	644	680	12	553	565	n.d.	342	342	n.d.	378	378
DGMG	108	961	1069	51	868	919	n.d.	482	482	n.d.	488	488
MGDG	231	3021	3251	108	2975	3083	n.d.	2518	2518	n.d.	2789	2789
DGDG	939	3159	4098	466	3699	4165	43	3033	3076	16	3221	3237
Σ glycolipids (nmol/g)	1314	7784	9098	637	8095	8732	43	6377	6420	16	6876	6892
LPC	13	434	446	n.d.	372	372	n.d.	443	443	n.d.	534	534
PC	13	62	75	n.d.	49	49	n.d.	135	135	n.d.	80	80
LPE	2	61	63	n.d.	59	59	n.d.	100	100	n.d.	106	106
PE	1	4	5	n.d.	5	5	n.d.	33	33	n.d.	17	17
LPI	2	3	5	2	3	5	2	53	55	2	39	42
PI	4	9	13	4	7	10	5	125	130	4	90	94
LPS	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	12	12	n.d.	6	6
PS	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	13	13	n.d.	5	5
NALPE	10	8	18	4	61	65	6	52	58	3	35	38
NAPE	56	30	86	1	231	232	4	208	212	1	178	179
Σ phospholipids (nmol/g)	101	611	712	11	787	798	17	1174	1191	10	1090	1100
Σ total polar lipids (nmol/g)	1414	8396	9810	647	8882	9529	59	7551	7610	27	7966	7993

Values represent the sum of all species detected with electrospray ionization (ESI) tandem mass spectrometry (MS/MS) for a given lipid class. n.d. indicates that the signal of the lipid class was below the detection limit of the MS device. Abbreviations: MGMG: monogalactosylmonoacylglycerol; DGMG: digalactosylmonoacylglycerol; MGDG: monogalactosyldiacylglycerol; DGDG: digalactosyldiacylglycerol; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; LPI, lysophosphatidylinositol; PI, phosphatidylinositol; LPS, lysophosphatidylserine; PS, phosphatidylserine; NALPE, N-acyl lysophosphatidylethanolamine; NAPE, N-acyl phosphatidylethanolamine.

Table 4
Most prevalent acyl groups or acyl group combinations of free lipid (FL) and bound lipid (BL) extracts of flour, freshly mixed dough, fermented dough, and bread crumb.

Lipid class	Flour				Freshly mixed dough				Fermented dough				Bread crumb			
	FL		BL		FL		BL		FL		BL		FL		BL	
	FA	%	FA	%	FA	%	FA	%	FA	%	FA	%	FA	%	FA	%
MGMG	18:2	100	18:2	70	18:2	100	18:2	70	n.d.		18:2	73	n.d.		18:2	73
			16:0	11			16:0	11			18:3	13			18:3	13
			18:3	10			18:3	10			16:0	8			16:0	8
			18:1	9			18:1	9			18:1	6			18:1	6
DGMG	18:2	84	18:2	69	18:2	84	18:2	71	n.d.		18:2	72	n.d.		18:2	73
	16:0	16	16:0	18	16:0	16	16:0	16			16:0	14			16:0	13
			18:3	7			18:3	7			18:3	9			18:3	9
			18:1	7			18:1	6			18:1	5			18:1	4
MGDG	36:4	80	36:4	77	36:4	80	36:4	77	n.d.		36:4	77	n.d.		36:4	77
	36:5	12	36:5	11	36:5	12	36:5	11			36:5	11			36:5	11
	36:3	8	36:3	7	36:3	8	36:3	7			36:3	7			36:3	7
			34:2	3			34:2	3			34:2	3			34:2	3
DGDG	36:4	66	36:4	64	36:4	66	36:4	64	36:4	78	36:4	64	36:4	71	36:4	64
	34:2	14	34:2	15	34:2	14	34:2	15	36:5	22	34:2	15	36:5	29	34:2	15
	36:5	12	36:5	11	36:5	12	36:5	11			36:5	11			36:5	11
	36:3	4	36:3	4	36:3	4	36:3	4			36:3	4			36:3	4
	34:3	3	34:3	4	34:3	3	34:3	3			34:3	3			34:3	3
LPC	18:2	81	18:2	48	n.d.		18:2	50	n.d.		18:2	43	n.d.		18:2	53
	18:1	14	16:0	46			16:0	43			16:0	32			16:0	29
	18:3	3	18:1	7			18:1	7			18:1	13			18:1	8
	18:0	2									16:1	10			18:3	5
											18:3	3			16:1	5
PC	34:2	44	34:2	33	38:5	38	34:2	40	32:0	52	34:2	36	38:5	62	34:2	35
	36:4	26	36:4	15	32:0	35	36:4	19	38:5	48	32:2	10	42:6	38	36:4	11
	36:3	8	34:1	7	44:5	27	36:3	7			36:4	10			32:2	8
	34:1	6	32:0	6			32:0	7			34:3	9			34:3	7
	32:0	5	36:3	6			34:1	7			34:1	7			34:1	7
	36:2	3	36:2	2			38:1	3			32:1	5			36:3	5
	36:5	2	36:5	2			36:2	3			36:3	5			32:1	4
	34:3	2	34:3	2			34:3	2			36:2	4			32:0	4
							36:5	2			32:0	3			36:2	4
															38:1	2
															36:5	2
LPE	18:2	67	18:2	47	17:1	100	18:2	51	17:1	100	18:2	34	n.d.		18:2	50
	16:0	14	16:0	23			16:0	22			16:1	20			16:0	16
	18:1	10	14:4	9			14:4	8			18:1	15			16:1	10
	20:0	8	20:0	5			20:0	4			16:0	14			18:1	8
			18:1	4			18:1	4			14:4	5			18:3	4
			18:3	3			18:3	3			20:0	3			14:5	4
			14:5	3			14:5	3			18:3	2			20:0	2
							20:5	2			14:5	2			14:5	2
											14:3	2				
PE	36:4	62	36:4	61	28:1	100	36:4	62	n.d.		32:2	35	n.d.		32:2	33
	28:1	14	36:3	15			36:3	14			34:3	22			34:3	20
	36:3	13	34:1	10			34:1	9			34:1	13			36:4	15
	40:2	11	36:5	7			36:5	8			36:4	11			34:1	13
			40:2	6			40:2	7			36:2	6			36:2	7
											36:3	4			36:3	5
											36:5	4			36:5	4
											34:4	4			34:4	3
LPI	18:2	50	18:2	27	18:2	55	18:2	28	18:2	49	16:0	29	18:2	52	16:0	29
	18:1	13	16:0	24	22:1	13	16:0	22	18:1	14	18:1	23	18:1	16	18:1	22
	16:0	11	18:5	18	18:1	13	18:5	21	16:0	10	16:1	18	16:0	10	16:1	18
	22:1	10	16:2	8	16:0	7	16:1	9	22:1	9	18:2	16	22:1	10	18:2	16
	14:0	7	18:1	7	22:2	4	18:1	6	18:3	5	18:0	8	22:2	6	18:0	7
	22:2	6	16:1	5	14:0	3	16:2	5	14:0	5			18:3	4	18:3	2
	22:0	2	14:1	3	18:3	3	16:3	5	22:2	4			22:0	2	18:5	2
			18:3	3	22:0	2	18:3	3	22:0	3						
			18:0	3												
			16:3	2												

(*continued on next page*)

Table 4 (*continued*)

Lipid class	Flour				Freshly mixed dough				Fermented dough				Bread crumb			
	FL		BL		FL		BL		FL		BL		FL		BL	
	FA	%	FA	%	FA	%	FA	%	FA	%	FA	%	FA	%	FA	%
PI	36:4	26	34:2	28	36:4	38	34:2	26	36:4	32	34:1	27	36:4	39	34:2	26
	34:2	11	36:4	5	34:2	16	36:3	6	34:2	13	34:2	22	34:2	18	34:1	25
	44:7	8	34:3	4	36:3	7	36:4	5	36:3	7	36:1	14	36:3	7	36:1	13
	38:5	6	34:1	3	36:5	6	34:3	5	34:3	6	32:1	11	36:5	5	32:1	11
	40:1	6	34:0	3	34:3	4	44:7	5	36:5	5	36:2	6	32:0	4	36:2	6
	38:4	5	40:3	3	36:2	3	31:1	5	38:5	3	34:3	2	36:2	4	34:3	3
	36:3	4	36:3	3	38:5	3	40:3	4	34:1	3	32:2	2	34:3	2	32:2	2
	42:8	3	38:5	3	32:0	3	44:4	4	32:2	3			34:1	2		
	42:5	3	36:0	2	32:1	2	32:3	3	36:0	3			32:2	2		
	34:0	3	44:7	2	44:6	2	38:5	3	36:2	3			38:5	2		
	38:2	3	38:4	2	38:3	2	44:1	3	36:1	3						
	44:9	3	44:4	2	40:7	2	36:1	3	38:1	2						
	36:2	3	31:1	2	42:5	2	34:0	3	42:5	2						
	36:5	2	36:5	2			32:1	3	32:0	2						
	32:3	2	28:0	2			34:1	3	38:2	2						
	42:2	2	38:0	2			42:2	3	32:1	2						
	38:1	2	38:2	2			44:6	3								
			42:2	2			38:6	3								
			36:6	2			36:5	2								
							32:0	2								
							42:1	2								
							42:4	2								
LPS	n.d.		18:2	81	n.d.		18:2	100	n.d.		16:1	39	n.d.		16:1	36
PS			16:0	19							18:1	35			18:1	34
											18:2	16			18:2	17
											16:0	10			16:0	10
											18:3	1			18:3	2
	n.d.		44:12	100	44:12	100	44:12	65	44:12	100	34:2	61	n.d.		34:2	62
							40:2	35			34:3	10			34:3	11
											34:1	10			32:2	9
											32:2	8			44:12	7
											32:1	6			34:1	6
											44:12	2			32:1	5
NALPE	18:2 [18:2]	31	18:2 [18:2]	42	16:3 [18:1]	49	18:2 [18:2]	41	16:3 [18:1]	67	18:2 [18:2]	45	16:3 [18:1]	59	18:2 [18:2]	46
	16:3 [18:1]	17	18:2 [16:0]	20	18:3 [18:1]	47	18:2 [16:0]	16	18:3 [18:1]	26	18:2 [16:0]	18	18:3 [18:1]	32	18:2 [16:0]	16
	18:3 [18:1]	11	20:0 [18:2]	8	16:3 [18:2]	4	20:0 [18:2]	7	16:3 [18:2]	7	20:0 [18:2]	7	16:3 [18:2]	6	20:0 [18:2]	8
	18:2 [16:0]	10	20:0 [16:0]	8			20:0 [16:0]	6			20:0 [16:0]	6	16:2 [18:1]	3	20:0 [16:0]	7
	20:0 [18:2]	6	18:1 [18:2]	6			18:1 [18:2]	5			18:1 [18:2]	5			18:1 [18:2]	6
	20:0 [16:0]	5	20:1 [18:2]	6			20:1 [18:2]	5			20:1 [18:2]	5			20:1 [18:2]	6
	16:3 [18:2]	4	18:2 [18:1]	4			18:3 [18:2]	4			18:3 [18:2]	4			18:3 [18:2]	4
	18:1 [18:2]	3	18:3 [18:2]	4			18:2 [18:1]	4			18:2 [18:1]	4			18:2 [18:1]	4
	16:0 [18:2]	4	18:1 [16:0]	3			16:3 [18:1]	3			16:3 [18:1]	3			18:1 [16:0]	3
	18:3 [18:2]	3					16:0 [18:2]	3			18:1 [16:0]	2				
	18:2 [18:1]	3					18:1 [16:0]	2								
	20:1 [18:2]	3					16:3 [18:3]	2								
							16:3 [18:2]	2								
NAPE	18:2 [36:4]	33	18:2 [36:4]	33	16:0 [36:4]	38	18:2 [36:4]	36	16:0 [36:4]	20	18:2 [36:4]	36	16:0 [36:4]	44	18:2 [36:4]	36
	18:2 [34:2]	26	18:2 [34:2]	30	16:0 [34:2]	29	18:2 [34:2]	29	16:0 [34:2]	17	18:2 [34:2]	30	16:0 [34:2]	36	18:2 [34:2]	30
	18:2 [36:3]	7	18:2 [36:3]	7	16:0 [36:3]	17	18:2 [36:3]	7	16:3 [34:2]	16	18:2 [36:3]	8	16:0 [36:3]	20	18:2 [36:3]	7
	18:1 [36:4]	4	18:1 [36:4]	5	16:3 [34:2]	15	18:1 [36:4]	5	16:3 [36:4]	14	18:1 [36:4]	5			18:1 [36:4]	5
	18:1 [34:2]	3	18:1 [34:2]	5			18:1 [34:2]	4	16:0 [36:3]	9	18:1 [34:2]	4			18:1 [34:2]	4
	20:0 [34:2]	3	20:0 [34:2]	4			18:2 [36:5]	3	16:2 [34:2]	9	18:2 [36:5]	3			18:2 [36:5]	3
	18:2 [36:5]	3	16:0 [36:4]	3			18:3 [36:4]	3	16:3 [36:3]	9	18:3 [36:4]	3			18:3 [36:4]	3
	16:3 [34:2]	3	18:2 [36:5]	3			18:2 [34:1]	2	18:2 [34:2]	7	20:0 [34:2]	3			20:0 [34:2]	3
	18:3 [36:4]	2	18:2 [34:1]	3			18:3 [34:2]	2			18:2 [34:1]	2			18:2 [34:1]	2
	16:3 [36:4]	2	18:3 [36:4]	2			18:2 [34:3]	2			18:3 [34:2]	2			18:3 [34:2]	2
	18:2 [34:1]	2	18:3 [34:2]	2							18:2 [34:3]	2			18:2 [34:3]	2
	18:3 [34:2]	2	18:2 [34:3]	2												
	18:2 [34:3]	2														

Values between parentheses represent percentages in which lipid species occur within a given lipid fraction. In case of N-acylphosphatidylethanolamine (NAPE) and its lysoform N- acyl lysophosphatidylethanolamine (NALPE), the first acyl group corresponds to the fatty acid (FA) attached to the *sn*-3 position, whereas the acyl groups or acyl group combinations between square brackets represent the FAs attached to the *sn-1* and/or *sn*-2 position(s). For a given lipid class, only acyl groups or acyl group combinations that were present in (nmol) levels above 1% are reported. n.d. indicates that the signal of the lipid class was below the detection limit of the MS device. Abbreviations: MGMG: monogalactosylmonoacylglycerol; DGMG: digalactosylmonoacylglycerol; MGDG: monogalactosyldiacylglycerol; DGDG: digalactosyldiacylglycerol; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; LPI, lysophosphatidylinositol; PI, phosphatidylinositol; LPS, lysophosphatidylserine; PS, phosphatidylserine; NALPE, N-acyl lysophosphatidylethanolamine; NAPE, N-acyl phosphatidylethanolamine.

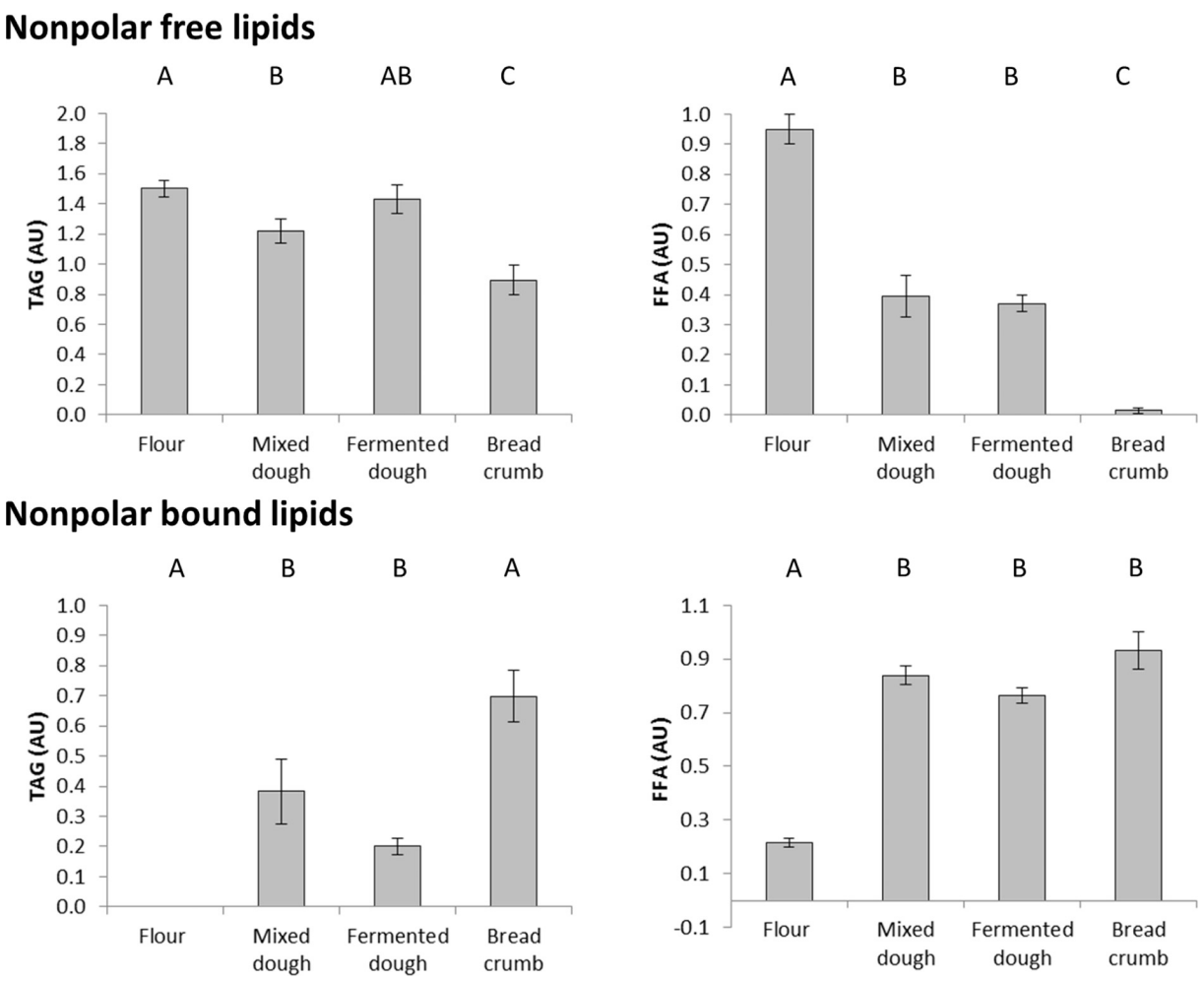

Fig. 2. Free lipid (FL) and bound lipid (BL) levels of extracts from flour, freshly mixed dough, fermented dough, and bread crumb, expressed as peak areas relative to that of the internal standard cholesterol [expressed as arbitrary units (AU)], determined with HPLC-ELSD. injection volumes were 2.0 µl and 5.0 µl for the elution and detection of FLs and BLs, respectively. Bars with the same capital letter within a given lipid class are not significantly different ($\alpha = 0.05$). Error bars represent the deviation from the mean for single measurements on three separate lipid extracts. Abbreviations: TAG, triacylglycerols; FFA, free fatty acids.

3.1. Flour

The lipid levels in FL and BL extracts of wheat flour were 5.8 and 6.5 mg/g, respectively (Table 2). In the FL extract, 78% of these lipids were nonpolar (FFA and TAG, see Fig. 2), while the BL extract contained no nonpolar lipids (Table 2). Although FFA are slightly more polar than TAG (Pareyt et al., 2011), we classified them as nonpolar species. In FL and BL extracts, glycolipids accounted for 93 and 95% of the polar lipids, respectively (Table 2). Finnie et al. (2009) and Finnie, Jeannotte, Morris, and Faubion (2010) reported that the glycolipid-to-phospholipid ratios in (i) FL and BL extracts of soft wheat flour cultivar Alpowa and hard wheat flour cultivar Overley and in (ii) BL extracts of soft wheat flour from six near-isogenic wheat lines were about 80-to-20. However, these researchers included neither NAPE nor NALPE in their analyses, which would of course have lowered that ratio somewhat. In contrast to our results and those of Finnie et al. (2009) and Finnie, Jeannotte, Morris, and Faubion (2010), Salt et al. (2018) in a recent paper reported a glycolipid-to-phospholipid ratio of about 30-to-70 for a TL extract of hard wheat flour cultivar Hereward. The high abundance of phospholipids in their study was mainly due to the presence of LPC, which made up 40 to 65% (or 1145 to 1612 nmol/g dm flour) of the polar lipids, depending on the year of harvest. In contrast, Finnie et al. (2009) and Finnie, Jeannotte, Morris, and Faubion (2010) reported extractable LPC levels of 1.96 to 3.32% (or 112 to 158 nmol/g dm flour) and 2.42 to 2.74% (or 78 to 90 nmol/g dm flour), respectively, of the polar lipids in the extracts mentioned above. In addition, extractable LPC made up only 4.60% (or 446 nmol/g dm flour) of the polar lipids in our study (Table 3). It is of note that Salt et al. (2018) subjected flour samples to a (probably dry) heat treatment (100 °C, 12 min) prior to lipid extraction. Such treatment may have facilitated the extraction of starch internal lipids and, thus, of LPC, which makes up about 70% of the wheat starch internal lipids (Morrison, 1981). Thus, overall our measured absolute values of LPC in flour are in good agreement with those reported in literature. Table 3 shows that the glycolipids in the flour FL extract mostly consisted of DGDG (71% of the free glycolipids), whereas the glycolipids in the BL extract of flour were dominated by both MGDG and DGDG (39 and 41% of the bound glycolipids, respectively). In the FL fraction, 36:4 made up 66% of the acyl group combinations of DGDG (Table 4). The latter acyl group combination was also the most common in DGDG (64%) and MGDG (77%) in the BL fraction (Table 4). Extractable phospholipid levels in flour were very low. NAPE was the most abundant (56%) phospholipid in the FL fraction, followed by PC (12%), LPC (12%), and NALPE (10%) (Table 3). While 18:2 was the most prevalent FA at the *sn*-3 position of free NAPE, 36:4 (33%) and 34:2 (26%) were the most abundant acyl group combinations at the *sn*-1 and *sn*-2 positions (Table 4). As palmitic acid (16:0) and linoleic acid (18:2) make up a respective 21 and 58% of the wheat FA population (Delcour & Hoseney, 2010), 34:2 and 36:4 most likely represent the acyl group combinations 16:0/18:2 and 18:2/18:2, respectively. Bound phospholipids were predominantly LPC (71%), PC

(10%), LPE (10%), and NAPE (5%) (Table 3). The predominant FAs of bound LPC were 18:2 (48%) and 16:0 (46%).

In what follows, we explore how dough development affects the flour lipid population.

3.2. Freshly mixed dough

A first remarkable observation was that the extractable TL content of freshly mixed dough was higher than that of flour. This was ascribed to the increased extractability of nonpolar lipids as a result of the mixing action. Indeed, the level of free nonpolar lipids (FFA and TAG) decreased from 4.54 mg/g in flour to 3.32 mg/g in dough, but the level of bound nonpolar lipids (FFA and TAG) increased from 0.00 mg/g in flour to 2.27 mg/g in dough (Table 2 and Fig. 2). This additional release of previously non-extractable nonpolar lipids most likely results from disintegration of flour particles during flour hydration and dough kneading.

In addition, extractable FL and BL levels in freshly mixed dough were significantly ($P < .05$) lower and higher than in flour, respectively (Table 2). These observations are in line with those of an earlier report by our group which was based on HPLC-ELSD analyses of flour and dough lipids (Gerits et al., 2013). Dough development thus led to increased BL levels (9.6 mg/g) at the expense of FL levels (3.9 mg/g) (Table 2). Indeed, both the levels of polar and nonpolar lipids in the free fraction were lower while those in the BL extract were higher in dough than in flour (Table 2 and Fig. 2). Such redistribution of lipids from FL to BL extracts during dough development is known as lipid-binding (Carr et al., 1992; Gerits et al., 2013; Olcott & Mecham, 1947; Ponte et al., 1964) (see section 1). It results from transfer of lipids from the surface of starch granules to gluten (Chung & Tsen, 1975; Gerits et al., 2013; Olcott & Mecham, 1947). The interactions between lipids and gluten proteins likely depend on the type of gluten protein (gliadins *versus* glutenins) and the lipid structure (Fig. 1). Gliadins have an isoelectric point of 7.8, whereas that of glutenins is much lower (5.8) (Lambrecht, Rombouts, Nivelle, & Delcour, 2017). Thus, at the pH of freshly mixed (5.8) and fermented (4.8) doughs (Jayaram et al., 2013), gliadins are positively charged, while glutenins carry little if any charge. Hence, phospholipids, which are either zwitterionic (*i.e.* PC, LPC, PS, and LPS) or negatively charged (*i.e.* NAPE, NALPE, PI, LPI, PE, and LPE) (Fig. 1) preferentially interact with gliadins through electrostatic interactions, whereas glycolipids, which are neutral (Fig. 1), preferably associate with glutenins *via* hydrogen bonds and hydrophobic interactions. A similar observation was made by McCann, Small, Batey, Wrigley, and Day (2009). Furthermore, nonpolar lipids may interact with glutenin proteins *via* hydrophobic interactions (McCann et al., 2009). Fig. 3 shows a schematic representation of the gliadin-lipid and glutenin-lipid interactions in freshly mixed dough. To what extent such interactions strengthen the gluten network is still under debate (Gerits et al., 2015; Sroan & MacRitchie, 2009), but that they have some impact on dough properties is beyond doubt.

It is of further note that the total level of extractable polar lipids in freshly mixed dough (7.91 mg/g polar TL) was slightly lower than that in flour (8.07 mg/g polar TL) (Table 2). More specifically, dough mixing resulted in a decrease in the level of extractable polar lipids in the FL fraction (from 1.26 mg/g FL in flour to 0.58 mg/g FL in freshly mixed dough) and an increase in that of the BL fraction (from 6.81 mg/g BL in flour to 7.33 mg/g BL in freshly mixed dough). Indeed, the total levels of extractable glycolipids and phospholipids in freshly mixed dough (7.36 and 0.55 mg/g polar TL, respectively) were lower and higher, respectively, than in flour (7.66 and 0.42 mg/g polar TL, respectively). Overall, this resulted in a slightly lower total polar lipid content in freshly mixed dough (7.91 mg/g TL) than in flour (8.07 mg/g TL) (Table 2). These observations could be ascribed to elevated DGDG, NAPE, and NALPE levels (Table 3). While the decrease in free extractable DGDG as a result of dough mixing (from 940 nmol/g dm in flour to 470 nmol/g dm in freshly mixed dough) was equal to the corresponding increase in bound DGDG (from 3160 nmol/g dm in flour to 3700 nmol/g dm in freshly mixed dough), the higher level of bound NALPE and NAPE levels in dough than in flour could not entirely be explained by their lower level in the FL extract (Table 3). As mentioned above, and similar to what was the case for FFA and TAG, NAPE, and NALPE were probably physically entrapped within flour particles, which prevented their extraction from flour. The mere breaking up of flour particles by mixing might have resulted in extractability of some lipids. Development of dough was also associated with a strong decrease of the extractability of MGMG, DGMG, MGDG, LPC, PC, LPE, and PE as FLs. In contrast to what was observed for DGDG, NALPE, and NAPE, their bound levels in most cases were lower in freshly mixed dough than in flour (Table 3), which may be attributed to the physical entrapment or interaction with other dough constituents of certain lipids in the dough matrix.

As was the case for flour, the polar lipid fraction mainly contained glycolipids. Indeed, they accounted for 98 and 93% of FL and BLs, respectively. That DGDG (73%) was the most prevalent free glycolipid, while MGDG (37%) and DGDG (46%) dominated the bound glycolipids, was also the case for flour (see section 3.1) (Table 3). The most abundant acyl group combination of free DGDG (89%), bound DGDG (66%), and bound MGDG (77%) was 36:4 (Table 4). As mentioned earlier, 36:4 probably represents a 18:2/18:2 acyl group combination. FL extracts contained only very low phospholipid levels (Table 3). Bound phospholipids were mainly LPC (47%) and NAPE (29%). The most common FAs of bound LPC were 18:2 (50%) and 16:0 (43%). The most prevalent acyl group combination at the *sn*-1 and *sn*-2 positions of bound NAPE was 36:4, while 18:2 represented the most occurring FA at the *sn*-3-position (Table 4).

The following paragraph deals with the effect of fermentation and, hence, gas cell expansion, on the FL and BL populations of dough.

3.3. Fermented dough

FL levels were significantly ($P < .05$) higher in fermented than in freshly mixed dough (4.6 *vs.* 3.9 mg/g flour, respectively). In contrast, fermentation did not impact dough BL levels (Table 2). As in flour and freshly mixed dough samples, the FL fraction of fermented dough samples mostly consisted of nonpolar lipids (99%). In contrast, the BL fraction of fermented dough contained more polar (64%) than nonpolar (36%) lipids (Table 2). Interestingly, in both the FL and BL fractions, the level of extractable nonpolar lipids was higher in fermented than in freshly mixed doughs (Table 2). However, our HPLC-ELSD data did not support these observations. Indeed, there were no significant ($P > .05$) differences in the levels of free and bound TAG (or free and bound FFA) between freshly mixed and fermented doughs (Fig. 2). Nonetheless, for both the FL and BL fractions, we put forward the hypothesis that the increased extractability of nonpolar lipids from fermented dough results from stretching of the gluten network due to gas cell expansion. Such stretching may weaken the hydrophobic interactions between nonpolar lipids and glutenin proteins, thereby exposing some hydrophobic sites of the latter (Fig. 3). That nonpolar lipids can interact with glutenins *via* hydrophobic interactions has already been established by McCann et al. (2009).

While the analyzed levels of nonpolar lipids of fermented dough were higher than those from freshly mixed dough, polar lipid levels were lower in the former than in the latter (Table 2). This decrease could be attributed to a decrease in the extractability of glycolipids for both the FL and BL fractions of fermented dough. This phenomenon may be related to the above mentioned stretching of the gluten network. While it rendered some nonpolar lipids more extractable, the now available hydrophobic binding sites of the glutenin proteins may have been occupied by glycolipids. That glycolipids rather than nonpolar lipids would occupy these sites is probably related to the higher strength of glycolipid-glutenin interactions. Indeed, while nonpolar lipids interact with glutenins only *via* hydrophobic interactions,

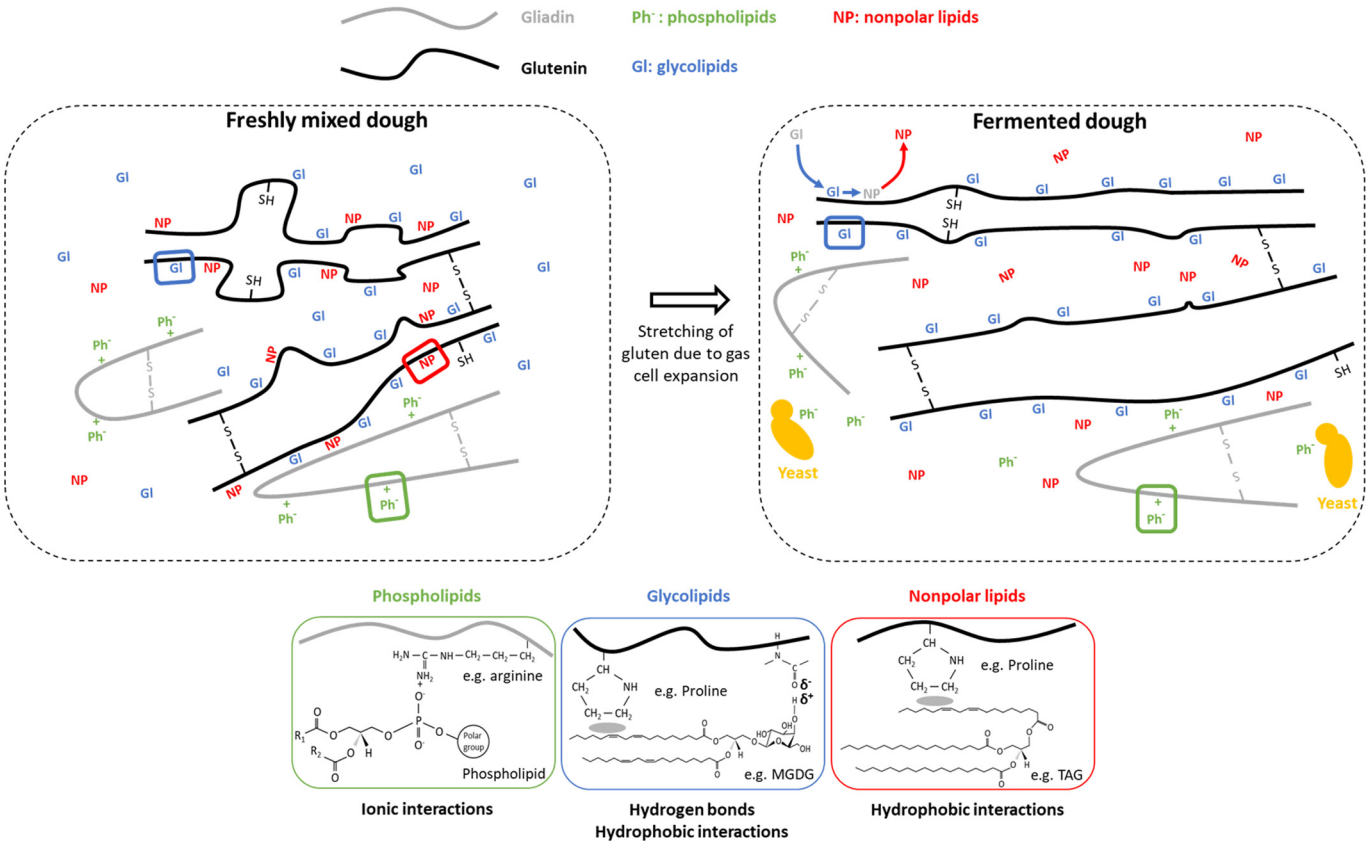

Fig. 3. Schematic representation of likely gliadin-lipid and glutenin-lipid interactions in freshly mixed and fermented doughs. Gliadins and glutenins have isoelectric points of 7.8 and 5.8, respectively (Lambrecht et al., 2017). Thus, at the pH of freshly mixed (5.8) and fermented (4.8) doughs (Jayaram et al., 2013), gliadins are positively charged, while glutenins carry little if any charge. Hence, phospholipids, which are either zwitterionic or negatively charged, preferentially associate with gliadins through ionic interactions, whereas glycolipids, which are neutral, preferably interact with glutenins *via* hydrogen bonds and hydrophobic interactions. Furthermore, nonpolar lipids probably associate with glutenins through hydrophobic interactions (McCann et al., 2009). During fermentation, gas cells expand and the gluten network is stretched. According to the 'train and loop' model of Peter Belton (1999), this results in flattening of the 'loops'. Also during fermentation, the total level of extractable nonpolar lipids [mostly triacylglycerols (TAG) and free fatty acids (FFA)] increased, while the total level of extractable glycolipids decreased. We hypothesize that stretching of the gluten network weakens hydrophobic interactions between nonpolar lipids and glutenin proteins, which causes nonpolar lipids associated with glutenins to be replaced by glycolipids. That glycolipids rather than nonpolar lipids would occupy these sites can probably be explained by the higher strength of the glycolipid-glutenin interactions. The extractability of phospholipids slightly increased during fermentation. Part of these phospholipids probably originated from yeast. In addition, similar to what was the case for nonpolar lipids, we speculate that stretching of the gluten network may have released some phospholipids associated with gliadin proteins in freshly mixed dough.

glycolipids interact with glutenins also through hydrogen bonds (Fig. 3) (Hoseney, Finney, & Pomeranz, 1970; McCann et al., 2009). Nonetheless, the glycolipids were still the dominant polar lipids, with glycolipid-to-phospholipid ratios of 73-to-27 and 87-to-13 in FL and BL extracts, respectively (Table 2). Dough fermentation led to complete loss of extractability of MGMG, DGMG, and MGDG and a substantial decrease of DGDG extractability as FL (Table 3). A similar decrease as a result of fermentation for all glycolipids was noted for the BL fraction (Table 3). Thus, a substantial amount of glycolipids became non-extractable during fermentation. The most common FA of MGMG was 18:2 (73%). The acyl group combinations 36:4 and 36:5 were the most abundant ones in free DGDG molecules (78 and 22%, respectively) (Table 4). The acyl group combinations 36:4 and 36:5 very likely represent the FA combinations 18:2/18:2 and 18:2/18:3, respectively. In contrast to what was the case for glycolipids, the levels of bound phospholipids slightly increased as a result of fermentation. First, part of these phospholipids most likely originated from yeast (cfr. Infra). Second, similar to what was the case for FFA and TAG, this may be due to gluten stretching, although in a less pronounced way (Table 3). Indeed, stretching of the gluten network during fermentation may have released some phospholipids previously associated with gliadin proteins in freshly mixed dough (Fig. 3). Exceptions were NAPE and NALPE of which the levels were slightly lower as a result of fermentation (Table 3).

The population of bound phospholipids in fermented dough was more heterogeneous than that in freshly mixed dough. It mainly consisted of LPC (37%), NAPE (17%), PC (11%), PI (10%), and LPE (8%) (Table 3). The most prevalent FAs of LPC were 18:2 (43%) and 16:0 (32%). While 18:2 was the most prevalent FA at the *sn*-3 position of NAPE, 36:4 (36%) and 34:2 (30%) were the most abundant acyl group combinations at the *sn*-1 and *sn*-2 positions (Table 4). The most common acyl group combinations of PC were 34:2 (36%), 32:2 (10%), 36:4 (10%), and 34:3 (9%). The most occurring acyl group combinations of PI were 34:1 (27%), 34:2 (22%), 36:1 (14%), and 32:1 (11%). The acyl group combinations 32:1, 32:2, 34:1, 34:2, 34:3, 36:1, and 36:4 probably for the most part represent the FA combinations 16:0/16:1, 16:1/16:1, 16:0/18:1, 16:0/18:2, 16:0/18:3, 18:0/18:1, and 18:2/18:2, respectively. Finally, the most abundant FAs of LPE were 18:2 (34%), 16:1 (20%), 18:1 (15%), and 16:0 (14%) (Table 4). It has been reported before that wheat contains the rather unusual FA 16:1 (Finnie et al., 2009).

Of course, some of the lipids mentioned above probably originate from yeast. Indeed, the yeast used here (*i.e. Saccharomyces cerevisiae*) had a dm content of 32% and a lipid content of 1.0% dm. Thus, each dough contained about 1.66 mg lipids originating from yeast. Apart from high levels of ergosterols and sphingolipids and trace amounts of

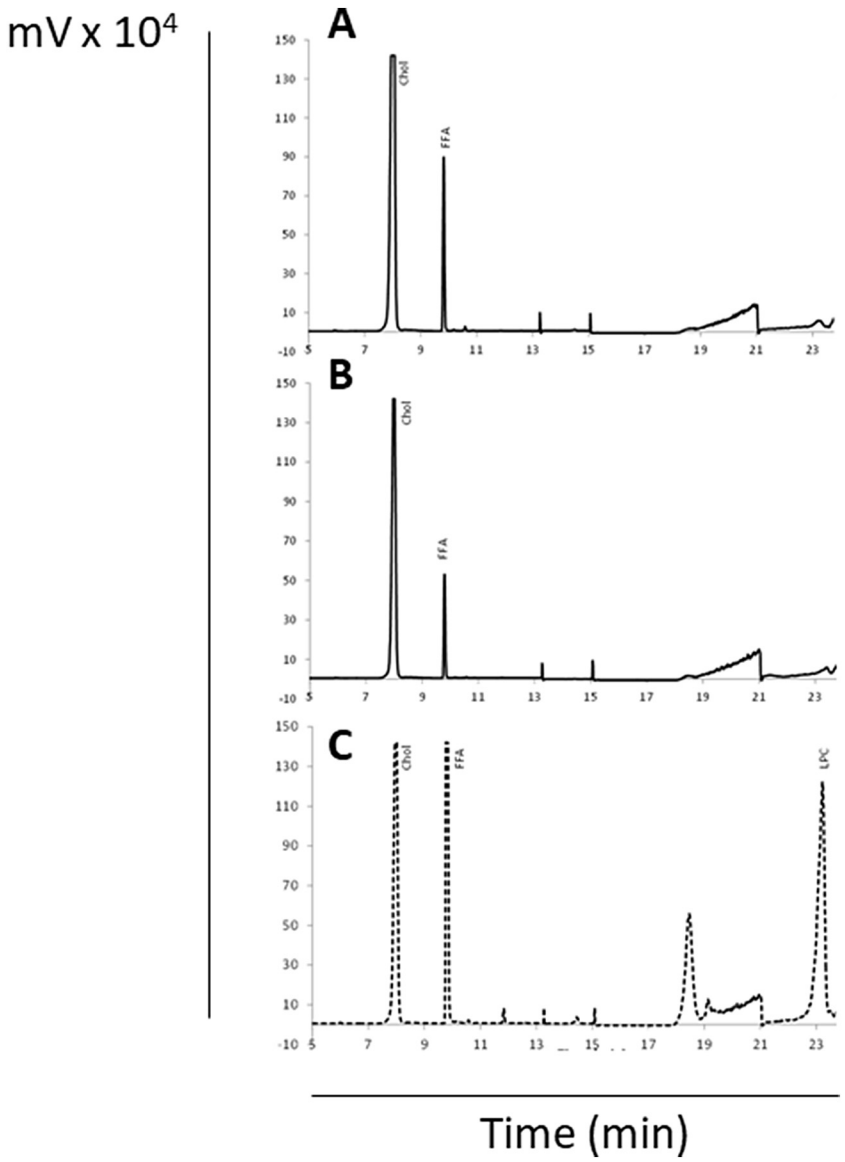

Fig. 4. High-performance liquid-chromatography (HPLC) profiles of bound lipid (BL) extracts of (A) native commercial wheat starch, (B) gelatinized commercial wheat starch, and (C) gelatinized starch with prior addition of palmitic acid. The injection volume was 15 µl. Abbreviations: Chol, cholesterol; FFA, free fatty acids; LPC, lysophosphatidylcholine.

diacylglycerols and TAG, lipids of (wild-type strains of)

S. cerevisiae consist of 20.3% PI, 14.9% PE, 14.3% PC, and 1.8% PS (Ejsing et al., 2009). Thus, each fermented dough piece contained approximately 0.027 mg PI/g dm, 0.020 mg PE/g dm, 0.019 mg PC/g dm, and 0.002 mg PS/g dm derived from yeast. The most occurring acyl group combinations of PI were 16:0/16:1 (31%), 16:0/18:1 (31%), 12:0/16:0 (9%), 16:1/16:1 (6%), 10:0/16:0 (5%), and 18:0/18:1 (5%), of PE 16:1/16:1 (47%) and 16:1/18:1 (41%), of PC also 16:1/16:1 (49%) and 16:1/18:1 (38%), and of PS 16:1/18:1 (46%) and 16:1/16:1 (23%) (Ejsing et al., 2009). Thus, after summing up the molar amounts of all different lipid species within a given lipid class, each fermented dough piece contained approximately 29 nmol PI/g dm, 25 nmol PE/g dm, 23 nmol PC/g dm, and 2 nmol PS/g dm derived from yeast. It follows that the increased measured levels of bound PI, PE, PC, and PS after fermentation partly originated from the presence of yeast. Moreover, our data are in agreement with the FA composition of yeast phospholipids. Indeed, during fermentation the relative abundance of bound PI (32:1), PI (34:1), PI (36:1), PE (32:2), PC (32:2), PS (34:2), and PS (32:2) strongly increased (Table 4). Interestingly enough, the species PE (34:2) was not detected in FL or BL extracts of fermented dough. Also, the relative abundance of the species PC (34:2) slightly decreased as a result of fermentation. It thus seems that PE (34:2) and part of PC (34:2) became non-extractable during fermentation.

In a final paragraph, we will discuss whether baking affects the distribution of the FL and BL populations of fermented dough.

3.4. Bread crumb

Bread crumb contained lower extractable FL and BL levels than fermented dough. This could mostly be attributed to poor extractability of nonpolar lipids levels from crumb (Table 2). Somewhat in contrast, Fig. 2 shows that the levels of extractable TAG and FFA in the FL fraction were lower and that those of the BL levels were higher than in fermented dough. The total level of extractable glycolipids was slightly higher after baking. This could be ascribed to an increase in the level of extractable glycolipids in the BL fraction (Table 2). While the level of extractable bound LPC was much higher after baking, opposite trends were observed for bound PC, PE, LPI, PI, NALPE, and NAPE (Table 3). The most abundant FAs of bound LPC were 18:2 (53%) and 16:0 (29%).

The mechanism responsible for some of the observed changes in

lipid population (*i.e.* the decrease of extractable FFA and NALPE in the FL and BL fraction, respectively, and the higher extractability of LPC in the BL fraction) during baking most likely is the formation of AM-L inclusion complexes (Putseys et al., 2010). Starch internal lipids consist of up to 90% of lysophospholipids, of which roughly 81% is LPC (Morrison, 1981). In flour, these lipids are initially present as AM-L inclusion complexes – which has been demonstrated for barley (Morrison, Tester, Gidley, & Karkalas, 1993), oat, maize, and rice (Morrison, Law, & Snape, 1993) starches – or they are tightly entrapped in between AM and amylopectin (Morrison, 1981). Starch gelatinization provokes dissociation of

AM-L inclusion complexes [at 96–100 °C for amorphous complexes and 105–125 °C for semi-crystalline complexes (Goderis et al., 2014, Karkalas, Ma, Morrison, & Pethrick, 1995, Kwaśniewska-Karolak, Nebesny, & Rosicka-Kaczmarek, 2008)] endogenously present in flour and, thus, promotes the release of LPC (Table 3). When the baked bread is cooled, AM-L inclusion complexes can again be formed (Conde-Petit, Escher, & Nuessli, 2006). Not only FFAs, but also NALPE, which both have a single FA (Fig. 1), can enter the hydrophobic cavity of an AM helix. Doing so renders these lipids non-extractable at temperatures below the dissociation temperature of AM-L inclusion complexes (see above), even with polar solvents such as WSB. Based on our findings, it thus seems that during cooling FFA and, to a less pronounced extent, NALPE, more readily form AM-L complexes than the LPC molecules initially present in such complexes in flour. That FFA more easily form inclusion complexes with AM than LPC during bread cooling is probably related to the higher degree of unsaturation of LPC FA moieties. Indeed, while the level of extractable (unsaturated) LPC (18:2) in the BL fraction was much lower in fermented dough (190 nmol/g dm) than in bread crumb (283 nmol/g dm), the increase of the level of extractable (saturated) LPC (16:0) during that same conversion was less pronounced (from 142 nmol/g dm in fermented dough to 155 nmol/g dm in bread crumb). That cis-unsaturated lipids have lower propensity for forming inclusion complexes with AM than saturated ones has already been established by Karkalas et al. (1995) and Putseys, Derde, Lamberts, Goesaert, and Delcour (2009). Thus, overall our observations strongly suggests that (probably for the most part saturated) FFA replace unsaturated LPC (18:2) molecules as the ligand in AM-L inclusion complexes during bread baking and cooling. To the best of our knowledge, this has never been reported before. To further examine this, lipids were extracted from commercial wheat starch after heating (and thus gelatinization) and subsequent cooling either in the presence or absence of palmitic acid (see section 2.2.2). The lipid composition of a control gelatinized starch sample, to which chloroform but no palmitic acid had been added, was not affected (data not shown). Fig. 4 shows that adding excess amounts of palmitic acid prior to gelatinization resulted in higher levels of WSB-extractable LPC after gelatinization. This suggests that palmitic acid more easily formed inclusion complexes with AM than LPC. In the absence of palmitic acid, the released LPC was probably again incorporated in the AM-L inclusion complexes for lack of structurally more suitable ligands (Fig. 4). Of course, while commercial starch probably has slightly different lipid and AM populations than the starch from the flour used here, such approach still has merits. In fact, it even illustrates that our finding may be more general, rather than being limited to the wheat cultivar examined here.

4. Conclusions

FL extracts from flour contained about 20% polar and 80% nonpolar lipids, while the BL fraction contained no nonpolar lipids. Glycolipids were much more abundant than phospholipids in both the FL and BL fractions of a soft wheat flour studied here. DGDG (36:4) was the most prevalent free glycolipid, whereas BL extracts were dominated by MGDG (36:4) and DGDG (36:4). NAPE (18:2 FA at sn-3 position and acyl group combinations 36:4 and 34:2 at sn-1 and sn-2 positions) was the most abundant free phospholipid, while LPC (18:2) and LPC (16:0) were the most prominent phospholipids in the BL fraction. In freshly mixed dough, the levels of BLs were much lower than in flour, while the opposite was true for those of BLs. This was mainly caused by lipid-binding, a phenomenon in which lipids previously present in the FL extract interact with gluten proteins and by doing so end up in the BL fraction. Part of these lipids even became completely non-extractable. This was found to be the case both for polar and nonpolar lipids. At the same time, an increase of the levels of some nonpolar (FFA and TAG) and polar (NAPE, and NALPE) lipids was observed in the BL extract, without a concomitant decrease in their levels in the FL fraction. This is probably due to flour particle disintegration as a result of the kneading action. As a result of fermentation, more nonpolar lipids were extracted as either FLs or BLs. In addition, fermentation also caused a slight increase in bound phospholipid levels. We theorize that stretching of gluten molecules releases nonpolar lipids and (certain) phospholipids that could not be extracted from freshly mixed dough. In contrast, although the glycolipids were still the dominant polar lipids in the FL and BL extracts, their extractability was lower after fermentation. We speculate that glycolipids may occupy some of the earlier unavailable binding sites of gluten proteins. Baking decreased and increased the extractability of FFA and LPC in the FL and BL fractions, respectively. This phenomenon can likely be explained by dissociation and reformation of AM-L inclusion complexes during baking and cooling, respectively, of the breads. Indeed, our findings strongly suggest that during heating LPC (18:2) molecules are released due to the dissociation of AM-L inclusion complexes, and are replaced by (probably saturated) FFA such as palmitic acid during cooling.

The general approach and ESI-MS/MS methodology to study wheat lipid distribution throughout the different stages of bread making used here can be exploited in future efforts. On the one hand, our findings regarding the (re)distribution of wheat lipid species throughout bread making may be valuable when assessing the bread making quality of different wheats. On the other hand, it will still be necessary to investigate whether our findings also hold true when comparing wheat cultivars of different harvest years). Furthermore, the present work opens perspectives for specifically targeting wheat flour endogenous lipids by lipases (Gerits et al., 2014; Gerits et al., 2015; Schaffarczyk et al., 2016).

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