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Cutignano A., Mamone G., Boscaino F., Ceriotti A., Maccaferri M., Picariello G. (2021). Monitoring changes of lipid composition in durum wheat during grain development. JOURNAL OF CEREAL SCIENCE, 97, 1-10 [10.1016/j.jcs.2020.103131].

Availability: This version is available at: https://hdl.handle.net/11585/880201 since: 2022-03-29

Published:

DOI: http://doi.org/10.1016/j.jcs.2020.103131

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Journal of cereal science 2021 Volume 97, n. 103131

The final published version is available online at: https://dx.doi.org/10.1016/j.jcs.2020.103131

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Floriana Boscaino, -Investigation and Methodology Marco Maccaferri -Investigation and Methodology Gianfranco Mamone - Funding acquisition

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1 Monitoring changes of lipid composition in durum wheat during grain development

2

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21 Abstract

The lipid fraction of durum wheat (*Triticum turgidum* ssp. *durum* (Desf.) Husn.), cultivar Svevo, was
 characterized during grain maturation. Growing grain kernels were harvested at five key stages of development

between 5 and 30 days post anthesis (dpa). Lipid evolution was assessed during kernel filling by gas 24 25 chromatography-mass spectrometry (GC-MS) analysis of triacylglycerols and methylated free fatty acids (FFA) 26 as well as by ultra-high performance liquid chromatography coupled to high resolution electrospray ionization 27 tandem mass spectrometry (UHPLC-HRESI-MS/MS) lipid analysis. Major triacylglycerols (TAG) were detected 28 already at early developmental stages, albeit at low abundance. The decrease of FFA during kernel filling 29 corresponded to accumulation of predominantly linoleate (C18:2n6)-containing C52 and C54 TAG. Fatty acid 30 composition of polar lipids including phospholipids and galactolipids also showed the progressive dominance of 31 linoleate, typically since two weeks after anthesis. Conversely, minor sphingolipids (ceramides and 32 glycosylceramides) were constituted mainly by saturated long chain FA, also oxygenated, combined with a 33 restricted set of sphingoid bases, and might play a signaling role during grain development.

34

Keywords: *Triticum turgidum* ssp. *durum* (Desf.) Husn., durum wheat, grain maturation, lipidomics, mass
 spectrometry, Q Exactive

37

38 **1. Introduction**

Cereals represent staple crops worldwide and the main source of food for humankind. Among them, wheat (*Triticum* spp.) plays a central role as it is transformed in flour that is used to prepare bread, pasta, pizza and a huge variety of baked foods. Based on the genetic asset, wheat is commonly classified as soft (*Triticum aestivum* L.) or durum (*Triticum turgidum* ssp. *durum* (Desf.) Husn.). Genomic differences determine distinct biochemical composition in terms of starch, proteins and lipids, rendering soft wheat flour mainly suited for the preparation of bread and baked products and durum wheat for making semolina and pasta.

Durum wheat has a tetraploid AABB genome (4 x 7, 4*n*). The ancient domestication and the subsequent breeding practices have selected the modern durum wheat, under the pressure of agronomical behavior and, more recently, pasta quality features. The main technological properties of durum wheat affecting pasta quality are undoubtedly related to the starch and protein content. However, minor components of the wheat caryopsis, such as lipids and xanthophylls, significantly affect nutritional and technological traits: for instance, nonpolar lipids influence the rate of starch gelatinization in pasta, glycolipids act as endogenous surfactants in dough, while carotenoids determine the "yellow index" of durum wheat.

52 On average, the lipid content of wheat kernels varies within the 2.4-3.8 % range (as dry weight), depending on 53 the cultivar and abiotic factors (Lafiandra et al., 2012). Durum wheat contains slightly higher levels of lipids 54 than the soft counterpart. Lipids are unevenly distributed in the caryopsis, as they are particularly concentrated 55 in the embryo (germ) and in the outer teguments. However, outer layers and embryo of the mature wheat caryopsis account for only 7-8% and nearly 6.0 % of the dry weight, respectively. Nevertheless, the nutritional 56 57 value as well as the cancer chemopreventing and antioxidant properties of lipids they contain have attracted increasing interest (González-Thuillier et al., 2015; Mahmoud et al., 2015). The greatest kernel compartment is 58 59 represented by the starchy endosperm (80-83%) which is low in lipids, while the protein-rich aleurone layer constitutes about 6.5% of the caryopsis (Barron et al., 2007). Within the endosperm, lipid concentration 60 progressively decreases from the outer to the inner layers (Liu, 2011). 61

62 Generally, when the grain is milled and sieved, outer layers, germ and aleurone are removed and recovered 63 together as the bran, thereby lowering the lipid content of refined flour or semolina up to 1.0-1.5 % dry weight. 64 During milling, lipids partly migrate from the germ and aleurone to the endosperm particles and they 65 redistribute with a fairly constant ratio of neutral, phospho- and glyco-lipids (Morrison, 1994). Despite their low 66 content, lipids significantly impact functionality of flour and dough by interacting with gluten proteins and 67 starch. In particular, polar lipids (i.e. glycolipids and phospholipids) seem to be favorable to baking 68 performance while nonpolar lipids (i.e., triacylglycerols, TAG) and free fatty acids (FFA) appear to be 69 detrimental (MacRitchie, 1977). Furthermore, lipids influence processing and storage properties of cereal-70 based products. Pioneering works carried out in 60's by thin layer chromatography (TLC) and chemical analysis 71 highlighted the gross changes in lipid composition during wheat maturation (Daftary and Pomeranz, 1965; 72 Klopfenstein and Pomeranz, 1968). Afterwards, wheat lipids were characterized by combining chromatographic

fractionation and GC analysis (Stokes et al., 1986). The assessment of the galactolipid content enabled discrimination between T. *durum* and *T. aestivum* (Qin et al., 2019). Recent mass spectrometry (MS)-based lipidomic investigations addressed the qualitative and quantitative determination of lipid classes and FFA content (Geng et al., 2015; González-Thuillier et al., 2015; Narducci et al., 2019; Wetzel et al., 2014), providing more detailed information about lipid distribution in wheat grain compartments. However, most of MS-based studies have explored only the lipid composition of mature wheat kernels, while the evolution of class-specific FA-composition during grain filling has not been investigated in detail, so far.

80 The complete genome of durum wheat has been recently sequenced and released, utilizing the Svevo 81 accession, chosen as a reference cultivar (Maccaferri et al., 2019). Metabolic and storage proteins of the same 82 accession have been recently characterized by proteomic approaches (Arena et al., 2017; Mazzeo et al., 2017). 83 As a part of a large research project aimed at defining the global biomolecular asset by state-of-the-art integrated omic technologies, herein the lipid fraction of Svevo durum wheat was characterized during grain 84 85 maturation. Plants were grown under controlled conditions and filling grains were collected at various stages of 86 development between 5 and 30 days post anthesis (dpa) to assess lipid evolution by ultra-high performance liquid chromatography coupled to high resolution tandem mass spectrometry (UHPLC-HRESI-MS/MS), with 87 88 special focus on acylglycerides, glycolipids, phospholipids and FFA.

89

90 2. Material and methods

91 2.1 Plant material

Durum wheat cultivar Svevo was experimentally grown in early spring 2015 in a growth chamber under completely controlled conditions of photoperiod, air, temperature and humidity, as well as horizontal laminar air flux, as previously detailed (Mazzeo et al., 2017). The growing cycle lasted for three months. Developing kernels were harvested at specific developmental stages, namely at 5 (embryo increase and kernel development at 1/3 of full developmental size, water-ripening stage), 11 (half-full size, water-ripening stage), 97 16 (kernel full development, water-ripening stage), 21 (milk-ripening stage) and 30 (dough stage) dpa, 98 according to typical reference stages (CerealDB, http://bio-gromit.bio.bris.ac.uk/cerealgenomics/cgi-99 bin/grain3.pl). Samples were harvested in triplicate and are named as follows: 5 dpa (T1), 11 dpa (T2), 16 dpa 100 (T3), 21 dpa (T4) and 30 dpa (T5). Immediately after harvesting, developing kernels were stored at -80 °C.

101

102 2.2 Sample preparation

103 Whole kernels were lyophilized, ground to a powder using a mortar and the obtained lyophilized powder kept frozen at -26°C until analyses. Lipids were extracted adapting the Bligh and Dyer method of total lipid 104 105 extraction. Briefly, 100 mg aliquots of kernel powder were extracted in glass vials, according to the following 106 steps: addition of 0.2 mL water and vortexing 10 min to form a wet slurry; addition of 0.75 mL chloroform-107 methanol (1/2, v/v) and 10–15 min vortexing; addition of 0.25 mL chloroform and vortexing for 5 min; addition 108 of 0.25 mL water and vortexing for 5 min; centrifugation for 10 min (3000×g, 4 °C, 15 min). The lower organic 109 layer was collected by aspiration. The aqueous phase was discarded as no lipids were detected by TLC, while 110 the pellet and interfacial layer were re-extracted twice with the same procedure described above. The organic 111 extracts were combined, dried under N₂ stream and stored at -80 °C until use. The yields of lipidic extracts from 112 100 mg of lyophilized kernels from each sampling period were: 1.0 mg (T1), 2.3 mg (T2), 3.6 mg (T3), 3.8 mg (T4) and 1.7 mg (T5). 113

114

115 2.3 High performance thin layer chromatography (HPTLC)

Before analysis lipids were re-dissolved in 1 mL of chloroform–methanol (2:1, v/v) and aliquoted. Lipid extracts were separated by HPTLC using silica gel 60 plates (Merck, Darmstadt, Germany), placing 2 μL of solution for each T1-T5 sample. TAG-rich wheat germ oil, purchased at a local herbalist's shop, was run by comparison. The developing eluent was petroleum ether/diethyl ether (80:20, v/v). Lipids were visualized by exposure to iodine vapors or by charring the plates previously sprayed with 10% (w/v) phosphomolybdic acid in ethanol.

122 2.4 TAG analysis by GC-MS

123 Raw extracts from each sampling were dried and resuspended in 2 mL of *n*-heptane. Each sample was analyzed 124 using a gas chromatograph (model 7890 A; Agilent Technologies, Santa Clara, CA) coupled with a quadrupole 125 mass spectrometer 5975 C (Agilent). Separation was carried out with a Rtx-65TG (mod. 17008) capillary column 126 (30 m x 0.25 mm i.d.; 0.10 mm film thickness) from Restek (Bellefonte, PA, USA). High-purity helium was used 127 as the gas carrier at 0.80 mL/min. Samples (1 μ L) were injected through an MPS2 autosampler (Gerstel, 128 Mülheim an der Ruhr, Germania) at 370 °C in splitless mode. Experimental chromatographic conditions were as 129 follows: the initial temperature (220°C) was raised to 320 °C at a rate of 15°C /min and then to 355°C at a rate 130 of 7°C /min, holding at 355°C for 20 min. Spectra were recorded in full scan mode from 29 m/z to 1050 m/z 131 with 0.2 s/scan. TAG were identified through the mass spectra, by matching with previous data reporting the 132 expected species and comparing the retention times with an in-house developed retention time library based 133 on commercial standards. The results are the average of three replicates, expressed as area percentage.

134

135 2.5 Free fatty acid methyl ester (FAME) analysis by GC-MS

136 A small aliquot corresponding to 1/10 of lipid extract from each harvested sample was methylated with 137 ethereal diazomethane (CH₂N₂) freshly prepared from DIAZALD[®] (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) 138 by using a glassware apparatus obtained from Aldrich (Merck Life Science, Milano, Italy; Macro Diazald® Kit, 139 Cat. No. Z100250). Each T1-T5 aliquot was dissolved in methanol (MeOH, 200µL) in a glass vial and 1mL of a 140 cold ethereal solution of CH_2N_2 was added. The reaction mixture were left at r.t. for 3 hrs. Samples were 141 successively dried under N₂ stream and redissolved in MeOH (200 μ L) before GC-MS analysis (2 μ L sample 142 injection volume) with an ion-trap MS instrument in EI mode (70 eV) (Thermo, Polaris Q) connected with a GC 143 system (Thermo, GCQ) by a 5% phenyl/methyl polysiloxane column (30 m \times 0.25 mm \times 0.25 μ m, Agilent, VF-144 5ms) using high-purity helium as the gas carrier. The following temperature gradient was applied: Initial 160 °C 145 holding for 3 min; then 5°C/min up to 260 °C followed by 30 °C/min up to 310°C, holding for 3 min at 310 °C; 146 split flow 10 mL/min; full scan m/z 50–450. FAME were identified by comparison with authentic standards.

147

148 2.6 Lipid analysis by UHPLC-Q Exactive-ESI-MS/MS

149 Crude lipid extracts obtained by equal amount of cereal grains for each sampling time as described above were 150 dissolved in 1 mL MeOH/Isopropanol 50:50 (v/v) and diluted 1:20 in the LC-MS initial elution solvent. 151 Chromatographic separation was performed on an Infinity 1290 UHPLC System (Agilent Technologies, Santa 152 Clara, CA, USA), equipped with a Kinetex Biphenyl column (2.6 μm, 150 × 2.1 mm) (Phenomenex, Castel 153 Maggiore, Bologna, Italy) at 28 °C. Eluent A: Acetonitrile/H₂O 60:40, 10 mM ammonium formate, 0.1% FA; 154 eluent B: Isopropanol/Acetonitrile 90:10, 2 mM ammonium formate, 0.1% FA. All solvents were LC-MS grade. The elution program consisted of a gradient from 20 to 40% B in 6.5 min, then to 50% B up to 13 min, reaching 155 156 90% B at min 16, holding for 1 min and returning back to 20% B in 1 min. A re-equilibration step of 5 mins was 157 included prior to each analysis. Flow rate was 0.3 mL/min. Samples were run in triplicates and the injection volume was 10 µL; the column temperature was set at 40°C and the autosampler was maintained at 10°C. MS 158 159 analysis was performed on a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, 160 San Jose, CA, USA) equipped with a HESI source. Source parameters were as follows: spray voltage positive 161 polarity 3.2 kV, negative polarity 3.0 kV, Capillary temperature 320 °C, S-lens RF level 55, Auxiliary gas 162 temperature 350 °C, Sheath gas flow rate 60, Auxiliary gas flow Rate 35. Full MS scans were acquired in the 163 range 200-1200 m/z at 70000 of mass resolution. For MS/MS analysis a data dependent ddMS2 Top10 method 164 was used; Mass Resolution was 17500, AGC Target 1e5, Acquisition Time 70ms. Mass fragmentation was 165 obtained with a stepped normalized energy (NCE) of 16-20 and 20-40 in positive and negative ionization mode, 166 respectively. A pool of in-house and commercial standard mix (SPLASH-Lipidomix, AvantiPolar Lipids) was used 167 as reference for MS/MS regioisomeric FA characterization. Raw LC-MS/MS data were processed with the 168 Xcalibur software (Thermo Scientific, version 3.1.66.10); lipid species were identified and quantified with the 169 support of LipidSearch software (Thermo Scientific, version 4.1.30). All data were manually double checked.

171 **3. Results and Discussion**

172 3.1 Sample preparation

Durum wheat cv. Svevo was selected for this study because it is a well characterized reference cultivar, widely used as a crossing parent worldwide. Grain samples were harvested at five developmental stages in the postanthesis period, namely 5 (T1), 11 (T2), 16 (T3), 21 (T4) and 30 (T5) dpa, corresponding to partial kernel development (T1-T2), full kernel development (T3-T4) and grain maturation and desiccation (T5) stages. After lyophilization samples were extracted by a chloroform/methanol mixture according to a modified Blight and Dyer method to recover both starch and non-starch lipids.

179

180 3.2 HPTLC screening

A preliminary screening carried out by HPTLC (**Fig. S1**) clearly evidenced the progressive accumulation of TAG and a decrease of FFA in wheat kernels particularly evident at T5. TAG were detected already at T1 stage, albeit at very low amount. Few spots of unresolved polar lipids appeared at lower Rf.

184

185 3.3 GC-MS analysis

186 TAG were identified and quantified by capillary GC-MS at the different stages of kernel development. In 187 agreement with HPTLC, TAG were detected already at T1 and progressively increased up to T5. In general, the 188 profiles were dominated by TAG containing 54 acyl carbons (C54), followed by C52 and C56 TAG by abundance. 189 Interestingly, the ratio of TAG groups at the early developmental stage (T1) was different, since C52 TAG were 190 more abundant than C54 ones, whereas C56 TAG were undetectable (Fig. S2). 191 Independent GC-MS analysis of FFA as methyl esters (FAME) confirmed that at the initial stage the main FA 192 were palmitic (C16:0) and oleic (C18:1) acids. Notably, the synthesis of polyunsaturated linoleic (C18:2n6) and 193 linolenic (C18:3n-3) acids started being significant already from T2. These FA became prevalent in the T3/T4

- 194 filling stages, while they dropped down dramatically during the T5 period (**Fig. S3**). These results are in line with
 - 8

the previous observations that the increased content of unsaturated FA was paralleled by the FFA declineduring grain maturation (Daftary and Pomeranz, 1965).

197

198 3.4 UHPLC-Q Exactive MS/MS analysis

An in-depth analysis of wheat lipidome was then performed using the high-resolution LC-MS Q Exactive platform. Software-assisted lipid profiling allowed the unambiguous identification of overall 24 lipid classes and 190 main individual species. Interestingly, raw lipid profiling disclosed a complex pattern of polar lipids occurring along with major TAG (**Fig. 1 and Fig S4**). Lipid compounds were identified and corresponding peak areas compared by software-assisted analysis, exclusively on kernel dry weight basis.

204

205 3.4.1 Triacylglycerols (TAG)

206 Overall, 64 species of TAG were identified and characterized for their FA composition. The predominant groups 207 were C52 and C54, which exhibited an evident positive trend from T1 to T5 (Fig. 2). A detailed investigation of 208 the FA composition revealed that the highest increment involved the species containing oleic and linoleic acids 209 (C54:3, C54:4, C54:5 and C54:6) followed by mixed C16/unsaturated C18 TAG (C52:2, C52:3 and C52:4) and to a 210 lesser extent by C56 series (C56:3, C56:4, C56:5) (Table 1). On the contrary, C42-C50 and odd FA-containing 211 TAG remained substantially unchanged or showed minor changes through the various maturation phases. TAG 212 were the most abundant and assorted class in terms of type and combination of FA on the three carbon 213 positions of glycerol. Furthermore, their constant increment from initial development to final desiccation stage 214 of wheat kernels is in agreement with their intrinsic role of storage reservoirs.

215

216 3.4.2 Diacylglycerols (DAG)

Diacylglycerols (DAG) as a class (**Fig. S4B and S5**) appeared early, already at T1, exhibited a sharp increase in unsaturated species from T2 to T3, peaked at T4, after which they dropped at full kernel maturity. This class of lipids plays a central biosynthetic role, being the building block for *de novo* formation of all membrane and

220 storage lipids in plants. Their decrease corresponds to a massive DAG-to-TAG biosynthetic conversion during 221 the last stage (T5), when the entire lipid biosynthesis tends to converge into TAG accumulation. More in detail, 222 the highest increment observed at the T3/T4 stages was recorded for the polyunsaturated FA-containing DAG 223 (PUFA-DAG), i.e. DAG 16:0/18:2 and 18:2/18:2 and slightly lesser 18:1/18:2. Conversely, DAG analogs 224 composed by palmitic and oleic acids maintain a basal level throughout kernel maturation. These latter species 225 are synthesized de novo through the acylCoA/phosphatidic acid (PA) pathway (Kennedy pathway) and as 226 precursors serve to the biosynthesis of polar and neutral glycerolipids containing C16:0 and C18:1 FA. The 227 occurrence of PUFA-DAG analogs starting from T3 stage suggests the activation/prevalence at this period of 228 other DAG biosynthetic pathways, including de novo DAG synthesis combined with phosphatidylcholine (PC)-FA 229 modification and acyl editing and PC-derived DAG synthesis (Bates and Browse, 2012). In fact, C18:1 FA is 230 desaturated to C18:2 and C18:3 on PC so that PUFA-PC can accumulate and in part be converted into PUFA-231 DAG, which in turn represent the committed precursors of PUFA-TAG.

232

233 3.4.3 Phospholipids (PL)

234 The most represented PC species was the 16:0/18:2 derivative (Table 2). However, PC 18:2/18:2 showed a 235 continuous increase up to final stage T5. This finding further supports the central role of PC 18:2/18:2 species 236 also in TAG biosynthesis. On the other side, the PUFA enrichment observed in phosphatidylethanolamines (PE), 237 phosphatidylinositols (PI) and phosphatidylglycerols (PG) during grain maturation (Fig. S4B) can be ascribed to 238 the utilization of PUFA-DAG for the formation of all these lipids. Together with DAG, the other key precursor for 239 de novo biosynthesis of all lipid classes is PA, typically in the form of PA 16:0/18:1 or 18:1/18:1. Indeed, in 240 wheat samples here examined PA occurred mainly as 16:0/18:2 and 18:2/18:2 molecular species, thus 241 suggesting a biosynthetic process involving PC with unsaturated FA (i.e. PC 16:0/18:2 and 18:2/18:2) as acyl 242 donor for the consecutive acylation steps of glycerol-3-phospate and lysophosphatidic acid (LPA), finally leading 243 to a DAG pool rich in PC-modified FA for TAG synthesis (Bates and Browse, 2012) (Table 2). Hence, it appears 244 straightforward the involvement of these PA as key intermediates in the biosynthesis of polyunsaturated lipids, 10

including TAG. An additional intriguing possibility is that PA may act as signaling molecules, produced under certain stress/stimuli (Wang et al., 2006). In fact, PA have been implicated in various plant processes which include responses to abiotic and biotic stresses (drought, chilling, nutrient starvation, ROS, wounding and microbial elicitation) and involvement in specific steps of growth and development, such as seed germination, seed aging, leaf senescence, root hair patterning, root growth, pollen germination, and pollen tube growth. However, a putative signaling role of PA in grain maturation has been never reported so far and it would deserve further investigation.

All PL, including PE, PG, PI showed a similar T3/T4 peak of relative abundance as monitored in LC-MS profiles (Fig. S6). Regiochemistry of PL was assigned according to previous interpretative studies (Hsu and Turk, 2009) and confirmed by a commercial standard mix run in our conditions on ESI-Q Exactive platform. The general strategy for PC, PE, PG and PI identification by MS/MS is based on the preferred loss of both carboxylate and ketene ions from position *sn*-2 of the glycerol backbone. Hence, the peak intensity of the resulting [M- $R_2CH=C=O$]⁻and R_2COO^- product ions are much higher than [M- $R_1CH=C=O$]⁻and R_1COO^- ions in product spectra of

either [M-H]⁻ or [M+HCOO]⁻ adducts, thus allowing to assign FA position on the glycerol backbone (**Table 2**).

258

259 Conversely, while fragmentation of PA ion at sn-2 is still favored, generating major loss of carboxylate with 260 corresponding $[M-H-R_2COOH] > [M-H-R_1COOH]$, successive CID fragmentations of these intermediate products 261 favor further loss of carboxylate from sn-1 of [M-H-R₂COOH] ion following a charge-driven process; in these 262 conditions, R_1COO^- ion net intensity resulted higher than R_2COO^- in product spectra, which is the opposite of 263 what observed with all the other PL. Therefore, based on an in-depth interpretation of all MS/MS data, 264 regiochemical distribution of FA was established for each identified PL species; regiochemical analysis allowed 265 to detect two couples of regioisomeric species, eluting at slightly different retention time (t_R) as reported in 266 Table 2, namely PA 36:5 (18:3/18:2 and 18:2/18:3) and PG 36:5 (18:2/18:3 and 18:3/18:2). All data were 267 indicative of a eukaryotic biosynthetic pathway for all glycerophospholipid classes. In fact, C18 acids were 268 found at both *sn*-1 and *sn*-2 glycerol positions, while C16:0 was exclusively located at the *sn*-1 position.

Apart from PA, whose composition and involvement in biosynthetic processes has been previously discussed, PC, PE, and PI showed prevalence of 16:0/18:1 species at the beginning (48.2-90.8 %) replaced by 16:0/18:2 and 18:2/18:2 species in later stages. In line with their plastidial biosynthetic origin, PG showed initially >67% of C16:0/16:0 species, supplanted by PUFA C18 species over time.

273

274 3.4.4 Lysophospholipids (LPL)

275 FA composition of lysophospholipids (LPA, LPC, LPE and LPG, Fig. S7) reflected the total natural FA abundance along with the observed variation in unsaturation of C18 FA during grain growth. However, they started to be 276 significant a few days post anthesis, since they were detected only in traces in T1 samples. Lysolipids including 277 minor monoacylglycerols (MAG) are considered "true" starch lipids, while full acylated lipids are non-starch 278 279 lipids, occurring in endosperm, aleurone and germ. Previous studies have excluded that they are simple 280 degradative forms of corresponding acylated lipids due to extraction artefacts. Thus, their biosynthesis is 281 supposed to be independently regulated from that of the corresponding PL. On the other hand, lysolipids are 282 located inside starch granules, tightly associated with amylose helix, so that their biosynthesis likely is 283 synchronous with starch formation. Indeed, previous studies carried on the same durum wheat cultivar 284 evidenced that expression of starch biosynthetic enzymes started at T2 and peaked at T3/T4 stages, which is in 285 agreement with major levels of lysolipids in our extract profiles (Arena et al., 2017). Coherently, starch granules 286 were detected by light microscopy in grain-cross section from 15 dpa in the endosperm cells, which 287 corresponds to T3/T4 in our harvested samples (Guillon et al., 2012).

288

289 3.4.5 Galactolipids (GL)

Digalactosylglycerols (DGDG) and monogalactosylglycerols (MGDG) (**Table 3 and Fig.S8**) were dominated by C18:2 FA containing species, which registered the highest level in T5. Evolution of GL through T1-T5 showed Gaussian profiles resembling those of PL. In fact, DGDG and MGDG species containing C16:0 and C18:1 FA, poorly represented in absolute levels in T1, account for 78.0 % and 89.1%, respectively, in relative terms, while 294 at T3 stage they dropped at 50.6% and 21.9%, respectively. In contrast, 18:2 containing individual species rose 295 at 46.7% for DGDG and 66.0 % for MGDG at T3, starting from T1 values of 22.0% and 5.8%, respectively. The 296 level of C18:2/18:2 species in both MGDG and DGDG continuously increased up to T5, paralleling the trend of 297 C18:2-containing TAG, in agreement with previous findings (Weber, 1970). In T5, species composed by 298 18:2/18:2 represented 47.3% of DGDG and 61.9% of MGDG.

299 Regiochemical analysis was carried out on samples processed according to our previous studies (Cutignano et 300 al., 2016) and was based on the general behavior of sodium adducts of glycoglycerolipids to produce 301 preferentially fragmentation of acyl chain at the glycerol *sn*-1 position. Extensive MS/MS data interpretation 302 revealed that both DGDG and MGDG were mainly synthesized by the eukaryotic pathway.

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304 3.4.6 Sphingolipids (SP)

305 Sphingolipids (SP), i.e. ceramides (Cer) and glucosylceramides (GlcCer) (Fig. 1), are evenly distributed in all parts 306 of wheat kernel (Geng et al., 2015). By our LC-MS analyses, 30 SP species were characterized (Table 4 and Fig. 307 **S9**). The FA composition of these minor lipids encompasses mainly saturated, long chain FA, even but not 308 exclusively in oxygenated form, in part contrasting with recent reports from wheat (Geng et al., 2015; Zhu et 309 al., 2013). Compared with other previous reports (Fujino and Ohnishi, 1983), we found some differences in SP 310 composition. In the 20 molecular species of ceramides detected, the most abundant FA were C16:0, C24:0, also 311 oxygenated, along with C18:1 and C24:1. Differently from ceramide composition recently reported in soft 312 wheat (Geng et al., 2015), in durum wheat here analyzed sphingoid long chain base (LCB) mainly occurred as 313 phytosphingosine (t18:0) along with sphingosine (d18:1), with minor contribution from sphingadienine (d18:2), 314 sphinganine (d18:0), and d20:1 sphingoid LCB. The main species of Cer resulted t18:0/24:0, t18:0/24:1 and 315 d18:1/24:0+O. Notably, in our LC-MS conditions ceramides of the same lipid group can be easily distinguished 316 as for LCB and FA composition based on both Rt and MS/MS fragments (Table 4). For example, as showed in 317 **Table 4**, three ions belonging to the group d42:1+O have been detected at t_{R} =9.51, 9.59 and 9.65 min for which 318 the same molecular formula $C_{42}H_{83}O_4N$ (calculated m/z 666.6395) can be inferred by high accuracy 13

measurement of the corresponding observed ion masses. Detailed analysis of MS/MS product ions allowed to distinguish the lipid species resulting from the combination of three different LCB and FA, namely: Cer(t18:0/24:1), Cer(d18:1+O/24:0) and Cer(d18:1/24:0+O), respectively. Ceramides could be revealed already at T1 stage and at an appreciable level in comparison with successive stages. On the contrary, biosynthesis of glycosylated forms appeared more active at later stages (T3 and T4). This is an interesting point that has been never discussed before.

325 Glycosylceramides (GlcCer) showed lower chemical diversity compared to Cer. The structural identity of the 326 monosaccharide residue was not investigated here, thus we generically refer to all of them as monoglycosyl 327 derivatives. However, in plants the monosaccharide unit is typically represented by D-glucopyranose in β -328 linkage to the C-1 hydroxyl group of the ceramide backbone. Occasionally, mannose has been found linked to 329 the sphingoid LCB. Overall, 10 species were identified, one of which was predominant, specifically that 330 containing d18:1/C16:0+O as the ceramide portion. Minor species typically showed the occurrence of monounsaturated or mono-oxygenated saturated FA (20:0, 22:0 and 24:0) that do not have a counterpart in 331 332 the ceramide pool. In general, it seems that there is no direct correspondence between Cer and glycosylated 333 analogs, since major GlcCer derived from preferential glycosylation of less abundant Cer species. This aspect 334 has been evidenced in previous papers (Fujino and Ohnishi, 1983) and suggests the origin of Cer and GlcCer 335 from biosynthetic pathways probably fueling two independent pools with different functions. In fact, in 336 contrast with main Cer species, the highest relative abundance of Cer d18:1/C16:0+O in T3/T4 stages matches 337 the highest peaks of the corresponding glycosylated products at the same stages (Fig S9). Indeed, plant 338 sphingolipids are not only structural components of the plasma membrane and other endocellular membranes, 339 but they also act as signaling molecules in response to biotic and abiotic stresses, despite this role is far for 340 being properly investigated (Ali et al., 2018).

341 SP profiling deserves an additional comment since none of the previous works took into consideration the 342 biosynthetic evolution of this class of lipids. Considering the total ion current of both total lipids and each 343 individual species, the trend is basically the same as the other lipids. However, it is quite worth noting that Cer

344 are actively synthesized in an appreciable amount soon after anthesis together with TAG, DAG and PA. This 345 may suggest a specific role of SP during the early phase of grain development; in effect, while DAG and PA are 346 clearly correlated to biosynthesis of TAG, the main lipids, Cer and other SP might function as signaling 347 molecules and perform regulatory roles in a series of biosynthetic and physiological processes including cell-348 wall formation (Lynch and Dunn, 2004) and cell type differentiation (Msanne et al., 2015). In effect, during the 349 initial stage of kernel development, cell division is very high to allow morphogenesis and organogenesis, thus 350 supporting a plausible involvement of SP as metabolic signals. However, determining the biological functions of 351 SP is challenging since each class, including Cer, GlcCer and sphingoid LCB plays a specific role either on its own or in homoeostasis with the others, being SP metabolites continuously converted into each other via 352 353 biosynthetic and degradative pathways (Pata et al., 2010).

354

3.4.7 Phytosterols 355

356 Wheat phytosterols represent an additional variegate class of structurally related compounds (Zhu and Nyström, 2019). Sitosterol is the most abundant component of wheat phytosterols, accounting for 51-54% of 357 358 total sterols with a constant proportion among the wheat varieties (Nurmi et al., 2008). These compounds 359 occur also as steryl glycosides formed through a β -glycosidic linkage between the C1 position of several 360 possible monosaccharides (most frequently D-glucopyranose) and the C3 position of sterol mojety. In this 361 study, sterols were detected as fatty acyl sitosteryl glycosides (AGIcSiE) with main FA, i.e. 16:0, 18:2 and 18:3 362 esterifying hydroxyl group at C6 of the monosaccharide unit (Fig. 1). These were minor compounds, found at 363 comparable amounts during all stages of grain filling although the chemical composition varied throughout the 364 growing stages, with a progressive increase of the C18:2 derivative (Fig. S10). Despite extensive research on the 365 occurrence of phytosterol esters in plants, acyl steryl glycosides are poorly characterized concerning structural variety and biological function (Ferrer et al., 2017). However, in consideration of their low abundance, they are 366 367 expected to not impact technological and functional traits of wheat flour and dough. Furthermore, glycosyl 368 phytosterols might be scarcely retained in the cooked pasta, leaching in cooking water due to a certain 15

solubility. On the other hand, the broad class of wheat phytosterols has been rather investigated for their
possible nutritional relevance (Nurmi et al., 2008).

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372 3.4.8 Other lipids

Finally, the lipidome of durum wheat also includes a series of additional compounds, such as waxes, tocochromanols, carotenoids (e.g. xanthophylls), free sterols, terpene alcohols and alkylresorcinols, which for the most have been already inventoried through dedicated workflows of purification and analysis (Geng et al., 2015; Lafiandra et al., 2012). Thus, these minor compounds have been not the focus of the present study.

377

378 **4. Conclusions**

379 This study represents the first lipidome characterization of durum wheat during grain maturation, over a period 380 covering 5-30 dpa that is from embryo development to full maturation. The opportunity to apply advanced 381 high-resolution MS methodologies allowed to monitor the evolution of individual lipid classes at the molecular 382 level over five key developing stages. The progressive increase in unsaturation degree of FA acyl chains during grain maturation has been described since long. On the other hand, here for the first time the trend in FA 383 384 composition for each of the main lipid classes and the dynamics ruling the relative balance of lipid classes have 385 been inferred at the key stages of grain filling by UHPLC-HRESI-MS/MS analysis. A general trend shared by all 386 lipid classes is the prevalence of palmitate and oleate-based lipids during the early developmental stages (T1 387 and T2), with an increment of linoleate and linolenate- containing species during the grain filling and 388 maturation stages (T3-T5). Except for TAG, the metabolic activation of lipid biosynthesis begins between T2 and 389 T3 stages, i.e. at the turn of two weeks (11-16 dpa), lasting until the dough stage. The biosynthesis of the 390 different lipid classes and, within each class, of specific components follows a seed maturation program that 391 can be explained only in part in the light of the current defined metabolic pathways. In line with fragmentary 392 data available for soft wheat and other cereals (i.e. maize) (Stokes et al., 1986; Weber, 1970, 1969), the current

393 findings provide an in-depth view into lipid metabolism during the process of grain filling and maturation, still 394 poorly studied from a biochemical standpoint. The preliminary nature of the present study, which could not 395 rely on biological replicates for appropriate statistical analysis of LC-MS data, implies some limitations for the 396 assessment of the reported values. At this stage, the aim and the implications of the research were to propose 397 an in-depth view into lipid metabolism during the process of grain maturation, still poorly investigated from a 398 (bio)chemical point of view. Follow-up of this study with other durum wheat cultivars will also be needed to 399 generalize our results. Furthermore, several metabolic and functional features of lipids await to be defined, 400 such as the involvement of key intermediates (i.e. PUFA-PC and -PA) in the possible pathways leading to 401 (poly)unsaturated TAG and the physiological role of bioactive lipids, such as sphingolipids. The combination of 402 lipidomic with partly available genomic, transcriptional, metabolomic and proteomic data, enables systems 403 biology approaches to assist breeding, improve agronomical practices and enhance grain quality. The global 404 biomolecular and metabolic perspective could be conveniently exploited in the next future for engineered 405 modulation of specific lipid components, such as dietary essential FA or bioactive sphingolipids in desirable larger quantity. In addition, the knowledge of the differences in lipid compositions of wheat cultivars may make 406 407 virtually possible to produce flours or recover bran and/or germ oil with purposely modified lipid compositions 408 suited for different end uses.

409

410 Acknowledgements

This work was partly supported by a grant from MIUR-CNR: Progetto Bandiera "InterOmics": Sviluppo di una
piattaforma integrata per l'applicazione delle scienze "omiche" alla definizione dei biomarcatori e profili
diagnostici, predittivi, e teranostici.

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490 **Table 1.** Identification, characterization and relative abundance of TAG species in wheat kernel across T1-T5 491 maturation stages. Values (%) are average of three replicates. Relative standard deviation was lower than 10 % 492 in all cases and was not reported. All species were detected as NH_4^+ adducts.

FA		Base				T1	T2	Т3	T4	T5
Group	Lipid Molecular	t.	Formula	Obs	Calc m/z					
Cloup	Species	•ĸ	Torritata	Mass				%		
Кеу		(min)								
42:0	TG(16:0/12:0/14:0)	15,91	C45 H86 O6	740.6768	740.6763	1,30	1,17	0,93	0,78	0,68
43:0	TG(15:0/14:0/14:0)	16,00	C46 H88 O6	754.6926	754.6919	1,43	1,21	1,08	0,93	0,63
43:1	TG(16:1/13:0/14:0)	15,91	C46 H86 O6	782.7234	782.7232	0,82	0,72	0,59	0,39	0,30
44:0	TG(16:0/14:0/14:0)	16,17	C47 H90 O6	768.7079	768.7076	3,10	2,56	2,39	2,05	1,47
44:1	TG(16:1/14:0/14:0)	16,08	C47 H88 O6	766.6921	766.6919	1,96	1,68	1,49	1,34	0,99
44:2	TG(16:1/14:0/14:1)	15,99	C47 H86 O6	764.6767	764.6763	0,80	0,66	0,61	0,53	0,34
45:0	TG(15:0/14:0/16:0)	16,25	C48 H92 O6	782.7234	782.7232	3,49	3,21	2,66	2,47	1,77
45:1	TG(15:0/14:0/16:1)	16,20	C48 H90 O6	780.7081	780.7076	2,42	2,15	1,74	1,66	1,20
45:2	TG(15:0/14:1/16:1)	16,11	C48 H88 O6	778.6931	778.6919	0,91	0,57	0,72	0,62	0,43
46:0	TG(16:0/14:0/16:0)	16,37	C49 H94 O6	796.7392	796.7389	6,42	5,90	4,59	4,13	3,40
46:1	TG(16:0/14:0/16:1)	16,33	C49 H92 O6	794.7238	794.7232	4,64	4,25	3,56	3,06	2,36
46:2	TG(16:1/14:0/16:1)	16,26	C49 H90 O6	792.7086	792.7076	2,47	2,26	1,80	1,55	1,35
47:0	TG(15:0/16:0/16:0)	16,46	C50 H96 O6	810.7543	810.7545	4,48	4,39	3,59	3,10	2,36
47:1	TG(15:0/16:0/16:1)	16,41	C50 H94 O6	808.7393	808.7389	4,80	4,48	3,41	3,03	2,58
47:2	TG(15:0/16:1/16:1)	16,36	C50 H92 O6	806.7242	806.7232	2,38	2,24	1,75	1,53	1,29
48:0	TG(16:0/16:0/16:0)	16,55	C51 H98 O6	824.7692	824.7702	5,13	6,06	4,57	3,93	2,69
48:1	TG(16:0/16:0/16:1)	16,51	C51 H96 O6	822.7545	822.7545	6,01	5,80	4,84	4,06	3,08
48:2	TG(16:0/16:1/16:1)	16,47	C51 H94 O6	820.7399	820.7389	4,27	4,06	3,29	2,78	2,20
48:3	TG(16:1/16:1/16:1)	16,41	C51 H92 O6	818.7240	818.7232	1,30	1,25	0,95	0,90	0,69
49:1	TG(15:0/16:0/18:1)	16,58	C52 H98 O6	836.7707	836.7702	3,54	3,36	2,65	2,20	1,66

49:2	TG(16:0/16:1/17:1)	16,54	C52 H96 O6	834.7549	834.7545	2,77	2,54	1,92	1,69	1,48
49:3	TG(16:1/16:1/17:1)	16,48	C52 H94 O6	832.7389	832.7389	0,75	0,73	0,57	0,49	0,44
50:1	TG(16:0/16:0/18:1)	16,66	C53 H100 O6	850.7863	850.7858	4,12	3,67	3,35	2,96	2,65
50:2	TG(16:0/16:1/18:1)	16,61	C53 H98 O6	848.7712	848.7701	4,33	4,22	4,25	5,27	5,10
50:3	TG(16:1/16:1/18:1)	16,56	C53 H96 O6	846.7561	846.7545	1,85	1,88	1,56	1,50	1,25
51:1	TG(16:0/17:0/18:1)	16,74	C54 H102 O6	864.8019	864.8015	1,45	1,41	0,97	0,77	0,64
51:2	TG(16:1/17:0/18:1)	16,71	C54 H100 O6	862.7870	862.7858	1,51	1,56	1,09	0,93	0,71
51:3	TG(16:1/17:1/18:1)	16,66	C54 H98 O6	860.7711	860.7702	0,75	0,72	0,56	0,45	0,40
52:1	TG(18:0/16:0/18:1)	16,78	C55 H104 O6	878.8139	878.8171	1,34	1,34	0,98	0,98	0,83
52:2	TG(16:0/18:1/18:1)	16,77	C55 H102 O6	876.8013	876.8015	2,60	2,66	2,65	2,97	3,55
52:3	TG(16:0/18:1/18:2)	16,71	C55 H100 O6	874.7865	874.7858	1,79	2,05	3,66	4,98	6,18
52:4	TG(16:0/18:2/18:2)	16,65	C55 H98 O6	872.7703	872.7702	0,80	1,44	5,72	8,72	9,50
52:5	TG(16:0/18:2/18:3)	16,59	C55 H96 O6	870.7542	870.7545	0,17	0,76	2,31	2,52	2,12
52:6	TG(16:0/18:3/18:3)	16,51	C55 H94 O6	868.7379	868.7389	0,08	0,36	0,50	0,26	0,17
53:0	TG(16:0/14:0/23:0)	16,89	C56 H108 O6	894.8488	894.8484	0,51	0,55	0,41	0,31	0,24
53:2	TG(17:0/18:1/18:1)	16,83	C56 H104 O6	890.8178	890.8171	0,54	0,51	0,36	0,30	0,28
54:0	TG(18:0/16:0/20:0)	16,95	C57 H110 O6	908.8634	908.8641	1,01	1,01	0,72	0,55	0,48
54:2	TG(16:0/18:1/20:1)	16,90	C57 H106 O6	904.8296	904.8328	0,98	0,99	0,89	0,85	1,04
54:3	TG(18:1/18:1/18:1)	16,86	C57 H104 O6	902.8170	902.8171	1,92	2,15	2,13	1,82	3,74
54:4	TG(18:1/18:1/18:2)	16,82	C57 H102 O6	900.8015	900.8015	0,74	1,17	2,38	2,43	5,13
54:5	TG(18:1/18:2/18:2)	16,77	C57 H100 O6	898.7859	898.7858	0,52	0,94	3,42	4,78	7,35
54:6	TG(18:2/18:2/18:2)	16,72	C57 H98 O6	896.7715	896.7702	0,31	0,97	3,96	5,29	7,04
54:7	TG(18:3/18:2/18:2)	16,65	C57 H96 O6	894.7540	894.7545	0,10	0,60	1,80	2,00	1,81
54:8	TG(18:3/18:2/18:3)	16,59	C57 H94 O6	892.7389	892.7389	0,02	0,34	0,47	0,32	0,26
55:0	TG(25:0/14:0/16:0)	16,98	C58 H112 O6	922.8790	922.8797	0,69	0,67	0,47	0,46	0,42
55:1	TG(16:0/16:1/23:0)	16,98	C58 H110 O6	920.8638	920.8641	0,50	0,54	0,41	0,33	0,31

55.2	TG(16·1/16·1/22·0)	16 97	C58 H108 OF	918 9/97	918 8/18/	0.27	0 22	በ 1 ዩ	0 17	0.15
JJ.2	10(10.1/10.1/23.0)	10,97	00 01100 00	510.0407	510.0404	0,27	0,22	0,10	0,17	0,13
56:0	TG(16:0/16:0/24:0)	17,03	C59 H114 O6	936.8948	936.8954	0,79	0,85	0,57	0,50	0,50
56:1	TG(16:0/16:1/24:0)	17,02	C59 H112 O6	934.8795	934.8797	0,78	0,75	0,51	0,44	0,42
56:2	TG(16:1/18:1/22:0)	17,01	C59 H110 O6	932.8639	932.8641	0,43	0,44	0,37	0,37	0,36
56:3	TG(20:1/18:1/18:1)	16,97	C59 H108 O6	930.8471	930.8484	0,19	0,23	0,29	0,30	0,41
56:4	TG(20:1/18:1/18:2)	16,94	C59 H106 O6	928.8330	928.8328	0,12	0,16	0,24	0,32	0,51
56:5	TG(20:1/18:2/18:2)	16,90	C59 H104 O6	926.8159	926.8171	0,12	0,12	0,28	0,31	0,67
57:0	TG(25:0/16:0/16:0)	17,06	C60 H116 O6	950.9097	950.9110	0,63	0,58	0,48	0,38	0,37
57:1	TG(25:0/16:0/16:1)	17,06	C60 H114 O6	948.8954	948.8954	0,54	0,52	0,44	0,35	0,33
57:2	TG(16:1/18:1/23:0)	17,05	C60 H112 O6	946.8793	946.8797	0,30	0,25	0,22	0,20	0,17
58:1	TG(16:0/18:1/24:0)	17,10	C61 H116 O6	962.9100	962.9110	0,49	0,56	0,41	0,40	0,33
58:2	TG(16:1/18:1/24:0)	17,09	C61 H114 O6	960.8955	960.8954	0,37	0,40	0,31	0,31	0,29
58:3	TG(16:1/18:1/24:1)	17,05	C61 H112 O6	958.8770	958.8797	0,11	0,12	0,14	0,16	0,16
59:1	TG(25:0/16:0/18:1)	17,13	C62 H118 O6	976.9255	976.9267	0,28	0,28	0,22	0,22	0,18
59:2	TG(25:0/16:1/18:1)	17,13	C62 H116 O6	974.9106	974.9110	0,22	0,22	0,19	0,15	0,14
60:1	TG(26:0/16:0/18:1)	17,18	C63 H120 O6	990.9407	990.9423	0,26	0,24	0,21	0,18	0,17
60:2	TG(26:0/16:1/18:1)	17,17	C63 H118 O6	988.9262	988.9267	0,22	0,22	0,18	0,19	0,16
60:3	TG(18:1/18:2/24:0)	17,15	C63 H116 O6	986.9091	986.9110	0,08	0,10	0,08	0,08	0,09

Table 2. Identification and relative abundance of PL species within each subclass (PA, PC, PE, PG and PI) in
wheat kernel across T1-T5 maturation stages. Regiochemistry *sn*-1/*sn*-2 is indicated. Values (%) are means of
three replicates. Relative standard deviation was lower than 10 % in all cases and is not reported.

FA	Lipid	Base					T1	T2	Т3	T4	T5
Group	Molecular	t _R	Formula	lon	Obs mass	Calc m/z					
Kev	Species	(min)		adduct					%		
,		()									
34:2	PA(16:0/18:2)	6,30	C37 H69 O8 P	M-H ⁻	671.4665	671.4657	58,35	42,95	44,87	50,50	48,50
34:3	PA(16:0/18:3)	5,86	C37 H67 O8 P	M-H ⁻	669.4519	669.4501	12,77	16,86	12,88	12,97	9,12
36:4	PA(18:2/18:2)	6,06	C39 H69 O8 P	M-H ⁻	695.4656	695.4657	22,41	27,41	32,37	31,62	37,69
36:5	PA(18:3/18:2) ^a	5,67	C39 H67 O8 P	M-H ⁻	693.4518	693.4501	6.40	40.70	0.00		4.60
36:5	PA(18:2/18:3) ^a	5,94	C39 H67 O8 P	M-H ⁻	693.4522	693.4501	6,48	12,78	9,88	4,91	4,68
				$M+H^+$	760.5855	760.5850					
34.1	PC(16:0/18:1) ^c	7,09	C42 H82 N O8 P	M+HCOO ⁻	804.5781	804.5760	48,17	28,90	13,54	8,56	9,52
				$M+H^+$	758.5708	758.5694					
34:2	PC(16:0/18:2)	6,51	C42 H80 N O8 P	M+HCOO ⁻	802.5625	802.5604	21,29	47,09	62,59	65,13	46,87
36:2	PC(18:1/18:1)	7,66	C44 H84 N O8 P	M+HCOO ⁻	830.5937	830.5917	29,19	6,57	4,12	3,36	8,27
				M+H ⁺	784.5857	784.5851					
36:3	PC(18:1/18:2)	7,06	C44 H82 N O8 P	M+HCOO ⁻	828.5789	828.5760	0,94	8,61	6,16	4,75	8,61
36:4	PC(18:2/18:2)	6,45	C44 H80 N O8 P	M+HCOO ⁻	826.5615	826.5604	0,42	8,83	13,58	18,20	26,72
34:1	PE(16:0/18:1)	6,82	C39 H76 N O8 P	M-H ⁻	716.5247	716.5236	90,82	35,95	13,52	15,58	39,12
34:2	PE(16:0/18:2)	6.24	C39 H74 N O8 P	M-H ⁻	714.5091	714.5079	0.34	25.11	33.54	27.86	17.52
24.2		5,2 0			742 4020	742 4022	0.01	,	4.05	2.24	1.00
34:3	PE(16:0/18:3)	5,78	C39 H72 N O8 P	IM-H	/12.4938	/12.4923	0,01	6,98	4,05	2,21	1,00
36:2	PE(18:0/18:2)	7,30	C41 H78 N O8 P	M-H ⁻	742.5419	742.5392	4,18	1,75	1,52	1,32	2,47
36:3	PE(18:1/18:2)	6,76	C41 H76 N O8 P	M-H ⁻	740.5246	740.5236	0,00	3,98	4,88	3,15	3,95
36:4	PE(18:2/18:2)	6,19	C41 H74 N O8 P	M-H ⁻	738.5089	738.5079	0,00	7,49	16,77	11,75	13,74
36:5	PE(18:3/18:2)	5,68	C41 H72 N O8 P	M-H ⁻	736.4934	736.4923	0,00	3,53	3,00	1,54	0,75

40:2	PE(22:0/18:2)	9,56	C45 H86 N O8 P	M-H ⁻	798.6039	798.6018	0,00	0,00	1,13	1,62	2,76
32:0	PG(16:0/16:0)	5,02	C38 H75 O10 P	M-H ⁻	721.5039	721.5025	67,34	26,93	29,36	22,41	12,06
34:1	PG(16:0/18:1)	5,48	C40 H77 O10 P	M-H ⁻	747.5189	747.5182	2,08	11,97	6,51	4,33	4,22
34:2	PG(16:0/18:2)	4,98	C40 H75 O10 P	M-H [⁻]	745.5038	745.5025	26,94	27,36	37,66	43,63	45,38
34:3	PG(16:0/18:3)	4,56	C40 H73 O10 P	M-H ⁻	743.4878	743.4869	0,00	19,06	9,99	7,76	3,50
36:3	PG(18:1/18:2)	5,43	C42 H77 O10 P	M-H ⁻	771.5196	771.5182	0,56	4,49	3,19	2,97	4,14
36:4	PG(18:2/18:2)	4,92	C42 H75 O10 P	M-H ⁻	769.5034	769.5025	2,24	5,83	9,91	15,26	27,11
36:5	PG(18:2/18:3) ^b	4,37	C42 H73 O10 P	M-H ⁻	767.4884	767.4869	0.84	1 25	2 20	2 65	2 50
36:5	PG(18:3/18:2) ^b	4,54	C42 H73 O10 P	M-H ⁻	767.4874	767.4869	0,04	4,55	5,50	3,03	5,55
34:1	PI(16:0/18:1)	4,82	C43 H81 O13 P	M-H ⁻	835.5332	835.5342	43,95	27,63	16,88	9,42	17,47
34:2	PI(16:0/18:2)	4,37	C43 H79 O13 P	M-H [⁻]	833.5200	833.5186	23,51	53,42	66,46	79,02	77,43
34:3	PI(16:0/18:3)	3,97	C43 H77 O13 P	M-H ⁻	831.5052	831.5029	32,54	18,95	16,66	11,57	5,10

499 ^{a,b} regioisomeric species percentages were combined; ^c PC percentage was calculated on peak areas of HCOO⁻ adducts

Table 3. Identification and relative abundance of GL species within each subclass in wheat kernel across T1-T5
 maturation stages. Regiochemistry *sn*-1/*sn*-2 is indicated. Values (%) are means of three replicates. Relative
 standard deviation was lower than 10 % in all cases and is not reported. All species were detected as HCOO⁻
 adducts.

FA		Base				T1	T2	Т3	T4	T5
Group	Lipid Molecular	t _R	Formula	Obs mass	Calc m/z					
Key	Species	(min)						%		
34:1	DGDG(16:0/18:1)	6,60	C49 H90 O15	963.6278	963.6262	55,59	7,44	4,51	3,07	1,66
34:2	DGDG(16:0/18:2)	6,05	C49 H88 O15	961.6126	961.6105	0,22	21,02	23,77	24,51	28,18
34:3	DGDG(16:0/18:3)	5,57	C49 H86 O15	959.5970	959.5949	0,12	18,51	11,80	9,90	3,53
36:2	DGDG(18:1/18:1)	7,11	C51 H92 O15	989.6450	989.6418	22,06	5,27	3,73	2,58	2,97
36:3	DGDG(18:1/18:2)	6,57	C51 H90 O15	987.6281	987.6262	0,04	7,97	6,76	5,18	5,37
36:4	DGDG(18:2/18:2)	6,00	C51 H88 O15	985.6118	985.6105	21,87	13,48	23,33	28,78	47,29
36:5	DGDG(18:2/18:3)	5,49	C51 H86 O15	983.5973	983.5949	0,08	11,95	16,60	18,07	8,52
36:6	DGDG(18:3/18:3)	5,01	C51 H84 O15	981.5819	981.5792	0,00	0,49	0,40	0,31	0,18
38:3	DGDG(18:2/20:1)*	7,55	C53 H94 O15	1015.6589	1015.6575	0,01	13,87	9,11	7,59	2,30
18:2	DGMG(18:2)	1,65	C33 H58 O14	723.3825	723.3809	86,35	55,24	64,66	67,86	87,55
18:3	DGMG(18:3)	1,55	C33 H56 O14	721.3663	721.3652	13,65	44,76	35,34	32,14	12,45
34:1	MGDG(16:0/18:1)	8,04	C43 H80 O10	801.5761	801.5734	10,93	4,05	0,66	0,00	0,00
34:2	MGDG(16:0/18:2)	7,24	C43 H78 O10	799.5593	799.5577	5,30	1,95	4,76	2,77	10,79
34:3	MGDG(16:0/18:3)	6,90	C43 H76 O10	797.5436	797.5421	16,32	12,38	7,84	5,77	1,89
36:2	MGDG(18:1/18:1)	8,51	C45 H82 O10	827.5917	827.5890	55,16	3,26	2,24	1,15	1,82
36:3	MGDG(18:1/18:2)	7,93	C45 H80 O10	825.5764	825.5734	1,37	4,93	6,37	5,13	7,21

36:4	MGDG(18:2/18:2)	7,36	C45 H78 O10	823.5596	823.5577	4,44	14,56	35,72	47,01	61,86
36:5	MGDG(18:2/18:3)	6,83	C45 H76 O10	821.5444	821.5421	0,03	14,54	23,90	25,08	12,59
36:6	MGDG(18:3/18:3)	6,32	C45 H74 O10	819.5281	819.5264	6,45	44,33	18,51	13,10	3,84
18:2	MGMG(18:2)	1,89	C27 H48 O9	561.3287	561.3280	99,67	42,08	58,13	59,16	81,63
18:3	MGMG(18:3)	1,75	C27 H46 O9	559.3124	559.3124	0,33	57,92	41,87	40,84	18,37

507 * regiochemistry not assignable.

Table 4. Identification and relative abundance of SP species within each subclass (Cer-Ceramide; CerG1-Glycosylceramide) in wheat kernel across T1-T5 maturation stages. Values (%) are means of three replicates. Relative standard deviation was lower than 10 % in all cases and is not reported. All species were detected as H^+ adducts.

		Base		Oha		T1	T2	Т3	T4	Т5
Group Key	Lipid Molecular	t _R	Formula	Obs	Calc m/z					
	Species	(min)		mass				%		
d34:0+0	Cer(t18:0/16:0)	5.91	C34 H69 N O4	556,5307	556.5299	2,17	3.10	1.18	1.68	0.33
420-0-0	$C_{r}(t10.0/10.0)$	0,01		612 5020	612 5025	2,17	1 10	0.54	0.20	0,00
d38:0+0	Cer(t18:0/20:0)	8,00	C38 H77 N 04	612.5930	612.5925	0,07	1,19	0,54	0,39	0,29
d40:0+O	Cer(t18:0/22:0)	9,21	C40 H81 N O4	640.6242	640.6238	6,86	6,28	2,89	2,86	2,15
d42:0+O	Cer(t18:0/24:0)	10,36	C42 H85 N O4	668.6552	668.6551	24,00	23,36	18,11	17,63	7,52
d42:0+2O	Cer(t18:0/24:0+O)	9,60	C42 H85 N O5	684.6503	684.6501	2,93	2,56	2,18	3,53	5,83
d42:1+0	Cer(t18:0/24:1)	9,51	C42 H83 N O4	666.6397	666.6395	19,80	18,77	15,27	12,64	17,10
d43:0+O	Cer(t18:0/25:0)	11,06	C43 H87 N O4	682.6713	682.6708	1,46	1,30	0,23	0,35	0,31
d44:0+O	Cer(t18:0/26:0)	11,67	C44 H89 N O4	696.6870	696.6864	5 <i>,</i> 83	3,60	3,76	4,02	10,15
d34:0	Cer(d18:0/16:0)	6,85	C34 H69 N O3	540.5359	540.5350	0,06	1,02	2,51	2,27	1,17
d34:0+O	Cer(d18:0/16:0+0)	6,18	C34 H69 N O4	556.5307	556.5299	0,00	0,95	2,30	0,54	0,02
d36:2	Cer(d18:0/18:2)	6,88	C36 H69 N O3	564.5358	564.5350	0,10	0,40	2,17	3,54	1,12
d42:1+0	Cer(t18:1/24:0)	9,59	C42 H83 N O4	666.6393	666.6395	6,92	5,46	5,18	5,50	8,84
d42:1+20	Cer(t18:1/24:0+O)	9,53	C42 H83 N O5	682.6351	682.6344	5,71	5,72	3,10	4,61	3,67
d34:1+0	Cer(d18:1/16:0+0)	4,76	C34 H67 N O4	554.5152	554.5143	0,02	1,68	13,14	14,49	10,59
d36:3	Cer(d18:1/18:2)	6,55	C36 H67 N O3	562.5203	562.5194	0,00	0,61	2,30	3,80	0,35
d38:1+O	Cer(d18:1/20:0+0)	8,01	C38 H75 N O4	610.5775	610.5769	1,87	1,27	3,41	2,47	2,57
d42:1+0	Cer(d18:1/24:0+0)	9,65	C42 H83 N O4	666.6393	666.6395	17,47	17,98	14,07	11,57	14,94
d34:3	Cer(d18:2/16:1)	4,54	C34 H63 N O3	534.4891	534.4881	0,48	1,41	3,49	3,16	1,78
d42:2+0	Cer(d18:2/24:0+0)	9,44	C42 H81 N O4	664.6244	664.6238	1,85	2,11	1,05	1,01	1,25
d38:1+O	Cer(d20:1/18:0+0)	6,73	C38 H75 N O4	610.5774	610.5769	2,40	1,23	3,11	3,93	10,00

d40:1+2O	CerG1(t18:1/22:0+0)	7,20	C46 H89 N O10	816.6570	816.6559	0,69	6,86	1,20	0,00	0,89
d42:1+2O	CerG1(t18:1/24:0+O)	8,31	C48 H93 N O10	844.6880	844.6872	45,21	19,89	3,95	3,52	0,20
d34:1+O	CerG1(d18:1/16:0+O)	4,76	C40 H77 N O9	716.5681	716.5671	20,71	18,79	42,65	43,80	32,80
d34:2	CerG1(d18:1/16:1)	4,80	C40 H75 N O8	698.5573	698.5565	1,18	4,96	1,71	1,33	0,67
d38:1+O	CerG1(d18:1/20:0+O)	6,73	C44 H85 N O9	772.6303	772.6297	2,23	2,25	6,50	8,71	22,15
d34:2+O	CerG1(d18:2/16:0+O)	4,54	C40 H75 N O9	714.5523	714.5515	20,81	19,24	16,01	14,32	7,66
d34:3	CerG1(d18:2/16:1)	4,54	C40 H73 N O8	696.5417	696.5409	0,03	15,99	13,24	11,64	6,48
d38:2+O	CerG1(d18:2/20:0+O)	6,52	C44 H83 N O9	770.6145	770.6141	7,78	5,94	5,39	5,69	4,22
d38:3	CerG1(d18:2/20:1)	6,51	C44 H81 N O8	752.6047	752.6035	0,00	3,62	3,05	3,29	2,77
d38:1+O	CerG1(t20:0/18:1)	6,72	C44 H85 N O9	772.6303	772.6297	1,36	2,45	6,30	7,71	22,15

516 Figure captions

517	Fig. 1. Representative structures of main lipids other than simple acylglycerols identified in durum wheat
518	kernel.
519	Fig. 2. UHPLC-ESI-MS/MS-based TAG species profile of wheat kernels across T1-T5 maturation stages. Peak
520	areas were compared on kernel dry weight basis. Values are average of three replicates. Standard deviation
521	was always below 10 % and was not reported.
522	
523	
524	





- Grain kernels were harvested at 5 key stages of development (5-30 days post anthesis)
- Lipidome evolution was assessed during kernel filling by UHPLC-Q Exactive-MS/MS
- Triacylglycerols and polar lipids steadily accumulated, while fatty acids declined
- Linoleic acid dominated in all glycerolipids
- Lipidomics of growing kernels complements the biomolecular inventory of durum wheat

Declaration of interests

 \boxtimes The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

□The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: