Genetic and environmental effects on the composition and properties of lipids in wheat flour

Byoung-hyun Min

Rothamsted Research / Quadram Institute Bioscience

A thesis submitted for the degree of Doctor of Philosophy to the
University of East Anglia

September 2017

ABSTRACT

Being valued at £3.4 billion per annum and producing 4 billion loaves a year, the UK breadmaking industry is vast. Due to this, much research has focused on improving breadmaking quality.

Breadmaking quality is significantly affected by flour lipids which play a crucial role at various stages of breadmaking. For example, during the initial stages of dough development, lipids can adsorb to the surface of gas bubbles, stabilizing them, allowing air retention in the dough. This stability helps provide the loaf volume, crumb structure and quality associated with UK bread.

Despite the importance of lipids in breadmaking, limited research has been carried out on grain and flour lipids. No evidence of significant genetic control of wheat grain lipids had been reported, and wheat lipids were not considered a suitable target for improving breadmaking quality.

Therefore, in this project, six wheat lines grown under three nitrogen conditions in 2012-13 were milled and analysed using a 'lipodomics platform.' This provided flour lipid profiles, allowing the use of multivariate statistics to identify the effects of genotype, environment or GxE effects on individual lipid species.

A previous project identified QTL (Quantitative Trait Loci) for milling and baking quality parameters using a doubled haploid (DH) population from a cross between two UK breadmaking cultivars, Malacca and Hereward. Four robust QTLs for gas cell number and loaf volume located on chromosomes 1B, 4D, 6A and 7A were selected, and near isogenic lines (NILs) with a good or poor quality allele in the Malacca background were obtained. Lipidomic analysis across two years identified lipids associated with these alleles, allowing the correlation of the lipid profile with the good or poor quality alleles at the four loci. The functional significance of any differences in lipid composition was explored by extracting dough liquor and analysing their surface properties.

Contents

ABSTRACT ..... **i**

Contents ..... **ii**

List of Figures ..... **viii**

List of Tables ..... **xi**

Abbreviations ..... **xii**

Acknowledgements ..... **xvi**

Chapter 1 Introduction **1**

1.1 Wheat 1

1.1.1 A Brief History 1

1.1.2 The development of wheat as a modern crop 1

1.2 Introduction to UK wheat breeding 4

1.2.1 Wheat in the United Kingdom 4

1.2.2 Brief introduction to breeding in the UK (Yield) 5

1.2.3 Breeding for quality in the UK 8

1.3 The Wheat Grain 9

1.3.1 Pericarp 9

1.3.2 Aleurone Layer 10

1.3.3 Starchy Endosperm 10

1.3.4 The Embryo (Germ) 11

1.3.5 Wheat Flour and Milling 11

1.4 The significance of improving wheat quality 12

1.5 Breadmaking and Quality 14

1.5.1 Historical perceptions of bread quality in the West 15

1.5.2 Breadmaking: an overview 16

1.5.3 CBP (Chorleywood Breadmaking Process) 16

1.5.4 Importance of various stages of breadmaking on final loaf quality 18

1.6 Wheat Proteins 19

1.6.1 The classification of wheat proteins 19

1.6.2 The Gluten Proteins 20

1.6.3 HMW glutenin subunits and their role in breadmaking 21

1.6.4 Puroindolines 23

1.7 Starch 25
1.7.1 Summary 25
1.7.2 Starch crystals 27
1.7.3 Starch granules 27
1.7.4 Wheat starch 29
1.7.5 Importance of Starch 30
1.7.6 Factors affecting starch and its impact on breadmaking quality 31
1.7.7 Enzyme activity on starch and the Hagberg Falling Number 32
1.7.8 Impact of starch damage through milling 32
1.8 Enzymes 32
1.8.1 Amylases 32
1.8.2 Proteases 33
1.8.3 Other enzymes 34
1.8.4 Lipases 35
1.8.5 Lipases in breadmaking 36
1.9 Lipids 37
1.9.1 Classification of lipids 37
1.9.2 Lipid biosynthesis 40
1.9.2.1 Fatty Acid Synthesis 40
1.9.2.2 Triacylglycerol synthesis 44
1.9.2.3 Biosynthesis of other Lipids 46
1.9.3 Introduction to the lipid classes in wheat 48
1.9.4 Effects of Saturations 51
1.9.5 The Key to Gas Cell Stability 52
1.9.6 Effects on Loaf Volume 52
1.10 Environmental effects on wheat grain lipids 54
1.11 Wheat genetics 54
1.11.1 Summary 54
1.11.2 Breadmaking quality QTLs 56
1.12 Final Remarks and Thesis Aims 56

Chapter 2 Materials and Methods 59
2.1 Materials 59
2.1.1 Wheat cultivars 59
2.1.2 Chemicals 59

2.1.3 Analytical standards 61
2.2 Methods 61
2.2.1 Sample preparation, growth and storage 61
2.2.1.1 Milling and Storage 61
2.2.1.2 Six UK cultivars 62
2.2.1.3 Malacca xHereward Near Isogenic Lines (NILs) 63
2.2.1.4 Malacca x Hereward Double Haploid (DH) population 63
2.2.1.5 Dough Liquor Preparation 64
2.2.2 Lipidomic Analysis 64
2.2.2.1 Lipid Extraction from Flour 64
2.2.2.2 Lipid extraction from dough liquor 64
2.2.2.3 Principles of acid catalysed esterification of fatty acids 65
2.2.2.4 Fatty acid esterification 67
2.2.2.5 Fatty Acid Methyl Esters (FAMEs) Gas Chromatography-Flame Ionization Detector (GC-FID) 67
2.2.2.6 Electrospray Ionization-tandem triple quadrupole Mass Spectrometry (ESI-MS/MS) 67
2.2.2.7 Quantitative Lipid Analysis 69
2.2.3 Quality control of lipidomic data 70
2.2.3.1 Determination of Calibration Factors for Lipid Groups 70
2.2.3.2 TLC-GC-FID 70
2.2.3.3 Fitting a predictive model 71
2.2.3.4 Fitted models for WGIN lipids (Chapter 3) 72
2.2.3.5 Fitted models for MxH NIL lipids (Chapter 4) 73
2.2.3.6 Determination of starch damage 76
2.2.4 Biophysical Analysis 76
2.2.4.1 First Ten Angstrom (FTA) Pendant drop 76
2.2.4.2 Measuring Surface Tension 77
2.2.4.3 Reducing contamination 78
2.2.4.4 Dough Liquor interfacial properties 78
2.2.5 Sodium Dodecyl Sulphate-Polyacrylmide Gel Electrophoresis (SDS-PAGE) 79
2.2.5.1 SDS-PAGE of Flour MxH DH population 79
2.2.6 Data Processing and Statistics 80
2.2.6.1 Multivariate Statistics 80
2.2.6.2 Procrustes Analysis 80
2.2.6.3 QTL analysis 81

Chapter 3 Genetic and Environmental effects on intrinsic wheat lipids 82
3.1 Introduction 82
3.1.1 A short description of each cultivar 82
3.1.2 Nitrogen treatments 85
3.1.3 Project Aims 85
3.2 Results 86
3.2.1 FAMEs analysis 86
3.2.2 Effects of genotype and environment on specific lipid classes 88
3.2.3 Effects of Genotype and Nitrogen on Lipid Classes 90
3.2.4 ANOVA on lipid class totals 91
3.2.5 Effects of Genotype and Nitrogen on Lipid Species 93
3.2.6 Multivariate Statistical analysis 97
3.2.7 Sample Description and Starch Damage 99
3.2.8 The effects of genotype and environment on the levels of saturated lipids 100
3.3 Discussion 102
3.3.1 Impact on PLs 102
3.3.2 Impacts on NLs 103
3.3.3 Effects of Environment and potential GxE effects 104
3.3.4 Differences between the cultivars 105
3.4 Concluding Remarks 106

Chapter 4 Investigating the genetic effects of the MxH NILs on the lipid profile of wheat flour 108
4.1 Introduction 108
4.1.1 Brief Introduction to the Malacca x Hereward Doubled Haploid Population 108
4.1.2 Generation of the NILs 109
4.1.3 Relating gas cell stability to cell number and loaf volume 111
4.2 Results and Discussion 113
4.2.1 Proportions of lipid classes across the two years 113
4.2.2 Univariate ANOVA analysis of data 116
4.2.3 Independent QTL effects on the lipid composition of the NILs 116
4.2.4 Allelic effects on PLs in Years 1 and 2 118
4.2.5 Effect of QTLs and alleles on GL in Year 1 and Year 2 124
4.2.6 Effect of QTLs and alleles on NLs in Year 1 and Year 2 125

4.2.7 Multivariate Statistical Analysis 127
4.2.8 CVA for full lipid datasets in Years 1 & 2 128
4.2.9 CVA for phospholipids 130
4.2.10 CVA for Galactolipids 132
4.2.11 CVA on Neutral lipids 136
4.2.12 Effect of year x QTL x allelic effects 139
4.2.13 CVA of the full dataset over the two years 140
4.2.14 Procrustes Analysis of the full dataset 143
4.3 Discussion 145
4.3.1 Degradation of PC-LPC 145
4.3.2 Impact of QTL x allele effects on the PL composition of the NIL population 145
4.3.3 Impact of QTL x allele effects on the NL composition of the NIL population 146
4.3.4 Impact of QTL x allele effects on the GL composition of the NIL population 147
4.3.5 Environmental effects 147
4.3.6 Conclusions 148

Chapter 5 Investigating the Functionality of the MxH NILs 150
5.1 Introduction 150
5.1.1 Bubble and Foam formation and stability 150
5.1.1.1 Drainage 151
5.1.1.2 Coalescence 151
5.1.1.3 Disproportionation 152
5.1.2 Bubble formation and stability in breadmaking 153
5.1.3 Studying the aqueous phase of dough 155
5.2 Development of a diluted DL extraction method 156
5.2.1 Diluted Dough liquor Preparation 156
5.2.2 Surface properties of diluted dough liquor 158
5.2.3 DL MS analysis of the various dilutions 160
5.3 Results 162
5.3.1 Dough Liquor surface properties of the MxH NILs in year 1 162
5.3.2 Surface Tension of Year 1 (2013) Material 163
5.3.3 Surface tension of Year 2 (2015) material 167
5.3.4 Elasticity Modulus of Year 2 (2015) material 170
5.3.5 Lipidomic analysis of diluted dough liquor fractions in Year 1 172
5.3.6 Lipidomic analysis of diluted dough liquor fractions in Year 2 173

5.3.7 The enrichment of specific lipid classes in the diluted dough liquor fraction 174
5.4 Discussion 177

Chapter 6 FAMEs analysis of the MxH DH population 180
6.1 Introduction 180
6.1.1 DH production 180
6.1.2 The MxH doubled haploid population 181
6.1.2.1 Simple Sequence Repeats (SSR) 183
6.1.2.2 Diversity Arrays Technology (DArT) 183
6.1.3 SDS-PAGE analysis 184
6.2 Results 185
6.2.1 FAMEs-GC analysis 185
6.2.2 QTL mapping of the fatty acids in wheat flour 186
6.3 Summary and Discussion 188

Chapter 7 Overall Discussion and Future Work 189
7.1 Discussion and Conclusions 189
7.2 Improvements and Future Work 191

Chapter 8 Appendix 194

Chapter 9 References 225

Chapter 10 Publication 238

List of Figures

Figure 1.1. The routes of expansion of wheat across Eurasia and Africa. 2
Figure 1.2 The pedigree of selected important wheat varieties in the development of modern wheat cultivars in the UK. 6
Figure 1.3 Simplified pedigrees of the key semi-dwarf varieties developed by Plant Breeding Institute in the mid-1980s. 7
Figure 1.4 Structure of wheat kernel……..…….12
Figure 1.5 2014 UK wheat crop by end-use category 13
Figure 1.6 Diagram displaying the different steps involved in the Chorleywood breadmaking process 18
Figure 1.7 SDS-PAGE of HMW subunits from a range of genotypes of wheat 22
Figure 1.8 The effects of proteins, protein + lipid, added PINA, PINB and protein on the foam stability. Taken from Dubreil et al., 1997 24
Figure 1.9 Structural sections of amylose and amylopectin, shown in Haworth representation. 26
Figure 1.10 Structure of various starch components 29
Figure 1.11 The oxidative deterioration of carotenoids by lipoxygenases. 36
Figure 1.12 The subdivision of wheat flour lipid classes and their proportions 37
Figure 1.13 Structures of the primary phospholipids present within the wheat grain. 39
Figure 1.14 Structures of the galactolipids and neutral lipids present within wheat 40
Figure 1.15 The overall reaction of fatty acid synthase 43
Figure 1.16 A generalized scheme for Triacylglycerol (TAG) biosynthesis in the developing seeds of oilseeds 45
Figure 1.17 The prokaryotic and eukaryotic pathways of glycerolipid synthesis. A- Prokaryotic pathway, B- Eukaryotic pathway 47
Figure 1.18 Gas cell formation and development during various stages of the breadmaking process . 53
Figure 1.19 The chromosomes of the wheat genome 55

Figure 2.1 Aerial image of the WGIN variety trial using over 25 cultivars. 62
Figure 2.2 Field plots of the Malacca x Hereward Near Isogenic Lines grown by the John Innes Centre, Norwich 63
Figure 2.3 Schematic for the acid catalysed esterification (A) and transesterification of lipids (B) 66
Figure 2.4 Reaction of dimethoxypropane as a water scavenger. 66
Figure 2.5 Diagram of electrospray ionization. 68
Figure 2.6 Schematic of the ESI triple quadrupole mass spectrometer 68
Figure 2.7 The fitted asymptotic exponential curve for the FFA data from the ESI-MS/MS and GC-FID for the WGIN lipids 2013. 72
Figure 2.8- The fitted asymptotic exponential curve for the TAG data from the ESI-MS/MS and GC-FID for the WGIN lines in 2013 73
Figure 2.9 Fitted curve for the FFA data from the ESI-MS/MS and GC-FID for the MxH NILs in 2013 and 2015 74
Figure 2.10 Fitted curve for the TAG data from the ESI-MS/MS and GC-FID for the MxH NILs in 2013 and 2015 75

Figure 2.11 (A) Diagram of FTA pendant drop (B) Droplet image taken using FTA pendant drop to measure the surface tension of dough liquor fraction. 77

Figure 3.1 Dry weights of fatty acids present in the wheat flour of six cultivars grown in three nitrogen conditions..... 87
Figure 3.2. Lipid profile (mol% of total lipids analysed) of the six UK wheat varieties grown under three different nitrogen conditions 100, 200 and 350kg/N/Ha, excluding galactolipids 90
Figure 3.3 Comparison of selected phospholipids (mol% of total lipids) between the six cultivars. ... 94
Figure 3.4 Comparison of LPC and PE (mol % of total lipids) between the six cultivars..... 95
Figure 3.5 Lipid compositions of selected neutral lipids between the three nitrogen conditions 96
Figure 3.6 Canonical Variates Analysis (CVA) for the six cultivars in three nitrogen conditions. 98
Figure 3.7 Starch damage analysis of the six cultivars grown in three nitrogen conditions 99
Figure 3.8 The % nanomoles of the total FFA and LPC between for Unsaturated and Saturated Fatty Acids. 101
Figure 3.9 Summary of the heritability of dietary fibre and other biological components in the wheat grain, based on the HEALTHGRAIN study. 105

Figure 4.1 Simplified schematic of the generation of the MxH NILs..... 110
Figure 4.2 Proportions of specific classes of lipids present in the four MxH NIL populations in Year 1 113
Figure 4.3 Proportions of specific classes of lipids present in the four MxH NIL populations in Year 2 114
Figure 4.4 Mol% of total lipids accounted by LPC + PC levels in the wheat grain across the two years. 115
Figure 4.5 Mol % of total lipids for the six lipid classes across the four bulked QTLs..... 117
Figure 4.6 Comparison of selected minor phospholipids (mol % of total lipids) between M and H NILs among the four QTLs in Year 1..... 119
Figure 4.7 Comparison of major phospholipids (mol% of total lipids) between M and H NILs among the four QTLs in Year 1..... 120
Figure 4.8 Comparison of minor phospholipids (mol % of total lipids) between M and H NILs among the four QTLs in Year 2. 121
Figure 4.9 Comparison of selected major phospholipids (mol % of total lipids) between M and H NILs among the four QTLs in Year 2..... 122
Figure 4.10 Galactolipids present on the four QTLs and their allelic combinations in year 1. 123
Figure 4.11 Galactolipids present on the four QTLs and their allelic combinations in year 2 124
Figure 4.12 Comparison of selected major neutral lipid species across the four QTLs and their allelic combinations in year 1..... 125
Figure 4.13 Comparison of major neutral lipid species across the four QTLs and their allelic combinations in year 2..... 126
Figure 4.14 Canonical Variates Analysis (CVA) for the four QTL and their allelic combinations for the full lipid dataset in year 1..... 128
Figure 4.15 CVA analyses for the four QTL and their allelic combinations for the full lipid dataset in year 2..... 129
Figure 4.16 CVA analyses for the four QTL and their allelic combinations for phospholipids in year 1 131
Figure 4.17 CVA analyses for the four QTL and their allelic combinations for phospholipids in year 2 132

Figure 4.18 CVA analyses for the four QTL and their allelic combinations for galactolipids in year 1 134
Figure 4.19 CVA analyses for the four QTL and their allelic combinations for galactolipids in year 2 136
Figure 4.20 CVA analyses for the four QTL and their allelic combinations for neutral lipids in year 1 137
Figure 4.21 CVA analyses for the four QTL and their allelic combinations for neutral lipids in year 2 138
Figure 4.22 CVA analyses for the four QTL and their allelic combinations for the full lipid dataset across the two years..... 142
Figure 4.23 CVA analyses for the four QTL and their allelic combinations for the GL dataset across the two years 142
Figure 4.24 Procrustes Analysis for the full lipid dataset across the two years 144

Figure 5.1 Schematic of bubble disproportionation. 153
Figure 5.2 Comparison of the surface tension (ST) of various diluted dough liquor fractions in Hereward flour across two years 159
Figure 5.3 Surface elasticity of the various diluted dough liquor samples in Hereward flour across two years. 160
Figure 5.4 The profile of selected lipids in various dilutions of dough liquor from Hereward material grown in 2012 and 2013 161
Figure 5.5 Predicted model for surface tension based on the results of the two-stage analysis, using the means of estimated parameter estimates according to the ANOVA on the sets of parameters 165
Figure 5.6 Predicted model for elasticity based on the results of the two-stage analysis, using the means of estimated parameter estimates according to the ANOVA on the sets of parameters. 167
Figure 5.7 Predicted model for surface tension based on the results of the two-stage analysis, using the means of estimated parameter estimates according to the ANOVA on the sets of parameters. 169
Figure 5.8 Predicted model for elasticity based on the results of the two-stage analysis, using the means of estimated parameter estimates according to the ANOVA on the sets of parameters..... 173
Figure 5.9 The lipid composition of selected lipids in the diluted dough liquor fractions of the first year MxH NIL material 172
Figure 5.10 The lipid composition of selected lipids in the diluted dough liquor fractions of the second year MxH NIL material 173
Figure 5.11 proportions of specific lipids present in the diluted dough liquor fraction of the MxH NILs in the year 1 (2013) dataset 175
Figure 5.12 proportions of specific lipids present in the diluted dough liquor fraction of the MxH NILs in the year (2015) dataset 176

Figure 6.1 Schematic for the generation of the MxH DH populations 181
Figure 6.2 Genotype Scores for the MxH DH mapping population 182
Figure 6.3 SDS-PAGE of HMW glutenin subunits 184
Figure 6.4 FAMEs analysis of the MxH DH population. 185
Figure 6.5 FAMEs analysis of the total fatty acids present in the MxH DH population 186
Figure 6.6 QTL plots with a significance threshold set as a LOD score of 1.5 187

List of Tables

Table 1.1 Proportions and amino acid compositions (g/100 g of protein of the major protein fraction of flour. 23
Table 1.2 The amylose content, size and shape of starch granules in major plant sources..... 28
Table 1.3 Grouping of wheat flour lipids by location, extraction and biochemistry. 38
Table 1.4 The various common fatty acids present in plants, their systemic, trivial and shorthand name. 41

Table 3.1 ANOVA table for the total values of the six lipid classes. 91
Table 3.2 A selected list of the p-values for F-test of cultivar, nitrogen and cultivar by nitrogen interactions of all the lipid species analysed in the study 92

Table 4.1 Background information on the MxH NILs. 111
Table 4.2 Loading vectors for the three CV dimensions for the CVA analysis of GLs in year 1..... 133
Table 4.3 Loading vectors for the three CV dimensions for the CVA analysis of GLs in year 2..... 135

Table 5.1 Recipes for various diluted dough liquor extractions for the 2012 and 2013 material. 157
Table 5.2 Data on the estimated parameters for the surface tension of the MxH NIL DDL for year 1. 164
Table 5.3 Data on the estimated parameters for the elasticity modulus of the MxH NIL DDL in year 1 166
Table 5.4 Data on the estimated parameters for the surface tension of the MxH NIL DDL in year 2 168
Table 5.5 Data on the estimated parameters for the elasticity modulusof the MxH NIL DDL in year 2 171

Abbreviations

ACP	**Acetyl Carrier Protein**
ACS	**Acyl-CoA synthetase**
AFM	**Atomic Force Microscopy**
AHDB	**Agriculture and Horticulture Development Board**
ANOVA	**Analysis of Variance**
ATP	**Adenosine Triphosphate**
AX	**Arabinoxylan**
BFP	**Bulk Fermentation Process**
CBP	**Chorleywood Breadmaking Process**
CCA	**Canonical Correlation Analysis**
CDP	**Cystidine 5'-diphosphate**
CMP	**Cytidine monophosphate**
CoA	**Acetyl Coenzyme A**
CTP	**Cystidine 5'-triphosphate**
CVA	**Canonical Variates Analysis**
DAG	**Diacylglycerol**
DArT	**Diversity Arrays Technology**
DATEM	**Diacetyl-tartrate ester of monoglyceride**
DDL	**Diluted Dough Liquor**
DEFRA	**Department for Environment Food and Rural Affairs**
DGAT	**Diacylglycerol Acyltransferase**
DGDG	**Digalacosyldiglycerol**
DH	**Double Haploid**
DL	**Dough Liquor**
DPC	**Diacyl-phosphatidylcholine**
ER	**Endoplasmic Reticulum**

ESCA	**Electron Spectroscopy for Chemical Analysis**
ESI	**Electrospray Ionization**
EST	**Expressed Sequence Tags**
FA	**Fatty Acid**
FAD	**Fatty Acid Desaturase**
FAE	**Fatty Acid Elongase**
FAME	**Fatty Acid Methyl Ester**
FATA/B	**Fatty Acyl-ACP Thioesterase A/B**
FFA	**Free Fatty Acid**
G3P	**Glycerol-3-Phosphate**
GC-FID	**Gas Chromatography-Flame Ionization Detector**
GL	**Galactolipid**
GOPOD	**Glucose oxidase peroxidase**
GPAT	**Glycerol-3-phosphate acyltransferase**
GxE	**Genotype x Environment**
Ha	**Hectare**
Het	**Heterozygote**
HFN	**Hagberg Falling Number**
HGCA	**Home Grown Cereals Authority**
HMW	**High Molecular Weight glutenin**
Hom	**Homozygote**
KAS I/II/III	**β-ketoacyl-ACP-synthase I/II/III**
LC-MS/MS	**Liquid Chromatography tandem Mass Spectrometry**
LMW	**Low Molecular Weight glutenin**
LOOH	**Linoleic Acid hydroperoxide**
LPA	**Lysophosphatidic Acid**
LPAAT	**Lysophosphatidic Acid Acyltransferase**
LPC	**Lysophosphatidylcholine**

LPCAT	**Lysophosphatidylcholine acyltransferase**
LPE	**Lysophosphatidylethanolamine**
LPL	**Lysophospholipid**
MAG	**Monoacylglycerol**
MGDG	**Monogalactosyldiglycerol**
MS	**Mass Spectrometer**
MUFA	**Monounsaturated Fatty Acid**
MxH	**Malacca x Hereward**
NABIM	**National Association of British and Irish Millers**
NADPH	**Nicotineamide adenine dinucleotide phosphate**
NCBI	**National Centre for Biological Information**
NDNS	**National Diet and Nutrition Survey**
NIL	**Near Isogenic Line**
NL	**Neutral Lipid**
nsLTP	**non-specific Lipid Transfer Protein**
ONS	**Office for National Statistics**
PA	**Procrustes Analysis**
PA	**Phosphatidic Acid**
PAGE	**Polyacrylamide Gel Electrophoresis**
PAP	**Phosphatidic Acid Phosphatase**
PBI	**Plant Breeding Institute**
PC	**Phosphatidylcholine**
PDAT	**Phospholipid:diacylglycerol acyltransferase**
PDCT	**Phosphatidylcholine:diacylglycerol choline phosphotransferase**
PE	**Phosphatidylethanolamine**
PG	**Phosphatidylglycerol**
PI	**Phosphatidylinostidol**
Pin	**Puroindoline**

PL	**Phospholipid**
PLA1/2	**Phospholipase A1/A2**
PS	**Phosphatidylserine**
PUFA	**Polyunsaturated Fatty Acid**
QTL	**Quantitative Trait Loci**
SEM	**Scanning Electron Microscopy**
SGAP	**Starch Granule Associated Protein**
SL	**Sulfoquinovosyldiacylglycerol**
SSL/CSL	**Stearoyl Lacctylates**
SSR	**Simple Sequence Repeat**
ST	**Surface Tension**
TAG	**Triacylglycerol**
TLC	**Thin Liquid Chromatography**
TQMS	**Triple quadrupole MS**
UDP	**Uridine diphosphate**
WE-AX	**Water Extractable Arabinoxylan**
WGIN	**Wheat Genetic Improvement Network**
WU-AX	**Water Unextractable Arabinoxylan**

Note: Abbreviations for the chemicals and reagents used in this study can be found in the Materials and Methods (Chapter 2.1.2)

Acknowledgements

First and foremost I would like to thank the LORD my God for his endless blessings during these last four years. Your love and grace has helped me through the difficult times and provided me with the strength to complete this chapter of my life.

I would like to express my gratitude and thanks to Professor Peter Shewry (Rothamsted Research-RRes) for giving me the opportunity to do this PhD, as well as for all the time, effort and help he has provided me throughout the last four years. I would like to sincerely thank the supervisory panel, Dr Richard Haslam (RRes) and Professor Pete Wilde (Quadram Institute Bioscience-QIB) for their expertise, guidance and advice over the last four years. Their skills and organization have made this PhD possible. I would like to thank all three for their valuable comments and corrections especially during the thesis writing. I also offer my thanks to the BBSRC CIRC (Crop Improvement Research Club) for funding this PhD.

My sincerest thanks to Dr Irene Gonzalez-Thuillier (RRes) for her patience and guidance while teaching me to use the lipidomics platform, and Dr Louise Salt (QIB) for her training and advice. I would like to thank both of them for their support both in and out of work! I would also like to thank Dr Stephen Powers (RRes) for his guidance and statistical expertise, Dr Simon Griffiths (JIC) for his advice and provision of the MxH DH population and NILs and Dr Luzie Wingen (JIC) for her help and provision of the genetic maps and marker data. I would also like to thank the many others at Rothamsted Research, Quadram Institute and the John Innes Centre who has helped me over the years.

A special thanks to my friends who have made my time both fruitful and enjoyable. Two individuals in particular, Ana Machado and Simone de Chirico, I must specially mention. I could not have asked for better friends and housemates, and I will always cherish the many memories we have together! I also thank Samuel Lee for his prayers and company during the breaks I had in Korea, and Suhyun Park for giving me hope and strength, even in the most difficult of times.

To my family, thank you. Your prayers and support were invaluable. To my grandparents who hold a special place in my heart, my mother in Korea and father in the UK, thank you. My brother Byoung-in, your help and support has been incredible.

Chapter 1 Introduction

1.1 Wheat

1.1.1 A Brief History

Throughout history, humankind has achieved some truly remarkable technological advances which have allowed us to survive, multiply and simply live the current lifestyle that we know, enjoy and take for granted. However as remarkable as the telephone, internet and gunpowder have been on shaping our history, the advances made in crop science have possibly been one of the most unrecognized achievements yet. It could be argued that the development of human society was made possible due to the vast improvements made in agricultural technology. Timely developments such as the introduction of fertilizers and the green revolution have allowed us to sustain the massive rise in human population that has occurred over the last two centuries.

Of the many plants that have been domesticated for human consumption, wheat has arguably had the greatest effect on society, its impact ranging from food production to religious significance. Wheat is grown on over 216 million hectares of land, more than any other crop in the world, and over 620 million tonnes of this crop is produced every year (FAOSTAT, 2014).

1.1.2 The development of wheat as a modern crop

Numerous types of wheat have evolved over approximately 10,000 years of cultivation. Early botanists such as Columella, from the classical period, and 16^{th}-17^{th} century herbalists, divided wheat into two groups. These were *Triticum*, the free-threshing wheats, such as our modern day bread wheat, and *Zea*, the hulled wheats such as einkorn, emmer and spelt.

Wheats belong to the *Poaceae* family (grasses) and occur in diploid, tetraploid and hexaploid form. The wild diploid *T.boeoticum* is the ancestor of domesticated einkorn, *T.monococcum* (AA genome) while tetraploid wheats include wild emmer (*T.dicoccoides*) (AABB) which is the ancestor of domesticated emmer (*T.turgidum* subspecies *dicoccon*). Durum wheat (*T.durum; T.turgidum* subsp. *durum*) mainly used for semolina and pasta was developed by the artificial selection of domesticated emmer wheat strains, which developed into a naked free threshing form. Bread wheat (*T.Aestivum*) and spelt (*T.spelta; T.dicoccum*) are both hexaploid (AABBDD).

By observing genetic relationships between wild and domesticated einkorn and emmer, it has been suggested that the region west of the Diyarbakir in south-eastern Turkey is where cereal domestication first began approximately 10,000 years ago (Heun et al., 1997, Ozkan et al., 2005 and Luo et al., 2007).

Domesticated einkorn and emmer began its dissemination across Asia, Europe and Africa (Figure 1.1). This spread led to gene exchange between various wild and domesticated emmer species together with wild grasses, notably *Aegilops tauschii* (genomes DD) leading to the emergence of hexaploid bread wheat (*T.aestivum*, genomes AABBDD) within the area ranging from Armenia to the south-western coastal areas of the Caspian Sea (Dvorak et al., 1998).

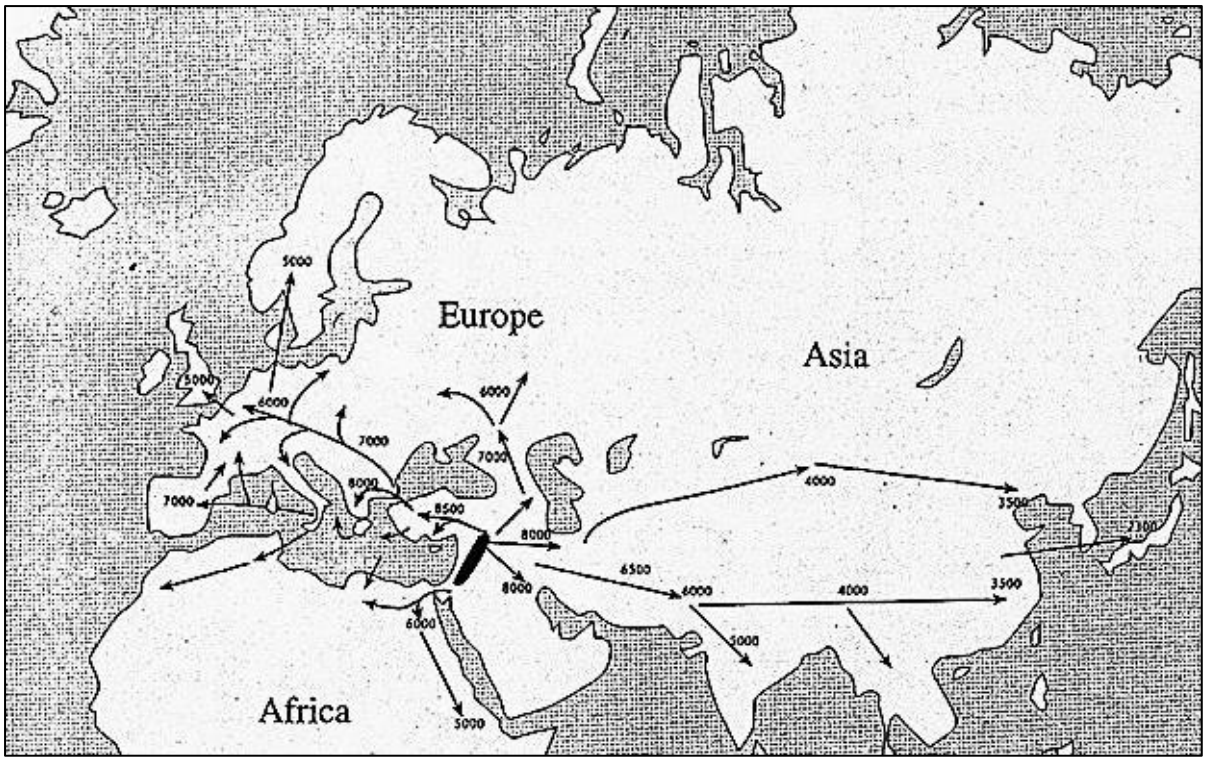


Figure 1.1. The routes of expansion of wheat across Eurasia and Africa. The date of the earliest wheat cultivation in different sites is indicated by years BP (Before Present- Present being 1950) (Taken from Bonjean and Angus, 2011)

Ploidy, the number of sets of chromosomes, can provide advantages to plants as polyploidy brings new genomes into a specie. For bread wheat, the introduction of the DD genome brought with it enhanced cold hardiness and certain distinct morphological features. Ploidy levels can also influence

various plant characteristics, for example, plants at higher levels of ploidy tend to have larger cell sizes, while wheats that exist within one ploidy level are more closely related to each other.

The success of bread wheat has largely been attributed to its adaptability to diverse environments and end use properties. This adaptability and diversity is rather surprising considering the genetic bottlenecks wheat has undergone from recent domestication and polyploidy speciation events (Dubcovsky and Dvorak, 2007). Currently approximately 95% of the wheat crop today is hexaploid bread wheat used for bread, cookies and pastries while the other 5% is made up of tetraploid durum wheat, grown for semolina products such as pasta.

Many of the useful qualities associated with wheat arose from the genetic changes associated with what is known as the domestication syndrome (Hammer, 1984). When wheat ripens, spike shattering occurs which scatters the grain. For agriculture this is generally an undesirable process leading to the loss of spike shattering through domestication, facilitating harvest. Also the tough glumes which tightly enveloped the seed to protect it during seed dispersal were lost, converting hulled grain into free-threshing varieties (Nalam et al., 2006; Jantasuriyarat et al., 2004). Other important traits associated with the wheat domestication syndrome include increased seed size, more erect growth and reduced seed dormancy. Various genes were found to be relevant to these traits such as *GPC-B1*, an early regulator of senescence with pleiotropic effects on grain nutrient content, affecting seed size. A recessive mutation at the Tg (tenacious glume) loci accompanied by the modifying effects of the dominant mutation at the Q locus, and mutations at several other locations are the primary genetic determinants of the free threshing trait (Simons et al., 2006; Uauy et al., 2006). Although many of the genes that played a role in the wheat domestication syndrome have now been investigated, there is still much to uncover. However, it is clear that genetic exchange and variation has played a crucial role in the success of wheat as a modern crop.

Wheat has a remarkably high rate of DNA replacement. In fact, it has been noted that indel polymorphisms (the insertion and deletion of bases in the DNA of an organism) from both chimpanzee and human genomes is less than 4% of the intergenic regions of wheat (Dubcovsky and Dvorak, 2007). There are cases where such genome evolution has led to useful wheat characteristics. For example, vernalisation is highly important in flowering plants where the seed is exposed to cold in order to accelerate the plants flowering process. The need for vernalisation was removed by inserting repetitive elements within the regulatory regions of the wheat *VRN1* and *VRN3* vernalisation genes, and by four large deletions within the VRN first intron (Yan et al., 2004; Fu et al., 2005). The method characterized different VRN-1 and VRN-3 alleles using markers listed in the publication. The markers amplified sequences in two specific regions, the promoter and first intron, where a series of insertions, deletions and duplications account for the different alleles and their function. Interestingly, there are

cases where such deletions have resulted in increased diversity in traits that affect processing properties. For example, deletions of the *Puroindoline A* and *B* genes on the A and B genomes of the plant are responsible for the grain hardness of durum wheat. Also, polymorphism such as the Puroindoline A deletion or the point mutation in Puroindoline B in the hexaploid wheat D genome dramatically affects grain hardness. This allows the classification of hexaploid bread wheat into the cultivars with a hard texture for breadmaking, and those with a soft texture for cakes, pastries, livestock feed and alcohol production, bioethanol and distillation (Giroux et al., 1998). Further information or the puroindoline genes can be found in section 1.6.5.

Various genes are known to control aspects of wheat quality and understanding this is important to underpin the improvement of quality by wheat breeding. This will result in benefits to grain processors and consumers and reduce the cost of importing high quality wheats, which usually account for about 15% of the grain used for milling and food processing.

1.2 Introduction to UK wheat breeding

Over thousands of years of breeding and selection, bread wheat has developed to become one of our primary food sources along with rice and maize. Even the bread wheat of merely 100 years ago differs to modern day wheat, as its physical appearance has been modified by the introduction of dwarfing genes.

In the future, the strains and challenges on global agriculture will increase. The continuous demand to sustain a rising population, the stresses of climate change, disease resistance and the demand of developing economies for a more westernized diet, are just some of the challenges wheat breeders need to address in the 21st century. Fortunately, recent developments in molecular biology, bio-informatics and marker technology has allowed us to learn more about the complex genome of wheat. Now, wheat breeding programmes have been provided with the tools to meet these rising challenges.

1.2.1 Wheat in the United Kingdom

In the UK, wheat is the major arable crop (by area) with an annual planting of approximately 1.9 million hectares. The east of England including East Anglia, the south-east and the east midlands together account for more than 58% of the wheat grown in the UK. The UK produces around 11-18 million tonnes of wheat a year, a figure mainly dependent on climatic factors. Most of the wheat grown in the UK is winter wheat, which is planted in the autumn generally between September and

November, and accounts for more than 95% of the UK grain used by millers. Spring wheat is planted between January-March and tends to have lower yields. The main source of imported wheat in the UK is the European Union from which over 1 million tonnes of wheat is imported, primarily from France and Germany. Imports from non-EU trading partners accounted for 493,897 tonnes of wheat, mainly from North America (Statista).

Wheat production in the UK is usually highly intensive with high levels of nitrogen fertilizer, fungicide and plant growth regulator input. Yields of over 12 tonnes per hectare are not unusual, and the world record wheat yield came from the UK in Northumberland with a yield of 16.52 tonnes per hectare, until it was very recently broken on the 17th February 2017 by a farmer in New Zealand who achieved a yield of 16.791 tonnes/ha. In addition to intensive farming practices, such yields are possible due to the mild UK climate in both winter and summer. The mild winters allow the crop to continue growing during the short days of winter, and the mild summers bring long grain filling periods and minimal exposure to drought.

1.2.2 Brief introduction to breeding in the UK (Yield)

The introduction of agricultural knowledge to northern Europe is believed to have come as late as 5000 BC (Murray, 1970), significantly later than China in 11,500 BC and the Levant in 9500 BC. Wheat is believed to have been introduced into the UK by Neolithic settlers around 2400 BC. However it was only during the time of the Roman Empire that wheat farming really developed and trading opportunities were established.

Over history, wheat yields rose, which was greatly accelerated after the 19th century due to the introduction of improved practices. In the Middle Ages changes in crop rotational practices with better agricultural equipment led to increases in yield. Although the estimate of yield in this period is inaccurate, wheat yields between 1200-1400 AD is believed to have reached as high as 540-605 kg per Ha (Bennett, 1968). With better rotations and increased stocking, yields are thought to have increased to around 750-1000 kg per Ha around the latter half of the 17th century. The 18th century saw considerable increases as arable management improved. Bennett estimates yields in this period reaching 1.7 t/Ha. By 1914, with agricultural intensification and the development of better varieties, yields rose to around 2 tonnes per hectare.

In the latter part of the 19th century entrepreneurial agriculturists practiced selection in wheat populations to produce superior lines. These were multiplied and sold as new varieties. However, these were small scale operations as there was little reward to incentivise their efforts. However some

of these early breeders imported varieties from overseas for commercialization, which would have significant impacts on UK wheat breeding.

The variety Sheriff (1873) started off wheat selection programmes in the UK, being used to develop many new varieties such as Sherriff's Bearded Red, Sheriff's Bearded White, Pringle (1875) and Sherriff's Squarehead (1882). Another variety Biffen was also widespread in the early parts of the 20th century. These newer varieties provided better yields and agronomic traits. Recognising the potential behind wheat breeding, the Plant Breeding Institute (PBI) was developed in 1912, as part of the Cambridge School of Agriculture.

In 1916 the variety Yeoman (Browick x Red Fife) was released. This variety was a significant milestone in UK wheat breeding due to its hard milling grain with high yield potential and superior breadmaking quality. Red Fife was a Canadian variety, more details of which can be found in Chapter 3. The variety Holdfast was a pedigree of Yeoman and White Fife which was released in 1936 with even higher yield potential and good breadmaking quality. Holdfast was an excellent cultivar whose dominance was not really challenged until the release of Maris Widgeon in 1964 over 25 years later.

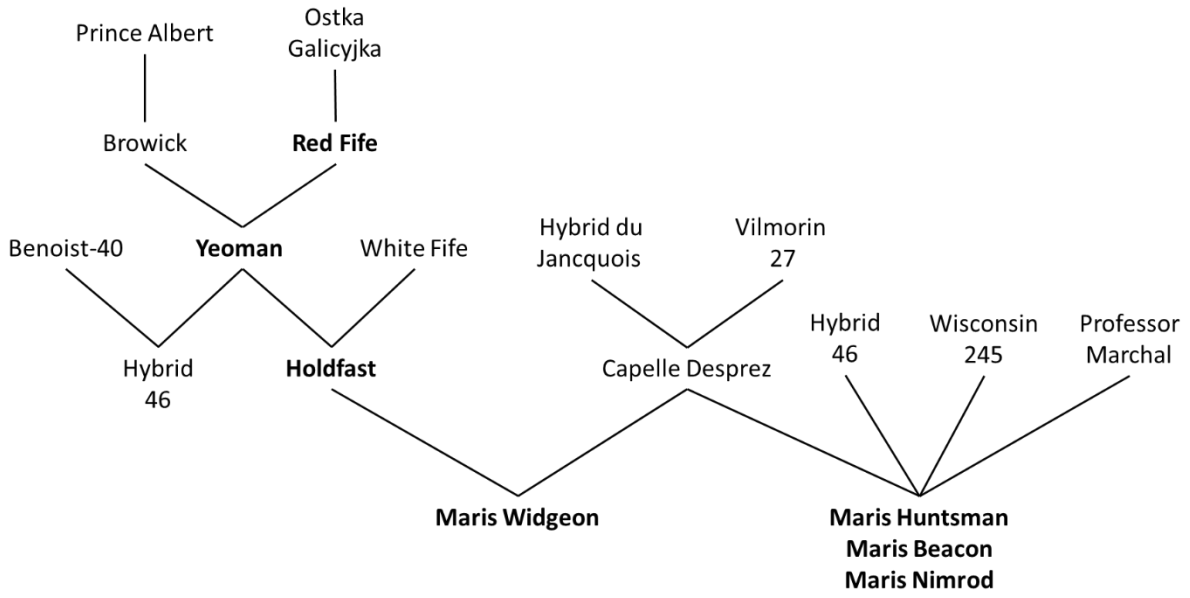


Figure 1.2 The pedigree of selected important wheat varieties in the development of modern wheat cultivars in the UK. Highlighted are the specific varieties described in this thesis.

The next significant milestone came from the introduction of Maris Huntsman, Maris Beacon and Maris Nimrod in the early 1970s. Maris Huntsman, which the PBI released in 1972, promised 20%

higher yields than some other varieties at the time and firmly established the PBI as the most significant wheat breeder for the next 30 years. Maris Huntsman provided excellent yield, as well as good foliar disease resistance, particularly against mildew and yellow rust, which certainly helped its statistics in field trials without fungicide treatment. The pedigree of Maris Huntsman and the other pre-1980 varieties mentioned in this section is displayed in Figure 1.2.

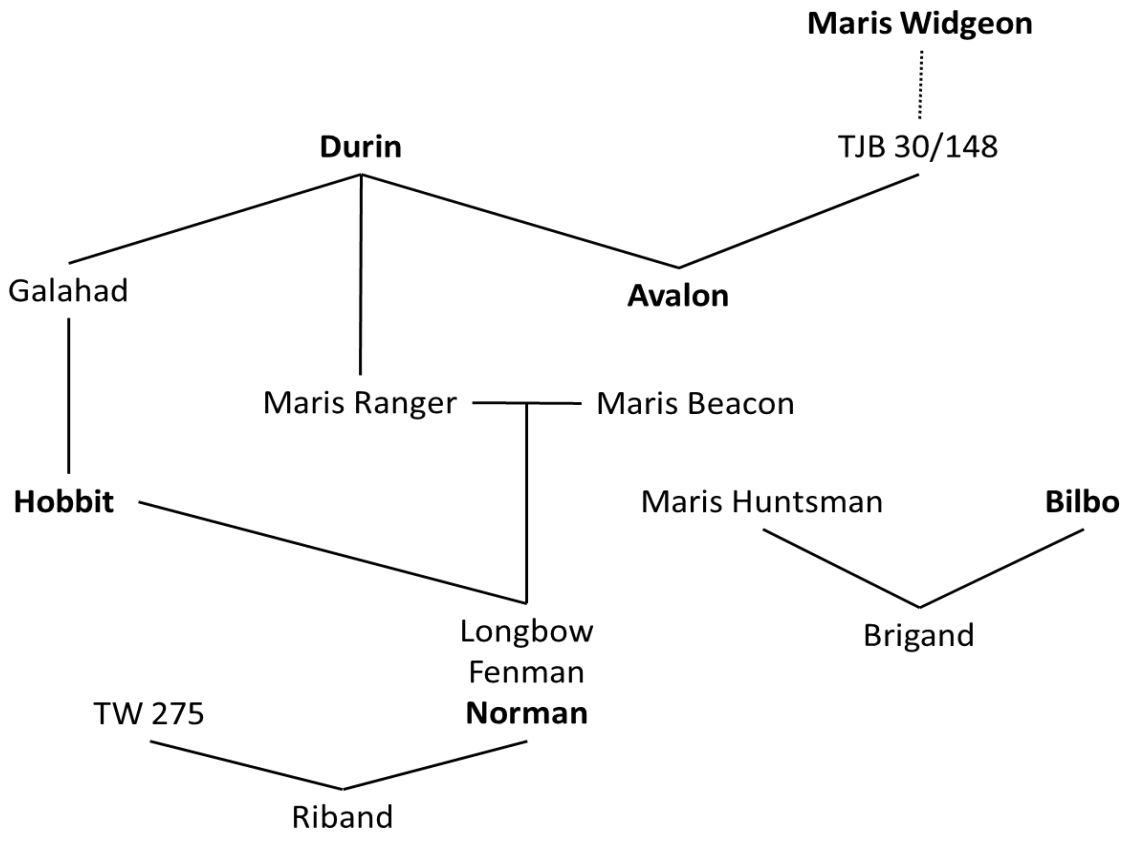


Figure 1.3 Simplified pedigrees of the key semi-dwarf varieties developed by Plant Breeding Institute in the mid-1980s. Notable varieties discussed in the chapter are highlighted in bold.

Relatively safe from commercial pressure, the PBI now had the luxury of developing new germplasm alongside the mainstream breeding programmes. A programme introducing the *Rht* dwarfing genes from US derived Japanese sources was implemented by Dr FGH Lupton. From this programme, with some difficulties, semi-dwarf lines were eventually developed which completely changed the landscape of UK wheat breeding. Initially released in 1977, the semi dwarf lines, via the variety Hobbit soon dominated UK wheat production covering over 80% of the UK acreage by the late 1980s. The varieties Durin and Bilbo alongside Hobbit, contributed enormously to UK wheat breeding in the 1980s and 1990s becoming the foundation for various semi-dwarf wheat breeding programmes. These three parental lines became the source of all dwarfing genes in wheat (all *Rht2*) for the next 20 years.

Although Hobbit only had short term commercial success due to its poor straw strength and the breakdown of its yellow rust resistance, it was introduced around the same time as better fungicides and plant growth regulators were developed. Combining these inputs and new varieties, farmers realized they could achieve yields of 10 t/Ha (Bonjean and Angus, 2011). New PBI varieties soon overcame the agronomic weaknesses of the initial semi dwarf varieties. These included Norman (1981), Longbow (1983) and Galahad (1983) which dominated the feed wheat market in the UK. PBI varieties covered over 80% of the wheat acreage in the late 1980s, including key breadmaking varieties such as Maris Widgeon, Avalon, Bounty, Mercia, Moulin and most notably Hereward. The pedigree of the semi-dwarf varieties described above is displayed in figure 1.3.

The largest market for wheat in the UK is now animal feed. As wheat yields have risen, the proportion of grain needed for bread, biscuits and other human consumption has decreased. Despite this, bread wheat tends to have a longer market life expectancy and attracts a premium price.

1.2.3 Breeding for quality in the UK

From the introduction of the Chorleywood Breadmaking process (CBP) in the UK in the 1960s (further details of which can be found below), the UK breadmaking industry needed very specific requirements. This process could utilize poorer quality wheat with lower protein content, while still providing relatively good quality loaves of bread. This has allowed the UK to use more home grown cereals, as continental European bread making wheat tends to perform less well in CBP conditions, due to their over-strong, high protein phenotype.

The first focused attempt to breed for breadmaking quality in the UK was through the variety Biffen in the early 1900s. This attempted to use Canadian varieties to introduce breadmaking quality to UK wheats, and reduce the UK reliance on imported wheat at the time. The varieties Holdfast and Yeoman were introduced (as described above) to improve breadmaking quality as well as yield. However, much of the selection at this point was done without any firm understanding of quality trait inheritance.

It was only in the late 1970s and early 1980s when associations between high molecular weight (HMW) glutenin subunits (Payne et al., 1981, 1984) and breadmaking quality were made. With the development of sodium dodecyl sulphate (SDS-PAGE) polyacrylamide gel electrophoresis, it was possible to increase the selection efficiency for breadmaking quality varieties in the lab in a way which was impossible before. Further details on HMW-glutenin subunits, their identification and their impacts on breadmaking quality are given below.

The major determinants of breadmaking quality in most varieties are the HMW subunits, with some quality-related alleles being traced all the way back to Red Fife. The first breadmaking variety to obtain a large market share, without possessing good quality HMW-glutenin subunits was Mercia, introduced in 1986. This was followed by Hereward in 1991 and Malacca in 1999. Due to the conservative nature of major UK bread businesses, there tends to be a single dominant variety grown in the UK at any one time.

All the cultivars discussed in this section have some relevance to the varieties used in this thesis. Understanding their pedigree and origin may shed more light on the reasons why certain results were obtained in this study.

1.3 The Wheat Grain

The wheat kernel is a single seeded fruit (caryopsis) which is complex in structure, consisting of several discrete tissues. The caryopsis is ovoid in shape with a longitudinal crease down the centre. The length of the kernel will vary depending on the type of wheat and growth conditions, but generally spans between 4-8 mm.

1.3.1 Pericarp

The outmost layer, the pericarp is around 50 µm thick, surrounds the entire seed and is composed of several layers. From the outside to inside, these layers include the epidermis, hypodermis and layers of cells termed cross cells and tube cells. Firmly joined to the layer of tube cells, the last layer of the pericarp is the seed coat. In white wheat, the seed coat is made up of two layers, a thick outer cuticle, and a thin inner cuticle. The seed coat or testa is around 5-8 µm thick. Beneath the seed coat is the hyaline layer or nucellar epidermis which is around 7 µm thick.

The pericarp makes up round 5% of the wheat kernel and is made up of approximately 6% protein, 2 % ash and 0.5% fat, with the remainder being non-starch polysaccharides. Millers term the outer pericarp (epidermis) as beeswing due to its pale membranous appearance after isolation. The inner portion of the outer pericarp consists of the remnants of thin walled cells. The inner pericarp is made up of cross cell, tube cells and intermediate cells. The cross cells are long and cylindrical being around 125 x 20 µm. They are placed perpendicular to the long axis of the kernel and are tightly packed together with little or no intercellular space. The tube cells are about the same size and have

their long axis parallel to the long axis of the kernel. These unlike cross cells are not tightly packed together and therefore have plentiful intercellular space.

1.3.2 Aleurone Layer

Beneath the seed coat is the starch-rich endosperm from which white flour is produced. The endosperm consists of the outer aleurone layer and the starchy endosperm, with the aleurone layer being tightly bound to the nucellar epidermis portion of the seed coat. The aleurone layer is generally one layer thick and completely surrounds the kernel, covering both the starchy endosperm and the germ. The aleurone cells covering the starchy endosperm are thick-walled, essentially cuboid, and free of starch at maturity. The average cell is between 20-75µm in diameter.

The aleurone layer is considered to be part of the bran by millers. The aleurone cell walls are 3-4 µm thick and cell wall sugars account for about 35-40% of the dry weight (Barron et al., 2007) The aleurone cell walls are composed of 29% β-glucan, 65% arabinoxylan and only 2% each of cellulose and glucomannan (Bacic and Stone, 1981). The aleurone layer stores some enzymes and is rich in ash, protein, total phosphorus, phytate phosphorous, and lipids. There is a higher concentration of niacin, thiamine and riboflavin in the aleurone than in other parts of the bran. The aleurone layer surrounding the germ is thin walled. These cells are around 13µm thick which is around a third of the thickness of the aleurone cells surrounding the starchy endosperm.

In total, the bran making up the pericarp, seed coat and aleurone makes up over 8% of the wheat kernel weight, encasing the endosperm and parts of the germ. The bran provides protection for the wheat grain, and ensures the seed will not grow immediately after harvest due to their waterproof properties.

1.3.3 Starchy Endosperm

The starchy endosperm is composed of three types of cells that vary in size, shape and location within the kernel. These are the peripheral, prismatic and central cells. The peripheral starchy endosperm cells are the first row of cells inside the aleurone layer. The prismatic cells are elongated, extend inwards and are about 150x50x50µm in size. The central starchy endosperm cells are more irregular in size and shape than the other cells.

The endosperm makes up 80-85% of the weight of the wheat kernel, containing mostly starch and protein. The storage proteins of wheat, the gliadins, glutenins, albumins and globulins make up about

10-14% of the weight of the wheat kernel. Further details on wheat proteins can be found below (Section 1.6).

The wheat endosperm cell walls account for approximately 2-3% of the dry weight of the grain and consist mainly of arabinoxylan (AX) (70%) and (1-3, 1-4)-β-D-glucan (β-glucan) (20%). Also present are small amounts of cellulose (2%) and glucomannan (7%). Arabinoxylans (also called pentosans) can be divided into two classes, water extractable arabinoxylan (WE-AX) and water unextractable arabinoxylan (WU-AX). Starch comprises of approximately 64-75% of the milled endosperm and occurs in the form of large lenticular A granules and the small spherical B granules. Further details on wheat starch can be found below.

1.3.4 The Embryo (Germ)

The germ or embryo is on the lower end of the wheat kernel and the opposite end of the crease. The germ consists of the embryonic axis (rudimentary radicle or root and plumule or shoot) and the scutellum which functions as the storage organ (Grundas, 2003). The scutellum is attached to the plumule which forms the shoot when the seed germinates.

The germ makes up 2.5-3% of the wheat kernel and is rich in proteins (~25%) and lipid (8-13%). The wheat germ has high levels of essential vitamins such as vitamin E (Fulcher et al., 1972), vitamin B and various enzymes. Due to this, there is a demand for the wheat germ which is sometimes sold separately.

The lipid content of the germ is dominated by non-polar lipids which make up 79-85% of the total germ lipid, with low levels of phospholipids and even lower levels of glycolipids (Hargin & Morrison, 1980). The non-polar lipids are typical of spherosome lipids such as TAG and FFA; the phospholipids include PI, PG while the galactolipid DGDG can also be found. There is a high proportion of unsaturated fatty acids of which linoleic acid C18:2 is the most abundant (Mattern, 1991). Further details on the lipids can be found in Chapter 1.10.

1.3.5 Wheat Flour and Milling

The milling of the wheat grain will vary depending on what kind of end product is desired. For general purpose white bread, millers will remove the bran and germ and extract as much of the endosperm as possible for white flour. Bran is generally undesirable due to its colour which contaminates the white flour, and the germ due to its high fat content which reduces the flour keeping quality.

High extraction flours contain around 85% of the original grain, with some of the bran and germ being removed. Wholemeal flour is made from the whole wheat grain. Due to the nutritional value of the components outside the starchy endosperm, wholemeal and high extraction flours are more nutritionally beneficial.

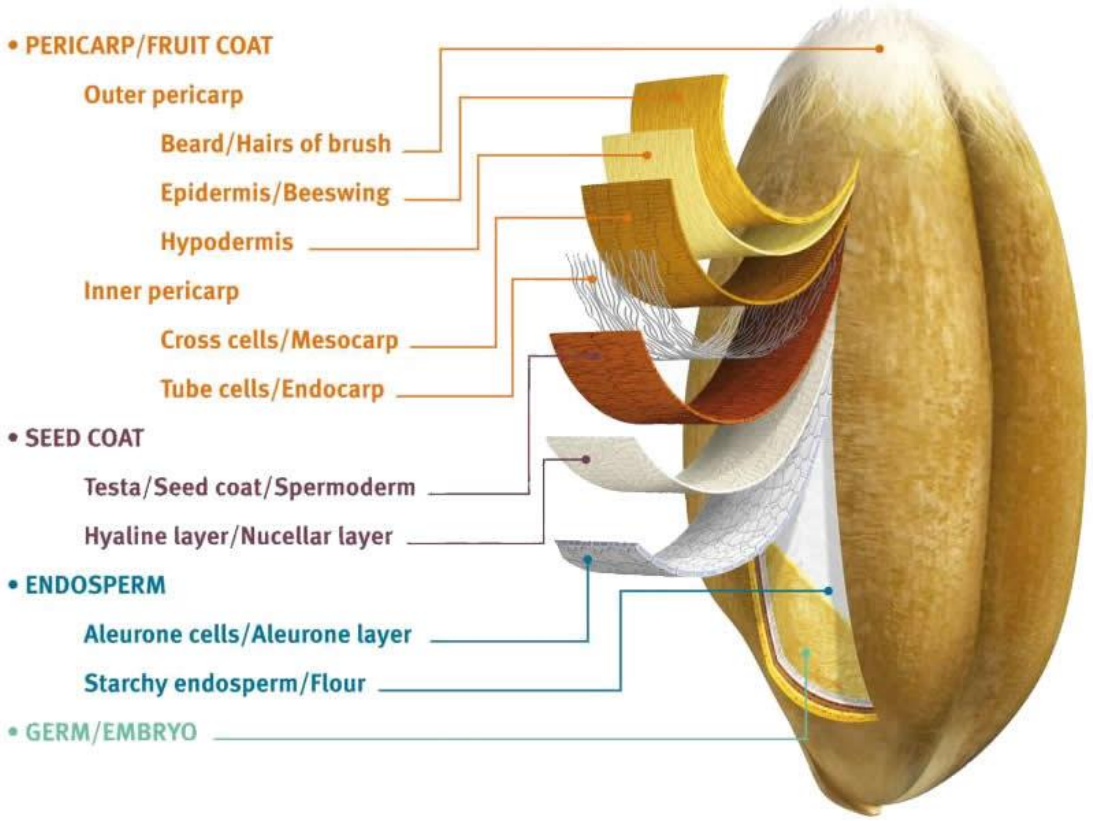


Figure 1.4 Structure of wheat kernel. Diagram shows the Pericarp, Seed Coat, Endosperm, Germ, and its components. Image taken from the Nabim website (http://www.nabim.org.uk/wheat-structure)

1.4 The significance of improving wheat quality

Although wheat is an excellent system to explore a range of natural genetic variation, its key importance comes from its economic significance. The global wheat market is vast. Wheat is responsible for one fifth of the calories consumed worldwide, and of the many wheat products that exist; bread is by far the most prevalent. Being the second largest industry in the UK food sector and a worldwide staple, its economic value is clear. The UK bread market alone is worth £3.4 billion per

annum and sells just under 4 billion units a year. This corresponds to nearly 12 million loaves sold every single day (www.bakersfederation.org.uk, 2014)

Only 25% of the wheat produced in the UK comprises Nabim (National Association of British and Irish Millers) group 1 and 2 varieties, suitable for breadmaking. Although class 1 breadmaking wheat is produced in much smaller quantities than the lower quality feed wheats (which make up 56% of the UK wheat crop), it attracts a premium and has a longer market life expectancy.

Due to this, breeders continually develop Nabim group 1 varieties and submit them for inclusion on the AHDB (Agriculture and Horticulture Development Board) recommended list. However, to be successful they require high protein content and good resistance to the most common diseases such as fusarium, septoria, mildew and rust. Additionally, they must have high first and second crop yields in various areas of the UK and most importantly show excellent breadmaking quality during their commercial trials.

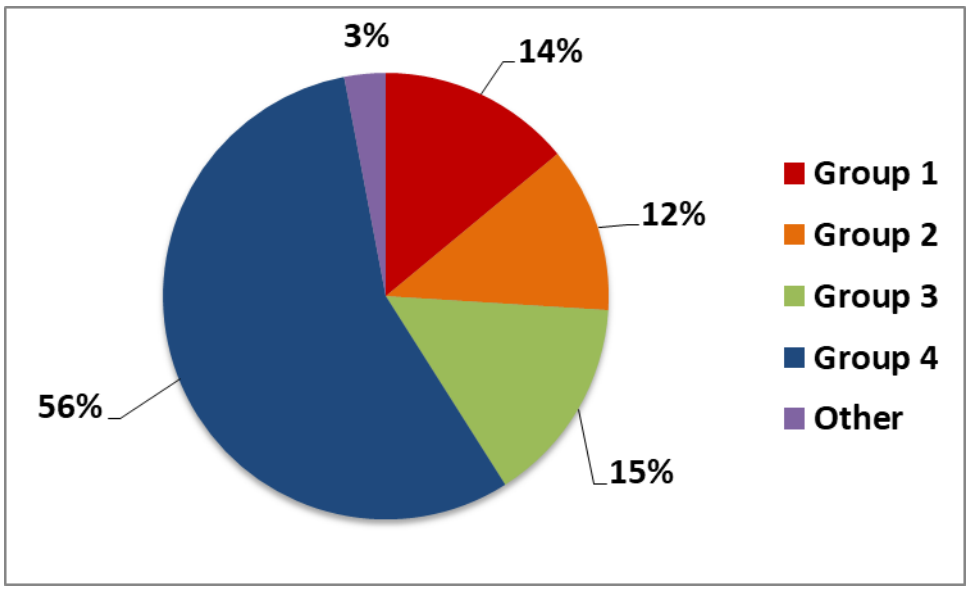


Figure 1.5 2014 UK wheat crop by end-use category. Group 1 & 2 indicate breadmaking wheat with Group 1 being consistent for milling and baking performance. Group 2 have breadmaking potential but have shown some performance variability. Group 3 is for soft varieties for biscuits and cakes. Group 4 wheat varieties are both hard and soft wheats used mainly for animal feed.

Consequently significant research has been carried out on the various components that determine breadmaking quality. However, breadmaking quality is a complex trait determined by a number of factors, most notably end use parameters such as dough strength and consistency, loaf volume and bread crumb structure.

With such large volumes of bread being produced and consumed in the UK on a daily basis, improvements in bread quality can have significant impacts on human health. Analysis of the UK Government's National Diet and Nutrition Survey (NDNS) in 2012 suggested that bread contributes to over 10 % of the adult daily intake of protein, thiamine, niacin, folate, iron, zinc, copper and magnesium, and around 20% of fibre and calcium intake, and over 25% of manganese intake in adults. Improving the quality of bread, can therefore help target nutritional deficiencies. As a major staple in our diets, small changes in bread quality could make large differences to the health of the general population.

However, bread consumption has been steadily falling over the last few decades with current average consumption being around 2-3 slices of bread a day. Apart from changes in public demand, there has been an increasing misconception about bread by the general public. This is seen very frequently in the UK where mass produced bread through the Chorleywood breadmaking process has become prevalent since the 1960s. Misconceptions include statements such as "mass produced bread is nutritionally different to home baked or artisan bread", "bread is fattening" and "white bread is bad for health" (FabFlour UK). The increase in public demand for healthier food is reflected on some of these statements. Since 1974 the consumption of white bread has dropped by 75% while the consumption of brown and wholemeal bread has risen by 85% (DEFRA/ONS). Factory produced bread in particular has received a lot of criticism for its 'bland flavour' and 'poor texture', however more criticism has been levied on the additional components used in the breadmaking industry, such as surfactants and enzymes (The Independent, 2006).

1.5 Breadmaking and Quality

As stated by Cauvain in his introductory chapter in the book "Breadmaking," quality is a difficult trait to pinpoint, simply due to the fact that quality is a very personal term. It can mean different things for each individual, changed by perceptions as much as our own sense of taste (Cauvain & Young, 2012).

Furthermore, with changing times and public perceptions, there have also been changes in the definition of quality, with different traits for each type of bread. For example, loaf volume is certainly not relevant for Middle Eastern flat breads, while the yellow pigmentation in durum wheat flour which is highly desirable in pasta products is undesirable in our standard white loaves.

1.5.1 Historical perceptions of bread quality in the West

Over history public perceptions have also affected our views of quality. For generations, white bread was the highly desired form of bread. Pure white loaves were first made possible by the improvements made in milling technology which brought about the fractionation of wheat grains. This originated from what was known as the French Process, where repeated milling and sifting allowed finer white flour to be produced than ever before. This concept was taken further by Austrian and Hungarian millers who created the *mouture en infini* (milling to infinity), a complex process involving more than 80 intermediate product streams that were manually conveyed between operations by scores of men. This process would yield over 10 products of various qualities with the finest being a flour whiter than was ever seen previously. Such efforts were driven by public demand at the time when white bread was a symbol of purity and hygiene for the wealthy upper and rising middle classes of European cities during the industrial revolution. Wholemeal bread or even bread made from old varieties was shunned by the upper classes and became commonly known as the bread of the poor. The Victorian phrase, "even beggars refuse rye bread' was born from this type of public perception.

This demand did not die out, and when white bread became more available in the 1880s and 1900s, demand for industrial white bread exploded. In the book by Aaron Bobrow-Strain, "White Bread: A social history of the store –bought loaf" he claims that industrial bread was a "perfectly shaped, perfectly clean, perfectly white spectacle of modern progress in an age where widespread anxiety about germs, gender roles and immigrants were rife."

The dominance of factory produced white bread continued. In the US, synthetic enrichment campaigns, fortifying the nutritionally deficient white breads with vitamins and minerals, championed industrial white loaves for reasons of national security, which gave industrial white bread another added appeal during World War 2. This nationalist pride associated with white bread contributed to the anti-communist movement and witch-hunting of the Red Scare in the early years of the cold war, giving factory produced white bread added appeal.

However, by the 1970s anti-war activists and ecologists started to campaign against white industrial bread as a "plastic" bread that was a "lethal symbol of militaristic hubris and cultural conformity" (Bobrow-Strain, 2012). From this period, public opinions slowly started shifting around the western world. The nutritional value of wholemeal and brown bread became more recognized. In the US, the rise of new elite niche market breads, along with the publicised nutritional deficiencies of the industrial white bread were significant factors on the decline of white bread in the 21st century. In the US, white industrial loaves gained another racial association, becoming associated with the infamous 'white trash icon.' In 2011 for the first time in US history wholemeal bread outsold white bread.

This demand for better quality bread has continued along with an increasing negative perception of industrially produced bread products. Rising claims of gluten intolerance, misconceptions on industrial additives, criticism on white bread's poor bland flavour and texture has resulted in increased demand for wholemeal as well as bread made from ancient or alternative cereals such as einkorn, spelt and rye. With such changes in public perception and demand, there is an ever increasing challenge on industry to improve breadmaking quality.

In this thesis, quality may be somewhat more simply defined. For the breadmaking industry, quality is a 'fitness for purpose' which equates to 'a product you can make consistently that will always bring back customers.' For industry, there are various quantifiable, measurable quality traits, such as loaf volume, height and gas cell number.

For the breadmaking process it is ultimately most important to be able to measure wheat quality and to predict the likely breadmaking performance (Cauvain and Young, 2009). While developing tools and decades of research have brought large improvements, that goal is yet to be achieved.

1.5.2 Breadmaking: an overview

The main components of industrial breadmaking are the same as bread baking done on a small scale at home, being a mix of flour, yeast, salt and water. The flour is hydrated, mixed with the other ingredients which bring the gluten proteins together to form a network. The aeration of the dough is initiated during mixing as the gluten network traps and retains air bubbles. The rheology of the dough is a crucial factor in controlling the initial development of the aerated structure. Overly strong dough will inhibit bubble expansion, while weak doughs will result in a collapse of the dough structure.

During the proving stage the dough is incubated at a warm temperature which allows the yeast to ferment sugars and release carbon dioxide. The CO_2 enters the air bubbles to form gas cells within the dough structure which expand throughout the dough. After proving, the dough is baked, usually around 230˚C (450 F gas mark 8), which denatures the gluten protein and gelatinizes the starch to form a solid matrix with the aerated crumb structure commonly associated with all forms of leavened bread.

1.5.3 CBP (Chorleywood Breadmaking Process)

The CBP was developed in 1961 by the British Baking Industries Research Association based at Chorleywood and is used to make most of the bread consumed in the UK (80% in 2009). Before the development of the CBP process, the bulk fermentation process (BFP) was commonly used in

industry. BFP is a traditional method, where ingredients are mixed together to form a dough and left to ferment for up to three hours. During fermentation the dough changes from a short dense mass into elastic dough.

Compared to bulk fermentation, CBP was able to produce bread within a shorter time, using wheat with lower protein content, which allowed greater use of home grown wheats. The CBP can produce the final sliced and packaged form of bread from flour in approximately three hours and a half. This was possible through the significant reduction in fermentation time, which was decreased from over three hours to less than one. This is achieved through the addition of ascorbic acid (Vitamin C), emulsifiers, yeast, enzymes and intense mechanical kneading by high speed mixers. The high speed mixing generates a high temperature which is cooled through a cooling jacket. Air pressure in the mixer headspace is controlled in order to produce the desired gas bubble size and number. Typical operating regimes are pressure followed by vacuum and atmospheric followed by vacuum. This pressure control during the mixing process is crucial as it affects the crumb texture in the final loaf.

The dough is divided in to individual pieces and left to proof for around 5-8 minutes, which is the intermediate proofing stage. Each dough piece is then moulded into tins and moved to a temperature and humidity controlled proofing chamber where it sits for 40-50 minutes, which is the second proofing stage. At this point the dough is baked for around 17-25 minutes at 230˚C, de-panned and then placed in a cooler for around 2 hours. This is the final loaf which can be sliced and packaged as required.

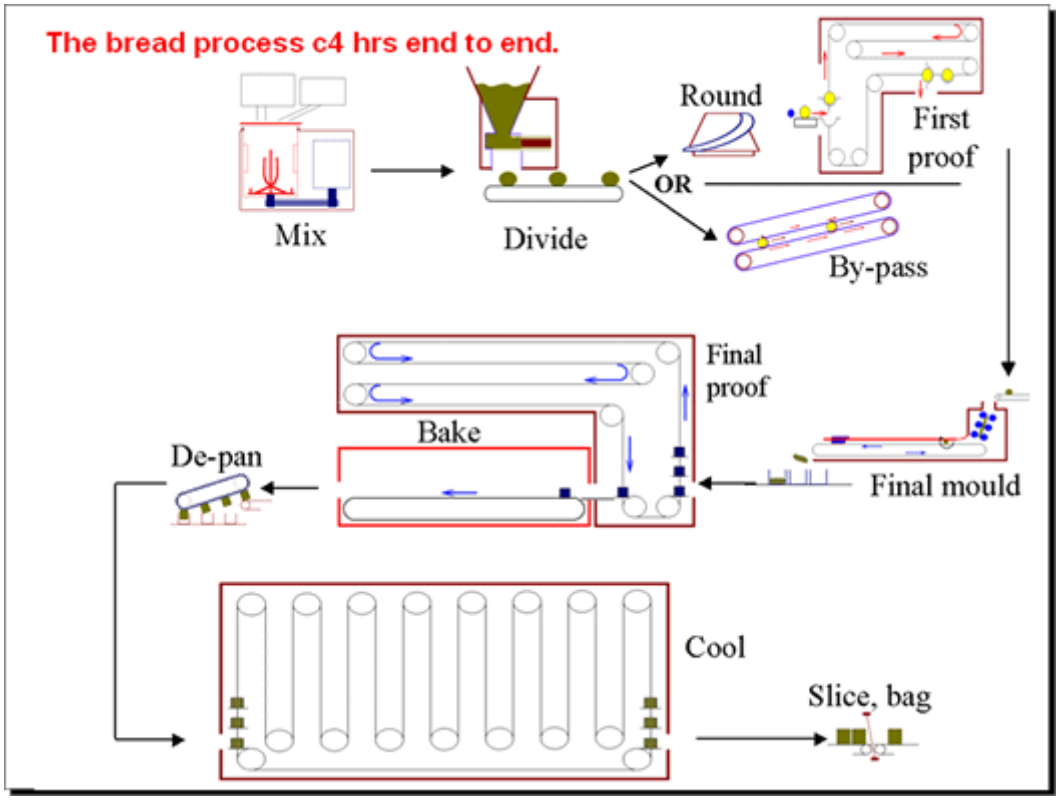


Figure 1.6 Diagram displaying the different steps involved in the Chorleywood breadmaking process.

CBP is not exclusive to the UK; it is used for over 80% of the factory produced bread in Australia, New Zealand and India. Contrary to public belief, CBP is not exclusive to white bread or industrial bread. Many smaller bakers also use the CBP process to mix their dough which they later process by hand. Many speciality, crusty and organic loaves are also actually produced through the CBP process.

Another breadmaking method mentioned in this study is the spiral white breadmaking procedure. Spiral white is the name for a common breadmaking process using a spiral mixer and white flour. Spiral mixers gently mix bread dough to allow it to develop the proper gluten structure without overworking the dough. This is achieved through the bowl which rotates as the spiral hook is spinning and kneading the dough. The spiral hook makes it so only a portion of the whole dough mass is kneaded at any given point in time, which keeps friction heat low, providing a more homogenous mix.

1.5.4 Importance of various stages of breadmaking on final loaf quality

Each stage of the breadmaking process can have a significant impact on breadmaking quality. With the amount of mechanical mixing affecting the strength of the gluten matrix, it has even been claimed

that in dough making processes such as the Chorleywood breadmaking process, 'about 90% of final bread quality is decided by what bakers chose to do in the mixer'(Cauvain and Young, 2002, 2007). This includes the choice of materials and formulation as well as how mixing will proceed.

Various molecular changes and interactions in flour components can play a significant role on breadmaking quality. Even components such as water and air can play a crucial role as water quantity is critical in providing a suitable dough rheology as well as underpinning essential molecular changes which occur during the mixing and proving stages of breadmaking.

However this thesis will focus on the molecular interactions behind the key components of wheat flour, all of which play a crucial role on breadmaking quality. Each of the components will be discussed and their importance on the breadmaking system will be laid out. The various factors playing a crucial role include proteins, starch, lipids, enzymes as well as the genetic control behind some of these mechanisms. Exogenous additives will be mentioned but discussed in relatively little detail.

1.6 Wheat Proteins

1.6.1 The classification of wheat proteins

Wheat proteins were first described in 1745, when Becarri described the isolation of gluten by washing wheat flour with dilute salt solutions (Becarri, 1745). 150 years later, Osborne introduced a more detailed protein extraction procedure which gave rise to what is now known as the "Osborne fractionation" (Osborne, 1924). Based on their solubility, proteins were sequentially extracted in four major groups: albumins (soluble in water), globulins (soluble in salt solutions), prolamins (soluble in 70-90% ethanol) and glutelins (soluble in dilute acids or alkali). The gluten proteins fall into two of these categories, the alcohol soluble gliadins being defined as prolamins and the alcohol-insoluble glutenins as glutelins.

The grain proteins of wheat comprise structural, metabolic, protective and storage proteins (Shewry, 2003). The structural, metabolic and protective proteins comprise water soluble (albumin, globulin) and water insoluble proteins which together account for around 30% of wheat flour proteins. Of these, non-membrane amphiphilic proteins have been reported to have effects on grain hardness and dough rheological properties (Dubreil et al., 1998). The storage proteins (gluten) are defined as prolamins

due to their solubility in aqueous alcohol, either in the native state or as reduced subunits, and their high contents of proline and amide nitrogen (from glutamine).

1.6.2 The Gluten Proteins

Washing wheat flour with water removes most of the starch, non-starchy polysaccharides and water-soluble constituents. The remaining viscoelastic and mainly proteinaceous mass is known as gluten.

Gluten is comprised of 80-85% protein, 5% lipid and some starch and non-starch carbohydrates (Wieser, 2007). Gluten is crucial to the formation of dough as it confers gas retention, good loaf volume and breadmaking quality. Gluten proteins are deposited in the mature wheat grain starchy endosperm where they form a continuous matrix around the starch granules.

Gluten proteins are classically divided into gliadins and glutenins. Gliadins are monomeric proteins consisting of single chain polypeptides which contribute to the viscosity and extensibility of dough (Wieser, 2007). They are a mixture of polymorphic proteins which are soluble in 70% alcohol and make up about half of the gluten proteins. They range from 30-80 kDa in mass and are divided into 4 groups (α, β, γ, ω) depending on their mobility in a low pH polyacrylamide gel. Gliadins are capable of associating with other gliadins and glutenins, mainly through hydrogen bonds (Veraverbeke and Delcour, 2002).

Glutenins on the other hand primarily contribute to the elasticity of gluten and dough. (MacRitchie, 1992; Wieser, 2007) The glutenin polymers are extractable in dilute acetic acid or alkalis, but if the inter-chain disulphide bonds are reduced, the component subunits become soluble in aqueous alcohol (similar to gliadins). They also have similar amino acid compositions to gliadins, with high levels of glutamine and proline and low levels of charged amino acids (Goesaert et al., 2005). Glutenins are comprised of subunits which can be separated into HMW and a LMW groups by SDS-PAGE after the disulphide bonds are reduced (Shewry and Miflin, 1986).

However, as the components in gliadin and glutenin fractions are related, it is also possible to classify them into three groups based on their genetic and structural relationships. These groups are the sulphur-rich, sulphur-poor and high molecular weight (HMW) prolamins. The sulphur-rich prolamins include the monomeric α-type gliadins, γ-type gliadins and polymeric low molecular weight (LMW) B and C-type subunits of glutenin. The sulphur poor prolamins contain the ω-gliadins and D-type LMW subunits and the polymeric HMW subunits of glutenins (Shewry and Halford, 2002).

The gliadins and glutenin subunits are encoded by genes located at several multigenic loci on the homoeologous group 1 and group 6 chromosomes. The genes encoding the high molecular weight

glutenin subunits occur on the long arm of chromosomes 1A, 1B and 1D and those encoding the low molecular weight glutenin subunits, ω and γ-gliadins occur on the short arm of chromosomes 6A, 6B and 6D (Payne, 1987).

Wheat prolamins are synthesized on ribosomes located on the rough endoplasmic reticulum. They pass via the translocation machinery into the lumen, with the N-terminal signal peptide being cleaved (Levanony et al., 1992). Within the lumen, protein folding and disulphide bond formation occurs without any further post-translational modifications (Shewry, 2003).

1.6.3 HMW glutenin subunits and their role in breadmaking

Wheat proteins are the most important determinants of breadmaking quality. Among these, the HMW glutenin subunits are particularly important as they account for 12% of the total grain protein content (Seilmeier et al., 1991; Halford et al., 1992; Nicolas, 1997), but 45-70% of the variation in baking performance within European wheats (Payne et al., 1987a, 1987b ; Branlard and Dardeyet, 2001). Furthermore, correlations between breadmaking quality and the amounts of HMW polymers enriched in HMW glutenin subunits have long been established (Huebner and Wall, 1976; Field et al, 1983).

The HMW subunits are encoded by the *Glu-1* loci, which are present on the long arms of the group 1 chromosomes (chromosomes 1A, 1B and 1D of hexaploid bread wheat). Each of these loci consist of two tightly linked genes, encoding one x type and one y type subunit, which differ in their amino acid sequences. However, although all loci encode 6 HMW subunit genes, commercial cultivars contain only three, four or five subunits due to the effects of gene silencing. Only the genes 1Bx, 1Dx and 1Dy are expressed in all cultivars while 1Ax and/or 1By are only expressed in certain cultivars. The gene 1Ay is never present in cultivated hexaploid bread wheat, but can be present in wild and cultivated diploid and tetraploid species (Waines and Payne, 1987; Levy et al, 1988; Margiotta et al., 1998). HMW subunits are generally numbered in order of their mobility on SDS-PAGE e.g. 1Dx2, 1Dx5 etc (Figure 1.6). As the x and y HMW subunit genes are tightly linked, the allelic forms of x and y type subunits are inherited together as ‘allelic pairs’.

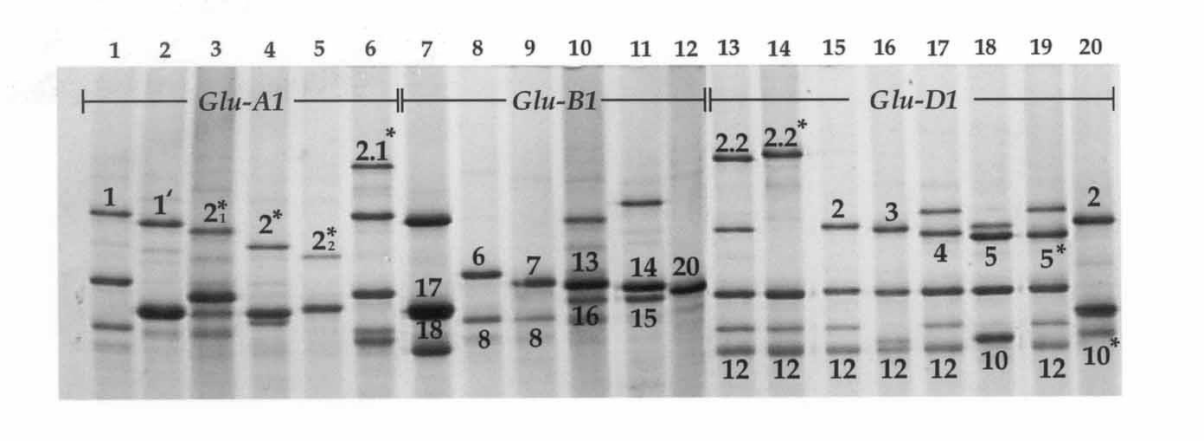


Figure 1.7 SDS-PAGE of HMW subunits from a range of genotypes of wheat. The allelic variation in the mobilities of proteins encoded by the Glu-A1, Glu-B1 and Glu-D1 loci are shown. The numbering is based on the scheme by Payne and Lawrence (1983) with subsequent modifications by other workers. Taken from Shewry et al (2003b) with permission.

The specific allelic forms of HMW subunits may also be correlated with quality parameters. On chromosome 1D, the subunit pair 1Dx5 + 1Dy10 is associated with the highest breadmaking quality, while other allelic pairs such as 1Dx2 + 1Dy12, 1Dx3 + 1Dy12 and 1Dx4 + 1Dy12 are associated with poor quality. For chromosome 1B, the allelic pair 1Bx17 +1By18 is superior. The presence of the allelic subunits 1Ax1 or 1Ax2 resulted in an increase in the proportion of HMW glutenin subunits. This led to the conclusion that an increase in HMW glutenin subunits caused improvements in breadmaking quality.

Apart from such qualitative variation among the subunits, HMW glutenins can also affect breadmaking quality by exerting quantitative effects. By comparing the SDS-PAGE patterns of HMW subunits from different cultivars, it has been shown that some allelic forms of subunits such as 1Bx and 1By are expressed at higher levels than others. Some of these highly expressed subunits are associated with improved breadmaking quality, in particular the highly expressed form of 1Bx7 is associated with increased dough strength (Vawser and Cornish, 2004; Ragupathy et al., 2008).

			Soluble Proteins		Gluten Proteins		
	Wheat	Flour	Albumin	Globulin	Gliadin	Glutenin	Residue Protein
Proportion %		100	3-5	6-10	40-50	30-40	6-10
Amino Acid							
Asp	4.7	3.7	5.8	7.0	1.9	2.7	4.2
Thr	2.4	2.4	3.1	3.3	1.5	2.4	2.7
Ser	4.2	4.4	4.5	4.8	3.8	4.7	4.8
Glu	30.3	34.7	22.6	15.5	41.1	34.2	31.4
Pro	10.1	11.8	8.9	5.0	14.3	10.7	9.3
Gly	3.8	3.4	3.6	4.9	1.5	4.2	5.0
Ala	3.1	2.6	4.3	4.9	1.5	2.3	3.0
Val	3.6	3.4	4.7	4.6	2.7	3.2	3.6
Met	1.2	1.3	1.8	1.7	1.0	1.3	1.3
Cys	2.8	2.8	6.2	5.4	2.7	2.2	2.1
Ile	3.0	3.1	3.0	3.2	3.2	2.7	2.8
Leu	6.3	6.6	6.8	6.8	6.1	6.2	6.8
Tyr	2.7	2.8	3.4	2.9	2.2	3.4	2.8
Phe	4.6	4.8	4.0	3.5	6.0	4.1	3.8
His	2.0	1.9	2.0	2.6	1.6	1.7	1.8
Lys	2.3	1.9	3.2	5.9	0.5	1.5	2.4
Arg	4.0	3.1	5.1	8.3	1.9	3.0	3.2
Trp	1.5	1.5	1.1	1.1	0.7	2.2	2.3
NH3	3.5	3.9	2.5	1.9	4.7	3.8	3.5

Table 1.1 Proportions and amino acid compositions (g/100 g of protein of the major protein fraction of flour.

1.6.4 Puroindolines

Puroindolines are proteins with a molecular mass of around 13000 M_r and occur in two major isoforms, Pin-A and Pin-B, which exhibit approximately 55% sequence homology (Gautier et al., 1994; Morris et al., 1994). Whereas both isoforms are present in the starchy endosperm, only Pin-B is found in the aleurone layer. Puroindolines have a unique tryptophan rich domain which is comprised of five tryptophan residues in Pin A (Trp-Arg-Trp-Lys-Trp-Trp-Lys) and three residues in Pin B (Trp-Pro-Thr-Lys-Trp-Trp-Lys) (Douliez et al., 2000).

Puroindolines play a role in breadmaking quality by determining grain hardness, which may be related to their lipid binding properties. Hardness is determined largely by the binding of proteins to the starch granule surface and the Pins may influence this binding strength (Day L et al., 2006, Chichti, E

et al., 2015). On the other hand, the reduction of adhesive strength results in softer kernels (Oda and Schofield., 1997; Morris et al., 1994). Hard wheats are required for breadmaking as milling results in higher levels of starch damage, which determines water absorption and consequently breadmaking performance.

Puroindolines may also play a role in determining the crumb structure of bread. The crumb structure of bread is determined by the network of gas cells formed in the dough which are incorporated during the mixing step and expanded from fermentation during proving. Gas will become transported from small to large bubbles, a phenomenon known as disproportionation, as well as coalesce to form a fragile foam-like structure in the risen dough. These gas cell walls are formed from the starch-gluten matrix of dough which act as the primary stability mechanism, while a liquid film called the liquid lamellae forms around the gas cells and the matrix (MacRitchie and Gras, 1983; Gan et al., 1995). The properties of this liquid layer are crucial for gas cell stability in the dough. The puroindolines may improve the foaming properties of lipid-damaged foams and positively affect the crumb structure of bread (Dubriel et al, 1998). It has therefore been hypothesized that puroindolines function to bind wheat flour neutral lipids (Wilde et al., 1993) and prevent them from destabilizing the fragile liquid films that line the gas cell; however there is little evidence to suggest this phenomenon occurs during breadmaking itself.

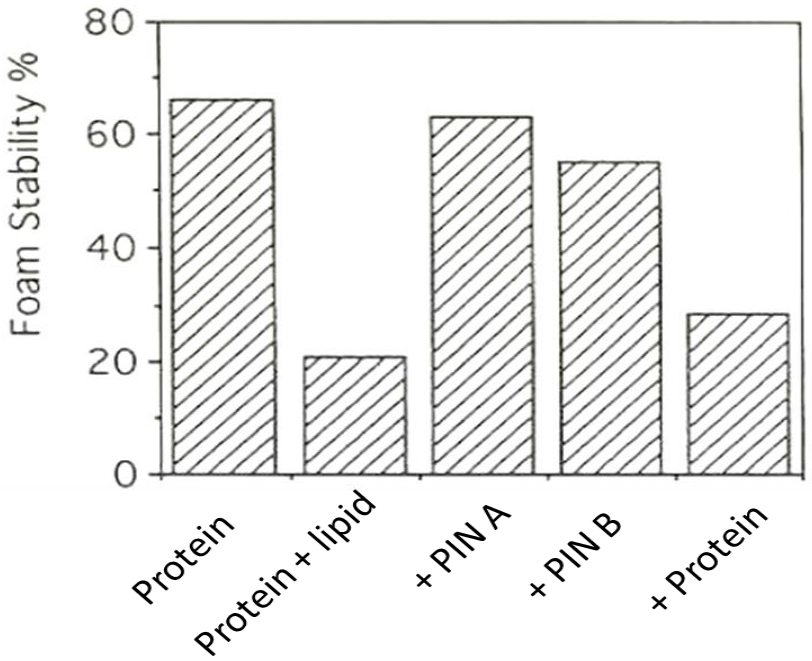


Figure 1.8 The effects of proteins, protein + lipid, added PINA, PINB and protein on the foam stability. Taken from Dubreil et al., 1997

Puroindolines have been reported to interact with polar lipids in vitro to improve foaming properties (Dubreil et al., 1997), which may be beneficial to breadmaking quality. Lysophospholipids (LPLs) such as Lysophosphatidylcholine (LPC) could form complexes with puroindolines during dough mixing and fermentation, synergistically enhancing foam stabilization (as discussed above) and therefore bread crumb structure (Pauly et al., 2013). Phospholipids and glycolipids on the surface of water-washed wheat starch granules in the endosperm display strong affinity for puroindolines and possibly provide lipid "bridges" between the surface of starch granules and puroindolines (Feiz L et al., 2009). Lipids may also play a role in grain hardness as soft wheats have higher levels of polar lipids (Greenblatt GA et al., 1995, Oda and Schofield, 1997).

1.7 Starch

1.7.1 Summary

Starch is the major stored carbohydrate in most plants, including the grain endosperm, and is the most abundant component in wheat flour. Starch plays a vital role nutritionally, providing energy value of approximately 1550 kJ/100g, a value comparable to that of proteins.

Starch comprises two polymers, amylose and amylopectin. Amylose is a linear molecule of α-D-glucose linked by α 1,4 bonds. Amylose has a molecular weight that varies between 80,000 and 1,000,000 (500-6000 anhydroglucose units). Its molecular weight can vary for different plant species as well as different stages of grain maturity. The linear nature of amylose gives it unique properties, such as its ability to form complexes with iodine, organic alcohols, or fatty acids. These complexes are called clathrates or helical inclusion compounds.

Although amylose is a linear molecule, this is not true for all molecules. 25-55% of the molecules have secondary chains which are attached by the occasional α 1, 6 branch points (Takeda et al., 1987). The level of branching also increases as a function of the molecular weight of amylose (Banks & Greenwood, 1975). A study by Hizukuri et al., 1997 found that an average of two to eight branch points existed per amylose molecule, while their the side chains ranged from four to over 1000 glucosyl units in length. Further fractionation of β-limit dextrin revealed that branched amylose had tiny clusters of short chains rather than a pure comb structure.

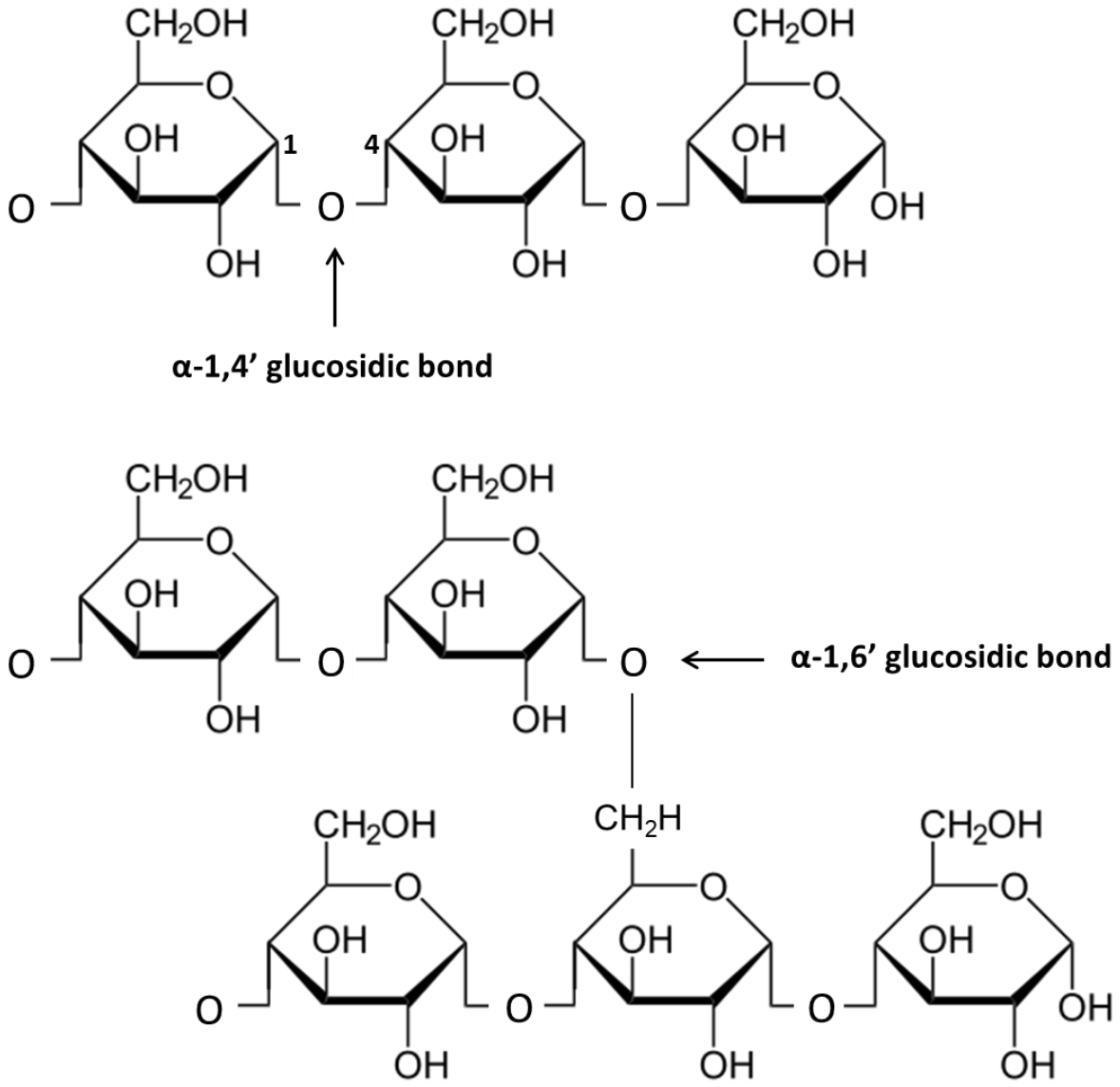


Figure 1.9 Structural sections of amylose and amylopectin, shown in Haworth representation. α-1,4' and α-1,6' glucosidic bonds are displayed. Hydrogen atoms attached to carbon atoms in the rings are not shown.

Like amylose, amylopectin is composed of α-D-glucose linked primarily by α-1,4 bonds. However, amylopectin is branched to a much greater extent than amylose, with 4-5% of the glycosidic bonds being α 1,6 bonds. This branching level means that average chain units in amylopectin are only around 20-25 glucose units long. The molecular weight of amylopectin has been reported to be as high as 10^8. Amylopectin is a huge molecule, one of the largest found in nature, that has around 617,000 glucose residues and more than 30,000 chains with an average degree of polymerization of 20. The structures of amylose and amylopectin are shown in Figure 1.7.

Amylopectin comprises three types of chains. A chains are composed of glucose linked α-1,4 linkages; B chains are composed of glucose linked α 1,4 and α 1,6 linkages; and C chains are made up of glucose with α 1,4 and α 1,6 linkages plus the reducing group. Therefore, A chains do not carry branches, while B chains do. C chains are branched and have the only reducing group (unbound C-1) in the molecule.

For this project, starch was an important component due to its interaction with lipids. Starch contains a significant amount of lipids which consist mainly of Lysophospholipids (LPLs). Lipids are also known to interact with the surface of the starch granule and starch surface lipids are believed to play an important role in the gas cell stability of dough during the breadmaking process.

1.7.2 Starch crystals

Starch is biosynthesized as a semi-crystalline granule and occurs in various polymorphic forms and levels of crystallinity. The conformation of amylose and amylopectin determines the crystalline or amorphous state of the starch crystals. A- and B- type fibrillary or lamellar crystals are prepared by the deacetylation of amylose triacetate fibres (Wu & Sarko, 1978) or by precipitation from dilute solutions of low polymerization degree amylose (Buleon et al., 1984).

The A and B type structures are believed to be based on parallel double stranded helices, which can be present in a right hand form or a left hand form (Hsein-Chih & Sarko, 1978; Imberty et al., 1988). However, differing forms of amylose with different complexes form various crystalline structures. For example, V-amylose (amyloses obtained as single helices) co-crystallizes with compounds such as iodine or fatty acids and are not included in the amylose helix. Amylopectin is believed to support the framework of the crystalline regions in the starch granule and the branch points do not induce extensive defects in the double helical structures mentioned above.

1.7.3 Starch granules

The starch granule is made up of alternating amorphous and semi-crystalline shells which are between 100-400 nm thick, which are termed “growth rings” (French et al., 1984, Gallant and Sterling, 1976). Amylose and amylopectin are deposited in granules containing several million amylopectin and even larger numbers of amylose molecules. The semi-crystalline structures of starch mentioned above densely pack the inside of starch granules.

The shape and size of starch granules are highly dependent on its botanical origin, with sizes ranging between 1 – 100 µm. However, the most interesting feature is the granule shape which is highly variable between different plants (Table 1.1).

Source	Amylose content (% total starch)	Granule type	Average Size (μm)	Shape
Wheat normal	25–29	A-granule	30	Discs
		B-granule	2–3	Perfect spheres
Maize normal (wild)	25–28	One type	30	Polyhedral and rounded
Maize waxy	0.5	One type	15	
Maize high amylose	60–73	One type	5–25	Highly elongated irregular filament
Oat	0.4-0.9	A-granule	15	Compound
		B-granule	2–3	Oval
Potato normal (wild)	18–21	One type	40	Large oval
Potato amylose free	1	One type	40	Large oval

Table 1.2 The amylose content, size and shape of starch granules in major plant sources.

Characteristic forms of starch granules may be observed where high contents of amylose are present (over 50%). Apart from amylose and amylopectin, starch granules contain a wide range of minor components such as proteins, lipids and phosphorous. Granule-bound proteins are observed inside as well as on the surface of starch granules. Lipids are present in low amounts (up to 1.5%) in many starches, especially cereal starches, mainly as FFA or LPLs (Morrison, Milligan & Azudin, 1984). The lipids present in cereal starch granules form complexes with the amylose fraction. In cereals, the proportion of amylose that is complexed with lipid varies from 13-43%.

Some cereals including wheat, oat and barley, contain two populations of starch granules, the large A-granules and the small B-granules, whose biosynthesis occurs at different stages of development.

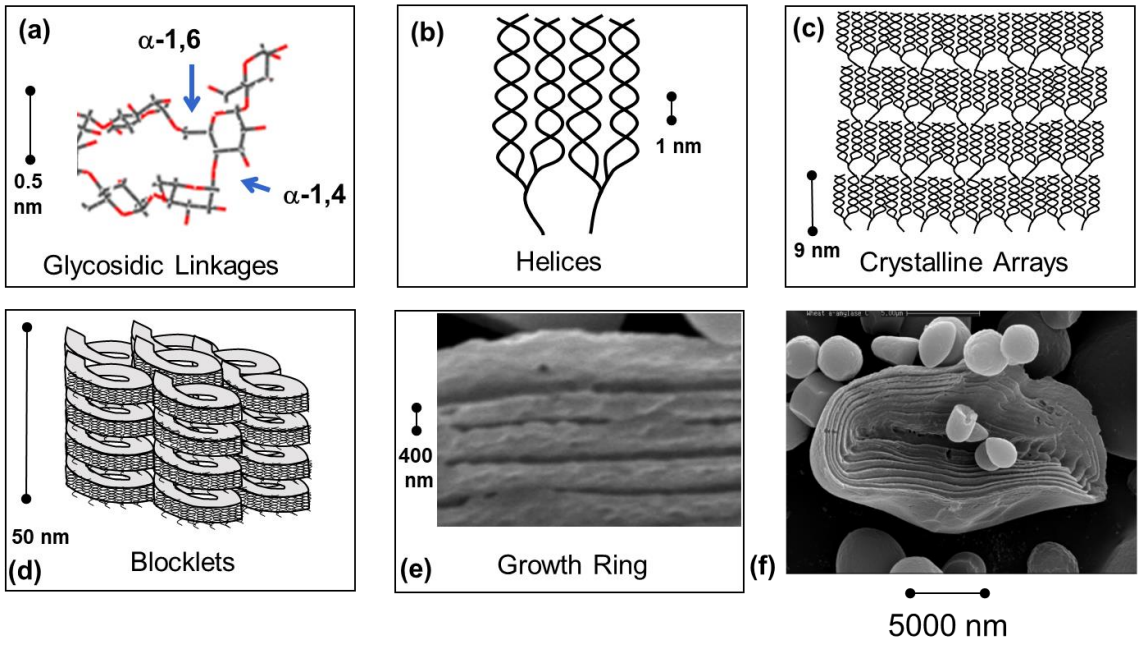


Figure 1.10 Structure of various starch components. A glycosidic linkages. B- Starch helics, C- Starch crystalline arrays. D- Starch blocklets, constrcuted by crystalline and amorphous lamellaes. E- Growth rings for starch crystals. F- Starch granules.

1.7.4 Wheat starch

Wheat starch accounts for 63-66% of the wheat kernel weight, being higher for soft wheats compared to hard wheats (Toepfer et al., 1972). Wheat starch comprises two types of granules, large lenticular (lens-shaped) A granules which are around 25-40 µm long, and small spherical B granules with a diameter of around 5-10 µm. Wheat starch granules have concentric shells, which are revealed through scanning electron microscopy (SEM) after enzyme treatment (French, 1984).

Light microscopy reveals pores and channels into the interior of the granules (Fanon et al., 1992). At the atomic resolution of AFM (Atomic Force Microscopy) 10-50 nm structures have been observed, interpreted as amylopectin side chain clusters. Non-contact AFM showed the rough surface of wheat starch granules with protruding surface structures of 200 nm in size or below (Baldwin et al., 1998).

The presence of carbohydrates, lipids and proteins on the wheat starch granule surface has been shown with many techniques such as Electron Spectroscopy for Chemical Analysis (ESCA). The proteins present on the starch granules, also known as surface granule associated protein (SGAP),

include proteins with molecular masses of 5, 8, 15, 19 and 30 kDa. These include the puroindolines (15kDa), non-specific lipid transfer proteins (nsLTPs) and a 60 kDa starch granule bound starch synthase. Apart from these proteins, there are two types of proteins with a molecular mass of 100 and 105 kDa, which are unique to *Triticum* (Douliez et al., 2000; Baldwin, 2001).

Wheat grain lipids can be divided into three categories: non-starch, starch-surface and starch-interior lipids. Non-starch lipids are defined as lipids external to starch, and consist of triacylglycerol (TAG), diacylglycerol (DAG) and phospholipids (PLs) (Finnie and Faubion, 2010; Ellis et al., 1998). The lipid content of wheat starch fractions of different sizes is proportional to the specific surface area of the fractions. This suggested that the lipid is concentrated near the surface of the granules (Whattam and Cornell, 1991).

Starch surface lipids include TAG, FFA, glycolipids and phospholipids (PL). Greater numbers of monoacyl lipids are present on the starch surface lipid fraction which aids in the formation of amylose-lipid complexes on the surface of starch granules (Finnie and Faubion, 2009). The amounts of starch surface polar lipids relates to the hardness of the endosperm. Glycolipids and PL were present in greater quantities on the surface of water washed starch from soft wheat than water washed starch from hard wheat (Greenblatt et al., 1995). Furthermore, a negative correlation between starch surface lipids and kernel hardness was found (Konopka et al., 2005).

Starch internal lipids are located inside the granules and are mostly made up of monoacyl lipids, such as LPLs which can exist as complexes with amylose in the native starch. LPC makes up the vast majority of the LPLs in wheat, comprising between 70-90% of the total LPL content. Starch internal lipids are not considered to play a role in breadmaking quality, at least during the dough mixing and proving stages (Rocha et al., 2012).

1.7.5 Importance of Starch

Starch is used in a wide number of industries. The pasting property of starch makes it suitable for gravies, soups, custards and desserts. Starch is also a common addition to various types of baked goods, in particular biscuits and cakes. The addition of starch to low protein flours improves the lightness in texture required in bakery sponges and pastries.

Starch can also play an important role in determining bread quality traits. A good example of this is bread staling, where the recrystallization of amylopectin is regarded as the most important factor

(Hebeda, 1996). Staling occurs after extended storage, and this led to the hypothesis that amylopectin recrystallization which occurs on the same time scale as staling, would be responsible (Gray and DeMiller, 2003), However this was based on a correlation not a demonstrated cause-and effect relationship, therefore this hypothesis must be approached with caution. Starch can be rapidly digested, resulting in a high glycaemic response and high insulin levels after consumption. Food factors that moderate the glycaemic responses to starch are therefore of interest.

Despite being the most abundant component in wheat flour, starch is not traditionally regarded as an important factor for determining quality differences between wheat varieties. However, reconstitution baking experiments, including various plant starches have shown the important role wheat starch plays in determining breadmaking quality parameters. Rye and barley starches were shown to be somewhat inferior compared to wheat starch (Hoseney et al., 1971). Starch from tapioca (from cassava tubers) gave good loaf volume but not crumb structure (Kusonose et al., 1999), while various wheat starches provided different baking results (D'Appolonia and Gilles, 1971).

1.7.6 Factors affecting starch and its impact on breadmaking quality

Compared to proteins, starch has received comparatively little attention for its impact on loaf volume. Various studies have reported different results on the role of starch in breadmaking quality. Matsoukas and Morrison, (1991) reported that starch granule composition, swelling and gelatinization properties were not related to baking quality in different Greek breadmaking varieties. However, in other studies, starch and protein quality were revealed to be important for hearth bread quality (Sahlstrom et al., 2006). Starches can also be expected to affect baking performance due to their physico-chemical properties such as crystallinity, granule size, distribution and gelatinisation behaviour. These properties can depend on the starch granule structure and the composition of the starch polysaccharides amylose and amylopectin.

The proportions of large A-type starch granules have been related to gas cell stability and crumb structure in the final loaf. It has been suggested that a greater proportion of large starch granules can cause gas cell coalescence and create a more open crumb structure (Hayman et al., 1998).

Starch can also play a crucial role in baking the dough. Starch granules in the dough become flat and folded when entering the oven and embed themselves into the gluten matrix (Glaszcznak et al., 2004). The gelatinization of starch during the initial stages of baking can provide the best results for breadmaking quality.

1.7.7 Enzyme activity on starch and the Hagberg Falling Number

The Hagberg falling number (HFN) test is an internationally standardized method to determine the level of α-amylase in various grains such as wheat and rye. The enzyme alpha amylase is produced in the early stages of germination (sprouting) and plays a crucial role breaking down complex starches into simple sugars for the developing embryo. The levels of α-amylase can increase by 1000 fold at the onset of germination, and due to such large increases, it takes just a few grains with high levels of α-amylase to affect the HFN of large quantities of grain. In breadmaking, high levels of α-amylase are undesirable as it leads to loaves with a sticky interior (DB Stevens et al., 1988).

HFN measures the α-amylase activity by measuring the time taken for a plunger to fall to the bottom of precision bore glass tube filled with heated mixture of wheat flour and water. The time taken for the plunger to fall is known as the falling number. Good quality flour with little enzyme activity will have scores between 300-600 seconds, while flour with poor quality scores of around 250 seconds or under are usually discarded. Therefore low HFN indicates excessive starch hydrolysis due to high enzymatic activity.

1.7.8 Impact of starch damage through milling

Milling results in starch damage of wheat flour, which also affects breadmaking and baking properties. The extent of this damage is greater in hard wheat, and is affected by the types of roller and their closeness. Damaged starch absorbs water and is also rapidly hydrolysed by amylase during fermentation (AACC, 1983b). Although high levels of amylase activity are detrimental to breadmaking quality, low levels of amylase, which limits substrate availability for yeast, also results in poor loaf volume, which is the reason why bakers add amylase to dough.

1.8 Enzymes

Enzymes are a key component of any plant for the synthesis of food and growth. Wheat enzymes are of some importance to the performance of flour in breadmaking, especially the amylases.

1.8.1 Amylases

Amylases are enzymes that catalyse the hydrolysis of starch into sugars. α-amylase, the major amylase in cereals, is an endohydrolase which acts slowly on damaged starch granules, but more

rapidly on gelatinised starch to produce a mixture of dextrins and other oligosaccharides. During the breadmaking process α-amylase is active during dough development due to its reaction with damaged starch and also in the baking process where it attacks gelatinised starch until it is heat-inactivated. Calcium ions act as enzyme activators, while phytic acid, present in bran, acts as an inhibitor due to its reaction with calcium. The impact of α-amylase on starch and the breadmaking process is described above.

β-amylase is the second major amylase in wheat and acts on the α-1,4' glycosidic bonds near the non-reducing ends of the amylose and amylopectin molecules to produce maltose. However, β-amylase is only able to achieve approximately a 60% conversion rate with amylopectin due to the α-1,6' linkages. The remaining material after this is usually called 'limit dextrin'. The α and β amylase content of the mature wheat grain is usually low, but when rainfall occurs before harvesting, the bound inactive form of β-amylase is converted to the active free form, which causes the levels of α-amylase to increase. β-amylase is mainly present in the endosperm of the immature wheat kernel. Upon maturation, although β-amylase can be found distributed throughout the endosperm, it is preferentially located near the subaleurone (Engel, 1947).

1.8.2 Proteases

Proteases can influence breadmaking quality by reducing the consistency of doughs and batters after mixing and resting. In wheat, the aleurone layer, the pericarp and embryo are the main sources proteolytic activity in the wheat grain. The protease content in the wheat grain can rise rapidly during germination. However, the endosperm contains very little proteolytic activity, and it is therefore unlikely to have a major effect in wheat flour. Therefore, in the baking industry small amounts of fungal proteases would be added to strong flours in order to reduce the mixing time and improve dough extensibility.

Proteases hydrolyse the peptide bond between the amino end of one amino acid residue and the carboxyl end of the adjacent amino acid residue in a protein. Endoproteases (proteinases) hydrolyse the peptide bonds along the protein chain resulting in the formation of two peptides while exoproteases remove amino acids one at a time from the end of the protein chain. These can be referred to as carboxypeptidases when they act from the carboxy terminus or aminopeptidases when they act from the amino terminus. However, in general, proteolytic enzymes are classified by their catalytic mechanisms as serine, metallo-, aspartic, and thiol or cysteine proteases. Endoprotease activity occurs mainly in the pericarp as well as the seed coat and aleurone layers during early development. However activity decreases over time and by maturity most of the activity is localized

in the germ tissue (Kruger 1972). Some activity can also be found in the endosperm, accounting for one quarter of the activity at maturity (Kruger and Preston, 1976).

The optimal activity for serine proteases occurs in the alkaline pH range, usually around 7.5 and 10.5. Classic examples of this type of proteinase include trypsin and chymotrypsin. The serine proteinases are responsible for all proteolytic activity during the early stages of wheat grain development. However, their activity falls at later stages. As serine proteases are localized in the developing and germinating embryo, this suggests that their physiological role is in protein metabolism rather than degrading storage proteins in the developing embryo.

Other proteases in wheat include metalloproteases which require the presence of a metal ion such as zinc at their active site. They are present during the later stage of wheat grain development. Aspartic proteases, which were earlier named "acid" or "carboxyl" proteases, are a widely distributed class of proteases present in animals, microbes, viruses and plants. Aspartic proteases contain two aspartic acid residues in their active site. They become active at acidic pH and are specific for peptide bonds between amino acid residues with large hydrophobic side chains. The best-known members of this group are pepsin, chymosin, renin, cathepsin D and yeast proteinase A. Aspartic proteinase activity is particularly prominent in wheat seedlings.

Cysteine proteases are enzymes where the nucleophile (electron donor) is the sylfhydryl group of a cysteine residue. The catalytic mechanism is similar to that of the serine-type proteases where the nucleophile and a proton donor or general base is required. The histidine residue present in the active site acts as a proton donor and enhances the nucleophilicity of the cysteine residue. The pH range in which these enzymes function is usually rather broad, with greater activity occuring below pH 7.0. Germinated wheat will contain cysteine proteinase activity.

1.8.3 Other enzymes

Other enzymes in wheat include cystases which act on various glucans, pentosans and polysaccharide-protein complexes in cell walls and starch granules. These belong to the family of endo- and exo-β-glucanases and –pentosanases. However, cystases are more important in the brewing industry than the baking industry, due to their effects on cell walls (HJ Cornell, 2012, p 67). They promote the breakdown of the cell wall allowing the entry of amylases and proteases for starch and protein hydrolysis which is crucial in brewing to convert the insoluble starch to soluble starch.

Phosphatases are esterases which belong to a family of hydrolases that hydrolyse phosphate esters such as 6-monophosphate esters, mainly present in wheat starch, Phytase is an important enzyme in wheat and is present in high levels in the bran. Phytic acid (inositol hexaphosphoric acid) binds various minerals such as calcium, zinc and iron making high levels undesirable in wheat. By reducing phytic acid levels, phytase reduces the complexation of these nutritionally-important minerals. Phytase activity is mostly locatlized in the endosperm and aleurone, together accounting for 73.6% of the phytase activity in the wheat grain (Peers, 1953).

Oxygenases such as lipoxygenase are present in high amounts in the germ especially the scutellum, together with various other enzymes of this type which catalyse the reactions where molecular oxygen is utilized to oxidise specific substrates (Blain and Todd, 1955). It is believed to improve dough rheological properties due to its activity with sulfhydryl groups. Other oxidases such as phenol oxidases, which act upon polymeric substances are also present in the wheat bran.

1.8.4 Lipases

Endogenous lipases are present in significant amounts in the wheat germ. They are esterases that hydrolyse ester bonds, particularly those which are present in TAGs, yielding mono-, diacylglycerols and free fatty acids. Lipase activity is important as free fatty acids are more susceptible to oxidation, and hence rancidity, than the same fatty acids present on a glycerol backbone. Some of the polyunsaturated fatty acids that are produced will be oxidized by wheat lipoxygenase.

Lipoxygenases catalyse the peroxidation of polyunsaturated fatty acids by oxygen. For this, a substrate with methylene-interrupted double bonds, both in a cis configuration is preferred. In wheat, lipoxygenases are only active on free fatty acids. Lipoxygenases affect flour colour and its acceptability by oxidizing the carotenoids. This reaction begins with the lipoxygenses catalysing the reaction of linoleic acid with molecular oxygen, to form a linoleic acid peroxy radical (LOO°). This radical reacts with a second linoleic acid forming linoleic acid hydroperoxide (LOOH) and a radical (L°). This linoleic peroxy radical may react with carotenoids (car-H) forming LOOH and a carotenoid radical. This carotenoid radical can react with molecular oxygen which can cause the production of a colourless product.

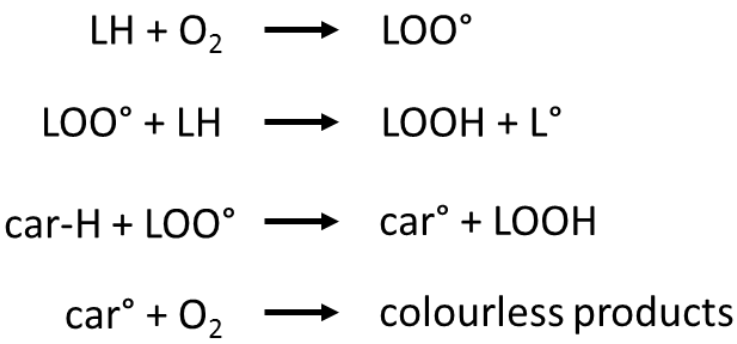


Figure 1.11 The oxidative deterioration of carotenoids by lipoxygenases.

1.8.5 Lipases in breadmaking

Wheat flour contains around 1-2% lipid material by weight, however a significant amount of flour lipids are bound within the starch granule and are not available as a substrate for lipases. Lipases will be active at the lipid-water/lipid-air interface and modify flour lipids such as TAG (Triacylglycerol) by cleaving off the fatty acids in the sn-1 and sn-3 position on the glycerol backbone (Figure 1.12)

During the breadmaking process, dough undergoes fermentation. During the early stages of fermentation, bread is generally regarded as a dispersion of gas cells, starch granules and lipid globules in a continuous protein (gluten) matrix. During the fermentation process, a foam like structure is generated, where gas cells in foam are encapsulated and stabilized by a liquid lamellae film consisting of surface active components such as lipids and proteins. The stability of these gas cells is crucial for breadmaking quality, as they ensure a good crumb structure and loaf volume.

In industry, emulsifiers such as diacetyl-tartrate ester of monoglyceride (DATEM) or the sodium or calcium salts of stearoyl lactylates (SSL/CSL) are used to help stabilize the gas cells and improve loaf volume. However, various studies have shown that the addition of phospholipases to replace some or all of the emulsifiers can still allow good gas cell stability and loaf volume (de Maria et al., 2007).

Lipolytic enzymes can be used to modify the lipids present within the dough, resulting in an increase of beneficial lipids and improving breadmaking quality. It is generally considered that hydrolysed polar lipids are beneficial to breadmaking quality, as they resemble emulsifiers and enhance the stability of gas cells. Baking lipases were first introduced in the mid-1990s and were only specific to non-polar lipids, such as triacylglycerol (EC3.1.1.3). Later on, phospholipase A1 (EC3.1.1.32) and A2 (EC3.1.1.4) lipases capable of modifying polar lipids were released to the market.

Lipolytic enzymes affect further stages of the breadmaking process. They can be used to achieve better dough handling, to increase dough strength and improve the machinability of dough. Lipases can improve the stabilization of gas cells in dough, even during the first stages of baking. Improved volume, oven spring and a finer and silkier crumb structure were all benefits seen with the addition of lipases. They were even observed to provide a whiter crumb. However, the use of excessive amounts of non-polar lipases was problematic in recipes with a high fat content as it led to higher levels of FFA generation which caused the development of rancidity in food products (Qi Si., 1997).

1.9 Lipids

1.9.1 Classification of lipids

Although wheat lipids make up a small fraction of the wheat endosperm and its derived flour (approximately 2.0-2.5%) they exert significant effects on breadmaking quality (Kim and Seib et al., 1993). The final lipid content and composition of wheat flour is dependent on a combination of parameters, including genetic and environmental factors as well as processing factors such as milling.

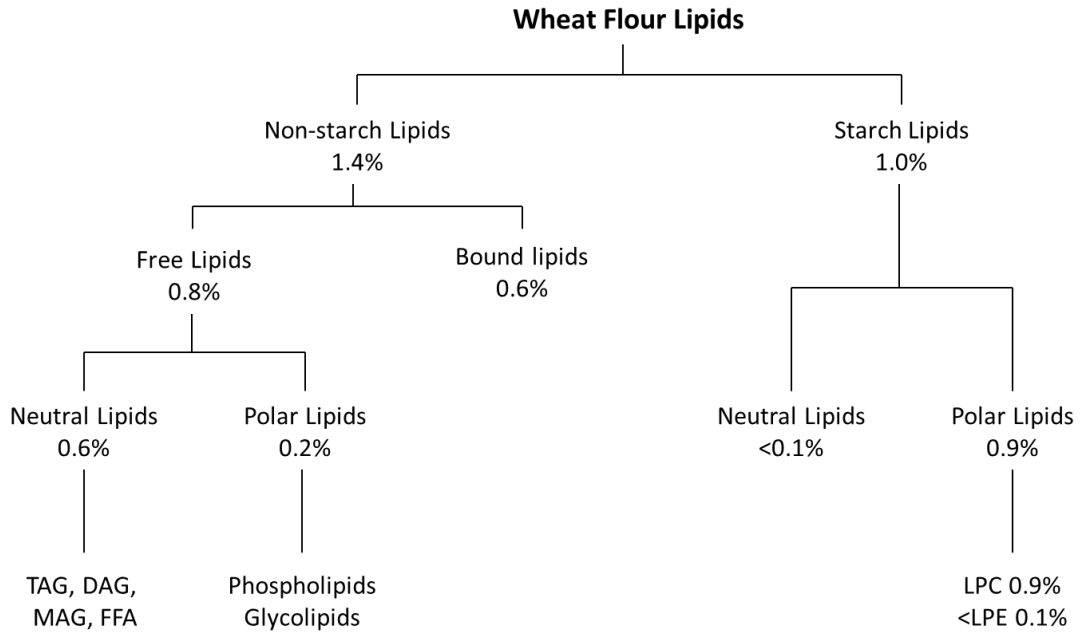


Figure 1.12 The subdivision of wheat flour lipid classes and their proportions. TAG – Triacylglycerol, DAG- Diacylglycerol, MAG- Monoacylglycerol, FFA- Free Fatty Acids, LPC – Lysophosphatidylcholine, LPE- Lysophosphotidylethanolamine.

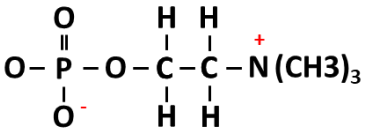
Wheat lipids can be classified in several ways. Classification by location was discussed in section 1.7.4 where wheat lipids were divided into starch surface, non-starch and starch internal lipids. However, they can also be classified based on their biochemistry and extractability in solvents of opposing polarities. These are summarized in Table 1.3.

Group	Lipid Category
By location	Nonstarch lipids
	Starch surface lipids
	Starch lipids
By extraction	Free lipids
	Bound lipids
By biochemistry	Simple lipids (Neutral lipids)
	Polar lipids (includes…)
	Glycolipids
	Phospholipids

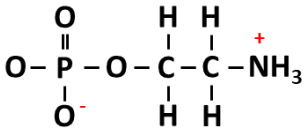
Table 1.3 Grouping of wheat flour lipids by location, extraction and biochemistry.

For this study, the lipids present in wheat were broadly classified into two groups, the polar lipids and the neutral (non-polar) lipids (NLs). The neutral lipids also called simple lipids are lipids yielding a maximum of two types of primary products per mole upon hydrolysis, whereas the polar lipids yield three or more primary products per mole when hydrolysed.

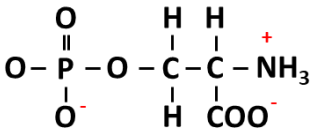
Most of the polar lipids in the wheat grain are present in the starchy endosperm with lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), phosphatidylethanolamine (PE) together with monogalactosyldiacylglycerols (MGDG) and digalactosyldiacylglycerols (DGDG) being the most prominent. The neutral lipids on the other hand are predominantly present in the embryo and aleurone tissues of the wheat kernel and consist of free fatty acids (FFA), monoacylglycerol, diacylglycerol and triacylglycerols (MAG, DAG and TAG) (Chung et al., 2009). The TAG in the embryo and aleurone layer of the wheat grain is present in discrete oil bodies which are surrounded by a monolayer of phospholipids (PC, PE and PI (phosphatidylinositol)) and oleosins (structural proteins). During the milling process, around 50% of the TAG present in the germ is transferred to the final breadmaking flour (Morrison and Hargin, 1981).



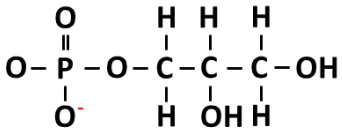
Phosphatidylcholine



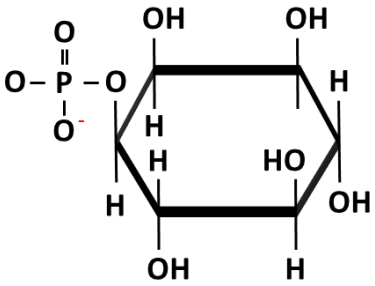
Phosphatidylethanolamine (PE)



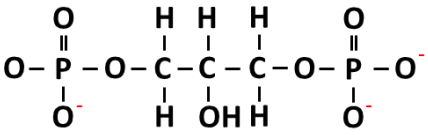
Phosphatidylserine



Phosphatidylglycerol (PG)



Phosphotidylinositol (PI)



Phosphatidylglycerolphosphate (PGP)

Figure 1.13 Structures of the primary phospholipids present within the wheat grain.

Wheat lipids can be further subdivided into 'free' and 'bound' lipids based on their extractability in solvents of opposing polarities. Free lipids are extracted with non-polar solvents such as petroleum ether or hexane, whereas more polar solvents such as water-saturated butan-1-ol or propan-2-ol: water (90:10) is required for the extraction of bound lipids. A further group of lipids present within the starch granule can only be extracted using a polar solvent at a temperature of 90˚C; this is due to the fact that high temperatures promote starch swelling and the subsequent lipid extraction from within the starch granule.

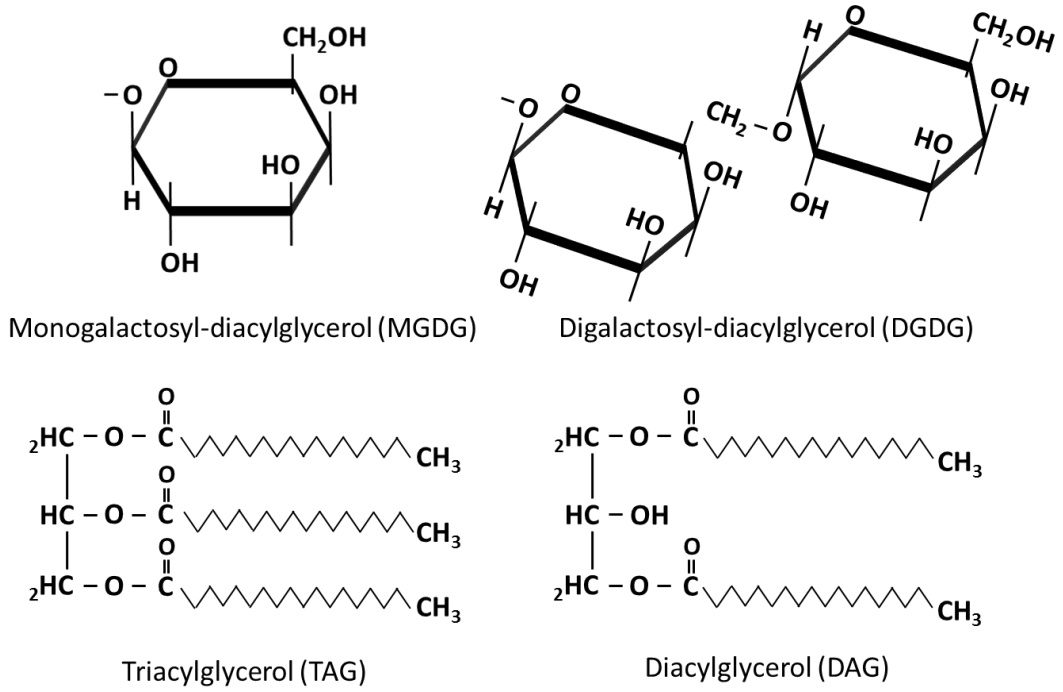


Figure 1.14 Structures of the galactolipids and neutral lipids present within wheat

1.9.2 Lipid biosynthesis

The maturing seed synthesises fatty acids at different rates, but by the dormant period, the fatty acid composition is the same as it is in the parent seed.

1.9.2.1 Fatty Acid Synthesis

In higher plants, the biosynthesis of lipids depends on acetyl coenzyme A (CoA). Plants differ in the molecular organization of the enzymes involved in fatty acid synthesis compared to other eukaryotes. In order to produce palmitic (16C) or stearic (18C) acid from acetyl CoA and malonyl-CoA, at least 30 enzymatic reactions are required. In animals, fungi and certain bacteria these reactions would be catalysed by a multifunctional polypeptide complex in the cytosol. However, plants undergo these reactions within the plastidial stroma using individual enzymes that are dissociable soluble components (Ohlrogge and Browse, 1995).

Malonyl-CoA is the central carbon donor for fatty acid synthesis. It is produced by acetyl coenzyme carboxylase (ACCase) from Acetyl CoA in a reaction requiring CO_2 and ATP (Figure 1.13 Step 1). Before malonyl-CoA is introduced to the fatty acid synthesis pathway, the malonyl group is transferred from CoA to an acyl carrier protein (Figure 1.13, Step 2). Acyl Carrier Protein (ACP) is an acidic protein around 9kDa which contains a phosphopantethein prosthetic group. The acyl chain which grows during the various reactions is attached to this group as a thioester. All reactions from this point on involves ACP until the 16 or 18 carbon chain fatty acid is exported out of the plastid or transferred to glycolipids.

Saturated Fatty Acids		
tetradecanoic	myristic	**14:0**
hexadecanoic	palmitic	**16:0**
octadecanoic	stearic	**18:0**
eicosanoic	arachidic	**20:0**
docosanoic	behenic	**22:0**
Monoenoic Fatty Acids		
cis-9-hexadecenoic	palmitoleic	**16:1 (n-7)**
cis-6-octadecenoic	petroselinic	**18:1 (n-12)**
cis-9-octadecenoic	oleic	**18:1 (n-9)**
cis-13-docosenoic	erucic	**22:1 (n-9)**
cis-15-tetracosenoic	nervonic	**24:1 (n-9)**
Polyunsaturated fatty acids		
9, 12-octadecadienoic	linoleic	**18:2 (n-6)**
6,9,12-octadecatrienoic	γ-linolenic	**18:3 (n-6)**
9,12,15-octadecatrienoic	α-linolenic	**18:3 (n-3)**
5,8,11,14-eicosatetraenoic	arachidonic	**20:4 (n-6)**
5,8,11,14,17-eicosatetraenoic	EPA	**20:5 (n-3)**
4,7,10,13,16,19-docosahexaenoic	DHA	**22:6 (n-3)**

Table 1.4 The various common fatty acids present in plants, their systemic, trivial and shorthand name.

To add two carbon units to the fatty acid chain, four enzyme reactions are needed. The first step is condensation. The malonyl-ACP thioesters enters a series of condensation reactions with acyl-ACP (or acetyl CoA) acceptors (Figure 1.13, Step 3). This reaction forms a C-C bond and releases the CO_2 that was added during the ACCase reaction turning CoA into malonyl-CoA. The removal of CO_2 during this condensation step essentially makes the reaction irreversible and helps to drive the reaction forward. Three different condensation enzymes are used depending on the length of the carbon chain. The first reaction is catalysed by β-ketoacyl-ACP-synthase III (KAS III) which uses acetyl-CoA and malonyl-ACP substrates in the manner described above. To produce chain lengths from 6-16 carbons, the enzyme KAS I is used and the final reaction between the 16 carbon palmitoyl-ACP and malonyl-ACP to produce stearate, utilises KAS II.

The second step is the first reduction step where the intermediate is reduced by β-ketoacyl-ACP reductase, a Nicotineamide adenine dinucleotide phosphate (NADPH) utilising enzyme which acts as the electron donor (Figure, Step 4). This enzyme has been purified from *Brassica napus* and functions as a tetramer with a Mr of 28 kDa (Slabas et al., 1992).

The third enzyme in the fatty acid chain elongation is β-hydroxyacyl-ACP dehydrase, which was purified from spinach leaves and found to be encoded by two genes in Arabidopsis (Harwood, 2010). This reaction causes the formation of a double bond and the release of a water molecule (Figure, Step 5).

The fourth step is the second reduction step where the enzyme enoyl-ACP uses NADH or NADPH to reduce the trans-2 double bond to form a saturated fatty acid (Figure 1.13, Step 6) (Fawcett et al., 2000).

De novo fatty acid synthesis in plants can be halted in various ways. There are two mechanisms for removing the acyl group from ACP. An acyl-ACP thioesterase hydrolyses the acyl-ACP products of fatty acid synthase and releases the free fatty acids. One of the two acyltransferases in the plastid transfers the fatty acid from ACP to glycerol-3-phosphate (G3P) or to monoacylglycerol-3-phosphate. The first acyltransferase is a soluble enzyme that prefers oleoyl-ACP as a substrate. The second acyltransferase is attached to the inner chloroplast envelope membrane and preferentially selects palmitoyl-ACP. Whether the fatty acid can be released from ACP by a thioesterase or an acyltransferase, determines whether it leaves the plastid or not. If a thioesterase acts on acyl-ACP, then the free fatty acid is able to leave the plastid.

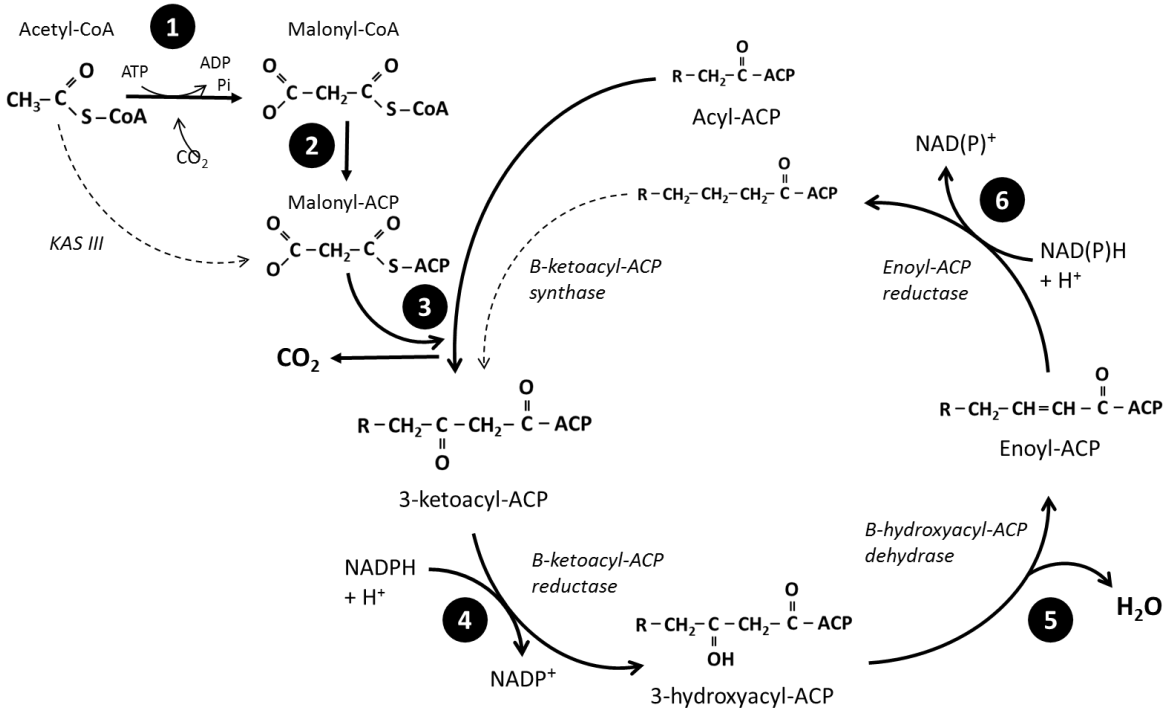


Figure 1.15 The overall reaction of fatty acid synthase

Exported fatty acids take part in the "eukaryotic" pathway of lipid synthesis whereas fatty acids retained in the plastid are used for the "prokaryotic" pathway (Browse and Somerville, 1991). Lipids produced by the eukaryotic pathway are enriched at both the sn-1 and sn-2 positions with 16C fatty acids, while the prokaryotic pathway produces thylakoid lipid molecules with 18C acids at the sn-2 position.

While the above has described the production of de novo saturated fatty acids in plants, in many plant tissues, over 75% of fatty acids are unsaturated. The soluble enzyme stearoyl-ACP desaturase allows double bonds to be introduced into fatty acids.

The desaturases work via an aerobic mechanism with oxygen being reduced by 4H, two from the substrate fatty acid and two from the reductant used. The genes coding for the desaturases are abbreviated as FAD (fatty acid desaturases) or fad, and are numbered according to what position the double bonds are introduced as well as the nature of the substrate.

Linoleic and α-linolenic acids are the most abundant fatty acids present in plants. They are generated by the desaturation of oleate via the introduction of a methylene interrupted double bond arrangement.

Fatty acid synthase produces stearoyl-ACP which acts as a substrate for a Δ9-desaturase which forms oleoyl-ACP.

In the prokaryotic pathway, oleate (18:1) is incorporated into the chloroplast membrane to be desaturated at position Δ12 via the FAD 6 enzyme and at position Δ15 via the FAD 7 and FAD 8 enzymes. On the plastid envelope, the enzyme Fatty Acyl-ACP Thioesterase A (FATA), FATB and unesterfied oleate is used to hydrolyse oleoyl-ACP to form oleoyl-CoA via the action of acyl-CoA synthase (Harwood, 1996). Within the plastid or on the endoplasmic reticulum, the incorporation of oleoyl-CoA into phosphatidylcholine to form linoleate is catalysed by FAD 2 (18:2). α-linoleate (18:3) is produced by the activity of FAD 3 which causes desaturation at the Δ15 position.

1.9.2.2 Triacylglycerol synthesis

Triacylglycerol (TAG) is composed of three fatty acyl groups which are esterified to a glycerol backbone at the sn-1, sn-2 and sn-3 positions. As the primary energy source in storage tissues, seeds can contain up to 75% triacylglycerol, and in certain fruits such as oil palm, triacylglycerol can make up around 90% of its dry weight (Hildebrand, 2012). Aside from its role as an energy store, TAG plays a major role in the bio-economy to provide a source of highly reduced carbon for both food and non-food applications.

TAG biosynthesis occurs in the endoplasmic reticulum and involves the acyl-editing of fatty acyl chains within the phospholipids of the ER. The specific reactions of triacylglycerol assembly and acyl-editing may be catalysed by one or more forms of an enzyme depending on the plant species. TAG droplets accumulate in the outer leaflet of the endoplasmic reticulum, forming deposits which eventually bud off to form an oil body around 0.5-2.5 micrometres in diameter. Surrounding the droplets is a monolayer of phospholipid with an amphiphilic oil body protein (oleosin) embedded in the triacylglycerol and phospholipid layer.

As discussed above, monounsaturated fatty acids are formed during the *de novo* biosynthesis of fatty acids. This occurs in the plastid with thioesterase catalysing the release of saturated or monounsaturated fatty acyl chains from the acyl-acyl carrier protein. After being exported from the plastid, the fatty acids are re-esterified to CoA to form acyl-CoA, the reaction being catalysed by acyl-CoA synthetase (ACS) on the outer membrane of the plastid (Figure 1.14, Step, 1). The FAs can then be further elongated on the endoplasmic reticulum via the action of fatty acid elongase (FAE) (Figure 1.14, Step 2).

The sequential acylation of the glycerol backbone in the sn-glycerol 3 phosphate pathway (Kennedy pathway) forms TAG, the details of which can be found below.

The fatty acid composition of TAG can be modified by the acyl editing mechanism that occurs on DAG at the PC level, but also by acyl exchange with the acyl-CoA pool. The acyl-CoA pool provides acyl-donor substrates for the acyltransferase reactions of TAG assembly in the ER.

The formation of TAG begins with the acyl-CoA dependent acylations of sn-glycerol-3-phosphate (G3P). Three acylations of the glycerol backbone occurs, and the removal of the phosphate group occurs just before the final acylation. These acylation reactions are catalysed by various enzymes. The first step occurs with Acyl-CoA:sn-glycerol-3-phosphate acyltransferase (GPAT) catalysing the acylation of the sn-glycerol-3-phosphate to produce lyso-phosphatidic acid (LPA) (Figure 1.14, Step 3). This process initiates the synthesis of all glycerolipid molecules. GPAT has a preference for the acyl-CoA substrate which leads to the asymmetric distribution of saturated and unsaturated fatty acids in the sn-1 and sn-2 positions of both phospholipid molecules as well as TAG.

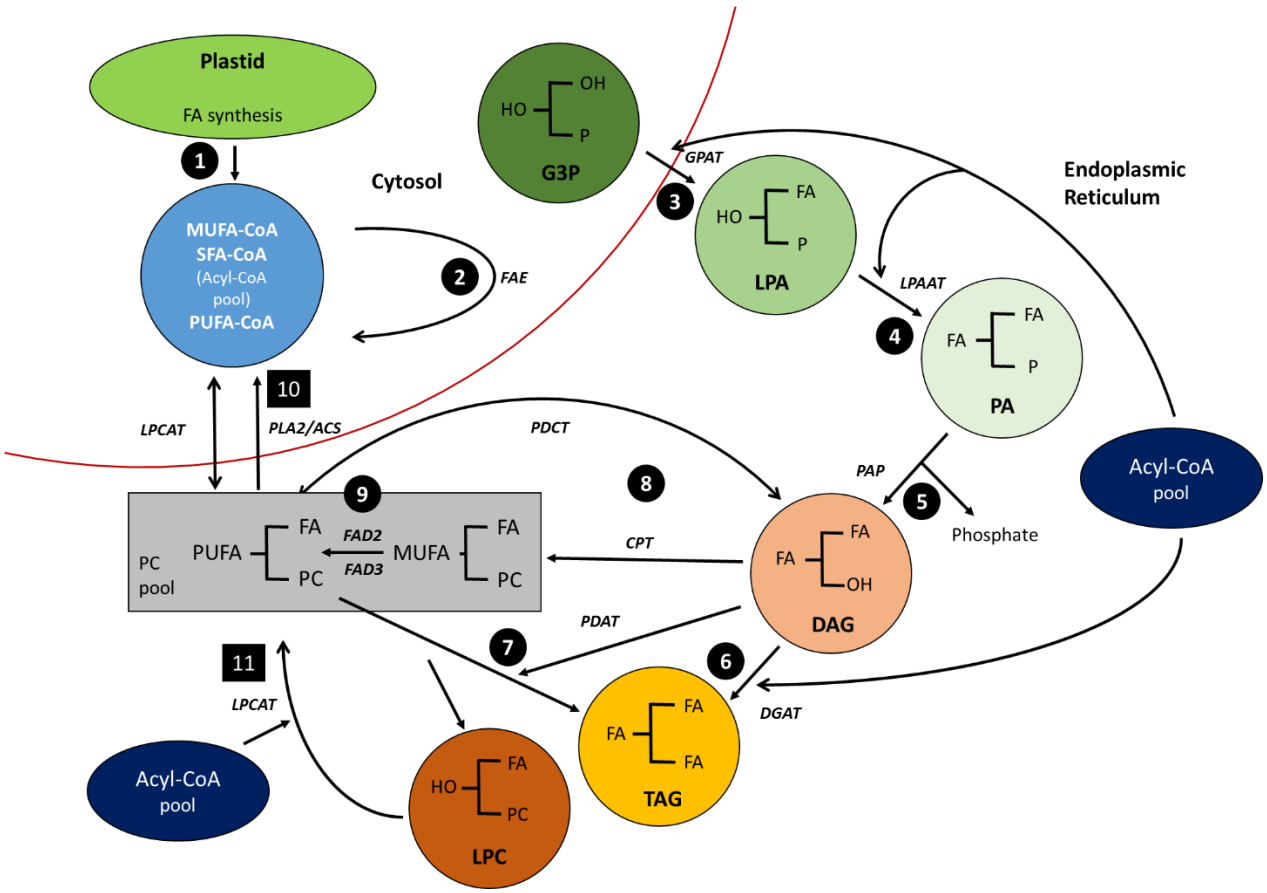


Figure 1.16 A generalized scheme for Triacylglycerol (TAG) biosynthesis in the developing seeds of oilseeds.

The second step involves the acylation of LPA into phosphatidic acid, which is catalysed by acyl-CoA:lyso-phosphatidic acid acyltransferase (LPAAT) (Figure 1.14, Step 4). Next the phosphate group is removed from phosphatidic acid which results in the formation of sn-1,2-diacylglycerol, a process catalysed by phosphatidic acid phosphatase (PAP) (Figure 1.14, Step 5). Two main PAPs exist in this process. PAP1 is associated with triacylglycerol biosynthesis which catalyses the dephosphorylation of phosphatidic acid and is dependent on Mg^{2+} ions. On the other hand, PAP2 is capable of utilizing a range of phosphate-containing substrates which include phosphatidic acid. Studies using Arabidopsis have shown that PAP1 and 2 are involved in providing diacylglycerol from the eukaryotic pathway in order to synthesize galactolipids in the plastid.

Finally, the last acyl-CoA dependent acylation is catalysed by acyl-CoA:diacylglycerol acyltransferase (DGAT) which results in the production of TAG from DAG (Figure 1.14, Step 6). Two DGAT families have been identified, which share no homology despite them both utilizing long chain acyl-CoAs. Structurally they show differences with DGAT 1 having many trans-membrane segments, while DGAT 2 was observed with just a few. Through studies, DGAT 1 and 2 have been shown to be present on different regions of the ER. DGAT2 plays a role in catalysing the incorporation of unusual fatty acids such as 18:1, 20:1 and 18:3.

TAG can also be synthesised by the activity of PDAT (phospholipid:diacylglycerol acyltransferase) which can transfer fatty acids at the sn-2 position of PC to DAG which generates TAG, meaning that the PC acts as the acyl donor and the DAG as an acyl acceptor. The unusual fatty acids from PC can also be transferred over to TAG through this reaction (Figure, Step 7).

1.9.2.3 Biosynthesis of other Lipids

Various other lipids can be synthesised between these steps outlined above. For example, the DAG synthesised from PA from the catalytic action of PAP can be converted into various phospholipids. Phosphatidylcholine can be synthesized via conversion from DAG by the action of sn-1,2 diacylglycerol:cholinephosphotransferase (CPT) and/or phosphatidylcholine: diacylglycerolcholinephosphotransferase (PDCT) (Figure1.14, Step 8). The monounsaturated fatty acids (MUFA) at the sn2 position of phosphatidylcholine can be desaturated by the actions of fatty acid desaturases (FAD) 2 and 3. The reaction that catalyses DAG to PC via PDCT can be returned to DAG into the linear part of the G3P pathway that leads into TAG, provided that the PC is enriched in PUFA (polyunsaturated fatty acids).

Phospholipase A2 (PLA2) can remove PUFAs from the sn2 position of PC. These PUFAs are then converted into acyl-CoA via the action of acetyl Co-A synthase (Figure 1.14, Step 10). Acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT) provides another method for moving PUFAs or unusual fatty acids into the acyl-CoA pool. The forward reaction catalysed by LPCAT uses acyl-CoA and lysophosphatidylcholine (LPC) as co-substrates to form PC and free CoA (Figure 1.14, Step 10). The reverse reaction is highly unfavourable, yet there is strong evidence to suggest that acyl-CoA binding proteins interact with acyl-CoA. Due to this, the free concentration of acyl-CoA may be reduced and this encourages the reverse reaction back to LPC.

Two forms of DAG can be produced. DAG is produced from PA by a specific phosphatase while a second form, a nucleotide activated form of DAG (CDP-DAG), is produced from the reaction of PA with cytidine 5'—triphosphate (CTP) (Figure 1.15, Step 1). Nucleotide activation is crucial as it provides the energy to drive the attachment of polar headgroups for glycerolipid synthesis in both the prokaryotic and eukaryotic pathways. When DAG is the lipid substrate, the polar headgroup is activated. Cystidine 5'-diphosphate (CDP)-choline, CDP-ethanolamine, and CDP-methylethanolamine can be substrates for phospholipid synthesis. Uridine diphosphate (UDP) galactose is a substrate for monogalactosyldiacylglycerol (MGDG) and UDP-sulfoquinovose is the substrate for sulfoquinovosyldiacylglycerol (SL) synthesis respectively. Digalactosyldiacylglycerol (DGDG) is synthesised from MGDG (Joyard et al., 1998).

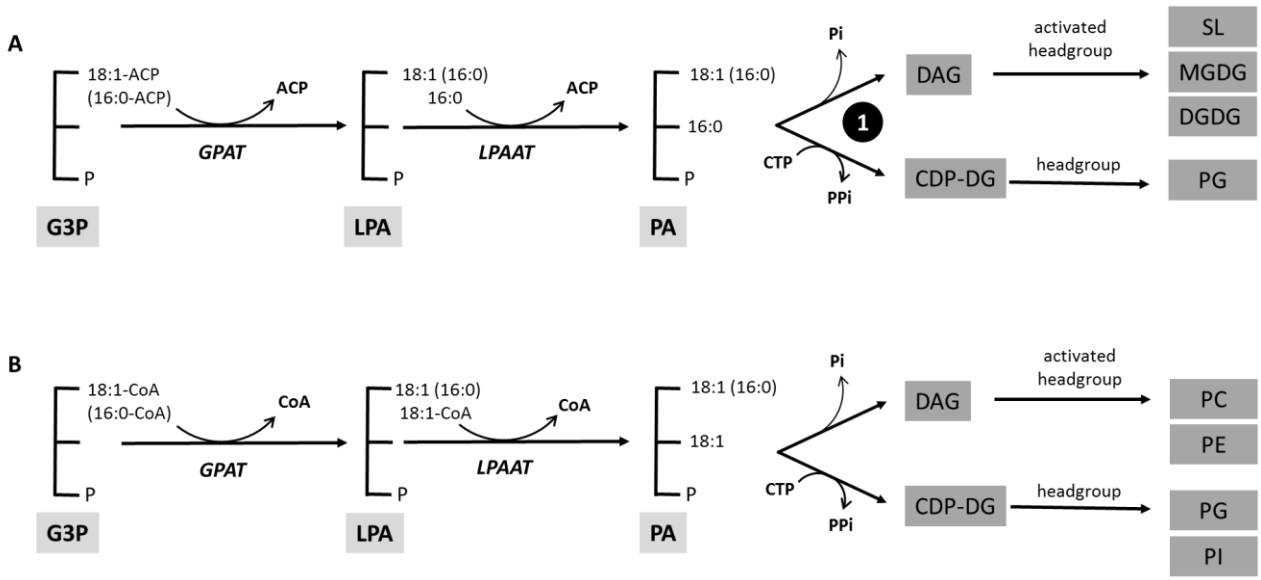


Figure 1.17 The prokaryotic and eukaryotic pathways of glycerolipid synthesis. A- Prokaryotic pathway, B- Eukaryotic pathway

CDP-DAG is the lipid substrate for reactions with myo-inostidol, serine and G3P that originates from the formation of phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylglycerol phosphate (the precursor of PG), respectively.

1.9.3 Introduction to the lipid classes in wheat

Phospholipids (PL)

In figure 1.10 lipids were classified based on their extractability in solvents of various polarities. Among the polar lipids, are the phospholipids which are a class of lipids that consist of two hydrophobic fatty acid 'tails' and a hydrophilic head consisting of a phosphate group. They are major components of all cell membranes. The phospholipids included in this study are lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylglycerol (PG). The structure of the phospholipids can be found in figure 1.11.

Lysophosphatidylcholine, also called lysolecithin, is derived from phosphatidylcholine. It results from the partial hydrolysis of phosphatidylcholine which removes one of the fatty acid groups. The hydrolysis is normally catalysed by phospholipase A2. The precise precursor of LPC in wheat is not established. However, a study by Liu et al., 2012 states that it is likely to be produced by the hydrolysis of diacyl phosphatidylcholine (DPC) catalysed by phospholipase A2 (PLA2) or phospholipase A1 (PLA1). The hydrolysation of DPC by phospholipase A2 has been reported in rice, and the fatty acid compositions of LPC and DPC are correlated (Tong et al., 2014). The relationship between DPC and LPC has not been established in wheat.

Phosphatidylcholines (PC) are a class of phospholipids with choline as a lipid head group. In wheat flour, PC comprises 8-10% of the total lipid content and plays a significant role on breadmaking quality. It is believed to promote loaf volume by helping to stabilize gas cells formed during the proving stage of the dough. PC plays crucial role as a pool for the biosynthesis of DAG, TAG and LPC. Details on the role of PC on the TAG biosynthesis pathway can be found above (Figure 1.14).

Phosphatidylethanolamines (PE) are also found in biological membranes and are synthesised by the addition of CDP ethanolamine to diglycerides, releasing cytidine monophosphate (CMP). It is mainly found in the inner cytoplasmic leaflet of the lipid bilayer. PE creates more viscous lipid membranes compared to phosphatidylcholine, due to its lower melting temperature. It is considered to play an important role as a precursor, substrate or donor in several biological pathways.

PG is a ubiquitous phospholipid that is present in biological membranes. In plants, PG is mostly present in the thylakoid membrane and is essential for the development of thylakoid membranes. It is considered to play roles in photosynthesis (Hagio et al., 2002), and cold sensitivity (Roughan, 1985).

As shown in Figure 1.15, phosphatidylglycerol (PG) can be synthesised via both the prokaryotic and eukaryotic pathways. Phosphatidic acid reacts with CTP, producing CDP-diacylglycerol while losing pyrophosphate. G3P reacts with CDP-diacyglycerol to form phosphatidylglycerol phosphate (PGP) while CMP is released. The phosphate group becomes hydrolysed forming phosphatidylglycerol.

Phosphoinositide's are derived from phosphatidylinositol (PI) by the phosphorylation of the inositol headgroup. The phosphate group of PI confers a negative charge at physiological pH. PI is the precursor for all phosphoinositide's which are generated by the condensation of CDP-DAG and D-*myo*-inositol catalysed by phosphatidylinostidol synthases (Heilmann, 2009). Unlike PC or PE which are structural components in the membrane, phosphoinositides are a minor component which plays important regulatory roles in the plant. These include the control of membrane trafficking, cytoskeletal remodelling, ion transport and signal transduction.

Galactolipids (GL)

When separating wheat lipids by their polarity, galactolipids are also part of the polar lipid fraction. However, they are structurally very different to PL. GLs are a type of glycolipid whose sugar group is galactose. They are primary components of plant membrane lipids where they substitute phospholipids when phosphate is conserved for other important processes. The galactolipids in this study include monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG).

MGDG and DGDG are galactolipids which are present in high amounts in the photosynthetic membranes of plants. As such they are crucial for the efficiency of photosynthetic light reactions. Galactolipids make up the bulk of thylakoid membranes and are integral components of photosystems I and II, the functional and structural units of protein complexes involved in photosynthesis.

In thylakoids, the ratio of MGDG to DGDG is crucial for the stability of the membrane bilayer. It has been shown that under various stress conditions such as drought and freezing, a proportion of MGDG is converted into DGDG and oligogalactolipids in order to prevent the fission of chloroplast membranes (Dormann, 2013). Furthermore, under phosphate-limited conditions, the amounts of DGDG in plant plastids increase and DGDG is exported to the extraplastidial membranes in order to replace phospholipids (Kelly A et al., 2016).

The biosynthesis of galactolipids is briefly introduced in section 1.10.2.4.

Neutral Lipids

Non-polar or neutral lipids were introduced in 1.10.1. They are lipids which yield a maximum of two types of primary products per mole upon hydrolysis. The NLs in this study include free fatty acids (FFA), diacylglycerol (DAG) and triacylglycerol (TAG).

Free fatty acids are the form in which a fatty acid leaves the cell to be transported for use in other parts of the system and may also be released by the hydrolysis of acyl lipids. Free fatty acid chains differ by length and are sometime classified as short, medium, and very long. Short chain fatty acids (SCFAs) are fatty acids with aliphatic tails of five or fewer carbons, while medium chain fatty acids are fatty acids with aliphatic tails of 6 to 12 carbons. Finally, long chain fatty acids are fatty acids with aliphatic tails of 13-21 carbons, which is the form free fatty acids are mainly found in wheat flour.

Free fatty acids FFA are predominant in wheat flour accounting for about 30% of the total lipids measured in this study. They have been reported to have negative impacts on breadmaking quality, and can be detrimental to loaf volume. Furthermore, because free fatty acids are readily oxidised they can cause rancidity in food products. High levels are therefore generally undesirable.

Diacylglycerol consists of two fatty acid chains covalently bonded to a glycerol molecule through ester linkages. Two forms exist, 1,2-diacylglycerols and 1,3-diacylglycerols. DAGs act as surfactants and emulsifiers in processed food products, often being found in bakery goods as well as beverages, margarine, confectionaries and candy.

DAGs constituted around 11% of the total lipids measured in this study, and can be derived from the glycerolysis of TAG. DAG plays an important role in the TAG biosynthesis pathway, which has been described in Chapter 1 (Figure 1.11). The synthesis of DAG begins with glycerol-3-phosphate which is primarily derived from dihydroxyacetone phosphate, a product of glycolysis. G3P is first acylated with acyl-coenzyme A to form lysophosphatic acid, which is then acylated with another molecule of acyl-CoA to yield phosphatidic acid. Phosphatidic acid is then finally dephosphorylated to form diacylglycerol.

Triacylglycerol is an ester comprising glycerol and three fatty acids. They are the main constituents of fats in animals and plants. TAGs can possess saturated fatty acids and unsaturated fatty acids. Unsaturated fats have double bonds between some carbon atoms and have a lower melting point and are more likely to be a liquid at room temperature. Saturated fats are saturated with hydrogen; they have no double bonds, tend to have a higher melting point and are solid at room temperature.

TAGs comprise around 23% of the lipids measured in this study (Chapter 3 Figure). Although they make up a significant portion of wheat grain lipids, over 50% of wheat TAG is lost during milling to

give white flour, due to their location in the germ/aleurone. Therefore, the TAG content observed in wheat flour is less than half of the wheat grain TAG content.

1.9.4 Effects of Saturations

Along with TAG, FFA makes up the majority of the lipids present in wheat flour, making up 25-35% of the lipid content (chapter 3, Figure 3.2). Aside from their abundance, FFAs are highly important due to the role they play in various areas of breadmaking quality, most of which are negative. High levels of FFA can cause rancidity in food products. In the case of polyunsaturated fatty acids, this is caused by the polyunsaturated fatty acids (PUFAs) acting as a substrate for wheat lipoxygenase (Castello et al., 1998).

However saturated FFAs are also believed to play a crucial role on gas cell stability. Through the use of lipases and surfactants, lipids which are more beneficial to gas cell stability can be generated within the dough system. Surfactants at the water/air interface are capable of forming a single or monolamellar phase system around gas cells. Three types of films can be created, the gaseous, expanded and condensed liquid films, all of which provide different properties. Among these, the condensed monolayers provide the most stable barrier against the coalescence of gas cells (Krog, 1981, Sroan & MacRitchie, 2009). It has been reported that saturated FFAs form condensed monolayers which are beneficial to breadmaking quality, while unsaturated FFAs form expanded monolayers which are detrimental to gas cell stability (Sroan & MacRitchie, 2009). Although these effects were observed from the actions of surfactants and lipases these effects are likely to be replicated by the intrinsic wheat lipids, albeit to a smaller scale.

A study by a group in Belgium (Gerits et al., 2014) used lipases to change the lipid distribution in the dough. These changes were then measured against increases in bread loaf volume. Pearson's correlation coefficients were placed between particular lipid classes and loaf volume, in order to see what lipid class changes would correlate with loaf volume. LPC was negatively correlated with loaf volume in this study. Although the study by Pauly et al., 2013 revealed LPC to form complexes with puroindolines during dough mixing and fermentation to enhance foam stabilization, this study was working with semi-sweet biscuit texture not breadmaking quality. Furthermore it provides no evidence in vivo for the enhancement of foam stability through LPC and puroindolines. Unfortunately to the best of the author's knowledge, no studies on the impact of saturated and unsaturated LPCs on breadmaking quality have been conducted.

1.9.5 The Key to Gas Cell Stability

It is known that lipids play a role in baking quality, most likely by influencing the gas cell stability of dough. Gas cell stability is of great importance due to the fact that it determines the extent to which bubbles coalesce, allowing the formation of the fine texture associated with UK sliced bread. Gas cells or bubbles can be stabilised by proteins, surfactants or lipids forming a stabilising layer on the surface of the bubbles. This stabilising layer is critical during dough proving, as when the gas cells come into contact, the risk of coalescence is markedly increased. The supporting dough network allows the gas bubbles (surrounded by liquid in the native dough) to come into contact. Lipids are considered to be crucial for gas bubble stability, with a significant effect on the loaf volume and crumb structure of bread.

It has been shown that above a critical concentration, polar lipids have a stabilizing effect on gas cells, increasing loaf volume, whereas non-polar lipids have a destabilizing effect on gas cells decreasing the loaf volume (MacRitchie & Gras 1973; Sroan & MacRitchie 2009). Furthermore, the monolayer at the gas-lipid interface plays a major role on the gas cell stability mechanism. Condensed monolayers are formed by the tight orientation of surface active molecules such as lipids and proteins, which can prevent the collapse of the lamellae and are thus desirable for gas cell stability. Long chain fatty acids and lipids such as DGDG are thought to be required for the formation of these condensed monolayers (MacRitchie 1990).

1.9.6 Effects on Loaf Volume

How do the endogenous lipids stabilize the gas cells and subsequently affect loaf volume? It has been observed that a phenomenon referred to as lipid binding occurs during dough mixing, which is the redistribution of lipids from a free to bound form (Carr, Daniels & Frazier, 1992). During dough development lipids are eluted from the surface of starch granules and become trapped in the gluten network where they subsequently interact. This process has a positive impact on the gluten network. Through this, the lipids are able to indirectly stabilise the gas cells in the dough. Polar lipids are also capable of directly stabilizing the gas cells in dough. Several authors have written about gas cell stabilization which they attribute to two different mechanisms, the gluten network, and the liquid lamella that surrounds the gas cells. It appears that the gluten network predominantly stabilises the gas cells at the early stages of breadmaking. However, after around 15 minutes, as fermentation begins to occur together with various baking discontinuities, a thin liquid lamella forms around the gas cells which become stabilized by the adsorbed surface active proteins or polar lipids. This provides a further mode of gas cell stabilization as well as a secondary durability mechanism. Although the

reasons have not been fully elucidated, it is generally believed that hydrolysed polar lipids resemble emulsifiers and therefore enhance the stabilization of gas cells. Further details on the liquid lamellae are provided in Chapter 5.

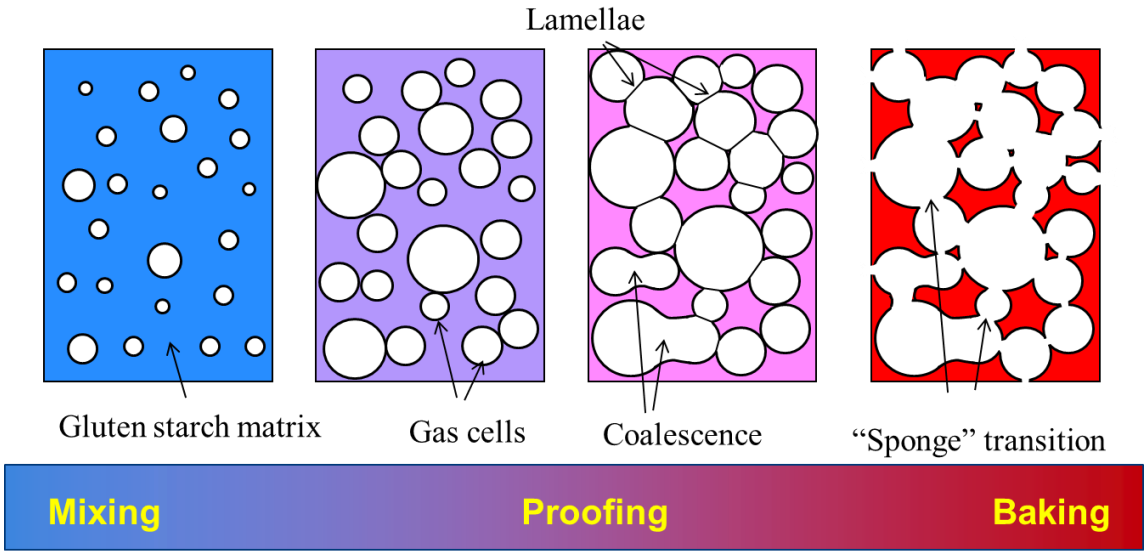


Figure 1.18 Gas cell formation and development during various stages of the breadmaking process. The liquid lamellae forms around gas cells as bubbles expand and come in to closer contact in order to prevent coalescence.

However, surface active proteins and polar lipids stabilize the gas cells in different ways. Surface active proteins form viscoelastic films to secure the gas cells, as do polar lipids. However, these components actually compete with one another for the gas cell interface and end up impairing each other's ability to sustain the gas cells (Mills et al., 2003).

Within the breadmaking industry, increasing interest has been directed towards using lipases to substitute for surfactants. This is due to the fact that lipases hydrolyse the endogenous wheat lipids in the dough to form surface active lipids (Aravidan et al., 2007). The addition of lipases is also capable of modifying the lipid population *in situ* without altering the other flour components.

The role of lipids in determining loaf volume was further shown by the addition of lipases to breadmaking (Gerits et al., 2014). It was found that the addition of lipases such as Lipopan F and Lecitase Ultra which caused the hydrolysis of galactolipids and phospholipids led to an increase in FFAs that strongly affected the bread loaf volume. The reason for why this increase in FFA affects loaf volume is unclear, as the addition of FFA to breadmaking has never shown an increase in bread loaf volume. This was shown in studies by de Stefanis and Ponte, 1976 as well as Sroan and MacRitchie, 2009, where linoleic acid, the most common fatty acid in wheat flour was added to native

and defatted flour in a breadmaking trial. In fact FFA was even shown to decrease loaf volume (Sroan and MacRitchie, 2009). Therefore, it is more likely that the residual molecule the FFA was derived from was responsible for improving loaf volume. On the other hand, all galactolipid types and even the lyso-forms of galactolipids were found to positively affect the loaf volume.

1.10 Environmental effects on wheat grain lipids

The effects of environment on crops have long been determined. Wheat is no exception. There have been numerous studies showing the effects of various environmental factors on the lipid profile of different wheat tissue. Cold conditions were observed to affect the lipid composition of thylakoid membranes in two wheat varieties (Vigh et al., 1985). Heat stress was associated with major lipid alteration in the lipid profile of wheat leaves (Naryanan et al., 2016). A study by Williams, Shewry and Harwood detected changes in the lipid composition of wheat plants when subjected to low nitrogen fertilizer application and an elevated CO_2 atmosphere (Williams et al., 1994).

However, there have been limited studies on the effects of environmental factors on the lipid composition of the wheat grain and flour. Considering the important role lipids play on breadmaking quality, observing environmental effects on wheat grain and flour lipids would be highly interesting. Furthermore, with breadmaking consistency being crucial for industry, the effects of a highly fluctuating parameter, environment, on a key quality component such as wheat flour lipids may be highly valuable.

1.11 Wheat genetics

1.11.1 Summary

Bread wheat has a hexaploid genome, with three pairs of 7 chromosomes which are derived from related grass species, giving a total of 42 chromosomes. Although possessing fewer chromosomes than the diploid human being (which has 46 chromosomes) the wheat genome consists of 17 billion bases, about five times as many as the human genome, and contains around 124,000 genes (IWGSC, 2014).

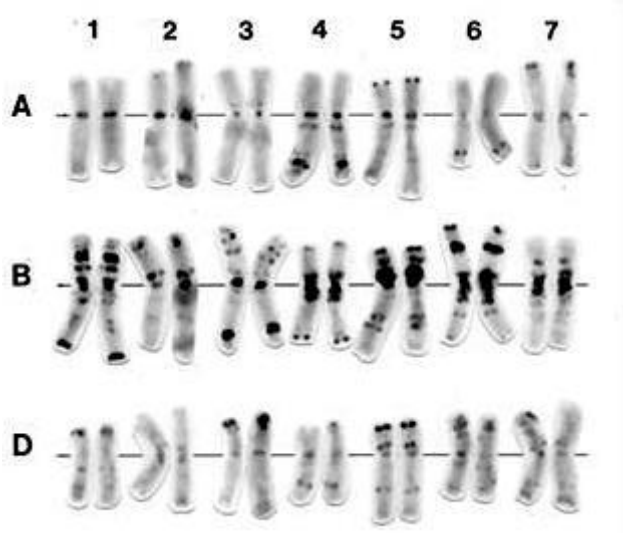


Figure 1.19 The chromosomes of the wheat genome. The wheat genome is hexaploid, with 3 sets (A, B and D) for each of its 7 chromosomes which have come from related species (Image taken from the triticae genome website http://www.triticeaegenome.eu/pillar.php?p=2)

In the future, a greater understanding of the wheat genome will allow us to identify more, if not all, wheat genes and their functions, including those responsible for breadmaking quality. Being hexaploid, there are challenges in sequencing the wheat genome, particularly in distinguishing between the three 'subgenomes'. These chromosomes also have extensive repeated DNA sequences making it difficult for algorithms to stitch together short sequence fragments into a coherent whole. However, synteny exists between the genomes of cereal species which can be exploited. Therefore, homologues of almost all the sequenced wheat genes are found in the rice genome (Goff et al., 2002), meaning that it is possible to use the rice genome as a model for wheat. This together with the development of large libraries of EST (Expressed Sequence Tags) will allow almost all major expressed genes in wheat to be added to the National Centre for Biological Information (NCBI).

Gene expression in the developing grain has been studied by analysing gene transcripts and proteomic analysis. Around 4500-8000 genes have been shown to be active in wheat in the developing grain, and analysis of around 1000 of these genes showed that 60% of them could be identified and classified according to their predicted function (Clarke et al., 2000). By analysing the proteome of the developing and mature grain, it has been estimated that by around 17 days post anthesis (dpa), there are 1298 detectable proteins, which fell to around 1125 around 28 dpa. Of these proteins, around 60% were prolamins of various types, the S-rich α, β and γ gliadins/LMW subunits of glutenin being the most abundant (Skylas et al., 2000).

1.11.2 Breadmaking quality QTLs

As discussed above, there are numerous studies relating wheat genetics to quality. Although the major genes that control endosperm texture and the genes controlling the HMW glutenin subunits play a vital role, it is likely that breadmaking quality is also affected by other minor genes. For example, although HMW glutenin subunits are believed to account for 45-70% of the variation in breadmaking performance within European wheat varieties (Branlard & Dardevet, 1985; Payne et al., 1987b, 1987a), there is still a great deal of variation left unaccounted for.

The use of newer technologies such as QTL analysis and marker assisted selection makes it possible to study a wider range of parameters that affect breadmaking quality, rather than focusing on the traditional baking performance indicators such as grain hardness, protein content and dough rheology.

Quantitative Trait Loci are stretches of DNA which contain or are linked to genes underlying a 'quantitative trait.'Kuchel et al., (2006) identified a number of QTL in a doubled haploid (DH) population based on the varieties Trident x Molineux. Details on doubled haploid production are given in Chapter 5. These QTL were related to dough rheology, loaf volume and crumb score as well as milling yield, flour protein content and colour. Some of the QTLs were related to the HMW and LMW glutenin subunits encoded by chromosomes 1A and 1B, but several other QTLs for traits such as loaf volume, crumb quality and flour colour were identified at other locations on chromosomes 2A, 3A, 6A and 7B. Similarly, Groos et al., (2007) used a population of 194 recombinant inbred lines from the cross Renan x Recital grown in three environments to map similar QTL on chromosomes 1A, 1B, 3A and 7B and novel QTL on chromosomes 2B, 5B, 6B and 7A.

Finally, another project funded by the HGCA investigating wheat functionality through breeding and end use identified several new novel QTL for breadmaking quality using three DH populations, Malacca x Charger, Shango x Shamrock and Malacca x Hereward (Millar et al., 2007). Further details of this project will be discussed in chapter 5.

1.12 Final Remarks and Thesis Aims

Breadmaking quality is a complex trait affected by various parameters such as mechanical handling, the choice of inputs and the actions of various biological components both exogenous and endogenous to white flour. Therefore, breeders and bakers both have an enormous task of satisfying the demands of an ever more demanding public within the UK, as well as a growing global population.

Although this introduction has focused solely on the breadmaking quality required by the UK breadmaking industry, different properties are required for other types of product. Various biological components within the wheat grain have been researched, with proteins, starch, enzymes, lipids as well as other minor components playing an important role on breadmaking quality, especially the HMW glutenin subunits.

Yet, despite HMW subunits accounting for over 45-70% of breadmaking performance, it has not answered some crucial questions associated with successful breadmaking wheat varieties over the years. In section 1.2.3 it was highlighted that the cultivars, Mercia, Hereward and Malacca had excellent quality while not achieving exceptional HMW subunit scores. Hereward in particular was incredibly successful, seeing widespread use in the UK as well as other European countries such as France and the Netherlands for over two decades, and is still favoured by some farmers to this day. This phenomenon has therefore been dubbed the "Hereward conundrum," and it would be highly interesting, not just from a scientific perspective, but an industrial perspective to solve this conundrum.

Clearly proteins alone are not solely responsible for providing excellent breadmaking quality. Lipids became an excellent candidate for research, due to their effects on gas cell stability in the dough, contributing to loaf volume and crumb structure. However, are lipids suitable candidates for improving breadmaking quality? Are they encoded by specific wheat genomes? Are there even genetic effects on the lipid composition of the wheat grain? Will these genotypic effects even be significant considering lipids make up a mere 1.5-2% of wheat flour? Are environmental effects going to be larger than these genotypic effects?

These are just some of the questions surrounding the potential of improving the lipid composition of the wheat grain to improve breadmaking quality. This will facilitate the improvement of wheat quality by breeders and may also reduce the need for improvers in the baking industry, contributing to the "clean labels" favoured by consumers. An improved endogenous lipid profile in wheat for breadmaking quality may have significant impacts on both consumer health as well as the baking industry. For industrial bakers, it may appease consumers that demand more transparency with the ingredients added to our food. While the wellbeing trend continues, and an emphasis on 'natural;' ingredients persist, being able to remove exogenous lipid sources, such as emulsifiers and surfactants in white loaves will be considered a step in the right direction by a scrutinizing public.

Overall, this project aims to identify the potential genetic effects on the lipid composition of wheat flour, to potentially identify the genetic control of a superior lipid profile for breadmaking quality. Due to the minor levels of lipids present in wheat, improvements will be small in scale. However small changes can make big differences. For bread, a staple food, widely consumed by the public on a daily basis, small improvements can have major additive effects. Considering the benefits improving

the lipid composition endogenous wheat lipids may have to breeders, bakers and our health, these improvements should certainly be worthwhile.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Wheat cultivars

The following lines of wheat were used in this project:

The cultivars (cv) Avalon, Cadenza, Crusoe, Hereward, Istabraq, Malacca (grown at Rothamsted Research, Harpenden, UK, 2013)

Malacca x Hereward Near Isogenic Lines (NILs) (grown at John Innes Centre, Norwich, United Kingdom, 2013; John Innes Centre, Norwich, UK, 2015)

Malacca x Hereward Doubled Haploid lines (DH) (grown at Rothamsted 2012).

2.1.2 Chemicals

Acetic Acid	Sigma Aldrich (Haverhill, UK)
Acetone	Thermo Fisher (Hemel Hempsted, UK)
alpha amylase	Megazyme (Leinster, Ireland)
Ammonium Acetate	Thermo Fisher (Hemel Hempsted, UK)
Amyloglucosidasae	Megazyme (Leinster, Ireland)
Bromophenol Blue	Sigma Aldrich (Haverhill, UK)
Butanol	Thermo Fisher (Hemel Hempsted, UK)
Calcium Chloride	Sigma Aldrich (Haverhill, UK)
Chloroform	Sigma Aldrich (Haverhill, UK)

Coomassie Brilliant Blue Dye	Sigma Aldrich (Haverhill, UK)
Dichloromethane	Thermo Fisher (Hemel Hempsted, UK)
Diethyl ether	Thermo Fisher (Hemel Hempsted, UK)
2,2 Dimethoxypropaane (DMP)	Sigma Aldrich (Haverhill, UK)
Dimethylsulfoxide (DMSO)	*
Dithiothreitol (DTT)	Sigma Aldrich (Haverhill, UK)
Ethanol	Sigma Aldrich (Haverhill, UK)
Glycerol	Sigma Aldrich (Haverhill, UK)
Hexane (Hex)	Thermo Fisher (Hemel Hempsted, UK)
Heptane (Hep)	Thermo Fisher (Hemel Hempsted, UK)
Hydrochloric Acid (HCl)	Sigma Aldrich (Haverhill, UK)
Isopropanol (Propan2-ol)	Thermo Fisher (Hemel Hempsted, UK)
Methanol (Met)	Thermo Fisher (Hemel Hempsted, UK)
Petroleum Ether (PEt)	Thermo Fisher (Hemel Hempsted, UK)
Potassium Chloride (KCl)	Sigma Aldrich (Haverhill, UK)
Primuline	*
Sodium Acetate (NaOAc)	Sigma Aldrich (Haverhill, UK)
Sodium Chloride (NaCl)	Sigma Aldrich (Haverhill, UK)
Sodium Dodecyl Sulphate (SDS)	Sigma Aldrich (Haverhill, UK)
Sodium Hydroxide (NaOH)	Thermo Fisher (Hemel Hempsted, UK)
Sulfuric Acid	Sigma Aldrich (Haverhill, UK)
Toluene	Thermo Fisher (Hemel Hempsted, UK)

Trichloroacetic Acid (TCA)	Fisher Scientific, (Leicestershire, UK
Trisaminomethane (Tris)	Sigma Aldrich (Haverhill, UK)

2.1.3 Analytical standards

For quantitative analysis the following standards were used:

C15:0 Pentadecanoic acid	Sigma Aldrich (Germany)
C45:0 Triacylglyceride	Sigma Aldrich (Germany)
Phospholipid Standards	Avanti Polar Lipids (USA)

24:1 (Cis) PC 1,2-dinervonoyl- *sn*-glycero-3-phosphatidylcholine

13:0 Lyso PC 1-tridecanoyl-2-hydroxy-*sn*-glycero-3-phosphatidylcholine

18:0-20:4 PI 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphatidylinositol

14:0 PE 1,2-dimyristoyl-sn-glycero-3-phosphatidylethanolamine

14:0 PG 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol)

14:0 PA 1,2-dimyristoyl-sn-glycero-3-phosphate

2.2 Methods

2.2.1 Sample preparation, growth and storage

2.2.1.1 Milling and Storage

Wheat grains were stored at room temperature, and approximately 50 grams of grain for each sample was milled using a Chopin CD1 mill, which complies with the NF EN ISO 27971 standard. Before milling, grains were conditioned to a 14% moisture content overnight by adding the appropriate amount of water to the wheat grains calculated using the equation below.

(16 – Current Moisture Content) x 0.5 = ml of water to add

With 16 indicating the % moisture content desired. Moisture levels were measured using the Bruker Minispec NMR. Straight grade flour was obtained for each sample after undergoing a break and reduction stage and sifting through a centrifugal sifter. Flour samples were stored at -20˚C.

2.2.1.2 Six UK cultivars

Six U.K. wheat cultivars were grown on the experimental farm at Rothamsted Research, Harpenden, U.K, in 2013 as part of the Wheat Genetic Improvement Network (WGIN) nitrogen use efficiency trial. These were five modern hard bread making cultivars (Avalon, Cadenza, Crusoe, Hereward, and Malacca) and one soft feed cultivar (Istabraq). Cultivars were grown in triplicate 9 × 3 m plots at three levels of nitrogen fertilization, 100, 200, and 350 kg/ha using ammonium nitrate. The 200 kg/ha (200N) is typical for intensive wheat production in the U.K, while the 100 kg/ha (100N) is more typical of less intensive systems used in many other countries. The highest application level of 350 kg/ha (350N) was above those typically used in crop production but was included to explore the effects of unusually high nitrogen availability. Cultivars were randomized to split-plots within the main plots of the nitrogen treatments.

Figure 2.1 Aerial image of the WGIN variety trial using over 25 cultivars. Details of the varieties grown can be found in Barraclough et al., 2010.

2.2.1.3 Malacca xHereward Near Isogenic Lines (NILs)

The lines were grown at Rookery Field, Church Farm, Bawburgh in 2013 and Deopham, near Wymondham in 2015, both located in Norwich, UK. Wheat lines were grown under standard agronomic conditions of about 200 kg/N/Ha. Details on the generation of the NILs can be found in Chapter 4.

Figure 2.2 Field plots of the Malacca x Hereward Near Isogenic Lines grown by the John Innes Centre, Norwich.

2.2.1.4 Malacca x Hereward Double Haploid (DH) population

The M x H DH population was developed by RAGT Seeds, United Kingdom. Grain material used in the SDS-PAGE identification were harvested by RAGT in 2005 and kept at room temperature. Flour samples used for the Fatty Acid Methyl Ester Gas Chromatography-Flame Ionization Detector (FAMEs GC-FID) analysis were grown at Rothamsted, harvested and milled in 2012 following the method described in section 2.2.1.1. Details on DH development are provided in Chapter 6.

2.2.1.5 Dough Liquor Preparation

Doughs were prepared by mixing specific quantities of flour, water and sodium chloride (NaCl) in a glass beaker. The exact recipes used for the development of a diluted dough liquor extraction method and the rheological analysis of diluted dough liquor are given in Chapter 5. Dough was mixed at 500 rpm using a magnetic stirrer for approximately 4-5 minutes, weighed into polycarbonate ultracentrifuge bottles (38-102 mm) with screw on titanium caps (Becman, UK) and incubated at 30˚C for one hour. Tubes were centrifuged in a pre-warmed (30˚C) fixed angle rotor at 41,000 g for 30 minutes, allowing the dough to be held at 30˚C for a total of 90 minutes, in accordance with standard bakery practice. The resulting supernatant (dough liquor) a straw coloured viscous material with a creamy pellicle, was then removed and the pellet discarded.

2.2.2 Lipidomic Analysis

2.2.2.1 Lipid Extraction from Flour

Non-starch lipids were extracted from flour samples as described by Finnie, Jeannotte and Faubinon *et al*, 2009 with some modifications. Flour (150mg) was added to a glass tube and was heated in boiling water for 12 mins to inactivate lipid-hydrolysing enzymes. Samples were then extracted sequentially with 1.5 ml of petroleum ether (PEt), water-saturated butan-1-ol (WSB), and propan-2-ol/water (90:10) (IW). The PEt and WSB extracts were washed by shaking with 0.88% KCl in a 1:1 ratio for 2 mins at 18000 rpm. The supernatants from the PEt and WSB extractions were combined, evaporated under nitrogen at 40℃, re-suspended in 1.5 ml of chloroform and washed with 1.5 ml of 0.88% KCl. The lower phase was retained, filtered through a 0.45µm Millex-FH filter (Merck Millipore, Germany), dried under a stream of nitrogen gas and re-suspended in 2ml of chloroform. The lipid extracts were then flushed with nitrogen and stored at -20 ℃.

2.2.2.2 Lipid extraction from dough liquor

Lipids were extracted from diluted dough liquor samples using the method described by Bligh and Dyer (1959) with some modifications. Firstly, 3.75 ml of chloroform:methanol (1:2) was added to 1ml of diluted dough liquor, the mixture was then shaken at 20˚C for 10-15 minutes at 250 rpm, vortexed and centrifuged at 2000 rpm for 10 minutes to enable the removal of the lipid in the lower layer. A

further 3.75 ml of chloroform:methanol:water (1:2:0.8) was added to the remaining precipitate. The supernatants from the first and second extractions were combined; 3.2 ml of chloroform and 3.2 ml of 0.88% KCl was added, followed by a rapid vortex and centrifugation at 2000 rpm for 5 minutes. The lower phase was removed and evaporated with N_2 while the upper phase was extracted using 2.5 ml chloroform. The upper phase mixture was vortexed and centrifuged at 2000 rpm for 5 minutes and the new lower phase was combined with the old one. Post-evaporation the dried sample was resuspended with 1ml of chloroform. Lipid extracts from dough liquor were flushed with nitrogen and stored at -20˚C.

2.2.2.3 Principles of acid catalysed esterification of fatty acids

In order for the fatty acids bound to lipids to be analysed by GC, the fatty acids need to be split off and converted into derivatives with a lower boiling point, such as alcoholic esters. The fatty acids in food and biological samples are therefore commonly analysed by gas chromatography (GC) of their fatty acid methyl esters (FAMEs). Various methods of esterification exist, however in this study acid was used as the catalyst.

Free fatty acids can be esterified and triacylglycerols can be transesterified through high temperatures with excess quantities of anhydrous methanol and an acidic reagent acting as a catalyst. The acid catalysed esterification process occurs in three stages. First the acid undergoes protonation which forms an oxonium ion. Then the proton is lost to become an ester. Although each step in the reaction is reversible, in the presence of a large excess of alcohol, the equilibrium point of the reaction is displaced so the esterification proceeds to near completion. Water inhibits the process as it is a stronger electron donor than aliphatic alcohols. As a result the formation of the intermediate is not favoured, causing esterification to not occur properly.

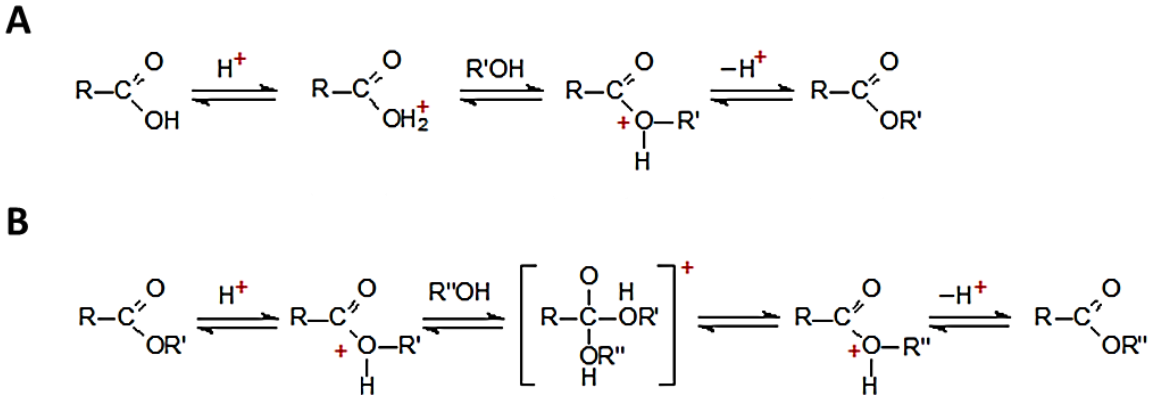


Figure 2.3 Schematic for the acid catalysed esterification (A) and transesterification of lipids (B)

Transesterification, also known as ester exchange, occurs in a similar fashion to esterification. Following the first protonation of the ester, the exchanging alcohol is added to form the intermediate which can be dissociated via the transition state to give the ester. Once again in conditions of excess alcohol, the equilibrium point of the reaction becomes displaced, so the ester conversion is almost 100%. Water is an inhibitor in this process too, as water can hydrolyse the intermediates to free acids.

As mentioned above, water inhibits both the esterification as well as the transesterficiation process. Therefore dimethoxypropane is added as a water scavenger in the reaction.

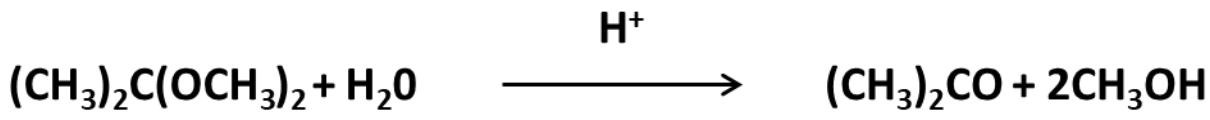


Figure 2.4 Reaction of dimethoxypropane as a water scavenger.

2.2.2.4 Fatty acid esterification

The total fatty acids in flour in this project were methylated by heating the samples at 80˚C for 2 hours with a 2 ml solution containing methanol/toluene/dimethoxypropane/H_2SO_4 (66:28:2:1 by volume) as described by Ruiz-Lopez et al., 2015. Methyl pentadecanoate (C15:0) was added to samples as an internal standard. After cooling, 1.5 ml of hexane was added, and FAMEs were recovered from the upper phase.

2.2.2.5 Fatty Acid Methyl Esters (FAMEs) Gas Chromatography-Flame Ionization Detector (GC-FID)

Methyl ester derivatives of total fatty acids were analysed by flame ionization detection using a gas chromatography−flame ionization detector (GC−FID, Agilent 6890, Palo Alto, CA, U.S.A.) with an AT-225 capillary column of fused silica (30 m length, 0.25 mm inner diameter, and 0.20 μm film thickness). The oven temperature cycle was set with a start temperature of 50 ℃, which was held for 1 min to allow for vaporized samples and solvent (hexane) to condense at the front of the column. The oven temperature was then raised rapidly to 190℃ at a rate of 40℃/min, followed by a slower increase to 220 ℃, which was held for 1 min, giving a total run time of 25 min and 50 seconds per sample. Hydrogen was used as the carrier gas. FAMEs were identified by comparison to known standards (37 FAMEs, Sigma, St. Louis, MO, U.S.A.) and confirmed by gas chromatography−mass spectrometry (GC−MS, Agilent 6890N, Palo Alto, CA, U.S.A.) 5 mg ml^{-1} of pentadecanoic acid 15:0 was used as an internal standard for quantification.

2.2.2.6 Electrospray Ionization-tandem triple quadrupole Mass Spectrometry (ESI-MS/MS)

ESI-MS/MS is a technique used in mass spectrometry to produce ions using an electrospray where a high voltage is applied to a liquid to create an aerosol (Brügger B et al., 1997). It is useful in order to produce ions from macromolecules and large non-polymeric molecules such as lipids, which overcomes the propensity of these molecules to fragment when they are ionized. ESI provides very little fragmentation which is advantageous as a molecular ion is always observed. However, it has the disadvantage of providing very little structural information if one simple mass spectrum is obtained.

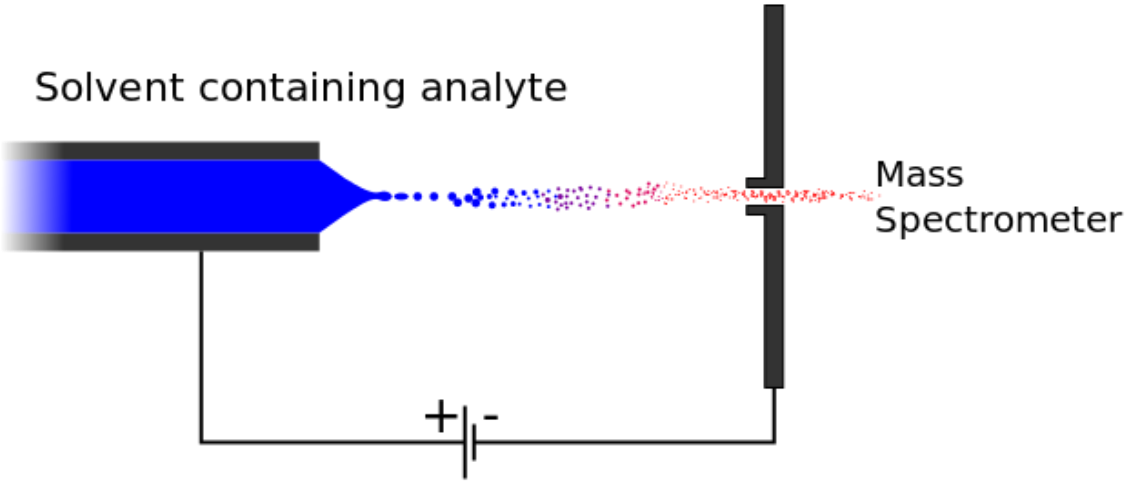


Figure 2.5 Diagram of electrospray ionization.

This however is overcome by tandem mass spectrometry which involves multiple steps of mass spectrometry selection, with fragmentation occurring between the stages. In a tandem mass spectrometer, ions are formed in the ion source and are separated by mass-to-charge in the first stage. Ions of a particular mass-to-charge ratio (precursor ions) are selected and fragment ions (product ions) are created by collision-induced dissociation. The second stage separates the resulting ions and detects them.

In the triple quadrupole mass spectrometer (QqQ) used in this study, the first and third quadrupoles act as mass filters and the second quadrupole causes analyte fragmentation through the interaction with collision gas. The mass filters Q1 and Q3 contains four parallel, cylindrical metal rods, which are both controlled by direct current (dc) and radio frequency (rf) potentials. The middle collision cell, q, is only controlled by the rf potential. The rf potential associated with the collision cell allows specific selected ions to pass through.

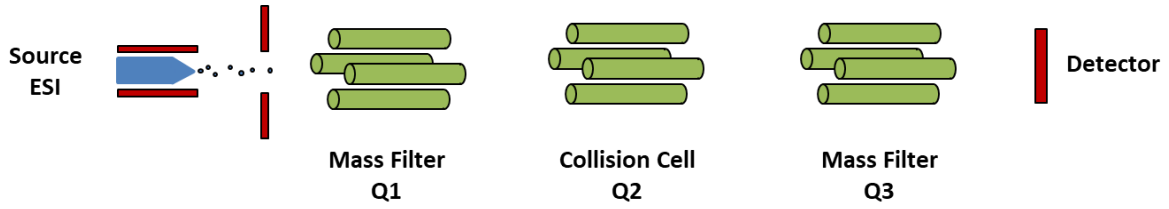


Figure 2.6 Schematic of the ESI triple quadrupole mass spectrometer

MS/MS allows for mass analysis to occur in a sequential manner in different parts of the instrument. The triple quadrupole MS (TQMS) follows a tandem in space arrangement, where the separation elements are physically separated and distinct, although there is a physical connection between the elements to maintain high vacuum.

The arrangement of the TQMS allows four different scans to be performed. In this study, two of these, the precursor and neutral loss scan were used. The precursor scan, selects a certain product ion in Q3, and the precursor masses are scanned in Q1. This method selects ions which have a particular functional group released by the fragmentation in Q2.

In a neutral loss scan both Q1 and Q3 are scanned together, however with a constant mass offset. This provides selective recognition of all the ions which has lost a given neutral fragment from the fragmentation in the Q2, which in this study was NH_4. The neutral loss scan is useful for selectively identifying closely related compounds in a mixture.

2.2.2.7 Quantitative Lipid Analysis

This study utilized Electrospray ionization−tandem triple quadrupole mass spectrometry (ESI−MS/MS, 4000 QTRAP, SCIEX) to quantify the major types of lipid in wheat flour: the PLs phosphatidylcholine (PC), phosphatidylethaloamine (PE), and lysophosphatidylcholine (LPC), the galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), and the neutral lipids (NLs) Free Fatty Acids (FFA), Dicylglycerol (DAG), and Triacylglycerol (TAG). Monoacylglycerols (MAGs) were not analysed due to their low levels in flour. Internal standards for polar lipids were obtained from Avanti (Alabaster, AL, U.S.A.) and were incorporated as 0.857 nmol of 13:0-LPC, 0.086 nmol of di18:0-PI, 0.080 nmol of di14:0-PE, 0.800 nmol of di18:0-PI, and 0.080 nmol of di14:0-PG, dissolved in chloroform. A total of 25 µl of sample dissolved in chloroform was combined with the standard and chloroform/methanol/400 mM ammonium acetate (300:665:3.5, v/ v/v) to make a final volume of 1 ml. The lipid extracts were added at a rate of 15 µl/min with an autosampler (HTS-xt PAL, CTC-PAL Analytics AG, Switzerland). Data acquisition and acyl group identification were as described by Gonzalez-Thuillier et al., 2015 with modifications. FFAs were analyzed by combining 25 µl of sample, 0.607 nmol of 15:0-FFA (Sigma-Aldrich, St. Louis, MO, U.S.A.) and propan-2-ol/ methanol/50 mM ammonium acetate/dichloromethane (4:3:2:1, v/v/ v/v) to a final volume of 1 ml, quantified by the Q1 ESI−MS negative mode.

DAG and TAG contents were quantified using the ESI−MS/MS method described by Li et al., 2014 with some modifications. For TAG, 15 µl of sample and 0.857 nmol of tri15:0-TAG (Nu-Chek Prep, Elysian, MN, U.S.A.) were combined with chloroform/methanol/300mM ammonium acetate

(24:24:1.75, v/v/v), and for DAG, 25 µl of sample and 0.857 nmol of 18:0−20:4-DAG (SigmaAldrich) were combined with propan-2-ol/methanol/50mM ammonium acetate/dichloromethane (4:3:2:1, v/v/v/v), to the final volume of 1 ml for direct infusion to the mass spectrometer. TAG and DAG were detected as $[M + NH_4]$ + ions by a series of different neutral loss scans, targeting the losses of fatty acids. The scans as well as the parameters used for the three NLs are shown in appendix figure 2.1. The data was processed using the LipidView software (SCIEX, Framingham, MA, U.S.A.), where isotope corrections were applied. The peak area for each lipid was normalized to the internal standard and further normalized to the weight of the initial sample. The ionization efficiency varies among acyl glycerol species with different fatty acyl groups, and response factors for individual species were not determined in this study. Consequently, the values are not directly proportional to the TAG/DAG contents of each species. However, the approach does provide a valid comparison of TAG/ DAG species between samples.

As the acquisition of MS data requires long periods of sample infusion, a quality-controlled approach was employed to remove any instrument or analytical variation from the data acquisition process. For example, the intensity of lipid species in samples was normalized to internal standards run with the biological extractions and separately. Furthermore, each sample infusion was replicated and the order of survey scans (Appendix Figure 2.1) was changed, for example i.e. the position of the PC 184 *m/z* head group scan was varied between the first and last positions within the analytical run.

2.2.3 Quality control of lipidomic data

2.2.3.1 Determination of Calibration Factors for Lipid Groups

PLs are more precisely determined by MS than FFAs and TAGs, which are overestimated and underestimated, respectively. Correction factors were therefore determined and applied to the data for FFAs and TAGs. Equal volumes of lipid extractions from over 28 flour samples were taken and quantified using two systems: ESI−MS/MS as described above and thin-layer chromatography−gas chromatography (TLC−GC).

2.2.3.2 TLC-GC-FID

TLC− GC was carried out by adding 50 µg of 45:0-TAG/15:0-FFA standard to each sample and separating the lipid extracts on silica gel TLC (plate thickness of 0.25 mm) using the solvent

hexane/diethyl ether/ acetic acid (150:50:2 by volume). The individual lipid classes were identified under ultraviolet (UV) light after spraying with primuline [0.05% (w/v) in 80:20 (v/v) acetone/water], and the TAGs and FFAs were scraped from the plate and directly methylated for FAME analysis. The quantity of each fatty acid was calculated in comparison to the internal standard and normalized for sample weight (grams of flour). The values (nanomoles per gram of flour) from the two procedures were compared in a scatter plot. The data for FFAs were directly compared, whereas for TAGs, the data for molecular species determined by ESI−MS/MS systems were compared to the sum of FAMEs determined by GC−FID. The sum of FAMEs provides a valid representation of the quantities of TAGs comparable to that measured by ESI−MS/MS.

2.2.3.3 Fitting a predictive model

The GenStat statistics package (18th edition, VSN International, Ltd., Hemel Hempstead, U.K.) was used to fit an asymptotic exponential model using the method of nonlinear least squares to estimate two parameters (the asymptote and the exponential rate) with standard error for both GC−FID (FAMEs) FFAs and TAGs in terms of the ESI−MS/MS (QTRAP) equivalents. The SigmaPlot package (13th edition, Systat Software, Inc., San Jose, CA, U.S.A.) was used to produce the picture of the fitted curve on the scatter plot. The mode allowed the prediction of values of FFAs and TAGs for one method given values for the other method.

2.2.3.4 Fitted models for WGIN lipids (Chapter 3)

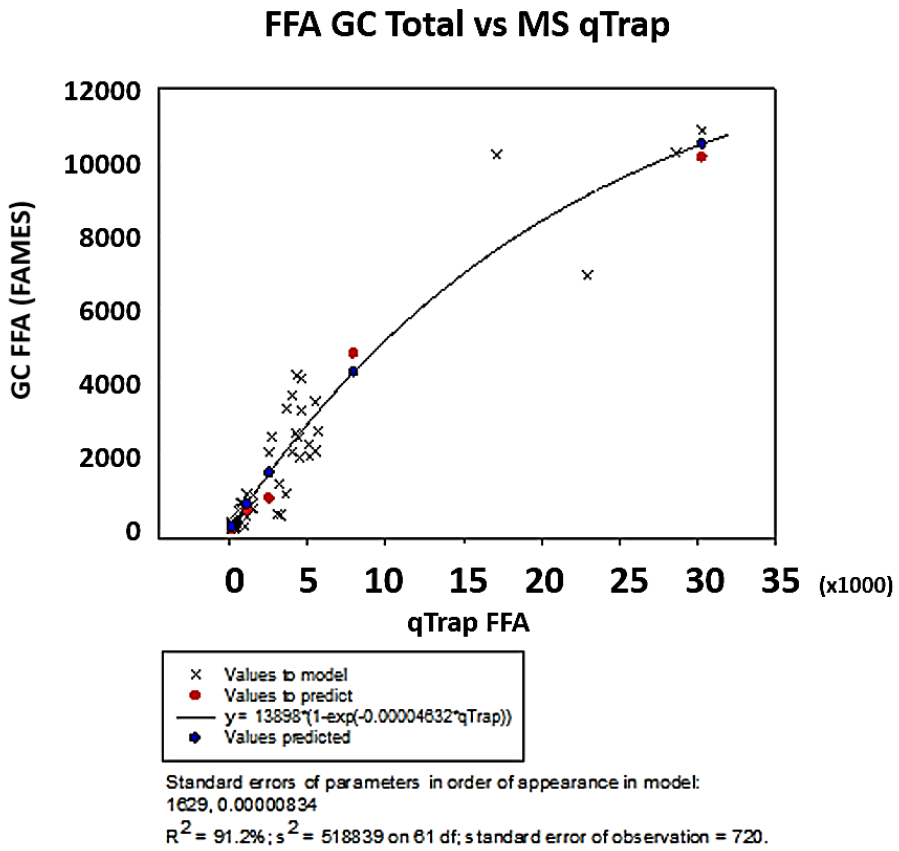


Figure 2.7 The fitted asymptotic exponential curve for the FFA data from the ESI-MS/MS and GC-FID for the WGIN lipids 2013. The equation of the curve used to model FFA FAMES GC (y) on QTRAP MS (x) was

$$y = A(1 - \exp(-Cx))$$

where A is the upper asymptote of the curve, and C is the exponential rate of approach to A. Note that the curve goes through the origin.

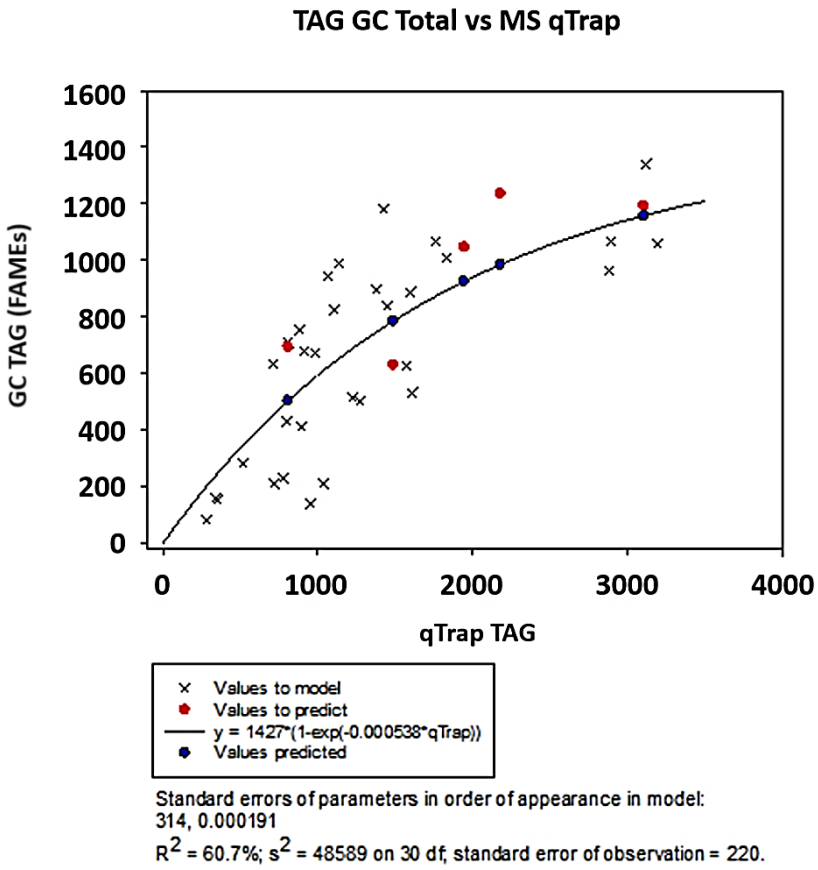


Figure 2.8- The fitted asymptotic exponential curve for the TAG data from the ESI-MS/MS and GC-FID for the WGIN lines in 2013. The equation of the curve used to model TAG FAMES GC (y) on QTRAP MS TAG (x) was

$$y = A(1 - \exp(-Cx))$$

where *A* is the upper asymptote of the curve, and *C* is the exponential rate of approach to *A*. Note that the curve goes through the origin.

2.2.3.5 Fitted models for MxH NIL lipids (Chapter 4)

The equation of the curve used to model GC FFA (*y*) on MS FFA (*x*) was:

$$y = C/(1 + \exp(-B*(MS\_FFA - M)))$$

where *C* is the upper asymptote of the curve, *B* is the exponential rate of approach to *C* and *M* is the value of *MS_FFA* providing a quantity of GC FFA which is half of the asymptote, *C*.

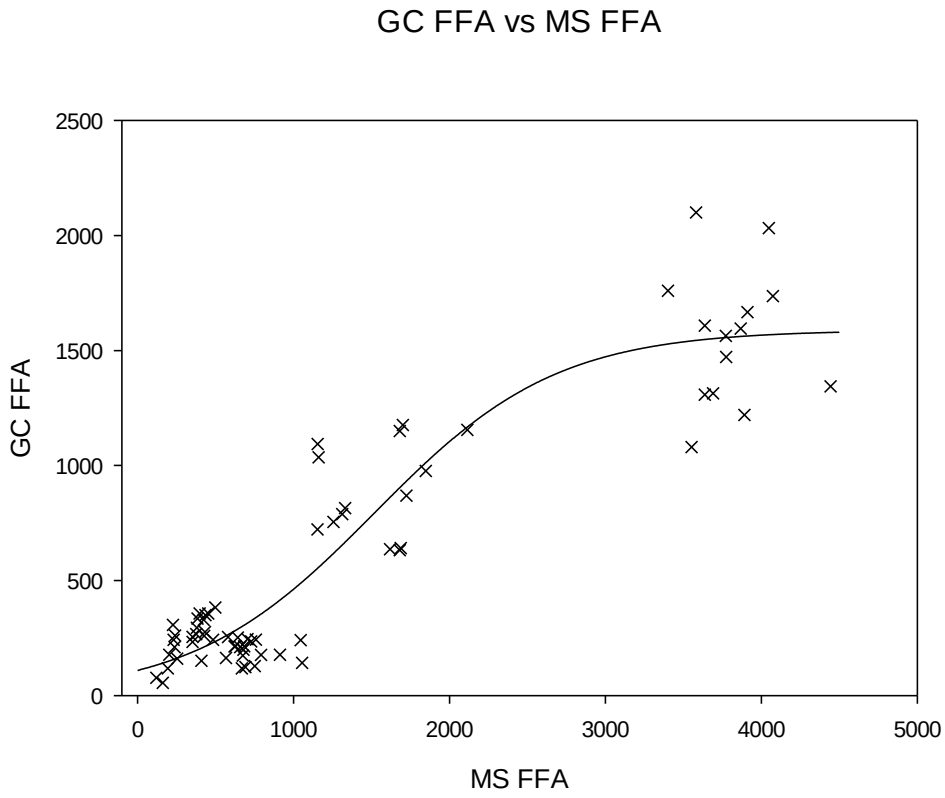


Figure 2.9 Fitted curve for the FFA data from the ESI-MS/MS and GC-FID for the MxH NILs in 2013 and 2015. The equation of the curve used to model FF FMEs GC (y) on QTRAP MS (x) was:

$$y = C/(1 + \exp(-B*(MS\_FFA - M)))$$

where *C* is the upper asymptote of the curve, *B* is the exponential rate of approach to *C* and *M* is the value of *MS_FFA* providing a quantity of GC FFA which is half of the asymptote, *C*.

For this model, the parameter estimates and standard errors are:

Parameter	estimate	s.e.
C	1589.4	63.6
B	0.001715	0.000215
M	1520	102.

The percentage of variance explained, R^2, was 87.1% and the standard error of observations was 202 given the estimated residual variation, s^2, of 40798 on 67 df.

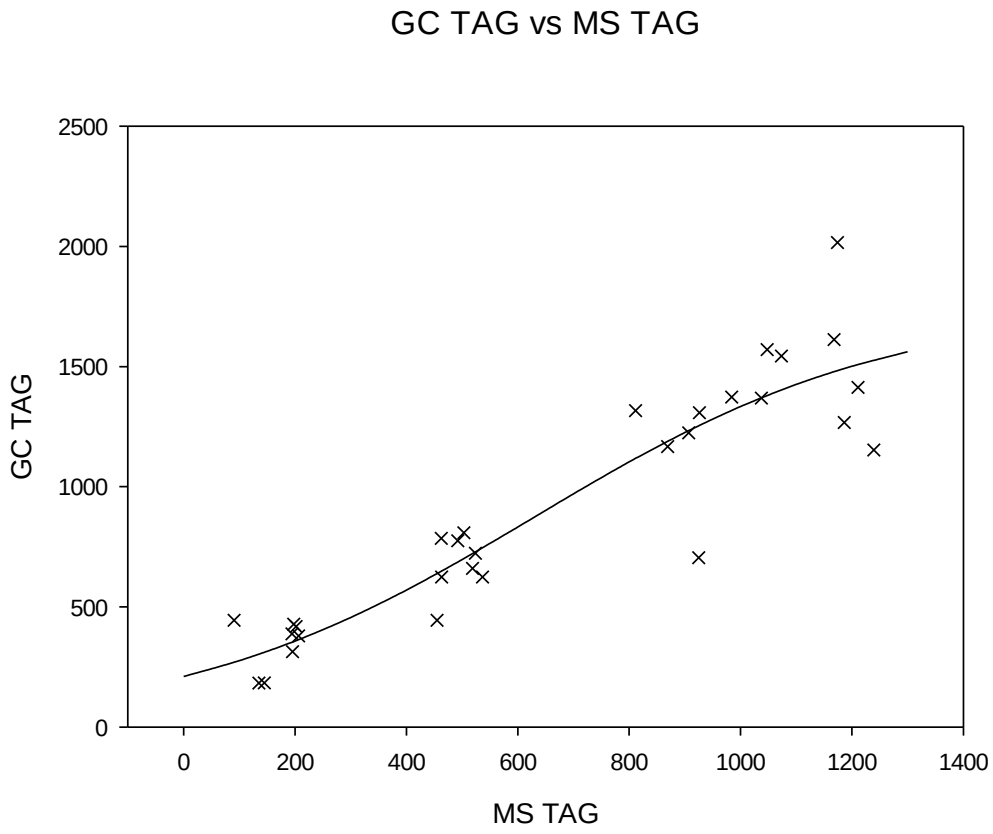


Figure 2.10 Fitted curve for the TAG data from the ESI-MS/MS and GC-FID for the MxH NILs in 2013 and 2015. The equation of the curve used to model FF FMEs GC (y) on QTRAP MS (x) was:

$$y = C/(1 + \exp(-B*(MS\_TAG - M)))$$

where *C* is the upper asymptote of the curve, *B* is the exponential rate of approach to *C* and *M* is the value of *MS_TAG* providing a quantity of GC TAG which is half of the asymptote, *C*.

For this model, the parameter estimates and standard errors are:

Parameter	estimate	s.e.
C	1752.	295.
B	0.003152	0.000857
M	632.	144.

The percentage of variance explained, R^2, was 83.8% and the standard error of observations was 199 given the estimated residual variation, s^2, of 39767 on 27 df.

2.2.3.6 Determination of starch damage

Determination of starch damage was necessary in order to establish whether the high levels of LPC in flour were non-starch lipids, or LPC leached from damaged starch granules. Starch damage was determined using the starch damage assay kit marketed by Megazyme International (Leinster, Ireland K-SDAM). Only the sodium acetate buffer (100 mM, pH 5.0) with calcium chloride (5 mM) and dilute sulphuric acid (0.2% v/v) were not supplied.

Sodium acetate buffer- 5.7 mL of glacial acetic acid (1.05 g/mL) was added to 990 mL of distilled water. The pH was adjusted to 5.0 by the addition of 2M (8g/100 mL) sodium hydroxide solution. 0.74g of calcium chloride dehydrate was dissolved into the mixture, the volume of which was adjusted to 1L and stored at 4℃.

Dilute sulphuric acid- Prepared by adding 2.0mL of concentrated sulphuric acid (98%) to 998mL of distilled water.

Wheat flour (100mg) was added to a thick-walled glass centrifuge tube which was then pre-equilibrated at 40°C for 5 minutes. The fungal α-amylase solution (50 U/mL) was also pre-equilibrated at 40°C for 5 minutes. 1 mL of the fungal α-amylase solution was added to each glass tube containing flour and this was mixed on a vortex for 5 seconds and left to incubate at 40℃ for exactly 10 minutes from the addition of the enzyme. 8 mL of dilute sulphuric acid solution was added to the tubes after 10 minutes and was shaken vigorously for 5 seconds to inactivate the enzymes and terminate the reaction. The tubes were centrifuged at 3000 rpm (1000 g) for 5 minutes and 0.1mL aliquots of the supernatant solution were added to the bottom of two new test tubes. 0.1mL of amyloglucosidase solution (2 U) was added to each tube which was then stirred on the vortex mixture and incubated for a further 10 minutes at 40℃. 4 mL of GOPOD (glucose oxidase/peroxidase) reagent solution was added to each sample tube as well as the glucose standard and the reagent blank tubes which were left to incubate at 40℃ for 20 minutes. The absorbances of all solutions were measured at 510 nm against a reagent blank.

2.2.4 Biophysical Analysis

2.2.4.1 First Ten Angstrom (FTA) Pendant drop

The pendant drop technique (Ambwani and Fort, 1979; Husband et al., 1998) measures the surface tension of a liquid through the formation of a droplet at the end of a Teflon coated hydrophobic needle. This provides information on the adsorbed layer formed at the interface. The surface tension

of the droplet can be measured through the gas-liquid interface and the liquid-liquid interface. This method has a high rate of reproducibility and requires low volumes of sample.

Due to surface tension forces, liquid droplets at the tip of a hydrophobic needle take on a spherical form. Gravitational forces distort the droplet into an elongated tear shape. The distortion of the droplet increases with effective drop density and decreases as surface tensions increase. If the liquid density is known, the surface tension of the droplet can be calculated using photographic images. Example image of a droplet can be found in Figure 2.9B.

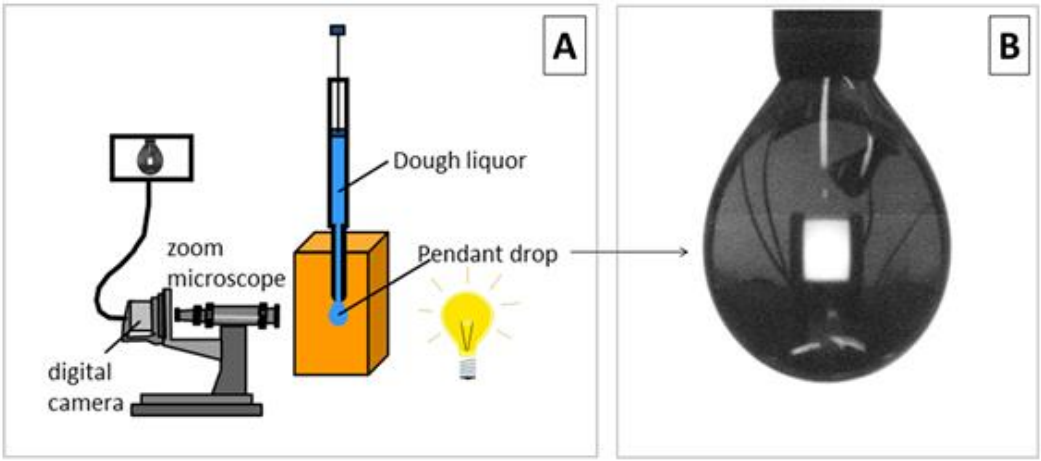


Figure 2.11 (A) Diagram of FTA pendant drop (B) Droplet image taken using FTA pendant drop to measure the surface tension of dough liquor fraction.

2.2.4.2 Measuring Surface Tension

The size of an oscillating pendant drop was measured over a period of time using video image analysis, allowing the surface tension of diluted dough liquors at the air/water interface to be calculated.

A 1.0ml Hamilton syringe (Hamilton Company, Reno, NV, USA) was thoroughly cleaned to remove any potential surface active molecules that could contaminate the surface tension measurements. The syringe was cleaned by taking up and expelling 1 ml of hot soapy water, ethanol and distilled water around 30 times in order to clean the syringe and remove any remaining detergent. The syringe components were dried using compressed air and assembled.

2.2.4.3 Reducing contamination

De-ionised water was taken up in the syringe which was fastened to the pendant drop apparatus. The needle was set up at the correct angle with the camera and light source to display the tip of the hydrophobic needle image on the software interface. Drops were made through the syringe to obtain a suitably shaped droplet of a magnification of 1.0. Snap shots of the droplets formed at the needle tip were taken using the video image analysis system. Shots were taken and the size of the droplet was measured every 5 seconds for 600 seconds to allow the surface tension measurements to stabilize. As the surface tension of water at normal air pressure is 72.8 mN/m, if the syringe was clean, the surface tension of water would remain thus for the duration of 10 minutes. If the surface tension decreased, it would indicate that the syringe contained traces of surface active molecules, which would make it unsuitable for biophysical analysis.

2.2.4.4 Dough Liquor interfacial properties

The diluted dough liquor fractions were taken up using a hydrophobic Teflon coated syringe. The syringe was fasted to a FTA 200 pulsulating drop densitometer (First Ten Angstroms, Portsmouth, Va, USA) where a suitable drop was left hanging in the air at the end of the needle.

The dilational rheology of dough liquor was determined by taking snapshots of a pulsulating droplet of about 10-13 µl every second for 600 seconds at room temperature. The size of the droplet corresponded to the concentration of the dough liquor fraction. The droplet size in each image was analysed by fitting the experimental drop profile to the Young-Laplace capillary equation to calculate surface tension, volume and specific area. Each sample was measured in triplicate, with three batches of dough prepared and each measured using the pendant drop three times as a function of time.

The surface dilatational elasticity of the dough liquor surface was determined by pulsulating the droplet volume at a frequency of 0.1 Hz, and amplitude of 3 µl. By measuring the change in surface tension ($d\gamma$) as a function of the change in surface area (dA) of the droplet, due to the change in volume, the surface elastic modulus G' could be calculated.

$E' = A.\, d\gamma/dA$

Samples were analysed in triplicate and the averaged elasticity modulus (G') values were plotted as a function of time.

2.2.5 Sodium Dodecyl Sulphate-Polyacrylmide Gel Electrophoresis (SDS-PAGE)

In order to determine the HMW glutenin subunit alleles of wheat, SDS-PAGE was used to separate proteins by electrophoresis. SDS-PAGE is a method for separating molecules based on the differences in their molecular weight, using a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulphate (SDS) to denature the proteins (Weber and Osborn, 1969). At the pH used, the SDS molecules are negatively charged and bind to the proteins at a 1:4:1 weight ratio. The detergent therefore confers a high net charge to the protein, which is independent of the original charge. The detergent destroys the secondary, tertiary and quaternary structure of proteins, denaturing them to form negatively charged linear polypeptide chains. The gel matrix separates the proteins by sieving. In an electric field from PAGE, the negatively charged polypeptide chains will travel towards the anode with different mobility. Therefore by measuring the distance travelled by the molecules in the gel, the relative weight of the proteins can be measured.

2.2.5.1 SDS-PAGE of Flour MxH DH population

The HMW glutenin subunit alleles present in the MxH DH population was determined using SDS-PAGE. The protein extraction buffer was prepared following the method from Alvarez et al., 2000: 0.0625M Tris-HCl at pH 6.8, 2% (w/v) SDS, 1.4% (w/v) dithiothreitol, 10% (v/v) glycerol and 0.002% (w/v) Bromophenol blue. All components for the protein extraction were obtained from Sigma Aldrich (Haverhill, UK). Grain samples were crushed using a pair of pliers and 10 mg of the crushed samples were soaked in the protein extraction buffer, placed in a 37˚C water bath for 1 hour and placed in boiling water for 2 minutes. After centrifugation in a microfuge, 15 µl aliquots of each supernatant were added to wells in a pre-purchased NuPAGE 4-12% Bis-Tris Gel, supplied by NOVEX (Thermo Fisher Scientific, USA). The gels were run at 200V for approximately 30 minutes.

The gel was removed and then fixed by adding the fixing reagent, made up with 7% trichloroacetic acid (TCA), 40% methanol for 20 minutes during shaking. The gel was dyed for 1 minute (40% diluted Coomassie Brilliant Blue dye, 40% methanol) and de-stained for 1 minute using destaining solution (10% acetic Acid, 25% methanol), and rinsed using 25% methanol for around 20 minutes. The gels were stored in water and kept at 4˚C.

2.2.6 Data Processing and Statistics

2.2.6.1 Multivariate Statistics

The method Canonical Variates Analysis (CVA) performs a linear discrimination between the factors (e.g. Genotype and Environment) and allows a low-dimensional representation of the differences to be made between them. Therefore (in two-dimensional plots) overall tentative significant differences, given technical replication, could be assigned using non-overlapping 95% confidence circles around the means of canonical variate (CV) scores per treatment combination, assuming multivariate normal distribution for the data.

The percentage data in the univariate analysis (ANOVA) required a logit transformation to ensure Normal distribution, which was checked by considering the residuals (histograms and Normal scores plots) following ANOVA. The same transformation is applied before applying CVA. Therefore the CVA assumed a multivariate normal distribution of the data, which was reasonable under the logit transformation.

The logit of the univariate percentage data accounted for any of the heterogeneity of variance in the multivariate analysis. This was checked (in the univariate setting) by considering residual plots. This was performed mainly by testing the residuals vs the fitted values from the ANOVAs to check for inconsistent variance of the residuals as the fitted values increase.

2.2.6.2 Procrustes Analysis

Procrustes analysis is used to compare the results of different configurations for a set of ordinates. It can provide information on how different combinations differ relative to each other between two time points. While this is normally pursued on principle coordinates analysis, in this study, an ordination for each set of data was obtained from the application of Canonical Variates analysis.

Procrustes works by rotating and rescaling one configuration of ordinates to most closely match a second configuration of the same object. The fit is then measured by the sum of squared distances between the corresponding objects in the two configurations. See Digby and Kempton (1987) for example and details.

Therefore, in this study, Procrustes analysis was used as a useful tool to see how datasets differed relative to each other across two years.

2.2.6.3 QTL analysis

QTL analysis as carried out by Luzie Wingen at the John Innes Centre (JIC). The QTLs were detected using the package QTL (vs. 1.35, B Roman et al., 2003) for R software as described in Horn et al., 2016. The genotype scores and genetic map was taken from Millar et al., 2007. The cross type "DH" was used and a single Q%L model was fitted as an initial QTL scan along the chromosomes, employing the extended Haley-Knott method on inputted genomes. The significance thresholds for QTL detection were calculated from the data distribution, however the detection threshold was lowered to 1.5 LOD scores as no significant QTL were detected. The final QTL LOD scores and effects were received from a multiple QTL model where more than one QTL was detected in the initial scan.

Chapter 3 Genetic and Environmental effects on intrinsic wheat lipids

3.1 Introduction

Various studies over the years have attempted to identify the effects of genotype or environment on a wide range of crop systems (Riedelsheimer et al., 2013; Tong et al., 2014). For example, in wheat, Hargin and Morrison showed that 4 different breadmaking varieties from around the world had differing lipid compositions (Hargin & Morrison, 1980. Liu et al compared the compositions of lysophospholipids (LPLs) in 58 breadmaking wheat varieties (Liu et al., 2017).

These studies revealed the potential for strong genotypic effects on lipids in the wheat grain. However, these studies compared highly varying wheats such as winter and spring wheats grown in America or the UK (Hargin and Morrison, 1980), or specific lipid classes such as LPLs (Liu et al., 2017).

Research has not addressed how varietal differences may impact the composition and content of grain and flour lipids in hexaploid bread wheat. Moreover, as stated in chapter 1, our understanding of grain lipid responses to environmental factors and genotype x environment (GxE) effects remain largely unknown. This study attempts to investigate the impacts of genotype and environment on wheat lipids, using a carefully selected set of UK wheat cultivars grown under three nitrogen conditions, quantifying 50 lipid species across six lipid classes including the major LPL lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), triacylglycerols (TAG), diacylglyercol (DAG) and free fatty acids (FFA).

Although lipids are minor components of wheat, making up 1.4-2.0% of the dry weight of wheat flour, it is clear they have important effects on the quality of the final end product. Details on the importance of lipids on the breadmaking process can be found in Chapter 1 (Section 1.10.4).

3.1.1 A short description of each cultivar

In order to identify any potential genotypic effects that influence grain lipid composition, six varieties of British wheat lines were selected; Avalon, Cadenza, Crusoe, Hereward, Istabraq and Malacca. All pedigree information for each cultivar was taken from the website below. (http://wheatpedigree.net/sort/renderPedigree/79382).

Avalon

Avalon was developed by the Plant Breeding Institute (Cambridge) and was released in 1980. Developed as a winter wheat, it has a complex pedigree including CI 12633, Capelle, Hybrid 46, Widgeon 4, Widgeon 5, Durin and Bilbo.

A closely related cultivar called Bounty (with an almost identical pedigree to Avalon, apart from Bilbo) was released in 1979 as a standard high quality breadmaking variety. However a year after its introduction, Avalon took over. Being a superior variety for both yield and protein content Avalon soon covered a remarkable 29% of the winter wheat area in the UK. Both Bounty and Avalon were derivatives of the variety Maris Widgeon whose quality traits were ascribed to the presence of the HMW glutenin subunit 1, the origins of which can be traced back to the cultivar Red Fife, a Canadian wheat from the early 19th Century.

Avalon replaced a number of excellent varieties, such as Maris Huntsman, a high yielding and highly successful variety over a number of years. Only with the introduction of Hereward, was Avalon replaced as the variety of choice.

Cadenza

Cadenza differs from the other varieties in this study as it was developed as a spring wheat. However, being winter hardy it is planted in the autumn. Developed by CPB Twyford Ltd, Cadenza has a pedigree including the varieties Axona and Tonic and was released in the United Kingdom in 1994. One of the parental cultivars Axona was a highly successful spring wheat variety, covering 13% of the certified seed area in 1996, which rose to approximately 25% in 2002. The other parental cultivar Tonic is an older spring wheat variety which was classified in category B for breadmaking quality and A for grain protein content. Also highly successful, Tonic covered 57% of the spring wheat acreage in 1988.

Cadenza itself was a successful variety grown extensively across Europe, including the UK, Ireland, France, Germany, Denmark, Ukraine and the Netherlands. Though it was only grown extensively in the UK between 1994 and 1998, it is still grown in areas of France and Germany to this day.

Crusoe

Released in 2012, Crusoe is the most modern of the six varieties in this study. Crusoe is from a cross between the varieties Cordiale and Gulliver which was developed by Nickerson International

Research, now part of Limagrain. With excellent second crop yields, disease resistance and protein quality, Crusoe quickly entered the Home Grown Cereals Authority (HGCA), now the AHDB (Agirulcultural and Horticultural Development Board) recommended list and was registered as a class 1 premium breadmaking quality wheat by NABIM (the National Association of British and Irish Millers)

Hereward

As the 'UK textbook breadmaking variety' between 1991 and 2010, Hereward was one of the most successful breadmaking wheat varieties in the UK. Even now it is still favoured by some UK farmers and is still grown in France, the Netherlands and Denmark. First developed by the Plant Breeding International Cambridge, Hereward is described as a cross between a sibling of the variety Norman and Disponent (Cereals DB, UK). However this parentage does not agree with its glutenin subunit composition.

Disponent was released in 1975 and had a good *Glu-1* score of 9 out of 10, and was marketed between 1975 and 1988. Despite having lower protein contents than some other important European varieties at the time (e.g. 2% lower protein content than the variety Tassilo), it provided the highest loaf volume among ten other important varieties grown in southern Germany, including Tassilo. Disponent was therefore an interesting variety for further research as it was believed to be capable of increasing yields without sacrificing breadmaking quality. This is because higher protein content is normally associated with lower yields. Therefore, Disponent's relatively low protein content, yet excellent breadmaking quality, could potentially allow the development of better yielding varieties while retaining quality.

The other proposed parent of Hereward is more uncertain being a sibling of the variety Norman. However, Norman was also a successful high yielding variety, released in 1981 by the Plant Breeding Institute. Norman was widely grown in the UK between 1981 and 1995.

Hereward was a major step forward in terms of combining high yield potential and breadmaking quality. This variety combined excellent breadmaking and milling characteristics placing it in the HGCA recommended list as Class 1, while retaining satisfactory yields.

As mentioned in Chapter 1, the Hereward conundrum exists as its success could not be explained by its protein content alone. Part of this is due to the HMW glutenin composition of Hereward which comprises the subunits 7+9 (Glu-1B) and 3+12 (Glu-1D), which does not explain its high quality.

Istabraq

Istabraq is the only soft wheat included in the study, classed in NABIM group 4 with all round good disease resistance. Sometimes used for cakes and pastries, it is primarily used as feed wheat. It was developed by Nickerson International Research GEIE and was released in 2004 as a progeny of the varieties Consort and Claire. It is still in distribution, through Limagrain, although its primary years of use ranged from 2004-2012.

Malacca

Developed by CPB Twyford, the breadmaking wheat Malacca is a progeny of the cultivars Riband, Rendevous and Apostle. One of the few breadmaking varieties to realise a large market share without possessing the good HMW subunit 1, Malacca was released in 1997 and was placed in group 1 by NABIM. Primarily grown in the UK and France, Malacca was in extensive use until 2009. However, as a breadmaking variety Malacca had the drawback of flour discoloration during storage, ultimately causing it to be replaced by newer varieties.

All of the cultivars in this study are excellent varieties which have been extensively grown in the UK; while differing considerably in their end use properties. Therefore these lines were considered to provide a good basis to study genetic variation in lipid composition.

3.1.2 Nitrogen treatments

The six cultivars were grown in three different nitrogen conditions to identify any effects of nitrogen nutrition on lipid composition. The nitrogen levels applied were 100, 200 and 350kg/N per hectare. This was to replicate the conditions of low input fertilization (100kg), standard intensive fertilization (200kg) and over-fertilization (350kg) in the United Kingdom. The varieties were grown as part of the 2013 Wheat Genetic Improvement Network (WGIN) field trials on plots of 9 x 3 metres in a randomised blocked field trial for each cultivar at a specific nitrogen condition.

3.1.3 Project Aims

Lipids in wheat flour are considered to play an important role on breadmaking quality. Yet little is understood on how genotype and environment affects the lipid composition of wheat flour.

To investigate this, the lipid compositions of these lines were determined with the MS-based 'lipodomics platform' in order to provide a full systematic survey of lipids, down to the specific molecular species. Details on lipid extraction from flour to its analysis via ESI-MS/MS can be found in the materials and methods (Chapter 2). The data was then analysed by single and multivariate statistics to identify the significant differences and similarities in their lipid profiles.

As mentioned in Chapter 1, this project also aimed to understand the 'Hereward Conundrum': the reason why the cultivar Hereward has such high and stable breadmaking quality despite its lack of "quality-associated" high molecular weight glutenin subunit alleles. This suggests that the quality may be determined by combinations of several alleles carried at loci that were not previously believed to be involved in quality variation. These alleles may control other biological components crucial for breadmaking quality, such as lipids. The identification of these alleles will become a key finding for plant breeders aiming to improve wheat quality.

3.2 Results

3.2.1 FAMEs analysis

To identify whether the lipid composition of wheat flour changes depending on cultivar and environmental effects, FAMEs (Fatty Acid Methyl Ester) analysis was performed on the flour samples. Description of FAMEs preparation and analysis can be found in the Materials and Methods (Chapter 2). Although far less specific than MS analysis, FAMEs analysis is a more accurate method to quantify the fatty acid content of all the lipids in wheat flour. It was therefore used as an initial method to determine differences in the fatty acid quantities between the various cultivars and different nitrogen conditions. (Figure 3.1)

The fatty acids (FA) 16:0 and 18:2 were the most abundant, making up 80-90% of the fatty acid profile for all cultivars in the three nitrogen conditions. The minor fatty acids 18:0, 18:1 and 18:3 made up between 10-15% of the measured lipids. This reflects the levels of palmitic acid and linoleic acid reported in the literature (Finnie et al., 2009, Gonzalez Thuillier et al., 2015, Min et al., 2017). Although other FAMEs such as 20:0 and 22:0 were identified, they were removed from the analysis based on their inconsistent low levels.

The study shows the impacts of both genotype and nitrogen input on the fatty acid profile of the six cultivars. There was a significant increase in the fatty acid dry weight as nitrogen application

increased. For N100, which represents under fertilized conditions, less than 10mg of fatty acids were detected per gram of fresh weight of flour in Cadenza, Crusoe, Hereward, Istabraq and Malacca. An increase in the levels of nitrogen input correlated with increased fatty acid quantity. N200 resulted in between 20-25mg of fatty acid dry weight while 350kg/N/Ha resulted in greater, but more varied quantities of fatty acids ranging between 15-35 mg.

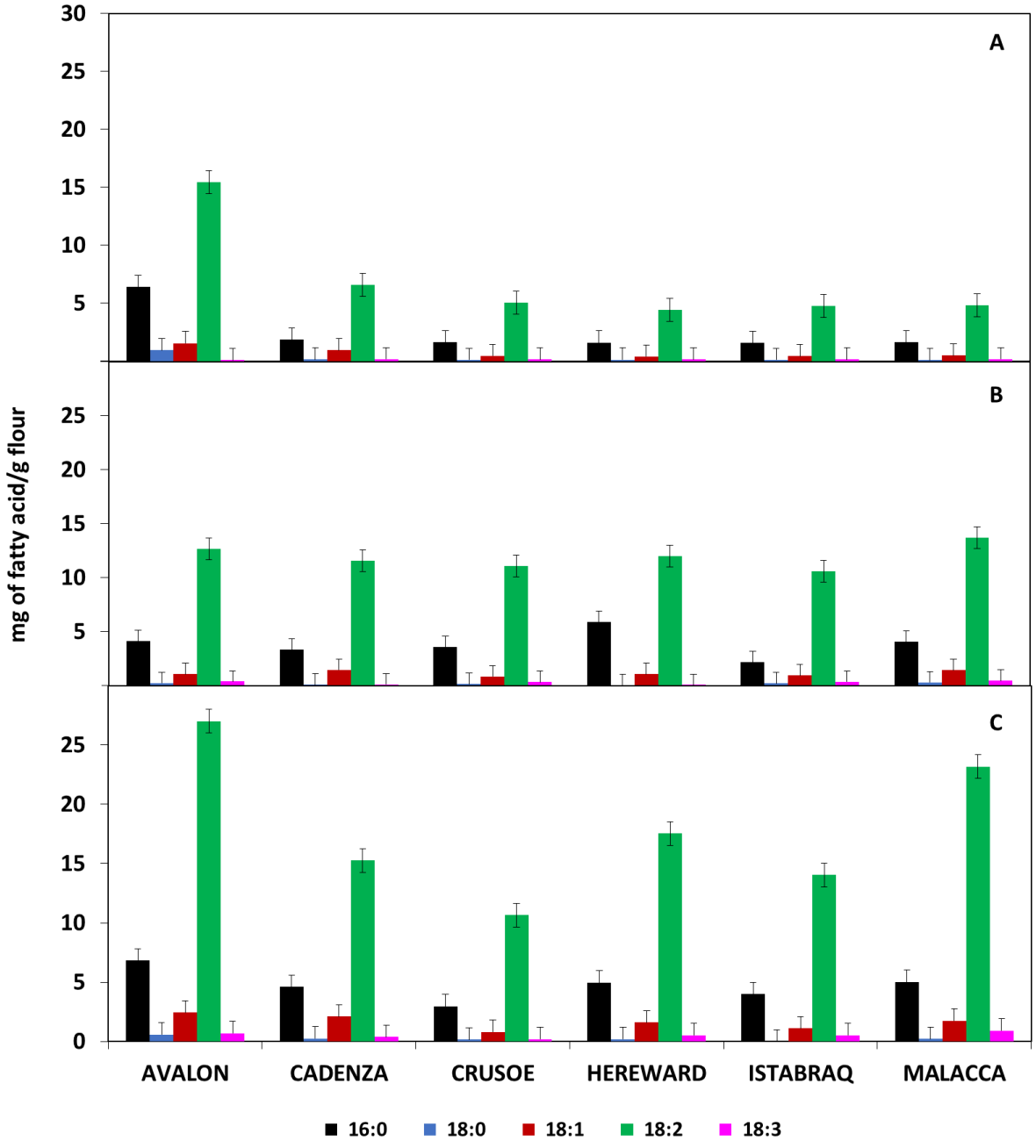


Figure 3.1 Dry weights of fatty acids present in the wheat flour of six cultivars grown in three nitrogen conditions. Five fatty acids were measured including 16:0, 18:0, 18:1, 18:2 and 18:3. A- the fatty acid profile at 100 kg/N/Ha, B- fatty acid profile at 200 kg/N/Ha, C- fatty acid profile at 350kg/N/Ha. Data are mean values of ±SE of five independent samples analysed by GC-FID profiling of FAMEs-.

Increases in nitrogen input also affected the variation in lipid profiles between the cultivars. This may imply a significant GxE effect, in this case for the cultivar x nitrogen effects on the fatty acid profile of wheat flour. While the differences in the fatty acid profiles between cultivars were far less apparent between N100 and N200, significant differences were seen at N350. All cultivars exhibited different fatty acid profiles at N350, with Avalon and Malacca showing particularly high fatty acid contents and Crusoe the lowest. Avalon was an exception to this; with N200 showing the least lipid diversity compared to other cultivars and N100 the highest.

The lipid profiles of the individual cultivars reacted differently to the increases in nitrogen. Avalon and Malacca did not just have the highest fatty acid content; they also were the most affected by nitrogen. Avalon in particular had lower fatty acid content at N200, compared to N100 while every other variety showed the lowest levels of fatty acids at N100. Furthermore, when over fertilized to N350, Avalon exhibited the highest level of fatty acids. Malacca, on the other hand, had the lowest fatty acid content among the cultivars at N100, which rose dramatically with increasing nitrogen. At N350, Malacca had the second highest fatty acid content, just below Avalon. Crusoe had the lowest fatty acid content, but also showed the least changes in fatty acid content with increasing nitrogen. In fact, the only significant difference for Crusoe occurred between N100 and N200. There was no significant difference in the fatty acid content of Crusoe between N200 and N350, making it the least affected cultivar by over fertilization.

Despite being the standard level of nitrogen fertilization in the UK, N200 was associated with the least lipid diversity among the cultivars in the three nitrogen conditions. N200 was the only level of nitrogen fertilization that gave no significant differences in the amounts of fatty acids between the six cultivars.

Both genotype and environment (cultivar and nitrogen) affected the fatty acid profile of wheat flour. Environmental effects were seen as increasing levels of nitrogen correlated with increasing fatty acid content. Although genotype showed little to no lipid variation at the standard level of nitrogen fertilization, N100 and N350 had significant effects on the fatty acid profile of wheat, indicating strong potential GxE effects. With genotype and environment affecting the fatty acid profile, it was likely that GxE effects would play a role in determining the profile of more specific lipid classes.

3.2.2 Effects of genotype and environment on specific lipid classes

To determine the effects of genotype (cultivar) and environment (nitrogen) on specific lipid classes within wheat flour, data acquisition and analysis was carried out using an ESI-MS/MS approach.

Studies over the years have attempted to identify the lipid composition of various biological components of wheat, including extensive lipid profiles of 165 lipid species (Narayanan et al., 2016). In flour, studies have been less extensive due to the difficulties in quantifying minor lipid classes, although Finnie et al., 2009 identified 146 polar lipid species. However, the aim of this study was to identify whether different genotype and environmental effects could affect the lipid composition of wheat flour, rather than providing a full systematic lipid profile of wheat flour. Therefore 50 lipid species in six major lipid classes, accounting for 90% of the total wheat flour lipid profile, were measured in this study. The lipids selected for analysis were LPC, PC, PE, FFA, DAG and TAG. Minor lipid components, comprising galactolipids and minor phospholipids, were not analysed. In the first year of the study difficulties were met with analysing the galactolipids and the more minor phospholipids, and were not accurately quantifiable.

The lipid data are presented as a mol% of the total lipids obtained by normalizing the peak area of each lipid to the internal standard which was further normalized to the weight of the initial flour sample. Further data in this study comparing the saturation and unsaturation index of white flour is presented as nanomoles of lipid/g flour.

Of the lipids extracted from white flour in this study, FFA were generally the most abundant class, accounting for an average of 31% of the lipids analysed. This was followed by LPC, TAG and DAG, which accounted for averages of 27%, 23% and 11% of the total lipids, respectively (Figure 3.2). The observed percentages agree with Chung et al., 2009 and the more recent study by Gonzalez-Thuillier et al., 2015 (which used samples grown at about 200 kg N/Ha), although in the present study TAGs were more abundant than FFA or LPC for Cadenza and Crusoe grown at 350N. Nevertheless, other authors have reported similar results, with TAGs being more abundant than FFAs in four wheat cultivars (Hargin and Morrison., 1980). The high levels of FFA present in the study were surprising as measures were taken in order to reduce the degradation of TAG into FFA. Flour samples were boiled in order to denature lipase activity in the flour, and isopropanol was used in the extraction procedure to further reduce the effects of lipases. Yet the significantly high levels of FFA observed may be reflective of the degradation of lipids that occurred during grain or flour storage. Further details on storage and its impacts on the lipid composition is discussed in (3.2.2…). Significantly higher levels of LPC were reported in this study compared to other studies, for example the study by Finnie et al., 2009 reported LPC to make up a mere 2-4% of the polar lipid fraction. The lack of starch damage eliminated the possibility of the leakage of starch internal lipids (see below) so the potential hydrolysis of PC to LPC during storage must be considered.

The proportions of individual species within lipid classes also varied. Among the ten FFA species, 16:0 and 18:2 comprised about 22% and 48% of the total FFA, respectively, while LPC 16:0 and LPC

18:2 represented 86% to 88% of total LPC, which is supported by the study by Liu et al., which reported that LPC 16:0 and 18:2 made up between 71.3-86.2% of the total LPC (Liu et al. 2017). Among the ten species of PC, PC 34:2 and PC 36:4 comprised 37% and 32%, of the total, respectively. TAGs comprised 13 species with TAGs 52:4, 54:5 and 54:6 comprising about 20%, 18% and 24% of the total, respectively.

3.2.3 Effects of Genotype and Nitrogen on Lipid Classes

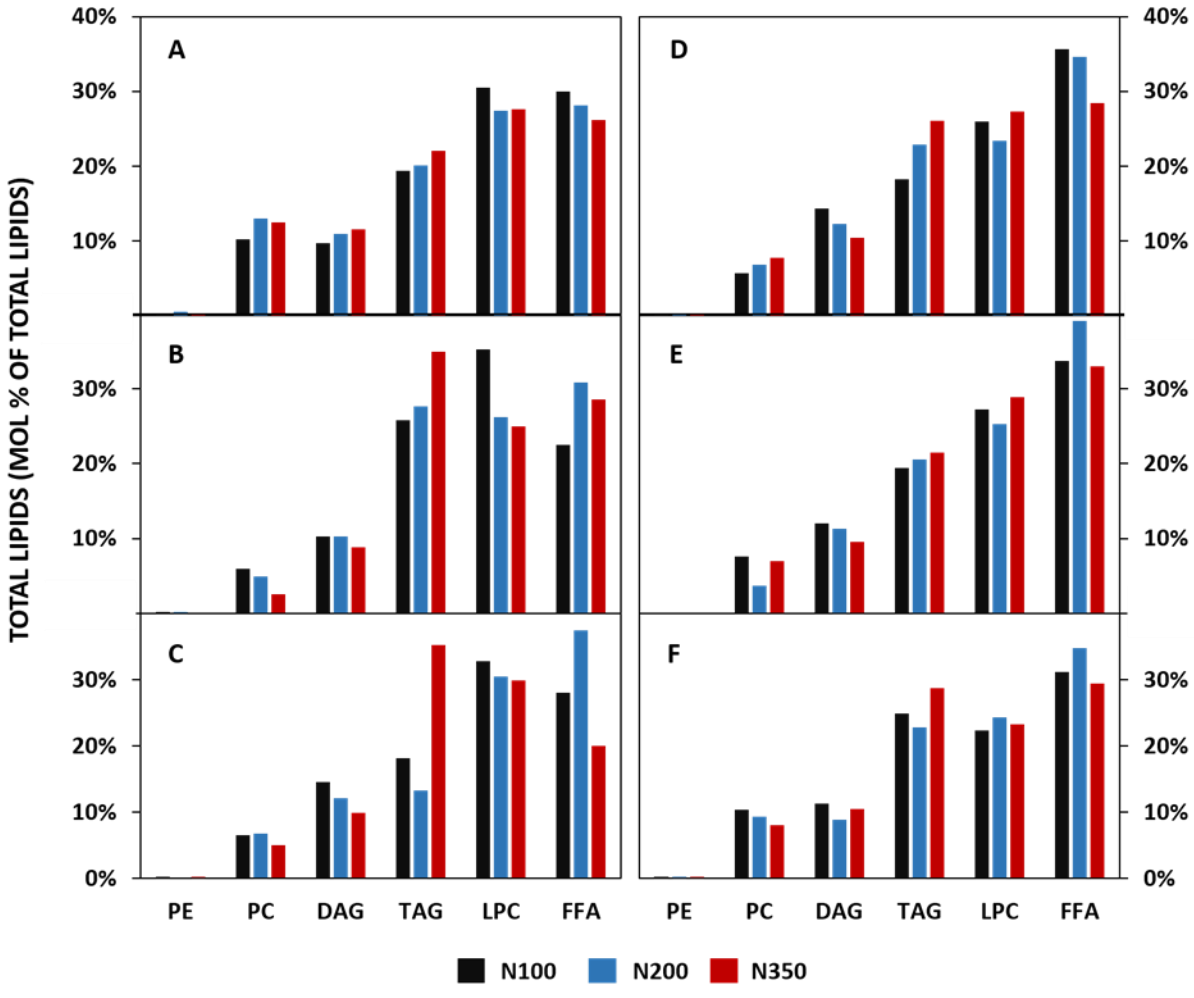


Figure 3.2. Lipid profile (mol% of total lipids analysed) of the six UK wheat varieties grown under three different nitrogen conditions 100, 200 and 350kg/N/Ha, excluding galactolipids. The lipids analysed are free fatty acids (FFAs), diacylglycerol (DAG), triacylglycerol (TAG), phosphatidylcholine (PC), lysophosphatidylcholine (LPC) and phosphatidylethaloamine (PE). A, Avalon; B, Cadenza; C, Crusoe; D, Hereward; E, Istabraq; F, Malacca.

Lipids	Cultivar	N	Cultivar x Nitrogen (GxE)
LPC Total	0.651	0.950	0.997
PC Total	**<u><0.001</u>**	0.661	0.261
PE Total	**<u><0.001</u>**	**0.029**	**<u>0.038</u>**
DAG Total	0.144	0.397	0.055
TAG Total	**<u><0.001</u>**	**<u><0.001</u>**	0.578
FFA Total	0.268	**0.024**	**<u>0.044</u>**

Table 3.1 ANOVA table for the total values of the six lipid classes. The significant effects for each lipid group ($p<0.05$, F-test) is highlighted in bold. When the interaction is significant ($p<0.05$) there is no need to consider the main effects means tables, so the term(s) to consider are underlined. The relevant means on the log scale are provided in the appendix figure 3.4.

3.2.4 ANOVA on lipid class totals

To test the main effects and interactions between the six cultivars and three nitrogen conditions, the total amounts of six lipid classes (LPC, PE, PC, FFA, TAG and DAG), together with total PL (LPC, PC, PE) and total NL (FFA, TAG and DAG) were analysed by Analysis of Variance (ANOVA) (Table 3.1).

No main effects of cultivar, nitrogen or cultivar x nitrogen were observed for total LPC, total DAG or total PL. However, cultivar strongly affected the levels of PC, PE and TAG, while nitrogen treatment had the greatest effects on total NL, FFA, TAG and PE. Although interactions between cultivar and nitrogen were less significant than cultivar and nitrogen alone, they did significantly affect the levels of PE and FFA (Table 3.1).

Although cultivar had the greatest effects on PC and PE, there were no effects on LPC and hence on total PL. In contrast, total NL was significantly affected by all three parameters (Cultivar, Nitrogen, CxN (Cultivar x Nitrogen)). The effects on NL possibly resulted from the effects on TAG which were greatly affected by both cultivar and nitrogen (See Table 3.2). However, there was no effect of cultivar x nitrogen on total TAG. Total FFAs were not affected by cultivar but were significantly affected by nitrogen and by cultivar x nitrogen effects.

	F-test p-values		
Term	Cultivar	N	Cultivar.N
LPC_18_0	**0.036**	0.080	**0.018**
PC_32_0	**<0.001**	0.762	0.204
PC_34_1	**0.014**	0.328	0.536
PC_34_2	**<0.001**	0.202	0.455
PC_34_3	**<0.001**	0.331	**0.009**
PC_36_2	**0.008**	0.642	0.230
PC_36_3	**0.003**	0.530	0.357
PC_36_4	**<0.001**	0.666	0.433
PC_36_5	**<0.001**	0.551	**0.041**
PC_36_8	**<0.001**	**<0.001**	**<0.001**
PE_34_2	**<0.001**	0.161	**0.044**
PE_36_3	**<0.001**	0.092	**<0.001**
PE_36_4	**<0.001**	**0.010**	**0.014**
DAG_32_0	**<0.001**	0.622	**<0.001**
DAG_34_1	**0.010**	0.058	0.414
DAG_36_2	0.136	**0.024**	0.058
DAG_36_5	**0.004**	**0.003**	0.722
DAG_38_0	**<0.001**	**0.019**	0.067
DAG_40_2	**0.003**	**0.035**	0.575
TAG_50_1	**0.018**	**<0.001**	**0.014**
TAG_52_2	**0.001**	0.053	0.214
TAG_52_5	**0.023**	**0.002**	**0.040**
TAG_54_3	**<0.001**	0.599	0.321
TAG_56_5	**0.003**	**0.003**	**0.002**
FFA_16_1	0.493	**0.005**	0.069
FFA_18_2	0.127	**0.008**	0.130
FFA_18_3	0.109	**0.004**	0.232
FFA_24_0	0.649	**0.029**	0.221

Table 3.2 A selected list of the p-values for F-test of cultivar, nitrogen and cultivar by nitrogen interactions of all the lipid species analysed in the study. Only the lipids significantly affected ($p<0.05$, F-test) by genotype (cultivar), environment (nitrogen) or GxE (CxN) were included. Full table with all lipid species can be found in the Appendix Table 3.1.

While ANOVA of the total lipid class values is a useful tool to identify which lipid classes are generally affected by genotype or environment, ANOVA of the individual 50 lipid species offers a more in depth analysis. (Table 3.2)

F-tests of cultivar, nitrogen and cultivar by nitrogen interactions of the entire lipid species analysed in this study identified all significantly affected lipid species. Although total LPC was unaffected by any parameter, LPC 18:0 was significantly affected by cultivar and by CxN effects. PC is particularly interesting as every one of the nine lipids species were strongly affected by cultivar. Among these, PC 32:0, 34:2, 34:3, 36:4, 36:5 and 36:8 were particularly strongly affected, with PC 36:8 being affected by cultivar, N as well as CxN effects (Table 3.1). Considering the important role PC is believed to play in the breadmaking process and in lipid biosynthesis, this is particularly interesting. As expected from the results of the total PE values, the individual species of PE were all very significantly affected, most notably by cultivar. PE 36:4 in particular was also significantly affected by all three parameters.

Like LPC, total DAG was shown to be unaffected by cultivar, N or CxN. However, among the individual lipid species, DAG 32:0, 34:1, 36:5, 38:0 and 40:2 were shown to be significantly affected by cultivar, while the latter three were also affected by nitrogen. DAG 32:0 is particularly interesting, being affected very strongly by cultivar and CxN. A total of five TAG species were significantly affected by cultivar and four species were affected by nitrogen. Three were affected by CxN effects. Considering how responsive total TAG was to cultivar and nitrogen, the individual lipid species were not as strongly affected as expected. Notably TAG 50:1, 52:5 and 56:5 were significantly affected by all three parameters. FFA was the major neutral lipid group that was only affected by the nitrogen input. No FFA species were affected by cultivar or by CxN effects while FFA species were significantly affected by nitrogen input.

Overall, of the six lipid classes investigated, PC and PE showed the greatest differences, with PC in particular being highly affected by genotype. However, other significant effects such as PE for cultivar, TAG for cultivar and FFA for nitrogen are highly interesting considering the impacts these changes may have on breadmaking quality (See Chapter 1, Section 1.10.4.5).

3.2.5 Effects of Genotype and Nitrogen on Lipid Species

Genotype affected the lipid composition of certain phospholipids and TAG. For phosopholipids (PLs), the cultivars Avalon and Malacca consistently showed the greatest mol % of total lipids among the six cultivars at any given level of nitrogen input. This agrees with the fatty acid profile observed from the

FAMEs analysis in Figure 3.1. On the other hand, Cadenza, Crusoe and Hereward consistently showed the lowest levels of PL for all six cultivars (Figure 3.3).

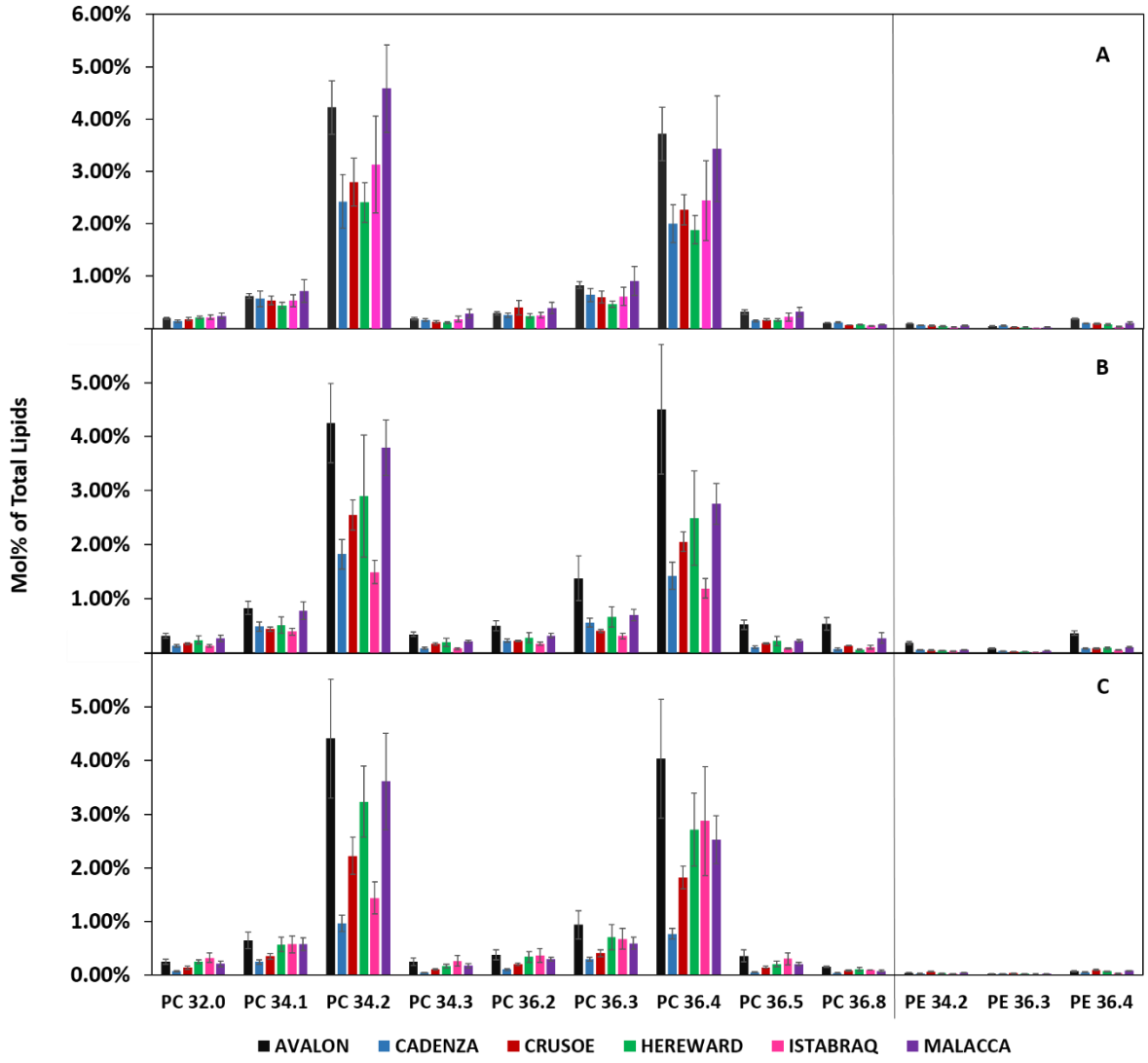


Figure 3.3 Comparison of selected phospholipids (mol% of total lipids) between the six cultivars. Nine species of phosphatidylcholine (PC) (32:0, 34:1, 34:2, 34:3, 36:2, 36:3, 36:4, 36:5 and 36:8) as well as three species of phosphatidylethanolamine (PE) (PE 34:2, 36:3 and 36:4) are displayed. Nitrogen conditions: A, N100 (100 kg/N/Ha); B, N200 (200kg/N/Ha); C, N350 (350kg/N/Ha). Data are mean values of ±SE of five independent samples analysed via ESI-MS/MS.

The proportions of individual PE species also varied significantly between cultivars and nitrogen inputs (Figure 3.4). In particular, PE 34:2 and 36:4 in Avalon showed some strong significant differences to the other cultivars at N100 and N200, but not at N350. Statistical analyses of the effects

of cultivar, nitrogen and cultivar x nitrogen on the individual PL species are shown in Table 3.2. All nine PC and all three PE species were significantly affected by cultivar, with four PC species and all three PE species being affected by cultivar x nitrogen. Of the individual lipid species, 13 out of 17 PL species were affected by cultivar while only 2 were affected by nitrogen conditions. 7 of the 17 PLs were affected by cultivar x nitrogen.

Very few nitrogen effects were observed between the cultivars, except for some aforementioned lipid species such as PC 36:8 and PE 36:4. Again Avalon was most affected with the quantities of these lipids increasing dramatically at N200. Although this is an interesting observation, PC 36:8 and PE 36:4 are present at low levels and are therefore unlikely play a large role in breadmaking quality.

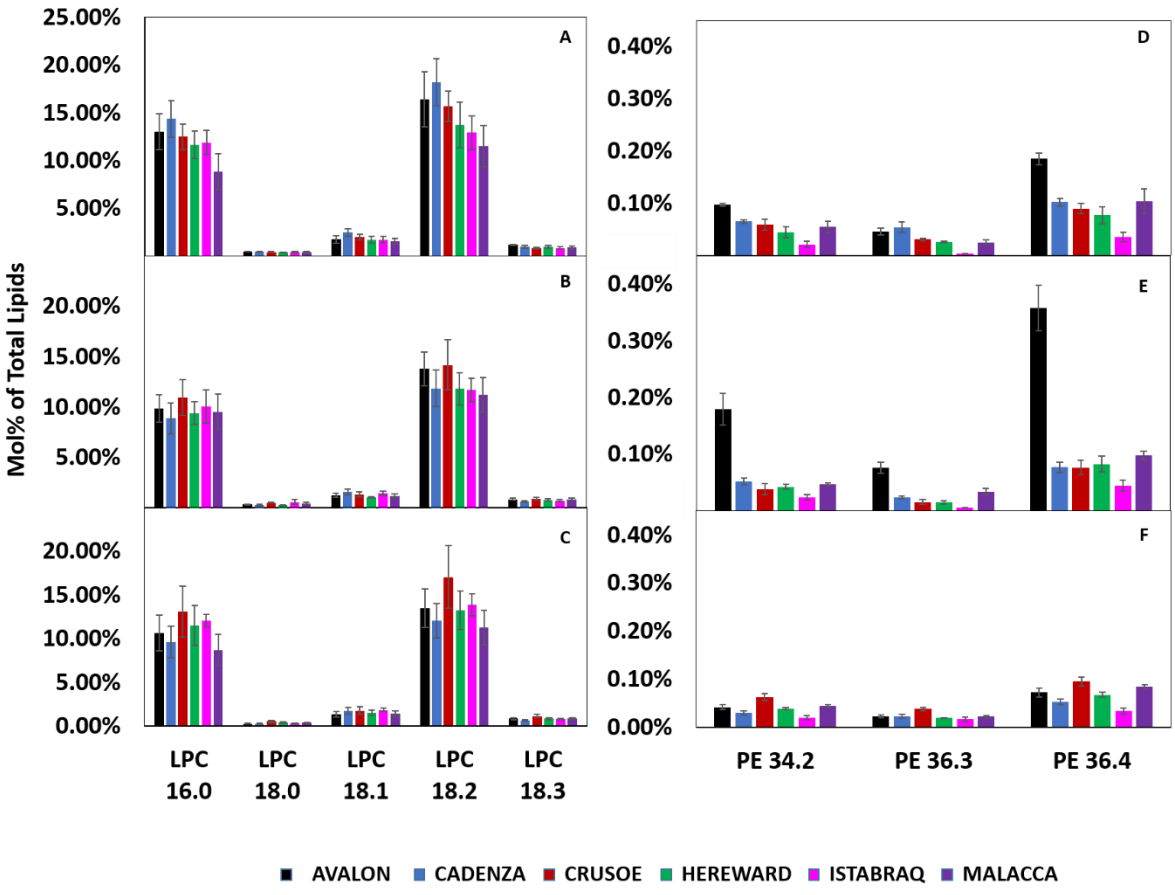


Figure 3.4 Comparison of LPC and PE (mol % of total lipids) between the six cultivars. The 5 species of lysophosphatidylcholine (LPC 16:0, 18:0, 18:1, 18:2 and 18:3) are displayed in this figure alongside the 3 phosphatidylethanolamines (PE 34:2, 36:3 and 36:4) which were not clearly visible in Figure 3.3. A&D, grown at 100kg/N/Ha; B&D, grown at 200kg/N/Ha; C&F, grown at 350kg/N/Ha. Data are mean values of ±SE of five independent samples analysed via ESI-MS/MS.

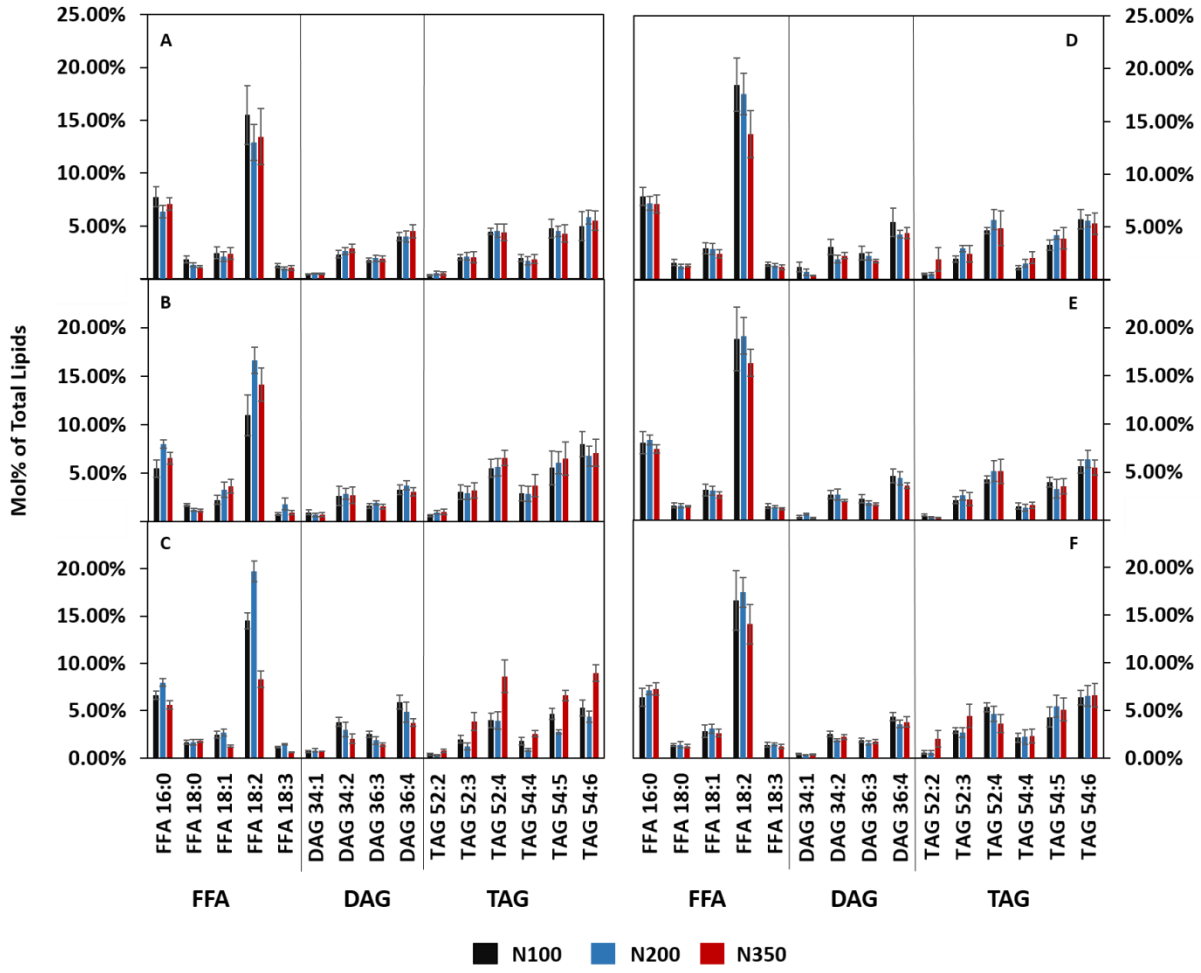


Figure 3.5 Lipid compositions of selected neutral lipids between the three nitrogen conditions. The lipids with the highest quantities were selected. Displayed in the figure are five species of free fatty acids (FFA 16:0, 18:0, 18:1, 18:2 and 18:3), four species of diacylglycerol(DAG) (DAG 34:1, 34:2, 36:3 and 36:4) and six species of triacylglycerols (TAG) (TAG 52:2, 52:3, 52:4, 54:4, 54:5 and 54:6). The data for the remaining minor neutral lipids are presented in appendix figure 3.2. A, Avalon; B, Cadenza; C, Crusoe; D, Hereward; E, Istabraq; F, Malacca. Data are mean values of ±SE of five independent samples analysed via ESI-MS/MS.

The compositions of NLs differed to a limited extent between cultivars, but greater differences were observed depending on nitrogen supply, particularly for Crusoe grown at N100 and N200 (Figure 3.5). Statistical analysis of the NL species showed that only five of the twelve TAG species (TAG 50:1, 52:5, 54:3 and 56:5) were significantly affected by cultivar and three of these (TAG 50:1, 52:5, 56:5) by nitrogen and cultivar x nitrogen (Appendix Figure 3.1). Six of the nine DAG species were also affected by one or two of the three parameters. Although ten species of FFA were determined, only four of these showed significant effects of nitrogen, with none being affected by cultivar or cultivar x nitrogen effects. The significant effects on DAG species are interesting considering that the ANOVA of the total DAG dataset provided no significant differences in general. The significantly

affected DAG species were DAG 32:0 which was significantly affected by cultivar, and DAG 36:5, 38:0 and 40:2 which were affected by both cultivar and nitrogen. Out of 31 NL species identified in this study, 11 were affected by nitrogen, while 10 were affected by cultivar. A cultivar x nitrogen effect was observed for four lipid species. It is important to note that the most of the NLs affected by nitrogen and cultivar affected were DAGs. However, FFA in particular seemed to be more affected by the nitrogen condition as none of the FFA species were affected by genotype, while major FFA classes such as FFA 18:2 were significantly affected by nitrogen

In broad terms, the analysis has shown cultivar effects to be predominant, especially on PC and PE, while nitrogen condition did not affect the PLs in a significant manner, aside from PC 36:8 and PE 36:4. The neutral lipids were more affected by nitrogen fertilization, with FFA in particular being mainly affected by nitrogen input while TAG was affected by both. DAG showed no significant effects based on ANOVA of total lipid classes, but ANOVA of the individual lipid species revealed it to be the most sensitive lipid class for both cultivar and nitrogen.

3.2.6 Multivariate Statistical analysis

Canonical Variates Analysis (CVA) was used to obtain a linear discrimination between the cultivar by nitrogen combinations and allowed a low-dimensional representation of the differences to be made (in two-dimensional plots). This allowed significant differences to be assigned by non-overlapping 95% confidence circles around the means of the CV scores for each treatment combination, assuming a multivariate normal distribution for the dataset. Further details on the CVA analysis can be found in 2.2.6.1. The magnitudes of the CV loadings on the variables (quantified lipids) indicate the relative importance of the lipids in the discrimination observed. In this way the effects of cultivar, nitrogen and cultivar x nitrogen on the lipid composition could be identified.

The first three CVs accounted for 66.17% of the total variance and the possible discrimination in the dataset. DAG 36:4, DAG 36:3, FFA 16:1 and PC 34:1 were the most important lipids for separation in the first CV dimension, TAG 52:5, FFA 18:2, TAG 54:7, TAG 54:3, FFA 24:0, TAG 50:2, LPC 16:0, DAG 36:4 and DAG 36:5 for CV2 and LPC 16:0, LPC 18:2, DAG 36:3, PC 34:1, TAG 52:5, DAG 36:5, FFA 18:2, FFA 24:0, DAG 32:0, PC 36:2 and FFA 18:1 for CV3. The full data regarding the loading vector values for the dataset, which determined the “important” lipids, are given in Appendix Table 3.3.

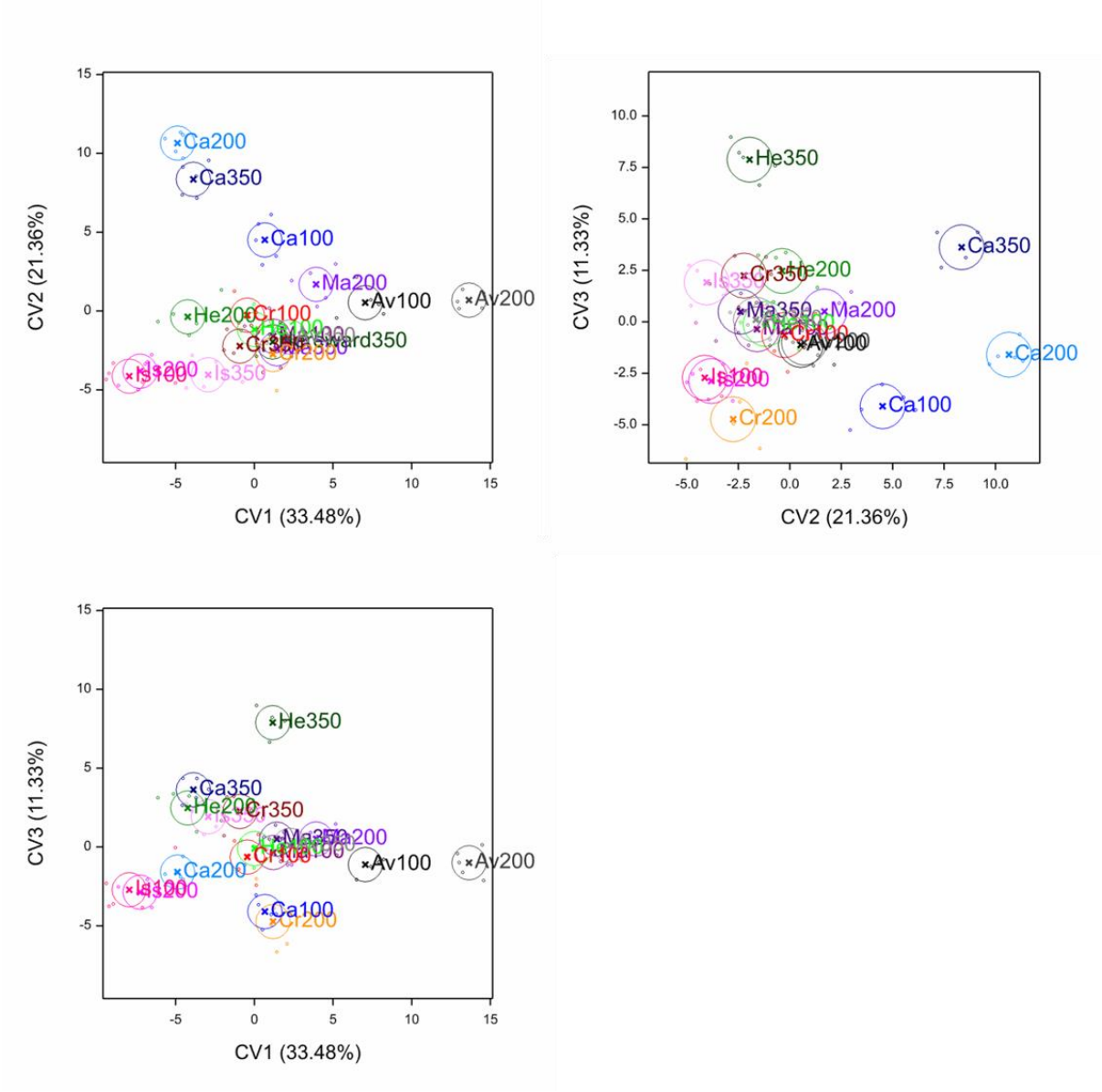


Figure 3.6 Canonical Variates Analysis (CVA) for the six cultivars in three nitrogen conditions. Included are Avalon(Av), Cadenza (Ca), Crusoe (Cr), Hereward (He), Istabraq (Is), Malacca (Ma) at 100N, 200N and 350N. CV scores (**o**) and means of scores (**x**) in the three dimensions are plotted. Although the circles indicate a 95% confidence interval, it should be borne in mind that the five replications were technical rather than biological in nature.

CV1 clearly separates Istabraq at all three nitrogen levels and Avalon grown at 100 and 200 kg/N/Ha from the other cultivars and nitrogen treatments (Figure 3.6). Cadenza is separated from the other cultivars in the CV2 dimension, particularly the samples grown at 200 and 350 kg/N/Ha. In broad terms the separation in CV1 and CV2 is determined more by differences between cultivars than between nitrogen treatments. Istabraq is particularly well separated from the other cultivars by CV3, and good separation was seen for Hereward grown at 350N and Crusoe grown at 200N. When

observing the effects of nitrogen condition on the CVA plots, cultivars grown at N350 did not necessarily show the greatest separation. N200 showed the greatest separation for cultivars such as Avalon, Cadenza, Istabraq and Crusoe. Hereward was the only cultivar that showed significant separation at N350. However, under fertilized conditions, N100, showed the lowest amount of separation between the cultivars.

3.2.7 Sample Description and Starch Damage

It was noted above that the levels of LPC observed were higher than in previous studies. There are various possible explanations, the most likely being the hydrolysis of PC into LPC via the actions of phospholipase A2, or two, the leaking of starch internal lipids into the samples. Lysophospholipids (LPLs) make up a significant proportion of wheat lipids, the total amounts being higher than those of starch surface glycolipids and phospholipids combined. LPLs are a major class of polar lipids that form complexes with the amylose in cereal starch granules (Hargin and Morrison, 1980). However, most LPLs are starch internal lipids which are strongly bound to starch granules and are therefore unlikely to affect dough processing before the gelatinisation of starch (Goesaert H, et al., 2005). In fact, it has been reported in literature that starch internal lipids do not play a role in dough processing properties (Rocha, et al., 2012). Due to this starch internal lipids were not measured in this study.

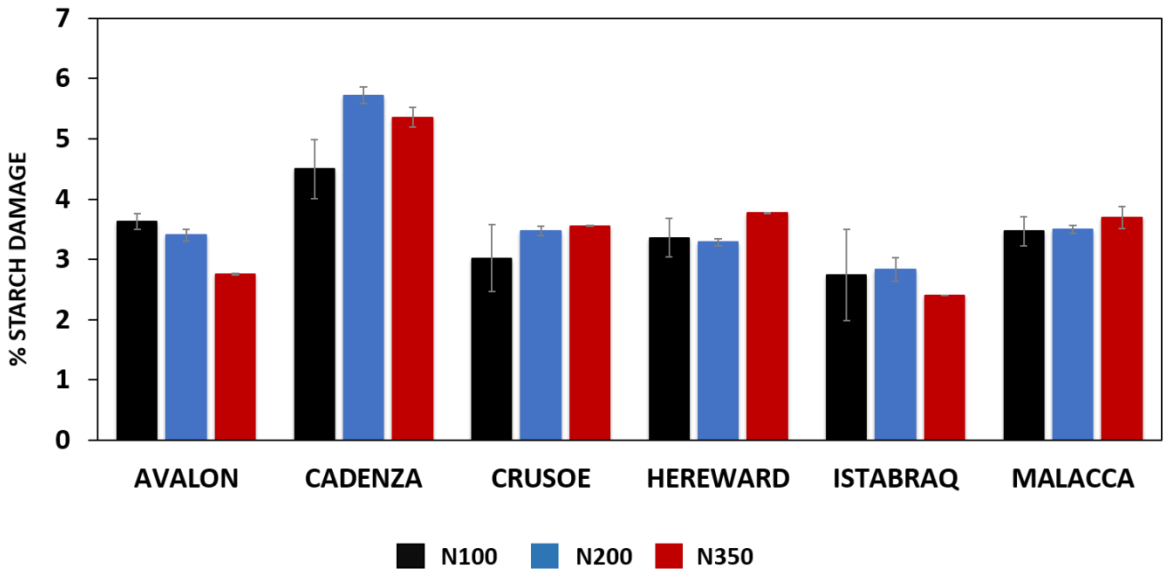


Figure 3.7 Starch damage analysis of the six cultivars grown in three nitrogen conditions. Starch damage levels were low being below 6% in all cases. Data are mean values of ±SE of three independent samples analysed using the Megazyme starch damage kit (Megazyme Leinster, Ireland).

Damage to starch granules in the wheat grain can cause the release of integral starch granule lipids which are rich in LPC. As LPC makes up over 90% of the LPL content of wheat, this made starch damage a strong candidate for the high levels of measured LPC. Starch damage may occur during milling, where harder wheat grains are more prone to increased starch damage. As breadmaking wheat has hard kernels (See Puroindolines chapter 1) this was a cause for concern. Starch damage was therefore measured using the starch damage kit marketed by Megazyme (Leinster, Ireland).

The results of the starch damage assay reflect the correlation of hard wheat and starch damage as Istabraq, the only soft wheat, had lower levels of starch damage of around 3% compared with the other hard wheat cultivars. However, even the hard breadmaking wheat cultivars had low levels of starch damage not exceeding 6%. For industrial millers, the acceptable level of starch damage is around 12-15%. No significant effect of nitrogen condition was observed for starch damage. Therefore, considering the low levels of starch damage detected, it is unlikely that the high levels of LPC observed are due to contamination from starch internal lipids.

3.2.8 The effects of genotype and environment on the levels of saturated lipids

Lipids play a crucial role on gas cell stability due to the formation of the liquid lamellae around the gas cells formed during dough proving (Details of which are provided in Chapter 1). However, it is not only specific lipid classes that play an important role in gas cell stability; saturation can also be important. Therefore the levels of saturated and unsaturated fatty acids in FFA and LPC were determined.

The saturated fatty acids species measured included 16:0, 18:0, 20:0, 22:0 and 24:0 while the unsaturated acids measured include 16:1, 18:1, 18:2 and 18:3. Due to inconsistent data 22:1 was removed from the analysis. For LPC, the unsaturated LPCs measured included LPC 18:1, 18:2 and 18:3, while the saturated LPCs included LPC 16:0 and LPC 18:0.

Investigating the effects of genotype and environment was crucial for FFA due to the role that saturation may play in various aspects of breadmaking quality. As mentioned in Chapter 1, high levels of FFA can lead to rancidity in food products. In the case of polyunsaturated fatty acids, this is caused by the polyunsaturated fatty acids (PUFAs) acting as a substrate for wheat lipoxygenase (Castello et al., 1998). The saturated: unsaturated ratio is also important due to its effects on gas cell stability. Details on the role saturated and unsaturated fatty acids play on breadmaking quality is discussed in Chapter 1.

Figure 3.8 displays the quantities of saturated and unsaturated fatty acids present in the six cultivars and three nitrogen conditions for FFA as well as LPC. There was a consistently high level of

unsaturated fatty acids present in almost every cultivar and nitrogen condition. This is consistent with various sources of literature (Gerits et al.,2014, Liu et al., 2017).

Among all the cultivars and nitrogen conditions, the only cultivar where unsaturated fatty acids were not significantly higher than saturated fatty acids was the cultivar Crusoe at 350kg/N/Ha. This phenomenon is particularly interesting considering that no significant differences in the levels of saturated and unsaturated free fatty acids were observed for Crusoe in other nitrogen conditions. Therefore, there is a possibility that this phenomenon is highly dependent on G x E interactions. There was only one significant effect of nitrogen among the cultivars which was for Avalon at N100 compared to N200 and N350.

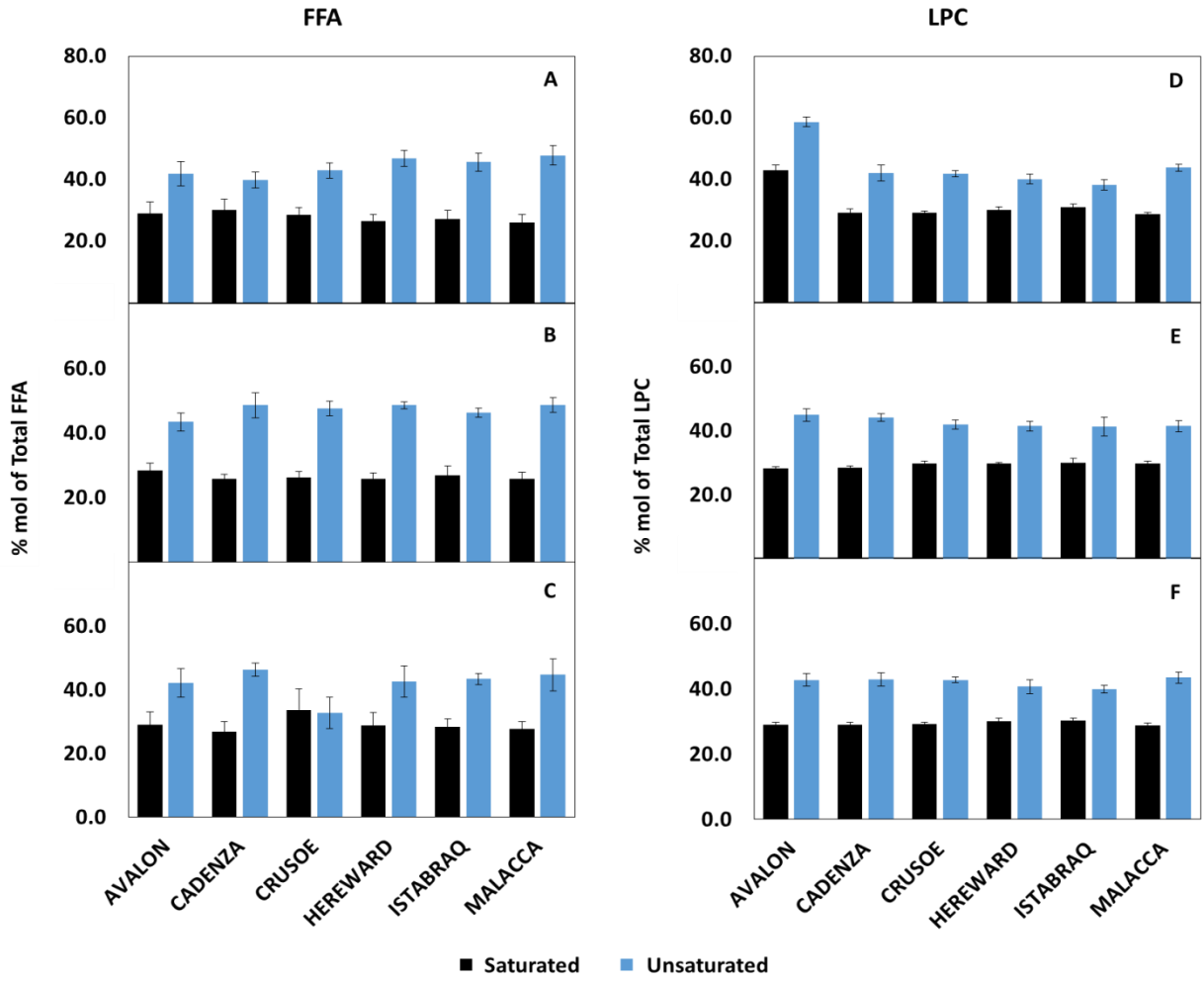


Figure 3.8 The % nanomoles of the total FFA and LPC between for Unsaturated and Saturated Fatty Acids. The saturated fatty acids for FFA and LPC are cumulative values for FFA 16:0 and 18:0 while the unsaturated FFA and LPC are cumulative values of 18:1, 18:2 and 18:3. A&D- N100, B&E- N200, C&F- N350. Data are mean values of ±SE of five independent samples analysed via ESI-MS/MS.

3.3 Discussion

The comparison of six cultivars grown at three levels of nitrogen input showed that cultivar and nitrogen had significant effects on the composition of specific lipid molecular species in white wheat flour.

3.3.1 Impact on PLs

The extent of lipid diversity observed was more surprising and interesting. The fatty acid profiles of the six cultivars at 350N showed surprisingly high diversity. Avalon and Malacca in particular showed the greatest amount of fatty acids compared to the other cultivars, and Crusoe had significantly lower levels of fatty acids. This was also observed when analysing the effects of genotype and environment on specific lipid classes. ANOVA of the total lipid classes showed PC and PE to be significantly affected by genotypic effects and F-tests of the p-values of the individual lipid species showed each and every one of the lipid species for PC and PE to be significantly affected by a cultivar effect.

Although PLs were most affected by cultivar, especially for the individual PC and PE species, little effect was observed for LPC. The proportions of LPC determined here was also higher than in other studies (Hargin and Morrison, 1980; Finnie et al., 2009). This may have resulted from the partial hydrolysis of PC by phospholipase A2 (PLA2) during flour storage.

However, we failed to detect increases in LPC in white flour stored over a period of 3 months (unpublished results). We therefore consider that it is more likely to have resulted from endogenous enzyme activity in the grain before milling, as LPC is produced from PC during germination (De la Roche et al., 1982).

The partial hydrolysis and degradation of lipid species during storage has not been noted in other studies. However, Liu et al., 2017 also suggests that LPC is produced from PC by the actions of phospholipase A1. With LPC levels being partially dependent on the potential degradation of PC, a significant secondary factor, it is possible that LPC would be less affected by the potential genetic differences present within the various cultivars. However, in the study by Liu et al., LPC was significantly affected by cultivar effects. However it must be noted that the study by Liu investigated 58 Chinese breadmaking wheat varieties.

Among the lipid classes, PC was the most affected by genotype effects. PC plays a crucial role in lipid biosynthesis as well as affecting breadmaking quality, which makes this particularly interesting. As well as being a precursor for LPC, the PC pool in the endoplasmic reticulum of plant cells plays a

crucial role in TAG synthesis. TAG can be synthesised by the activity of PDAT (phospholipid:diacylglycerol acyltransferase) which transfers fatty acids at the sn-2 position of PC to DAG which generates TAG, meaning that PC acts as the acyl donor while the DAG acts as the acyl acceptor. PC, DAG, TAG and to a lesser extent LPC, plays a crucial role in TAG synthesis in plants. PC is synthesised via conversion from DAG by the action of sn-1,2 diacylglycerol: cholinephosphotransferase (CPT) or phosphatidylcholine:diacylglycerolcholinephosphotransferase (PDCT). Further information on PC, its role in synthesising TAG and production of LPC can be found in Chapter 1 (Figure 1.14 & 1.15).

PLs have been shown to have a positive effect on loaf volume, (MacRitchie, F, 1983) acting as emulsifiers to improve the baking performance of wheat dough (Pomeranz et al., 1968; Pomeranz et al., 1970). Endogenous phospholipids together with DGDG and saturated FFA form condensed monolayers around the gas cells in dough. Of the phospholipids, PC has been reported to correlate with loaf volume, although the correlation was weak (Helmerich and Koehler, 2005). The role of PE has not been established, but one study showed it correlated negatively with loaf volume. However, PE plays an important role as a precursor, substrate and donor in the lipid biosynthesis pathway. PLs have also been shown to interact with gluten proteins, which may contribute to increased loaf volume and improved dough strength (McCann et al., 2009). The effects of cultivar and nitrogen on PC and PE demonstrated here may therefore have implications for breadmaking quality.

3.3.2 Impacts on NLs

The amount of TAG was significantly ($p < 0.05$, F-test) affected by both genotype and environment. Total TAG levels and 5 TAG species were significantly affected by genotype effects. These strong genotypic effects were highly interesting as it indicates possible genetic control of the TAG biosynthesis pathway. As mentioned above, PC plays a crucial role on TAG synthesis, however TAG is also produced from DAG via an acyl-CoA dependent acylation which is catalysed by acyl-CoA:diacylglycerol acyltransferase (DGAT). TAG biosynthesis involves multiple steps. These steps starting from fatty acid synthesis to TAG biosynthesis are discussed in Chapter 1, sections 1.10.2.1-3.

TAG is hydrolysed to DAG and FFA during germination and this may occur during flour storage at room temperature. High levels of FFA are undesirable for food production as oxidation may lead to rancidity while NLs have been reported to destabilize gas cells and negatively affect loaf volume (MacRitchie & Gras, 1973). Unsaturated FFAs in particular have been reported to reduce bread loaf volume (De Stefanis and Ponte., 1976; Sroan and MacRitchie., 2009). FFA do not usually accumulate in healthy living tissues and it is therefore likely that the high levels of FFA reported here result largely from TAG degradation, either in the grain or stored flour. The degradation of TAG into FFA

has been widely reported in various systems (Athenstaedt & Daum., 2006), and our own studies have also shown increases in FFA content together with decreases of TAG in wheat flour (Salt et al., 2017).

3.3.3 Effects of Environment and potential GxE effects

Although less clear than genotype or environmental effects, significant GxE effects were also observed throughout this study. The fatty acid profiles obtained by FAMEs analysis of the six cultivars changed significantly with increased levels of nitrogen fertilization. Not only was an increase in quantity observed, but there was also a greater diversity in the fatty acid composition. This was reflected in the ESI-MS/MS results where the greatest diversity among the lipid profiles was observed at N350. CVA analysis also showed greatest separation at N350 and N200 compared to N100.

No previous studies have reported the effects of nitrogen input on wheat lipids, therefore the reasons for this are unclear. However the lipid changes between cultivars according to the differences in nitrogen condition imply that each cultivar has its own unique mechanism for responding to different levels of nitrogen application. Avalon and Malacca showed significantly higher levels of lipid content, while Cadenza and Crusoe consistently had lower levels, especially at higher nitrogen conditions.

The large effect of environment and GxE effects on the lipid composition of wheat flour was not too surprising especially when considering how other biochemical components have been observed to respond to environmental effects (Figure ()). Furthermore, literature has shown how the lipid content of other wheat tissues responded to various environmental stresses; therefore it is not unreasonable to expect this would also be the case for the wheat grain. Studies later in the thesis show how significant environmental and GxE effects can be in the wheat grain and its implications will be discussed in more detail.

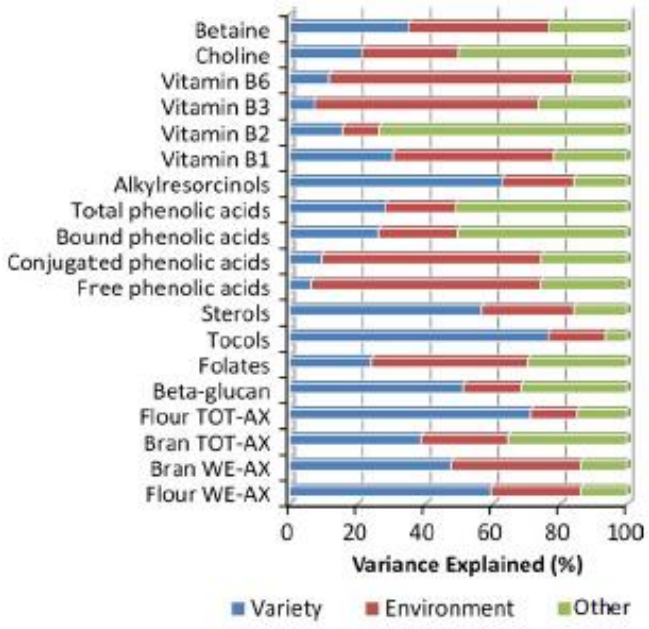


Figure 3.9 Summary of the heritability of dietary fibre and other biological components in the wheat grain, based on the HEALTHGRAIN study. "Other" includes the variance ascribed to Genotype x Environment interactions and/or error. Taken from Shewry and Hey, 2015.

3.3.4 Differences between the cultivars

Multivariate analysis showed significant differences between cultivars, with Cadenza and Istabraq being the most distinct, followed by Avalon. The other three cultivars, Crusoe, Hereward and Malacca showed no differences, except for Crusoe and Malacca at N200 and Hereward at N350. In broad terms, the variation in lipid composition between the cultivars was greatest at N200, which is the typical level of fertilization for intensive wheat production in the UK.

The separation of Istabraq is not surprising as it is the only soft wheat in this study. Several studies have shown a correlation between endosperm texture and the amount and/or type of lipid. For example, hard Australian wheat cultivars have higher levels of hexane-extractable free lipids than soft cultivars (Panozzo et al., 1990) while a strong inverse relationship was reported between hardness and total PL content in British cultivars (Morrison et al., 1989). Cadenza is a hard bread making wheat but differs from the other five cultivars in being a spring variety (although its winter hardiness led it to be generally sown in autumn). Hence, the differences in composition between Cadenza and the other cultivars could relate to the underlying pedigree, as spring and winter breeding programs use different sets of germplasm. Other authors have also reported differences in the lipid profiles of several

cultivars, with Hargin and Morrison (1980) comparing four bread wheat varieties and Beleggia et al (2013) four durum wheat cultivars.

Considering the significant differences observed between the lipid profiles of the six cultivars, this provides interesting opportunities for future work. The large differences in the lipid profile between Avalon and Cadenza makes the lipid profile of the well-established Avalon x Cadenza population an interesting candidate for investigation.

Unfortunately this study has not brought us closer to answering the Hereward conundrum. Overall, no significant effects were observed for Hereward or any of its lipid species. However considering the complex role lipids play on breadmaking quality, which has not yet been fully established, it is possibly too early to write off the Hereward lipid profile as being insignificant. It is possible that a good balance of various lipid classes are more beneficial than significantly higher or lower levels of a specific lipid class. This lipid stability may very well be a contributing factor for Hereward's success.

Future studies on the Malacca x Hereward double haploid (DH) population and near isogenic line (NIL) population may reveal some interesting differences that can shed further light on the Hereward conundrum.

3.4 Concluding Remarks

Lipidomic analysis of six UK breadmaking wheat cultivars grown in three nitrogen conditions showed significant effects of genotype, environment and GxE effects on the lipid composition of wheat flour. Although a wider range of cultivars, growth conditions, and lipid classes should be analysed for firm conclusions to be drawn, this study has shown that genotype plays a major role in determining the lipid composition of wheat. These differences may therefore be exploited in breeding to develop cultivars with superior lipid profiles.

Among the cultivars, Istabraq the only soft wheat, showed the greatest separation from the other cultivars. This was followed by Cadenza, a spring wheat (although usually planted in the autumn). Comparison of the lipid profiles showed that Malacca and Hereward were the most similar with the greatest differences being between Avalon and Cadenza.

Low nitrogen fertilisation resulted in significantly lower quantities of fatty acids, and the least variation in lipid profiles between the cultivars. On the other hand, the highest level of nitrogen input (overfertilization) did not give greater lipid diversity than the 200kg application, although they were associated with greater quantities. In addition 200 kg/N gave the greatest lipid diversity. Considering

this is the standard level of UK fertilization, but not the rest of the world, the lipid diversity of cultivars at N200 may be worth investigating.

We have therefore demonstrated effects of genotype and nitrogen nutrition on the content and composition of PL, TAG and FFA, which could have impacts on dough properties and bread making performance. Although this is the most detailed study so far reported, it should be noted that only a single harvest year was studied. Hence, year-to-year differences between grain samples due to environmental factors can be expected, including those demonstrated here related to genotype and agronomy. Consequently, the exploitation of differences in processing quality resulting from lipid composition by wheat breeders and wheat processors poses a challenge.

Chapter 4 Investigating the genetic effects of the MxH NILs on the lipid profile of wheat flour

4.1 Introduction

4.1.1 Brief Introduction to the Malacca x Hereward Doubled Haploid Population

As discussed in Chapter 1, the UK wheat supply chain has seen drastic changes over the past few decades with large increases in the use of home grown British wheat. Currently about 5 million tonnes of wheat are milled annually, to produce 4 million tonnes of flour, and the UK grows 85% of its breadmaking wheat, only importing 15%. These imports are necessary due to the requirement for higher quality than is available in the UK crop. However, the UK also exports similar volumes of wheat to that which is imported, to countries such as the Republic of Ireland, France and Morocco. Breeders have been very successful in developing markers for desirable characteristics which are controlled by major genes, which has facilitated selection for good agronomic properties and grain quality. However, as discussed in Chapter 1, many genes for many quality traits have not been identified, which may reflect the fact that they are influenced by the effects of many minor genes rather than a few major genes.

In 2001-2007 a project funded by the Home Grown Cereals Authority (HGCA), (Investigating Wheat Functionality through Breeding and End Use) developed doubled haploid populations to map QTLs for milling and baking parameters. (Millar et al., 2007)

The study also aimed to create a benchmark "quality map" for breadmaking performance to improve the understanding of raw material functionality and processing. Three recombinant doubled haploid (DH) populations were developed for this study (DH) between good breadmaking varieties that represented a range of quality characteristics. These were

- Malacca x Charger
- Malacca x Hereward (M x H)
- Shango x Shamrock

More details of these DH populations and their generation will be given in chapter 6.

4.1.2 Generation of the NILs

A range of novel QTLs for milling and breadmaking traits were identified using several approaches including single marker ANOVA and marker regression analysis. The QTLs were evaluated in terms of significance and robustness to select for those considered to have the greatest impact, the final total being 606. Of these, 179 novel QTLs were identified from the M x H DH population for a wide range of breadmaking quality characteristics ranging from water absorption to loaf volume, with robustness determined by comparing two years of study, 2005 and 2006.

The M x H population is particularly interesting to this study due to the identification of QTLs for gas cell stability. Both parental populations were highly successful UK breadmaking varieties, as discussed in Chapters 1 and 3. Furthermore, both Malacca and Hereward are unusual in that their quality cannot be explained by the presence of good HMW glutenin subunits. The mapped QTLs included several located on chromosomes 1B, 4D, 6A and 7A which were shown to influence gas cell number, loaf volume and gas cell wall thickness.

To elucidate the effects of these QTLs, sets of near isogenic lines (NILs) were developed at the John Innes Centre (JIC). NILs are pairs of lines that have identical genetic backgrounds except for allelic variation at a single QTL for the trait of interest. NILs therefore allow detailed comparisons of the effects of allelic variation at a single QTL, and can facilitate the mapping and characterisation of the underlying gene(s). In particular, they facilitate the identification of markers which are close enough to the gene(s) of interest to enable wheat breeders to select for the desirable alleles of the gene(s) in different breeding programmes.

NILs are created by crossing plants with allelic variation at the QTLs of interest with a standard line (usually one of the parental lines). In this case, the two M x H DH lines differing at a QTL of interest (Line MH100 for the QTL 1B, see Table 4.1) were crossed with the recipient parent Malacca (Mal4) and lines with the two alleles selected. Two more backcrosses were then carried out, selecting again for the two alleles, and the BC2 individuals were self-crossed. The homozygotes for the specific alleles were then selected, producing NILs with a recurring parent (Malacca) genomic background of at least 87.5%. (Figure 4.1)

The individual NILs used in this study are described in Appendix Figure 4.1. A total of 10 NILs (5 Hereward + 5 Malacca alleles) were selected for the 1B, 4D and 6A QTLs and a total of 8 lines (4 Hereward +4 Malacca) were selected for the 7A QTL. At all four QTLs, the Hereward alleles were associated with good breadmaking quality, while the Malacca alleles were associated with poor breadmaking quality (details of which are given in Table 4.1.).

The lines were multiplied in the field and those with the same alleles were bulked for analysis. The analysis was repeated for two years.

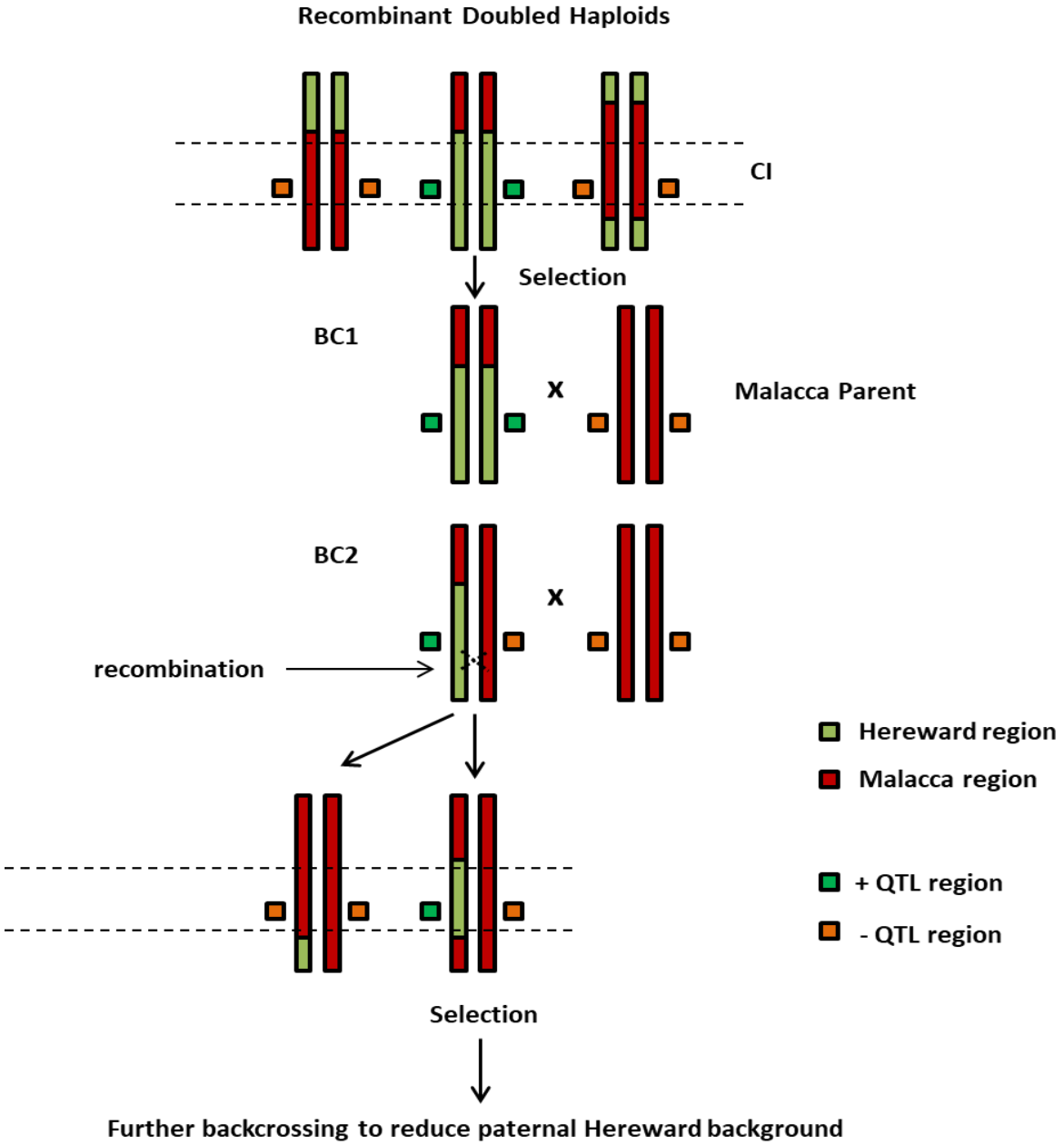


Figure 4.1 Simplified schematic of the generation of the MxH NILs. Among the recombinant double haploid (DH) lines those with positive QTL effects within the confidence intervals were selected. Selection of the recombined lines after BC2 were selected by marker assisted selection (MAS) BC-Backcross, CI- Confidence Intervals

Donor Line	Parental Line	Chromosome Location	Quality Trait
MH100	Malacca	1B	Cell Number
MH1	Malacca	4D	Loaf Volume/L*
MH 70	Malacca	6A	Cell Number
MH 60	Malacca	7A	Wall Thickness, Cell diameter/volume, Loaf volume

Table 4.1 Background information on the MxH NILs.

4.1.3 Relating gas cell stability to cell number and loaf volume

In the final loaf, around 70% of the volume can be made up by gas, and the size and distribution of the gas cells could have a large effect on the texture and sensory properties of the final product (Zghal et al., 2002). All stages of the baking process can influence the gas cell distribution and therefore the crumb structure of the loaf. The bubbles are entrapped in the dough due to high energy mechanical mixing. These bubbles are then expanded by CO_2 produced by yeast during dough proving. During proving, the bubbles expand and come into closer contact. Due to this, the gas cell walls become thinner and the system becomes more prone to destabilization.

The bubble network in the breadmaking process is considered to be a foam structure, which makes it susceptible to problems associated with foam stabilization which are prevalent in the breadmaking process. The final baking fixes the foam structure, solidifying it and setting it in place as the bubbles within the matrix rupture to form the crumb structure that we associate with UK sliced bread.

As foams are intrinsically unstable structures, surface active molecules are required to form an interfacial layer around the bubbles to stabilize them against coalescence leading to foam collapse. It is considered that gas cell walls are formed from the starch gluten matrix of dough lined by a thin liquid film containing surface-active materials (Gan et al., 1990; MacRitchie, 1976; Ornebro et al., 2000; Sahi, 1994). Further details of the mechanism of foam stability are given in Chapter 5.

Efforts have been made to elucidate how gas cells within a dough system become stabilized. During dough development the gluten proteins act as the primary stability mechanism for the dough, and phospholipids and galactolipids may interact with these, providing further stability (McCann et al., 2009). A secondary mechanism of gas cell stabilisation has also been proposed, the formation of a thin liquid lamellae. Lipids as well as other surface active components are believed to interact with the lamellae to provide a further stability mechanism for the gas cells.

The NILs were therefore analysed to determine their lipid content which may contribute to gas cell stability. A lipidomics approach, using ESI-MS/MS identified 88 lipid species over 8 lipid classes in this study across two years. These include 5 phospholipids, lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), 2 galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) and 3 neutral lipids, diacylglyerol (DAG), triacylglycerol (TAG) and free fatty acids (FFA).

The NILs harvested in 2013 were grown at Rookery Field, Church Farm, Bawburgh while the material harvested in 2015 was grown at Deopham, near Wymondham. Both farms are located in Norfolk and both crops were grown under standard agronomic practices, using 200 kg of nitrogen per hectare.

Mature grain from the replicated field trial was milled using a Chopin CD1 mill to produce white flour. This mill gives low levels of starch damage (determined as ranging from 5-7% in this study, Appendix Figure 4.2) which would minimise the extraction of intrinsic starch granule lipids which do not affect bread making quality (Rocha et al., 2012).

4.2 Results and Discussion

4.2.1 Proportions of lipid classes across the two years

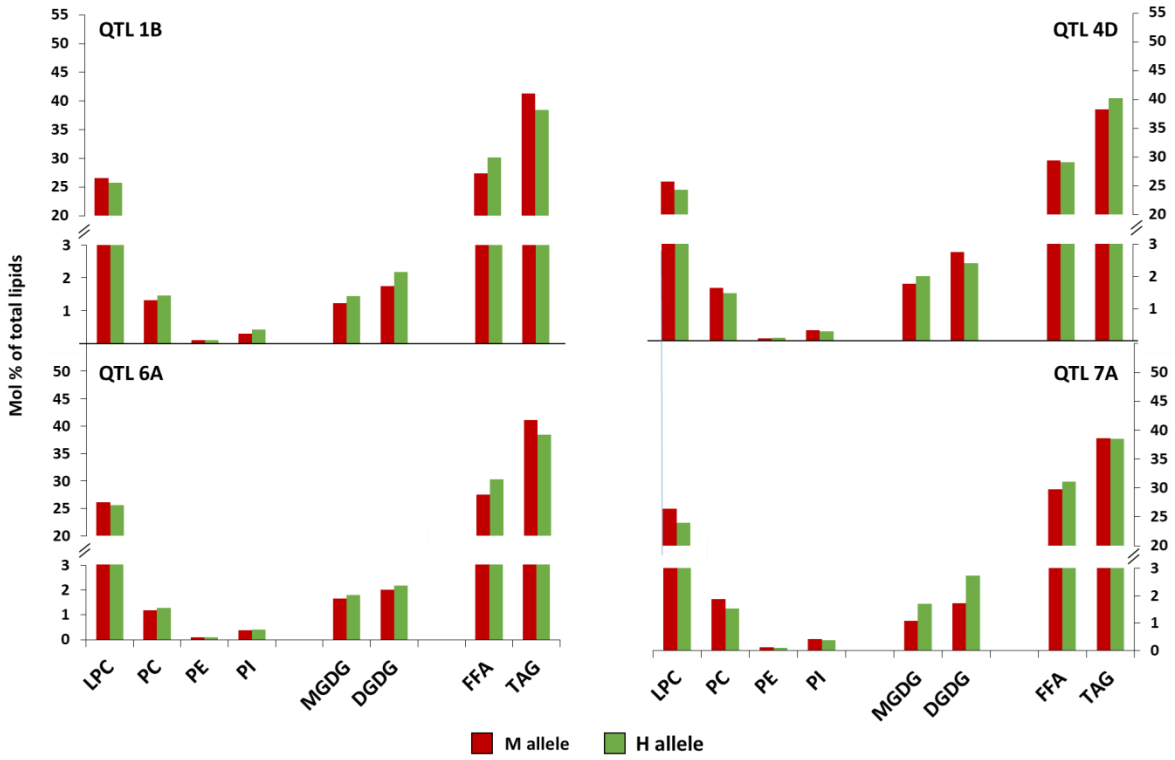


Figure 4.2 Proportions of specific classes of lipids present in the four MxH NIL populations in Year 1.

Year 1

TAG was present in the highest proportion making up over 35% of the total lipids, followed by FFA (25-30%) and LPC (20-24%). These three lipid classes comprised approximately 90% of the lipids present in flour. The galactolipids DGDG and MGDG made up 2-3% and 1-2% respectively. The major phospholipid PC made up approximately 1% of the lipid content. PI represented approximately 0.4%, and PE made up around 0.2-3% of the total lipid profile. (Figure 4.2) These lipids together make up over 92% of the total lipid profile present in the wheat grain (Gonzalez Thuillier et al., 2015; Finnie et al., 2009).

Year 2

Neutral lipids again made up the highest proportion of lipids with TAG accounting for 40-45% and FFA accounting for 30% of the lipid profile. These were followed by the major phospholipids which include LPC (10%) and PC (4.5-7%). The galactolipids were present in almost identical proportions to year 1; DGDG (2-3%), and MGDG (1-2%). Finally the minor phospholipid PI made up around 0.5% of the lipid profile, while the levels of PE made up around 0.2% of the lipid profile.

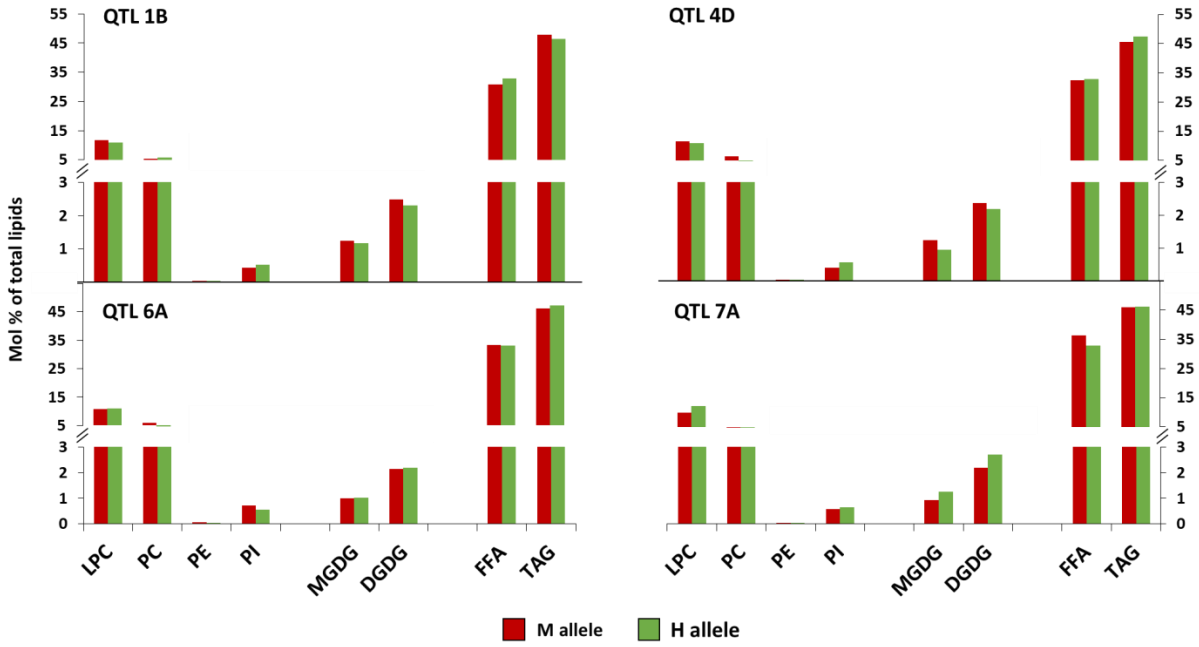


Figure 4.3 Proportions of specific classes of lipids present in the four MxH NIL populations in Year 2.

Comparison of years 1 and 2

Some differences were observed between the lipid profiles across the two years, with significantly lower levels of PC observed in year 1 and correspondingly lower level of LPC in year 2. The proportions of TAG and FFA agree with the study by Hargin and Morrison (1980) who analysed four wheat cultivars showing that TAG was present in higher amounts than FFA. However, other studies have reported higher levels of FFA than TAG in white wheat flour (Chung et al., 2009; Gonzalez Thuillier et al., 2015; Min et al., 2017). However, higher levels of TAG were reported for three of seven pearling fractions by Gonzalez-Thuillier et al.(2015) and higher levels of TAG was present in certain cultivars such as Cadenza and Crusoe grown at 350 kg/N/Ha (Chapter 3, Figure 3.2).

The levels of LPC observed in the year 1 material agree with the levels of LPC observed in Chapter 3 of this thesis, where significantly higher levels of LPC than PC were observed. Although the ratio of PC to LPC varied, there were significantly higher levels of LPC compared to PC in the grain pearling fractions that were most similar in composition to white flour (Gonzalez Thuillier et al., 2015). However, the ratio of LPC: PC in the second year material was more similar to what was reported in other studies. Finnie et al., (2009) reported similar LPC: PC ratios to the year 2 material, which had significantly lower levels of LPC compared to year 1. However, few studies have been reported of the lipid content and composition of white flour making these proportions difficult to compare across multiple studies.

The large differences in the LPC: PC ratio between the year 1 and 2 samples can be attributed to the degradation of PC by phospholipase activity, as discussed below and in chapter 3. This was not unexpected, considering the differences in the length of time the respective grain and flour samples were in storage before analysis. However, the combined levels of total LPC+PC were very similar between the two years, despite the large differences in their individual proportions.

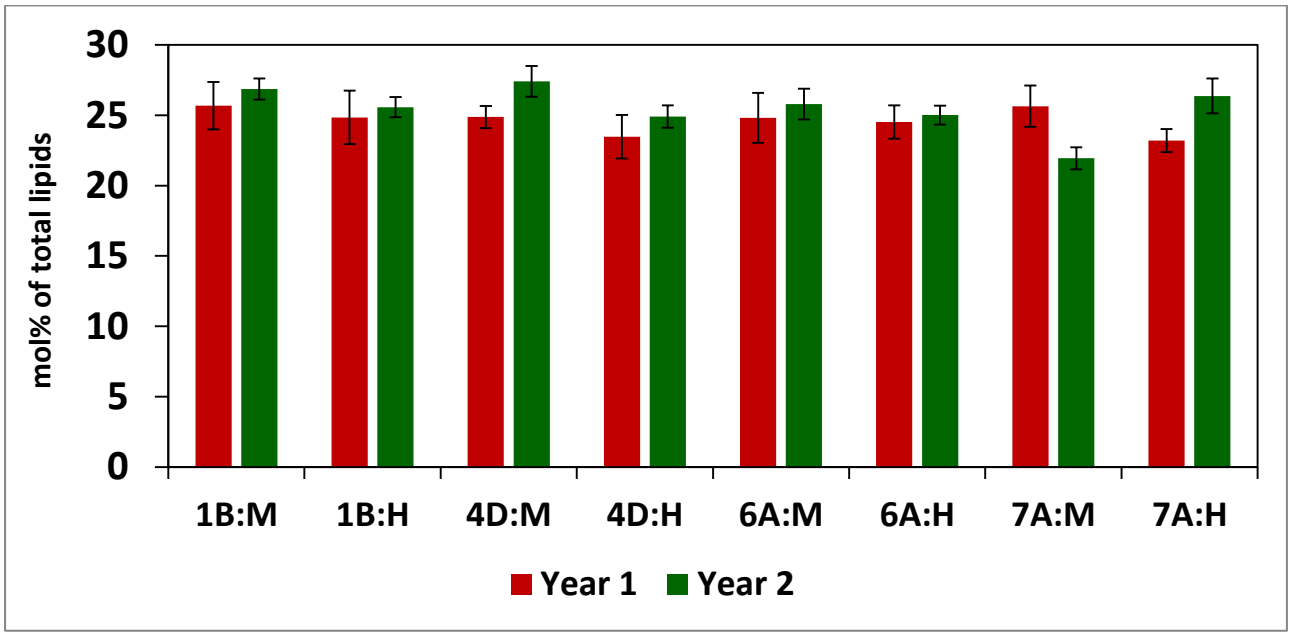


Figure 4.4 Mol% of total lipids accounted by LPC + PC levels in the wheat grain across the two years. 1B, 4D, 6A and 7A indicate chromosomal location, and H (Hereward) and M (Malacca) indicate the allele.

4.2.2 Univariate ANOVA analysis of data

Analysis of Variance (ANOVA) was applied to the percentage data for all lipid classes for each year of the study. In year 1, a total of 68 lipids were quantified, comprising 24 phospholipid species (LPC, PC, PI, PE, and PG), 9 galactolipid species (MGDG, DGDG) and 35 neutral lipid species (TAG and FFA). The main effects and interactions between the alleles (the specific Malacca or Hereward alleles) and QTLs could be tested (F-test). However, it must be noted that the five replicates in this study are from pools of material, bulked from the sets of NILs with the Malacca or Hereward alleles, and are therefore not true biological replicates.

ANOVA showed 31 lipids with a significant ($p<0.05$, F-test) interaction between the alleles and QTL factors (QTL x alleles). These include the phospholipids PC 34:1, PI 34:1, PI 34:2, PI 36:3, PI 36:4, PI 36:5, PI 37:2, PI 37:3, the galactolipids DGDG 36:3 and 36:5 and the neutral lipids FFA 13:0, FFA 18:2, FFA 18:3, FFA 19:0, FFA 20:0, FFA 21:0, FFA 22:1, FFA 23:0, FFA 24:0, TAG 33:0, TAG 37:0, TAG 50:1, TAG 53:3, TAG 53:4, TAG 54:2, TAG 54:3, TAG 54:5, TAG 54:9, TAG 55:5, TAG 55:6 and TAG 56:5.

A total of seven lipid species were significantly affected by independent allele effects only. These include the galactolipids MGDG 36:4, DGDG 34:2, DGDG 34:3 and DGDG 36:4, and the neutral lipids FFA 16:0, FFA 18:1 and TAG 54:4. While this is a good statistical interpretation of the data, it is important to note that due to the nature of the NILs, which were generated by backcrossing to the Malacca parent, the only significant genetic effects observed should be allelic effects associated with the Hereward alleles. Independent allelic effects are significant regardless of QTL, indicating a possible genetic effect. However, the QTL x allele effects needed to be interpreted with caution. This is because effects on lipid composition associated with the different QTLs, as opposed to the allelic variants at the QTLs, are likely to be associated with the background genes of the NILs or with non-genetic effects. Therefore, unless the allelic effects far outweigh the QTL effects, it is unlikely the significant differences are being contributed by genetic effects.

4.2.3 Independent QTL effects on the lipid composition of the NILs

In order to observe whether the QTLs show any independent effects on the lipid composition of the NILs, the flour for the sets of NILs for individual QTLs were bulked together. For example, the 5 lines with the Malacca allele and the 5 lines with the Hereward alleles were combined. This should essentially remove the main genetic differences between the NILs.

Very little variation in the lipid composition of the NILs was observed after bulking together the Malacca and Hereward alleles. Of the 6 lipid classes measured, comprising 57 lipid species, only 4 species (TAG 54:5, 54:6, PC 34:2 and 36:4) showed independent QTL effects. The QTL 6A was associated with lower TAG content than the other QTLs while both the 4D and 6A QTLs were associated with lower PC content than the other QTLs.

Overall little variation in lipid composition was observed between the QTLs after bulking the alleles, indicating that the backgrounds of the NILs are highly random. This suggests that any significant differences in lipid composition observed between alleles at the NILs are likely to occur due to the genetic effects of those specific alleles.

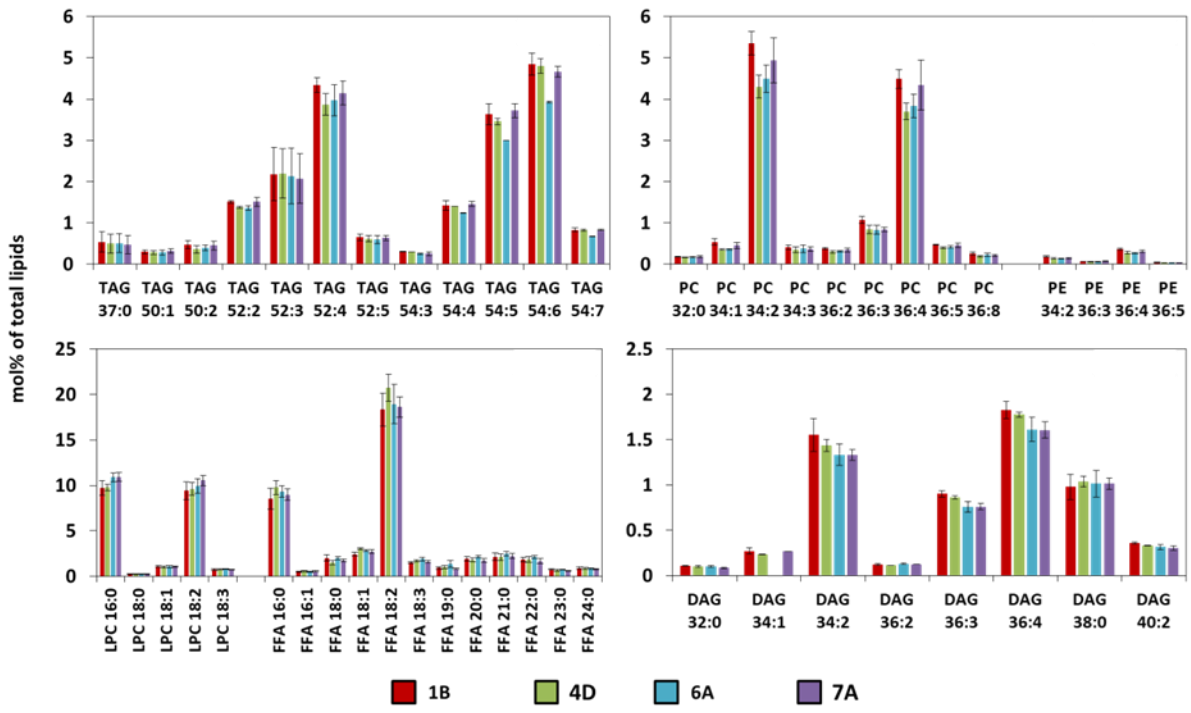


Figure 4.5 Mol % of total lipids for the six lipid classes across the four bulked QTLs. The alleles were bulked together, making the NILs lose their genetic variability.

4.2.4 Allelic effects on PLs in Years 1 and 2

To summarize the results of the ANOVA analysis, specific annotations are included in the figures. To aid understanding, a short description will be added here.

***** = Lipid species significantly affected by independent allele effects only ($p < 0.05$, F-test)

+ = Lipid species significantly affected by independent QTL effects only ($p < 0.05$, F-test)

○ **=** Lipid species affected by QTL x allele effects ($p < 0.05$, F-test)

▲ = Lipid species affected by QTL x allele effects, but specifically showing significant allele effects ($p < 0.05$, LSD).

It should be noted that it is impossible to observe ▲ alone. It will always be associated with ○ which indicates significant QTL x allelic effects.

This approach provides a robust method with which to analyse the statistical significance of the effects of the Hereward and Malacca alleles. Due to the nature of the NILs where the lines have been backcrossed to the Malacca parent to eliminate the effects of the genetic background, the only genetic effect on the lipid composition of the NILs should result from effects of the Malacca or Hereward alleles. Great care must therefore be taken with the interpretation of the QTL x allele effects as significant QTL x allele effects for a particular set of NILs (e.g. for QTL 7A) may reflect the differences between the QTLs rather than effects of the Malacca and Hereward alleles themselves. Therefore, unless the allelic effects are much greater than the QTL effects it is not possible to conclude that the genetic effects are genuine. In order to indicate where the allelic effects were significantly greater than the QTL effects a ▲ symbol was added.

In summary, while differences in lipid composition which are significantly affected by independent allele effects are more likely to be robust, care must be taken with QTL x allele effects as these are likely to result from factors outside the actual Malacca and Hereward alleles focused on in this study.

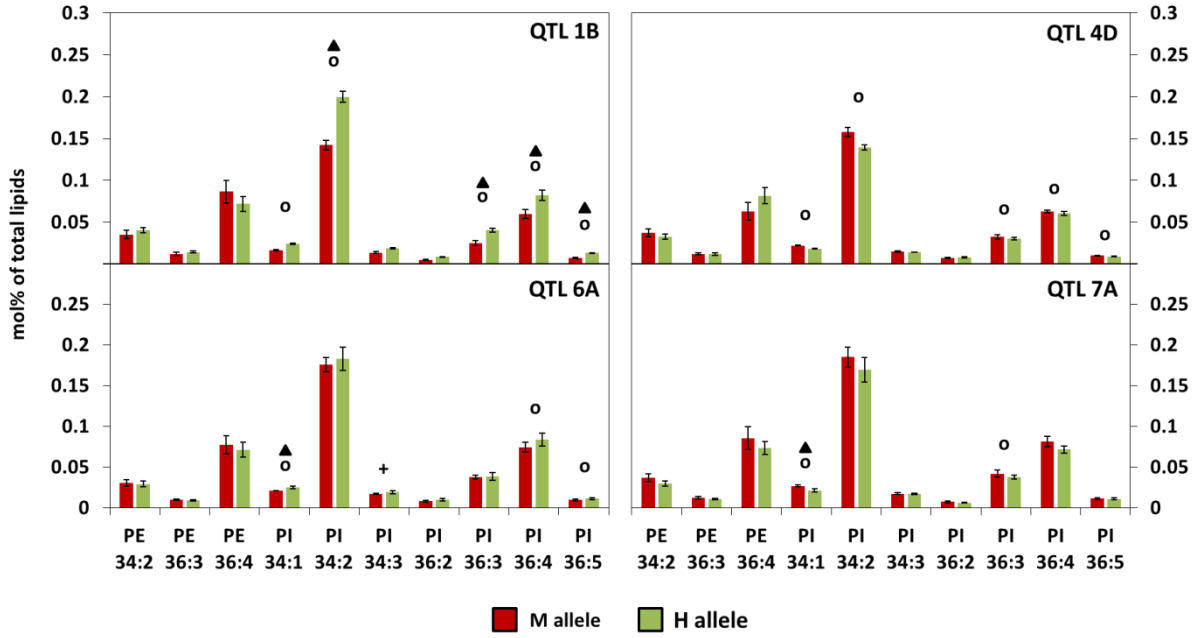


Figure 4.6 Comparison of selected minor phospholipids (mol % of total lipids) between M and H NILs among the four QTLs in Year 1. Three species of phosphatidylethanolamine PE (34:2, 36:3, 36:4) and seven species of phosphatidylinositol PI (34:1, 34:2, 36:2, 36:3, 36:4, 36:5) are displayed. Data are mean values of ±SE of five independent samples analysed via ESI-MS/MS.

Year 1

No independent allelic effects were observed for the minor PLs in the first year, however, a large number (6) of QTL x allele effects were observed. 5 of the 7 PI species showed significant QTL x allele effects with the alleles at the QTL 1B locus showing significantly stronger allelic effects. The Malacca allele was associated with significantly lower levels, while the Hereward allele was associated with higher levels of lipid. The only other allelic difference observed was of the QTL 6A locus on the minor PI specie 34:1.

Again no significant independent allelic effects were observed for the major phospholipids, indicating a lack of allelic control of the PLs. PC 34:1 showed significant QTL x allele effects, with significant allele effects on QTLs 4D and 6A, however, considering the very low levels of PC 34:1 it is unlikely that PC 34:1 will exert major effects. Despite being the most abundant PL, LPC was not affected.

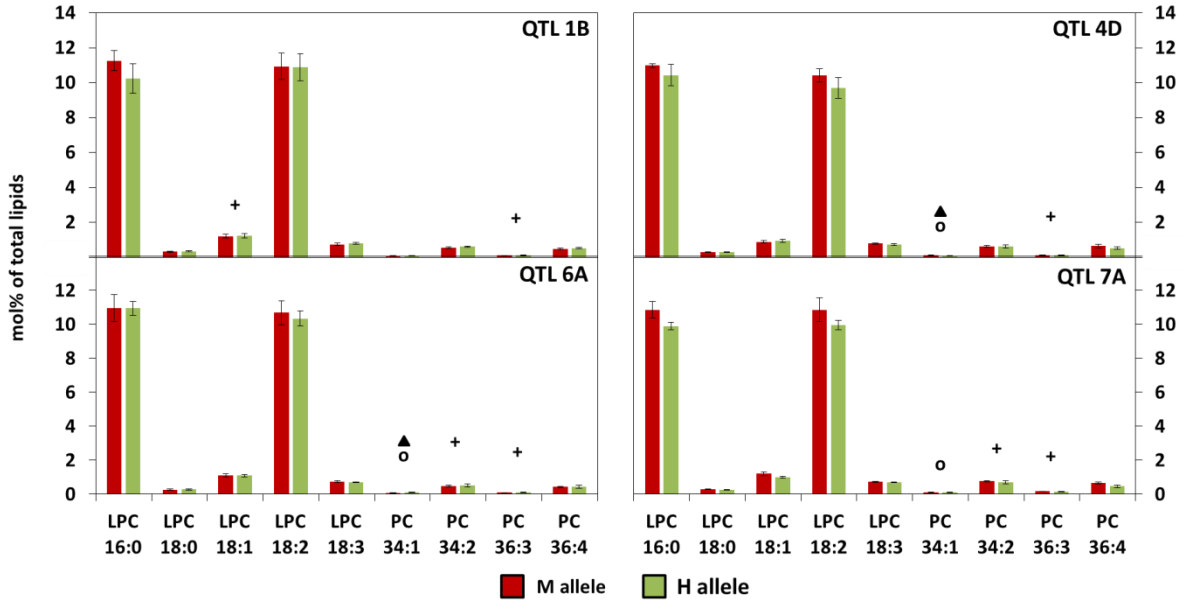


Figure 4.7 Comparison of major phospholipids (mol% of total lipids) between M and H NILs among the four QTLs in Year 1. 5 species of lysophophatidylcholine (LPC 16:0, 18:0, 18:1, 18:2, 18:3) and four species of phosphatidylcholine (PC 34:1, 34:2, 36:3, 36:4) are displayed. Data are mean values of ±SE of five independent samples analysed via ESI-MS/MS

Year 2

The effects of QTLs, alleles, and QTLs x alleles were different for the second year dataset, which was expected considering the large differences in the levels of PC and LPC observed between the two years.

No significant independent allelic effects were observed for the minor PLs in year 2. Four lipid species, PE 36:4, PI 34:2, 36:4 and 36:5, showed QTL x allele effects, compared to five in year 1. In year two, allelic effects were stronger than the QTL effects for PI 34:2 on QTLs 1B, 6A and 7A. Some QTL independent effects were observed for the minor PLs PE 34:3, PI 34:1 and PI 36:2 indicating they were affected regardless of the alleles present in the NILs.

Although few effects were seen in the first year, the major phospholipids showed more significant differences in the second year material. This was particularly the case for LPC 16:0 and 18:2 which showed significant QTL x allele effects for the QTL 7A.

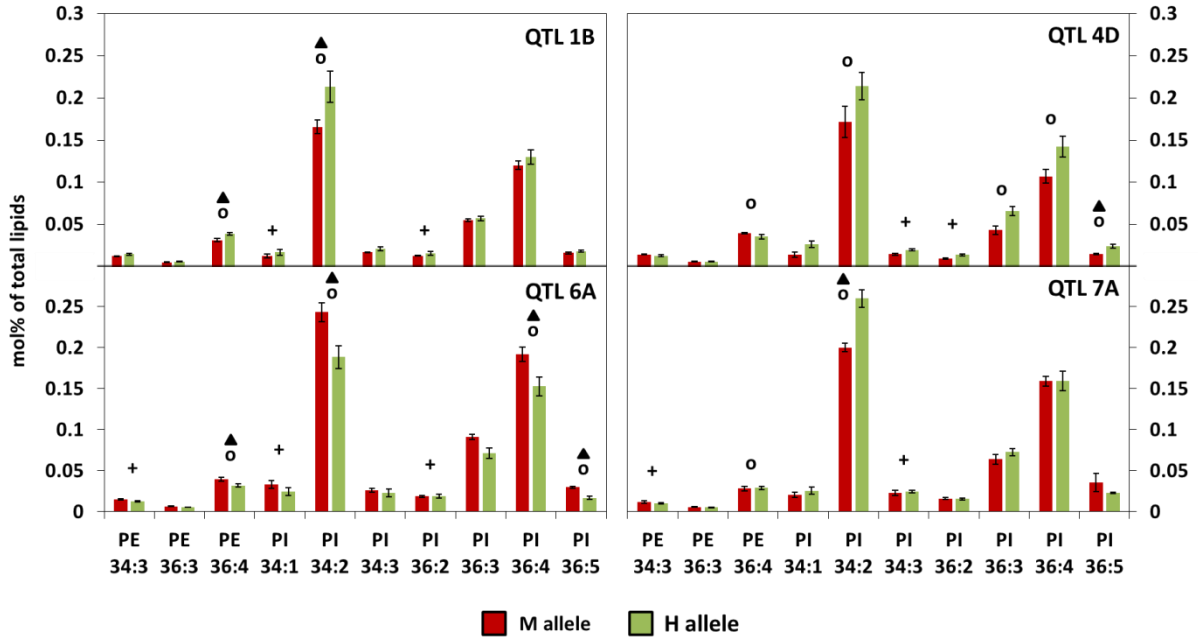


Figure 4.8 Comparison of minor phospholipids (mol % of total lipids) between M and H NILs among the four QTLs in Year 2. Three species of phosphatidylethanolamine PE (34:2, 36:3, 36:4) and seven species of phosphatidylinositol PI (34:1, 34:2, 36:2, 36:3, 36:4, 36:5) are displayed. Data are mean values of ±SE of five independent samples analysed via ESI-MS/MS.

Of the 8 species of PC in the second year data, 5 were affected by independent QTL effects and one, PC 36:2, was affected by QTL x allele effects (Appendix Figure 4.3b). However, the low level of PC 36:2 and the limited allelic effects observed makes it unlikely to be associated with allelic differences. .

Comparing the two years, although the phospholipids showed different effects of QTLs, alleles and QTLs x alleles, a number of broad similarities were observed. Generally the major phospholipids were affected by QTL effects alone, indicating significant non-genetic effects on phospholipids. Only the levels of LPC in year 2 showed allelic differences, with the Hereward allele being associated with greater lipid quantities. Due to the difference in the level of LPC and PC between years 1 and 2, it was interesting to see significant effects on the major LPC species, when the levels of LPC fell significantly in year 2.

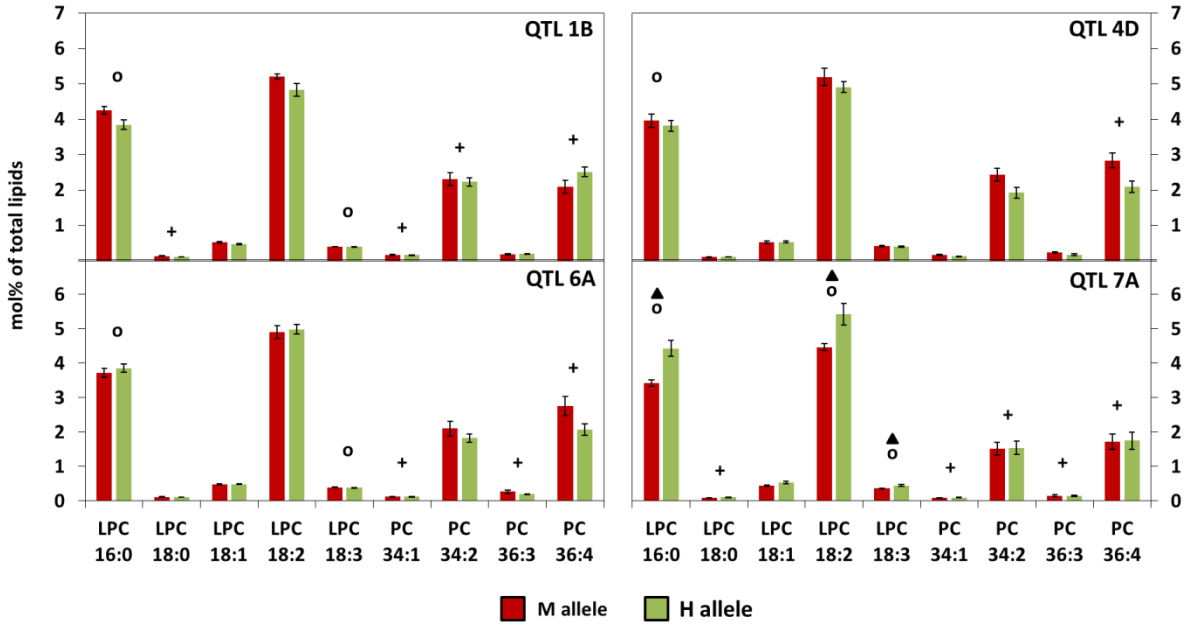


Figure 4.9 Comparison of selected major phospholipids (mol % of total lipids) between M and H NILs among the four QTLs in Year 2. Minor phosphatidylcholine species can be found in appendix Figure 4.3a.5 species of lysophophatidylcholine (LPC 16:0, 18:0, 18:1, 18:2, 18:3) and four species of phosphatidylcholine (PC 34:1, 34:2, 36:3, 36:4) are displayed. Data are mean values of ±SE of five independent samples analysed via ESI-MS/MS.

Among the minor PLs, PE showed almost no differences between the years, with only PE 36:4 showing weak QTL x allele effects in the year 2 dataset. PI was the phospholipid being significantly affected in both years, with 5 species affected in the first year and 4 species affected in the second year by QTL x allele effects mainly for QTL 1B. Although care must be taken with interpreting QTL x allele data, the allelic effects on QTL 1B were far larger than the differences between the other NIL sets. The allelic effects did not all correlate across the two years, with the exception of PI 34:2, yet it is interesting to observe that PI, a phospholipid strongly implicated in affecting breadmaking quality (Helmerich & Koehler, 2005) , was by far the most significantly affected phospholipid.

PI 34:2 is particularly interesting. It is the most abundant PI specie, present in higher levels than the next three most abundant PI species combined, and was consistently affected by allelic effects. The Hereward allele was consistently associated with a higher level of PI 34:2.

4.2.5 Effect of QTLs and alleles on GLs in Year 1 and Year 2

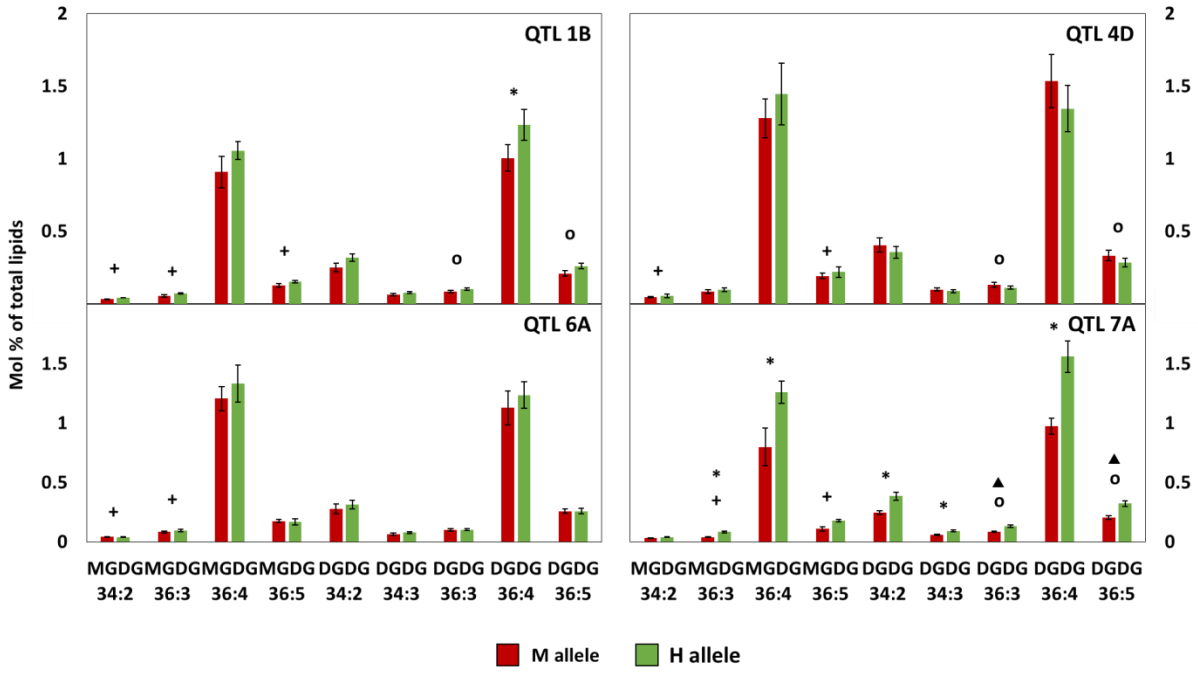


Figure 4.10 Galactolipids present on the four QTLs and their allelic combinations in year 1. Four species of MGDG (34:2, 36:3, 36:4 and 36:5) and DGDG (34:2, 34:3, 36:3, 36:4, 36:5) are displayed. Data are mean values of ±SE of five independent samples analysed via ESI-MS/MS.

Year 1

The galactolipids showed the highest number of independent allele effects among all of the lipids, with five species being affected by independent allelic effects. All of these significant independent allelic effects occurred between the alleles present on the QTL 7A. The affected lipids included MGDG 36:3, 36:4, DGDG 34:2, 34:3 and 36:4, with the Hereward allele being associated with higher contents of GLs compared with the Malacca allele.

On the other hand, some QTL x allele effects were observed for DGDG 36:3 and 36:5, where once again the only significant allele effects were observed for the 7A QTL. While care must be taken interpreting the QTL x allele effects, these were both strong allelic effects. Three significant independent QTL effects were observed for the MGDG species 34:2, 36:3 and 36:5, indicating that the effects on these lipids were regardless of the alleles.

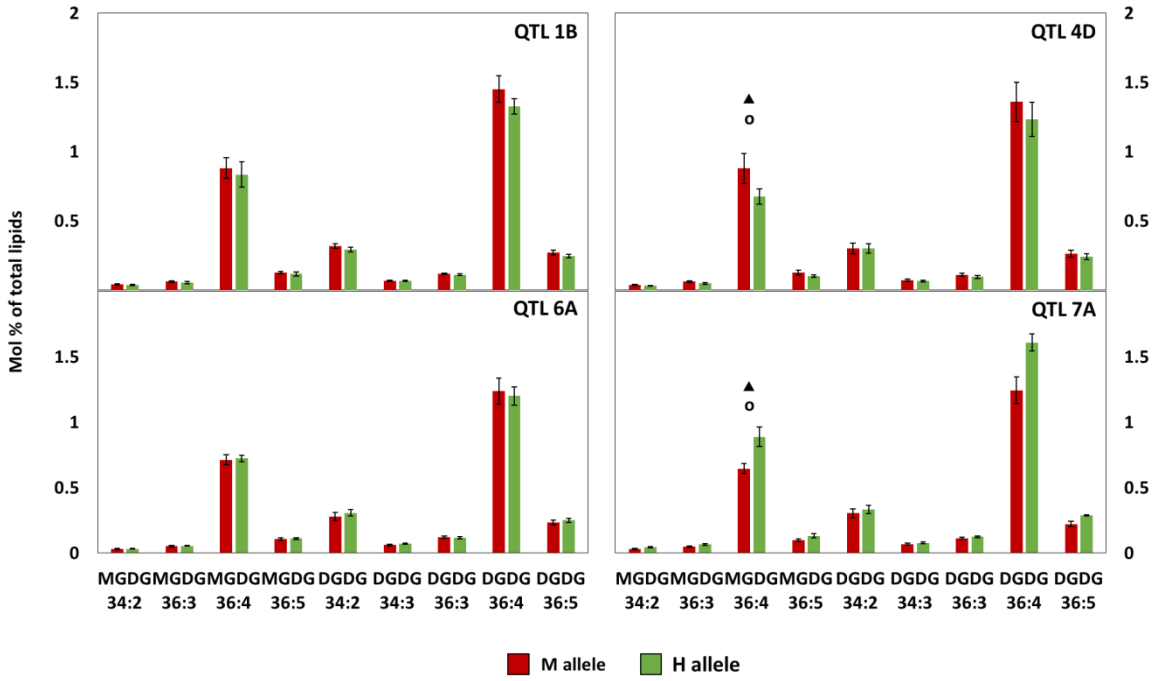


Figure 4.11 Galactolipids present on the four QTLs and their allelic combinations in year 2. Four species of MGDG (34:2, 36:3, 36:4 and 36:5) and DGDG (34:2, 34:3, 36:3, 36:4, 36:5) are displayed. Data are mean values of ±SE of five independent samples analysed via ESI-MS/MS.

Year 2

The second year material showed weaker effects than the first year. Only one GL specie; MGDG 36:4 showed any statistically significant differences. MGDG 36:4, the most abundant MGDG lipid specie, was significantly affected by QTL x allele effects at QTLs 4D and 7A. However, at QTL 4D the Malacca allele was associated with higher levels of lipid while the opposite was the case for QTL 7A. The effects of the QTL 7A and the Hereward allele at this QTL on MGDG 36:4 are consistent with the observations from year 1.Although not statistically significant, there was a difference between the levels of DGDG 36:4 between the Hereward and Malacca alleles on QTL 7A. While the results of these QTL x allele effects must be considered with caution, the allelic effects on QTL 4D and especially on QTL 7A were stronger than the QTL effects ($p < 0.05$, LSD) between the NIL sets.

Between the two years, the galactolipids showed the most significant allele effects, while showing the greatest consistency across the two years. Compared to the year 1 material, year 2 showed a significant effect on just one lipid (MGDG 36:4) and another potential candidate, DGDG 36:4. Yet the effects on lipids were consistent across the two years, and since the galactolipids affected were those present in the highest quantities, they are arguably the most important and interesting.

4.2.6 Effect of QTLs and alleles on NLs in Year 1 and Year 2

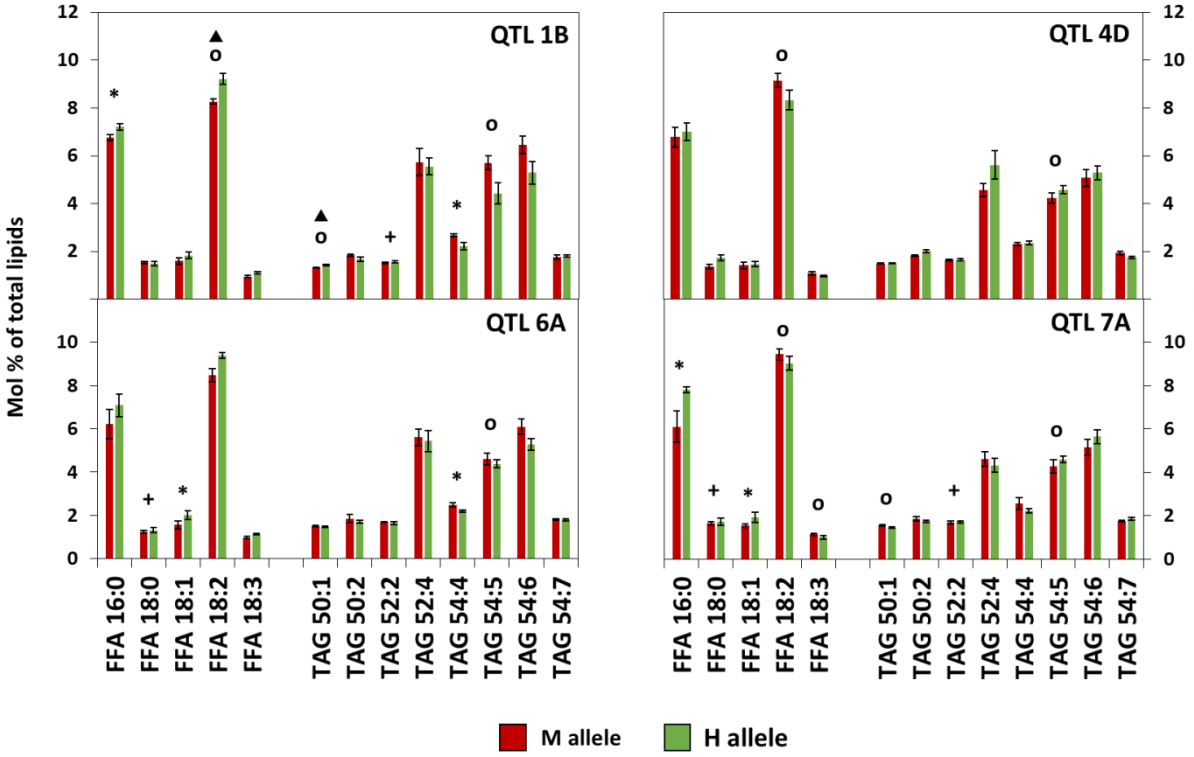


Figure 4.12 Comparison of selected major neutral lipid species across the four QTLs and their allelic combinations in year 1. Minor neural lipids can be found in the Appendix Figure 4.4a. Five species of FFA (16:0, 18:0, 18:1, 18:2, 18:3) and eight species of TAG (50:1, 50:2, 52:2, 52:4, 54:4, 54:5, 54:6 and 54:7) are displayed. Data are mean values of ±SE of five independent samples analysed via ESI-MS/MS.

Year 1

Of the 35 neutral lipid species identified in the first year material, 3 were affected by independent allelic effects, 21 by QTL x allele effects, and 6 by QTL effects alone. However only the major neutral lipid species that accounted for over 1% of the total lipids will be considered in this study. Data on the minor neutral lipids can be found in the appendix figure 4.4a.

The three lipid species affected by independent allelic effects were FFA 16:0, FFA 18:1 and TAG 54:4. The Hereward allele was associated with higher lipid content compared to the Malacca allele for FFA 16:0 and 18:1. On the other hand the content of TAG 54:4 was higher for the Malacca allele as opposed to the Hereward allele. Among the QTL x allele effects, only the alleles located at the QTL 1B locus showed significant differences. For the QTL 1B, the Hereward allele was associated with

lower levels of TAG 54:5, while the Hereward allele was associated with higher levels of TAG 50:1. Once again care must be taken when interpreting the QTL x allele data.

Year 2

In the second year dataset, there were fewer independent allelic effects or QTL x allele effects. Of the 34 TAG and FFA species observed in year 2, only FFA 18:2 and TAG 56:5 showed independent allele effects (appendix figure 4.4b). Only six molecular species showed QTL x allele effects while seven lipids showed independent effects of the QTL alone.

Among the major NLs, only FFA 18:2 showed independent allelic effects at QTL 4D with the Hereward allele being associated with higher lipid content. Only one QTL x allele effect was observed for FFA 18:1, with the Malacca allele being associated with higher lipid content.

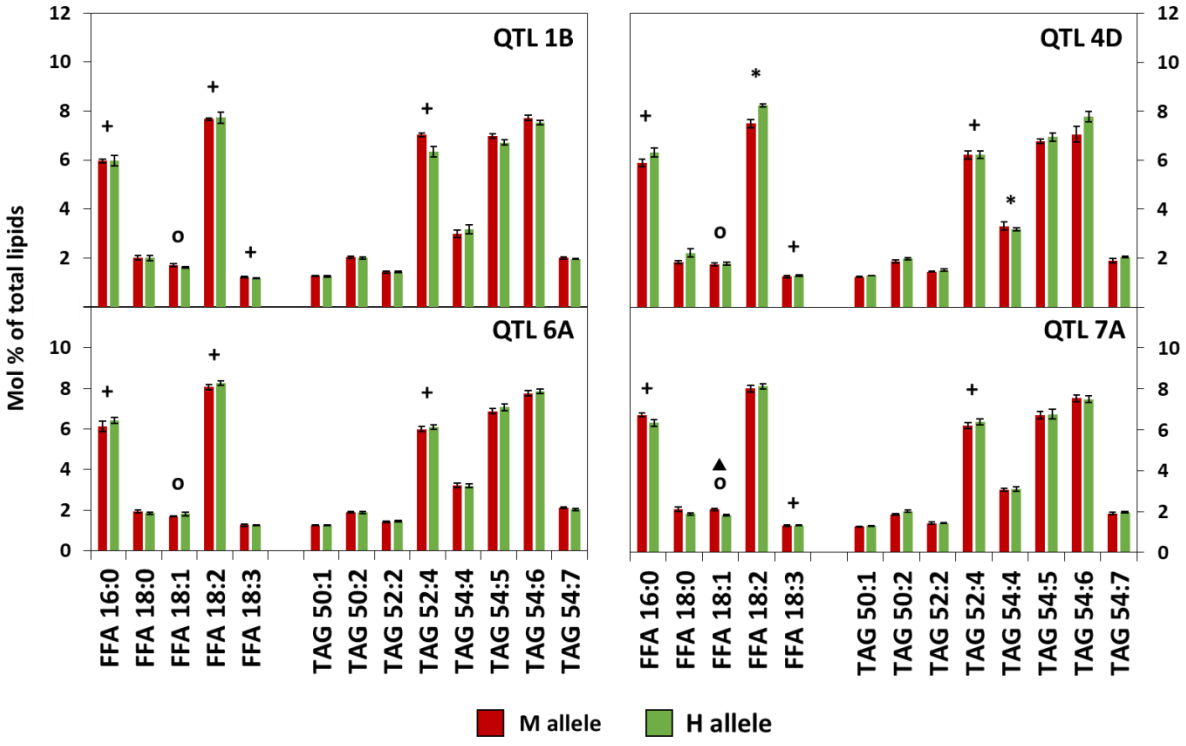


Figure 4.13 Comparison of major neutral lipid species across the four QTLs and their allelic combinations in year 2. Minor neural lipids can be found in the appendix Figure 4.4b. Five species of FFA (16:0, 18:0, 18:1, 18:2, 18:3) and eight species of TAG (50:1, 50:2, 52:2, 52:4, 54:4, 54:5, 54:6 and 54:7) are displayed. Data are mean values of ±SE of five independent samples analysed via ESI-MS/MS.

While DAG levels were analysed in the second year data, they were not included in this analysis because they were not studied in year 1, The DAG data for the second year are given in appendix figure 4.5.

Comparing the two years, the neutral lipids did not show many consistent differences. Allelic variation was observed mainly for major FFA species such as 18:2 and 16:0 and these effects were only observed in the second year**.**

4.2.7 Multivariate Statistical Analysis

CVA (Canonical Variates Analysis) was used to perform a linear discrimination between the allele x QTL combinations allowing low dimensional representation of the differences in two dimensional plots. This allowed tentative significant differences to be assigned by non-overlapping 95% confidence circles around the means of canonical variate scores per treatment combination, making the assumption of a multivariate normal distribution for the data. The magnitudes of the CV loadings on the percentage lipid variables are given in the appendix figure 4.6. These provide information on the relative importance of the original lipids in the discrimination observed. Multivariate analysis offers a useful tool to visualize the differences between the NILs in this study, and CVA maximises the discrimination in the dataset. Furthermore, the small differences that may have escaped a CVA analysis of the full data could be overcome by analysing the three lipid classes separately.

Also due to the nature of the NILs, CVA provides a way to observe whether the separation of the NIL sets is due to the presence of the Hereward or Malacca allele, or to other significant non-genetic effects. As the NILs were developed by backcrossing to the Malacca parent (Figure 4.1), the NILs possessing the Malacca alleles should not separate, while the Hereward alleles should separate from the Malacca alleles. While it is unlikely that a perfect representation of this separation will be observed due to the nature of lipid data, it should be noted that significant separation of the lines with the Malacca alleles should not be observed if strong allelic effects are present.

4.2.8 CVA for full lipid datasets in Years 1 & 2

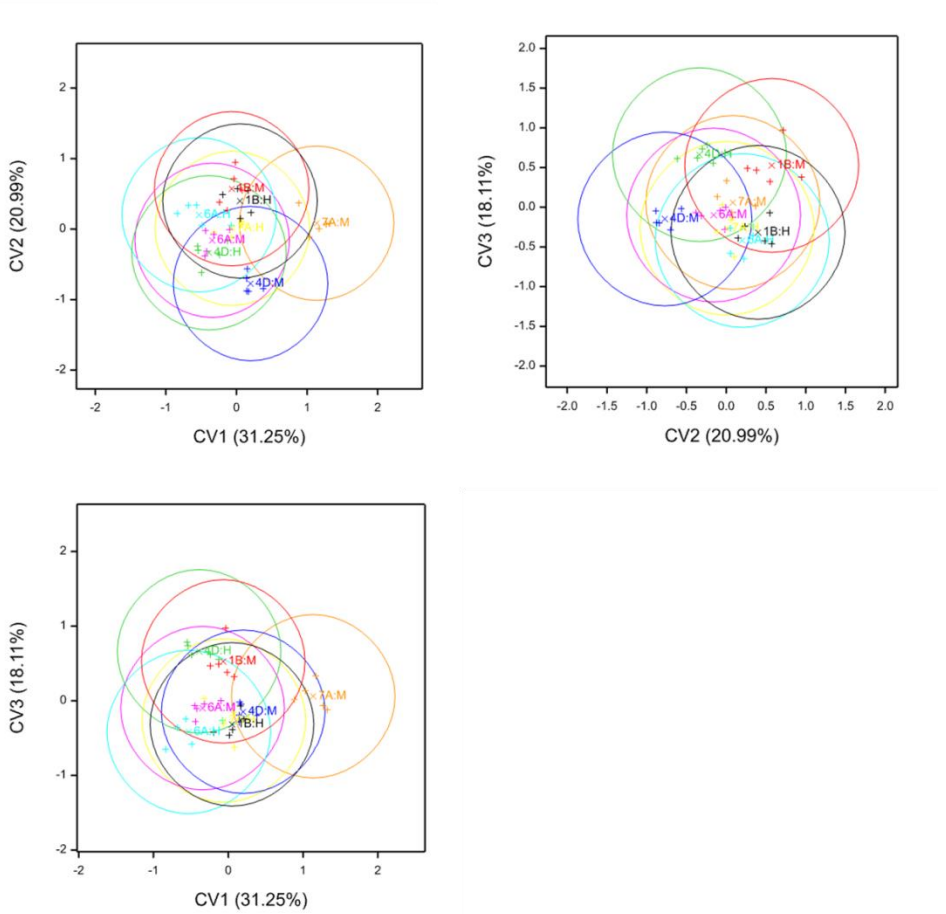


Figure 4.14 Canonical Variates Analysis (CVA) for the four QTL and their allelic combinations for the full lipid dataset in year 1. Included are QTL 1B, 4D, 6A and 7A with the alleles, M (Malacca) and H (Hereward). CV scores (**o**) and means of scores (**x**) in the three dimensions are plotted. Although the circles indicate a 95% confidence interval, it should be borne in mind that the five replications were technical rather than biological in nature.

In the first year material, the first three CVs accounted for 70.35% of the variation and possible discrimination, so it was sufficient to retain these and consider the CV scores in these dimensions. The CVA plots showed little statistical differences between the eight combinations of QTL and alleles. However, there was a possible separation of the 7A:M combination compared to the other seven combinations, as well as some potential differences for the 4D:M. The outputs of the CVA analysis are given in appendix figure 4.6a.

Similar observations were made for the second year material. In the second year the first three CVs accounted for 79.06% of the variation and possible discrimination in the dataset. The CVA plot

showed little statistical differences between the eight QTL and allelic combinations (Figure 4.14). However, some separation of the 4D:M NIL combination, consistent with the year 1 dataset was observed. While the slight separation of the Malacca NILs indicates lacking genetic effects, this may also be indicative of the lack of lipid variation in the total lipid profile.

The 1B:M and 1B:H combinations were both partially separated from the other six combinations. The output for the second year overall CV loading scores are given in appendix figure 4.6b.

The lack of variation shown by the multivariate lipid data may be due to greater variation in certain lipid classes such as the major neutral lipids compared to the minor phospholipids. This results in poorer overall discrimination when the full data are considered together. Therefore multivariate CVA analysis was performed on specific lipid classes, the PLs, GLs and NLs.

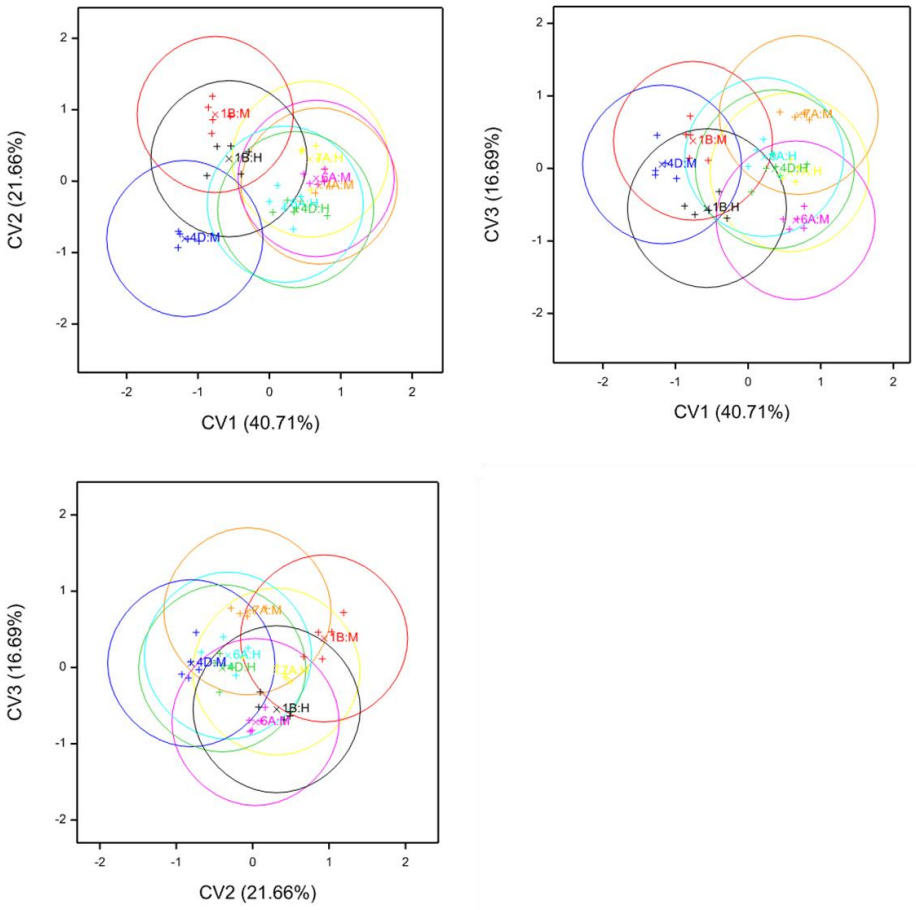


Figure 4.15 CVA analyses for the four QTL and their allelic combinations for the full lipid dataset in year 2. Included are QTL 1B, 4D, 6A and 7A with the alleles, M (Malacca) and H (Hereward). CV scores (**o**) and means of scores (**x**) in the three dimensions are plotted.

4.2.9 CVA for phospholipids

Year 1 PL CVA analysis

The first two CVs accounted for 65.96% of the variation and possible discrimination, so the third CV accounting for another 14.41% was retained, with the first three CVs accounting for 80.37% of the total variation. All outputs of the CVA analysis for specific lipid classes, with loading vector scores are given in appendix figure 4.7a. The lipids corresponding to the largest CV loadings by magnitude are important in the separation of the particular CV direction in the plot. Therefore lipids with loading values down to half of the highest loadings in the particular CVs are included.

While large separation could be seen between the NIL sets, there was no clustering of the Malacca allele. However, the CV3 dimension provided some separation of the Hereward alleles, especially on QTL 7A. Overall, the high level of separation observed in the CVA analysis with no clustering of the Malacca allele is indicative of a lack of allelic effects on the phospholipids.

Year 2 PL CVA analysis

In the second year dataset the first three CVs accounted for 90.13% of the variation and possible discrimination. The loading vector information is given in Table 4.3.

Once again the second year CVA analysis showed large separation between the NIL sets, which was greater than what was observed in year 1. However once again there was a lack of separation for the Malacca allele. While separation between alleles of the QTL 6A and 7A could be observed in the CV1 direction, and the alleles of QTL 1B and 4D in the CV2 direction, this variation in the lipid dataset cannot be associated with genetic effects.

Overall across the two years, there was no clustering of the Malacca alleles, which is surprising since the NILs were generated by backcrossing with the same Malacca parent. This suggests that although the individual NIL classes were well separated, this separation is unlikely to have resulted from direct effects of the alleles, and more likely to have resulted from differences in the backgrounds of the NILs or non-genetic effects.

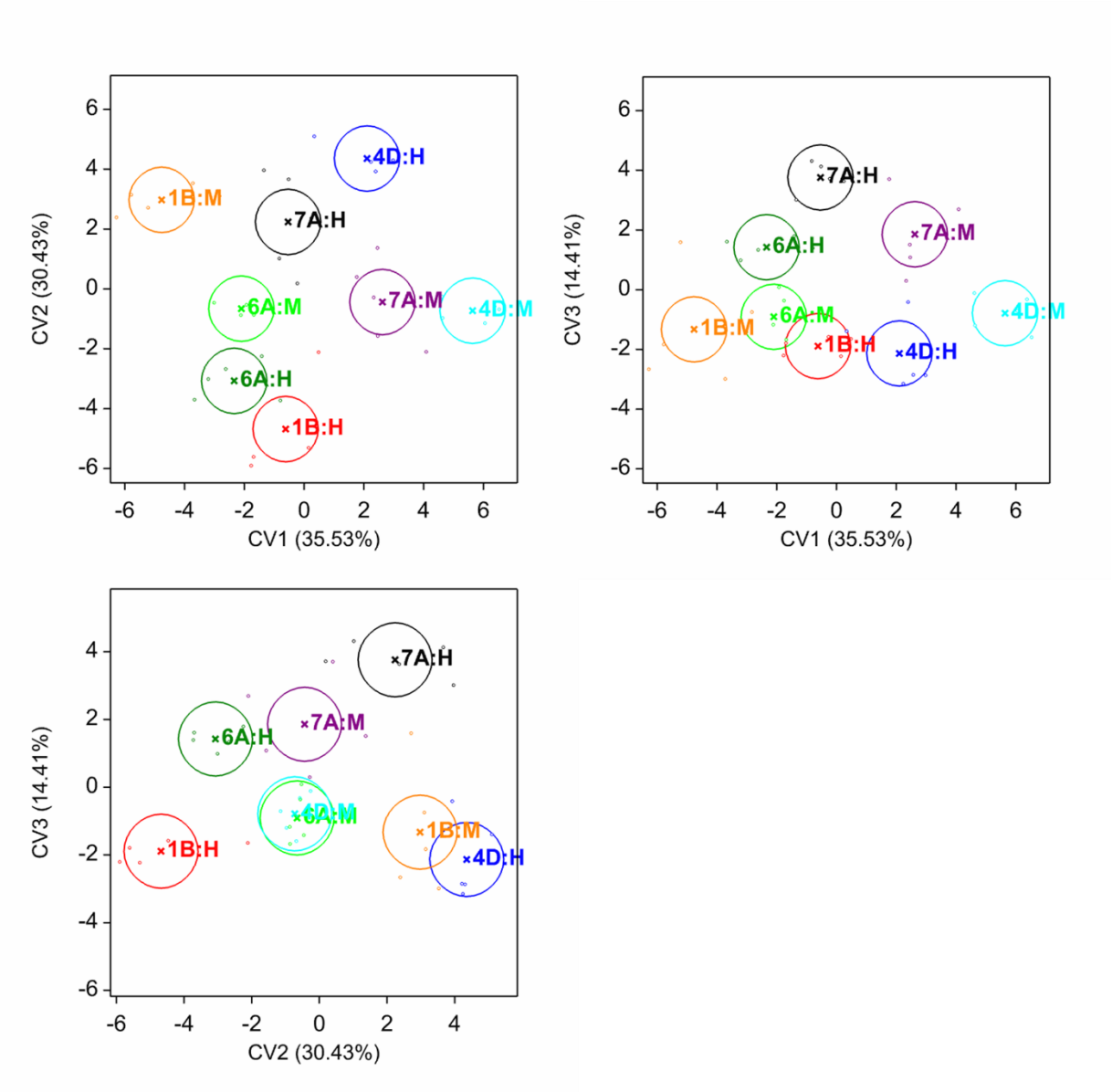


Figure 4.16 CVA analyses for the four QTL and their allelic combinations for phospholipids in year 1. Included are QTL 1B, 4D, 6A and 7A with the alleles, M (Malacca) and H (Hereward). CV scores (**o**) and means of scores (**x**) in the three dimensions are plotted.

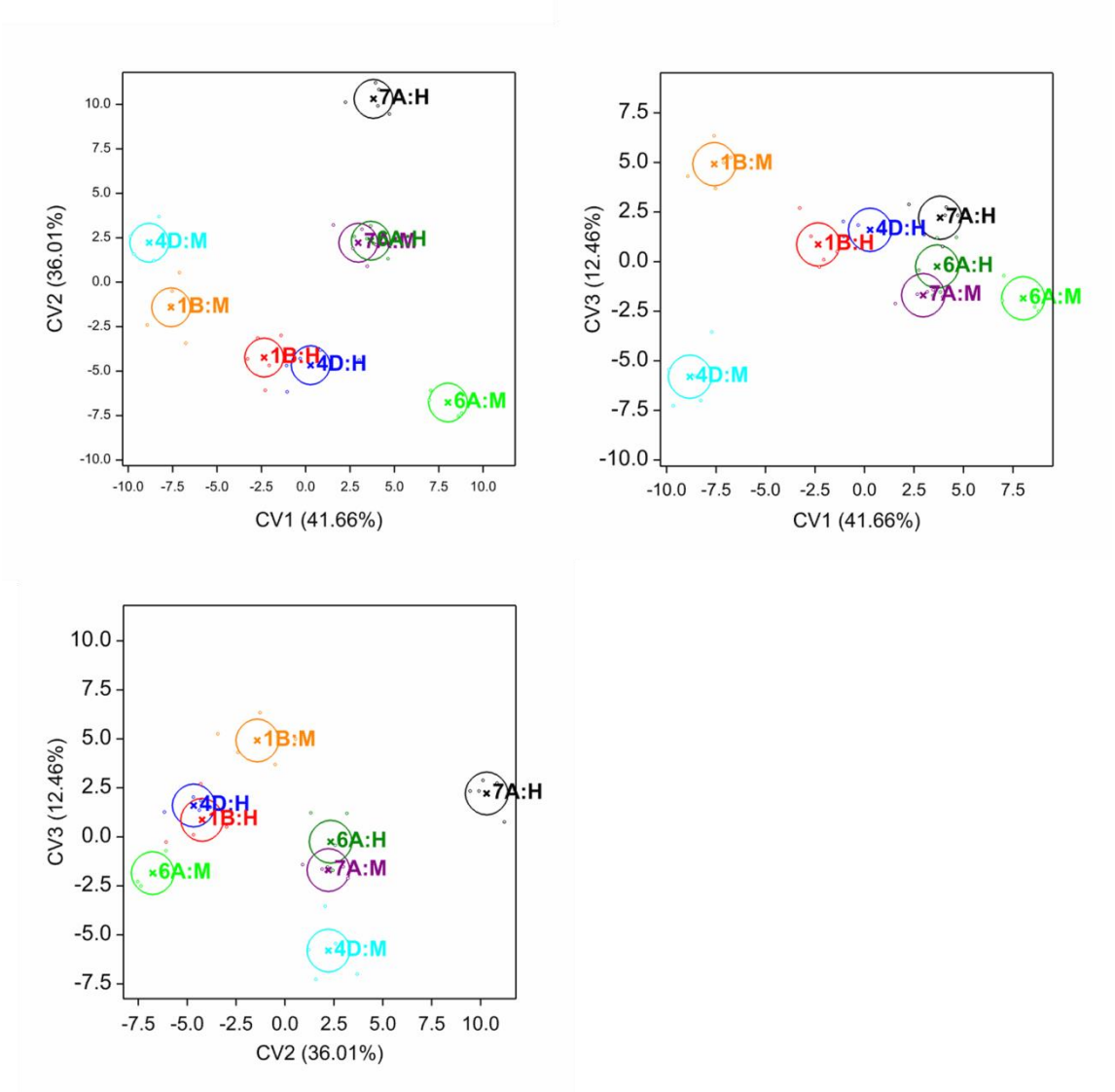


Figure 4.17 CVA analyses for the four QTL and their allelic combinations for phospholipids in year 2. Included are QTL 1B, 4D, 6A and 7A with the alleles, M (Malacca) and H (Hereward). CV scores (**o**) and means of scores (**x**) in the three dimensions are plotted.

4.2.10 CVA for Galactolipids

In the first year material, the first two CVs accounted for 66.74% of the variation and possible discrimination. Therefore a third CV was added, capturing a further 15.80% of the variation. These three CVs together accounted for 82.54% of the total variation.

	CV1	CV2	CV3
% variation	36.07	30.67	15.80
MGDG 34:2	1.759	0.713	-3.528
MGDG 36:3	2.826	3.864	0.316
MGDG 36:4	**6.054**	-1.923	3.878
MGDG 36:5	**-8.535**	0.677	-2.414
DGDG 34:2	**-12.104**	*-7.205*	2.501
DGDG 34:3	**9.351**	*6.214*	-10.156
DGDG 36:3	-0.905	-3.506	4.25
DGDG 36:4	**6.633**	2.941	5.401
DGDG 36:5	**-7.197**	1.302	0.022

Table 4.2 Loading vectors for the three CV dimensions for the CVA analysis of GLs in year 1. The lipids considered important in the separation seen in the CV1 direction are marked in bold, the CV2 direction is in italics and CV3 is underlined.

The CVA plot for galactolipids separated the sets of alleles at QTL 6A and 7A with 7A:M being mainly separated in the CV2 direction and 6A:H in the CV1 direction. The other NIL sets showed little differences, as shown by the overlapping confidence interval circles.

In the second year material, the first three CVs accounted for 87.19% of the variation and possible discrimination in the dataset.

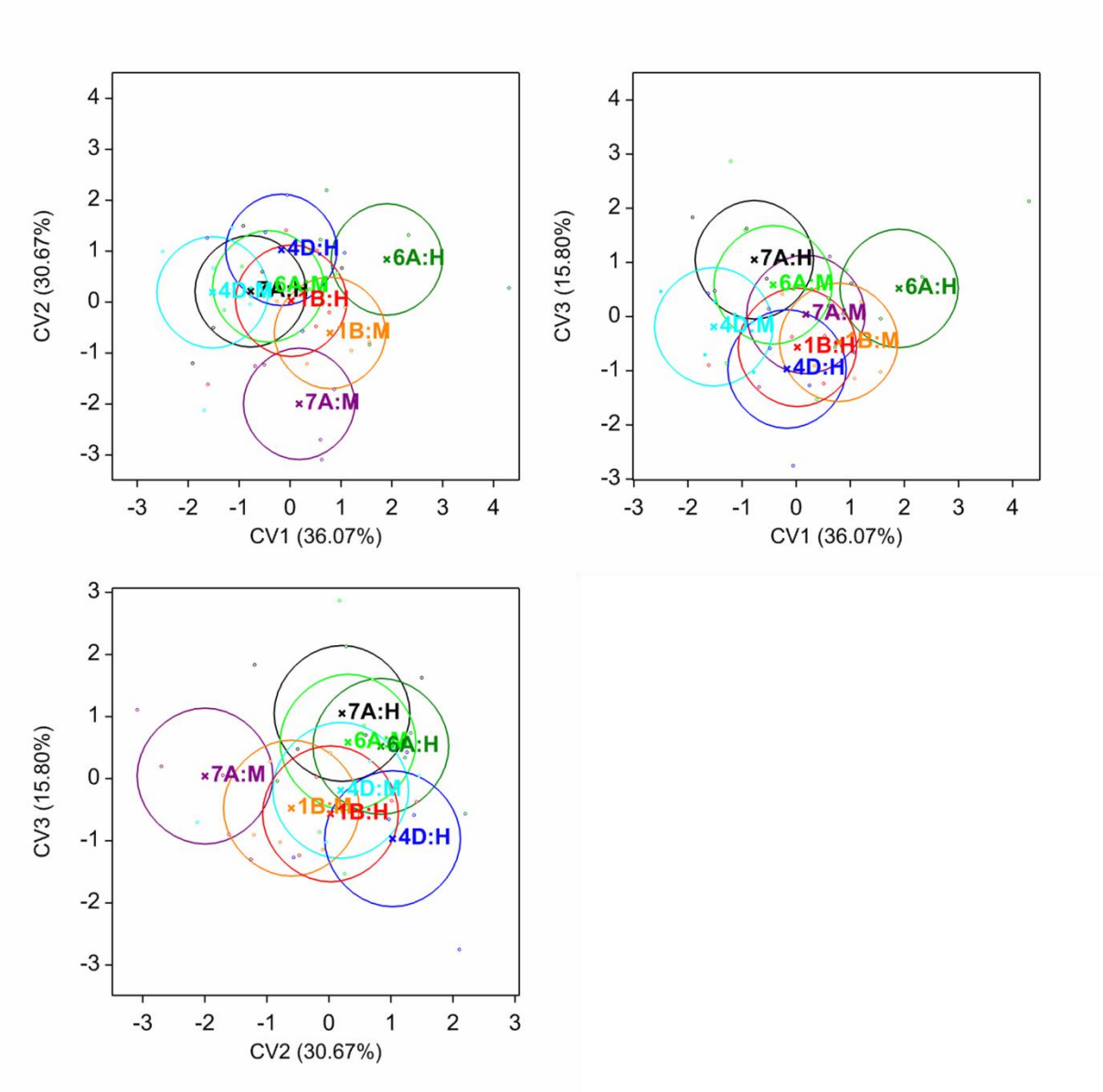


Figure 4.18 CVA analyses for the four QTL and their allelic combinations for galactolipids in year 1. Included are QTL 1B, 4D, 6A and 7A with the alleles, M (Malacca) and H (Hereward). CV scores (**o**) and means of scores (**x**) in the three dimensions are plotted

In the second year material, the first three CVs accounted for 87.19% of the variation and possible discrimination in the dataset.

As observed with the first year material, analysis of the second year data showed a lack of separation in the CVA dataset, with only two possible separations. The 4D:H allele was separated out mainly in the CV3 direction while the 7A:M was separated out mostly in the CV2 direction.

	CV1	CV2	CV3
% variation	41.00%	32.17%	14.02%
MGDG 34:2	1.825	0.978	-2.451
MGDG 36:3	-1.515	-0.56	-1.54
MGDG 36:4	**7.516**	0.241	1.213
MGDG 36:5	-4.383	-1.104	3.945
DGDG 34:2	-1.699	-3.899	-8.762
DGDG 34:3	0.808	2.869	7.064
DGDG 36:3	-3.307	-2.893	6.568
DGDG 36:4	**5.366**	*-14.321*	-5.013
DGDG 36:5	1.358	*18.171*	2.521

Table 4.3 Loading vectors for the three CV dimensions for the CVA analysis of GLs in year 2. The lipids considered important in the separation seen in the CV1 direction are marked in bold, the CV2 direction is in italics and CV3 is underlined.

When two year data was combined, only one consistent separation was observed. The slight separation of 7A:M from the other NIL sets in the CV2 direction. However, as discussed above, the separation of the Malacca alleles suggests that the separation of 7A:M was not due to the effects of alleles but due to non-genetic effects such as storage or instrument variation.

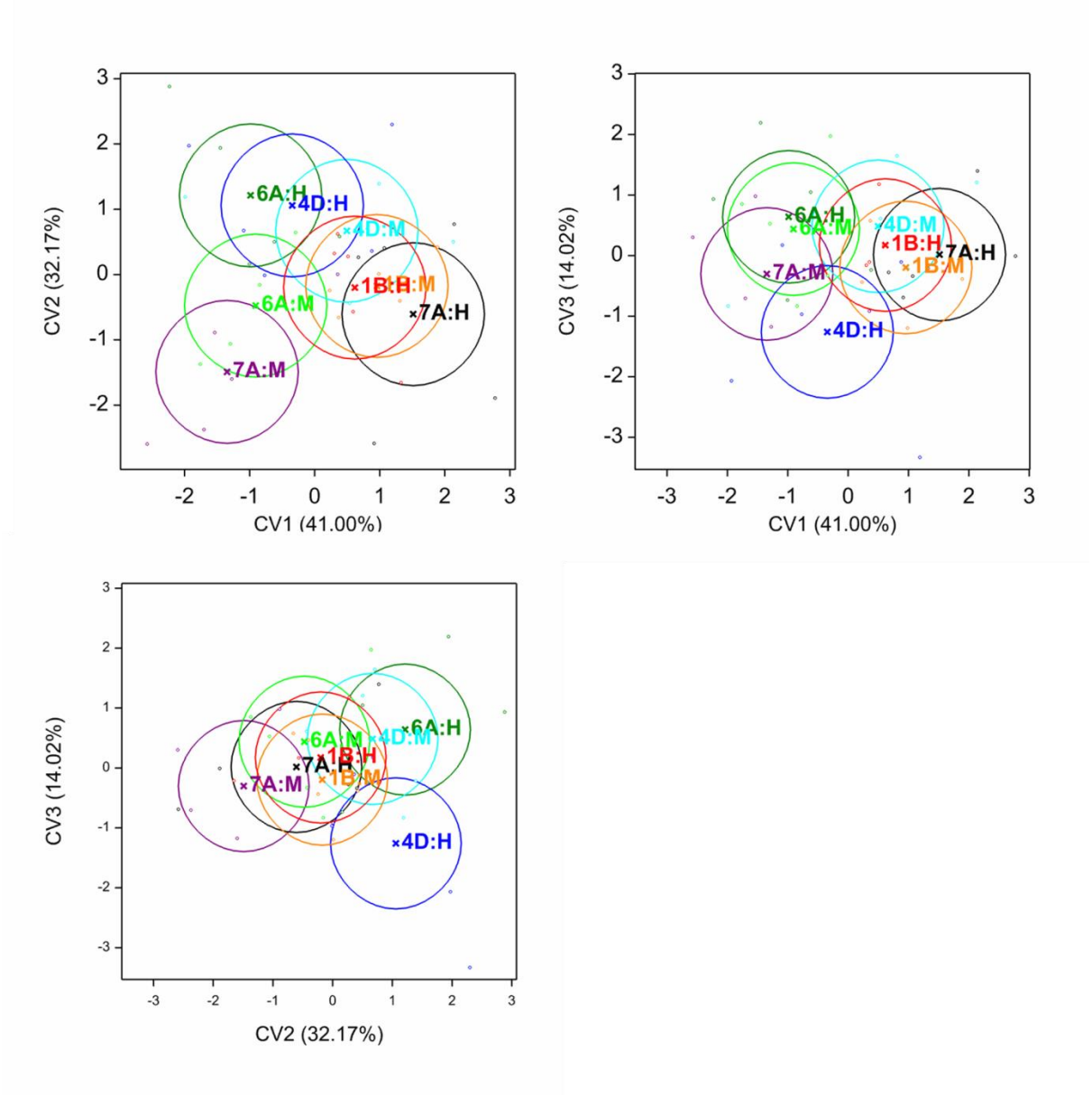


Figure 4.19 CVA analyses for the four QTL and their allelic combinations for galactolipids in year 2. Included are QTL 1B, 4D, 6A and 7A with the alleles, M (Malacca) and H (Hereward). CV scores (**o**) and means of scores (**x**) in the three dimensions are plotted

4.2.11 CVA on Neutral lipids

For the first year material, the first three CVs for the neutral lipids accounted for 92.17% of the variation and possible discrimination, so it was sufficient to retain the three.

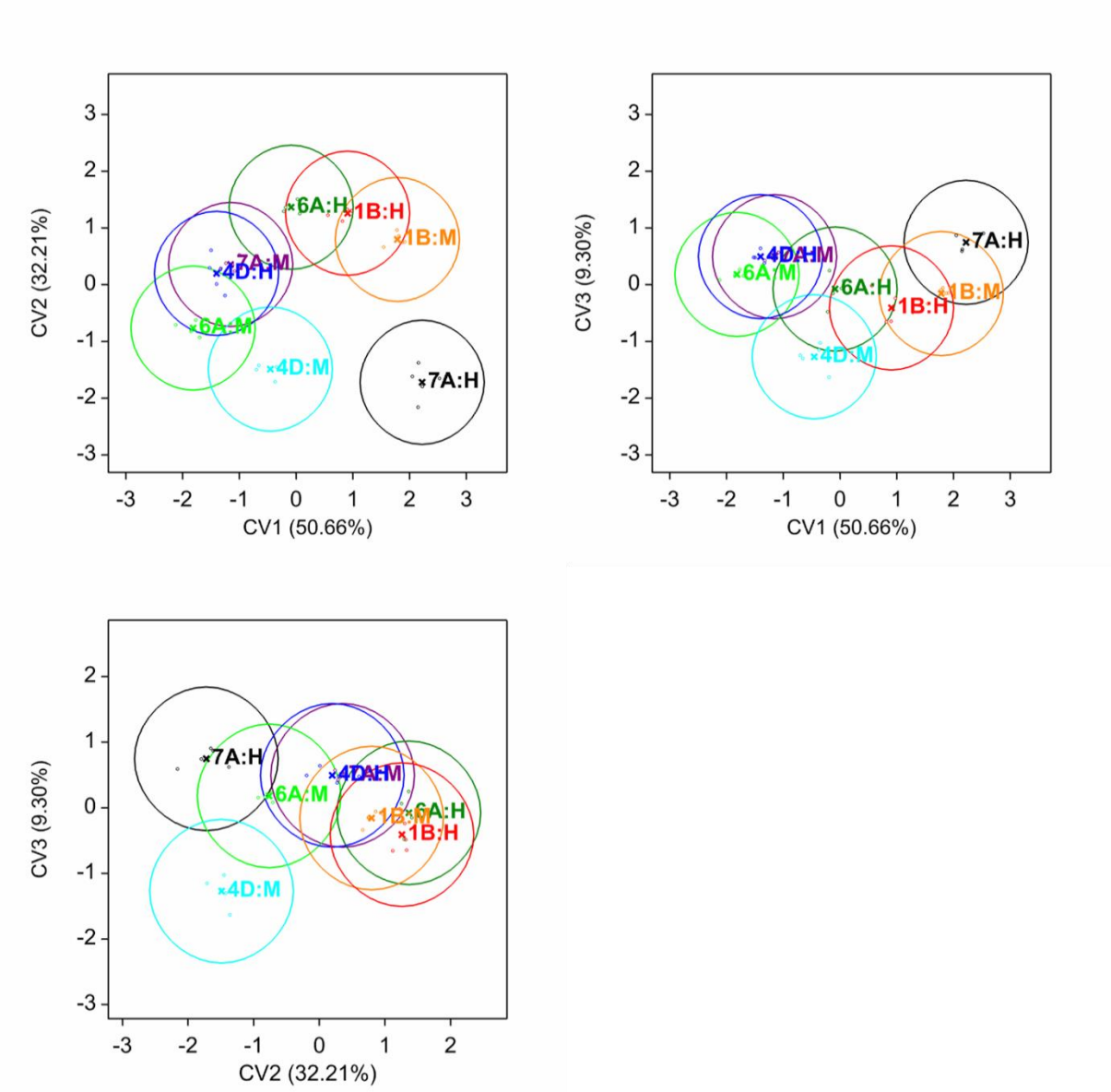


Figure 4.20 CVA analyses for the four QTL and their allelic combinations for neutral lipids in year 1. Included are QTL 1B, 4D, 6A and 7A with the alleles, M (Malacca) and H (Hereward). CV scores (**o**) and means of scores (**x**) in the three dimensions are plotted

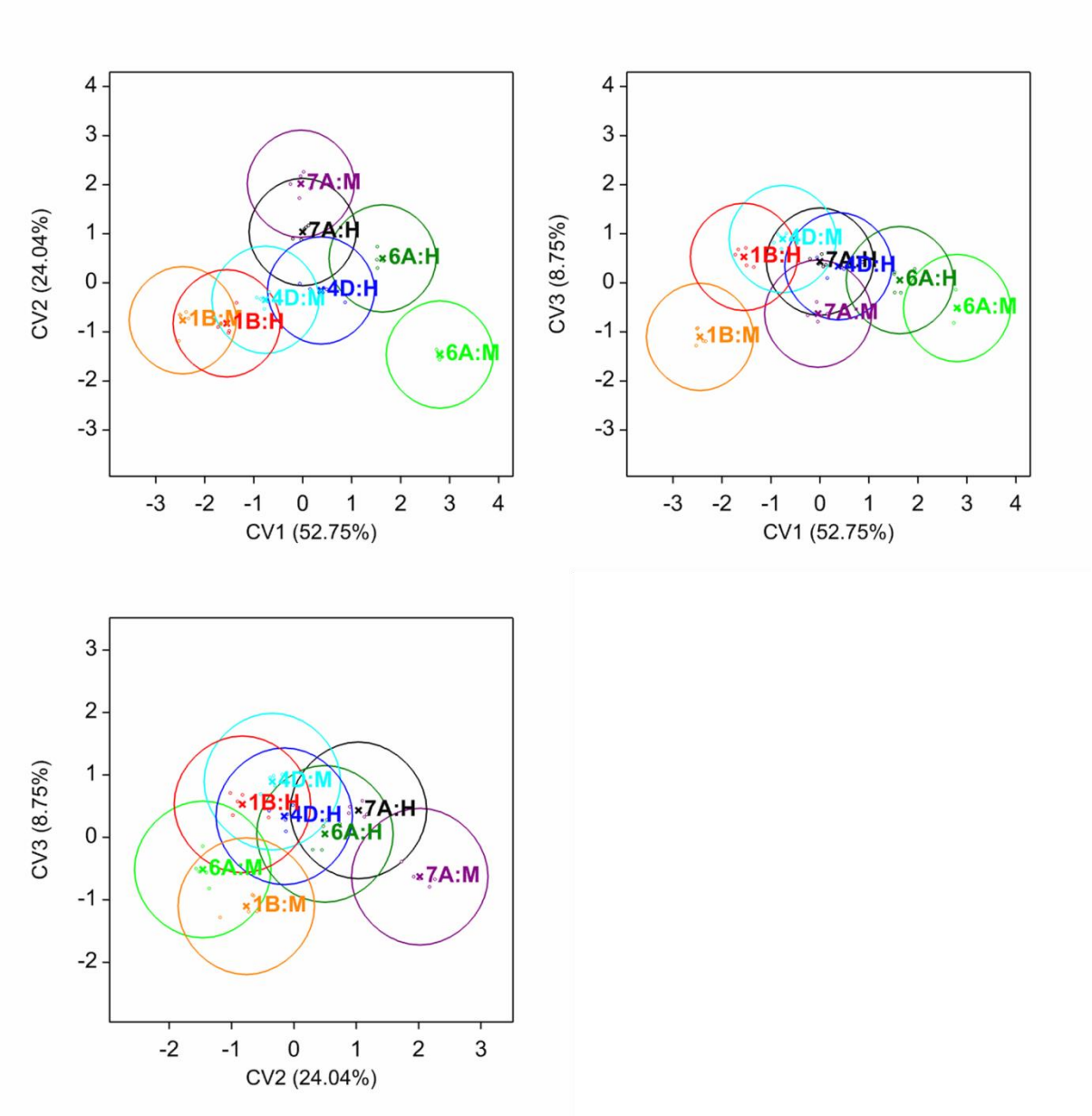


Figure 4.21 CVA analyses for the four QTL and their allelic combinations for neutral lipids in year 2. Included are QTL 1B, 4D, 6A and 7A with the alleles, M (Malacca) and H (Hereward). CV scores (**o**) and means of scores (**x**) in the three dimensions are plotted

In the first year material, the 7A:H allele was separated in the CV1 direction. However, the Malacca allele at QTL 4D was also separated indicating the lack of a true allelic effect.

The second year data showed separation of the Malacca alleles at QTL 7A and 6A. While the 7A:H allele was clearly separated in the first year, this was not the case in the second year where the Malacca allele showed greater separation.

Overall, across the two years, there was a lack of consistency in the results, with some separation of the Hereward alleles and a poor clustering of the Malacca alleles. While these differences between the two years may result from environmental factors, they may also reflect the effects of the neutral lipid DAG which was only measured in the second year. However, the contribution of DAG in year 2 did not result in increased separation of the QTL and allele combinations, apart from separation of the 6A Malacca allele in year 2 only. Overall the lack of consistency and the greater separation of the Malacca alleles indicate the lack of allelic effects on the neutral lipids across the two years.

CVA analysis of the three lipid classes across the two years showed some separation of the QTLs but not between the alleles at each QTL. As discussed above, since the NILs are produced by backcrossing to the Malacca parent, the CVA would be expected to show a cluster of overlapping points for the Malacca alleles, with the Hereward alleles being clearly separated from this cluster. The failure to observe this pattern indicates that the differences observed did not relate to allelic variation at the QTLs, but to differences in the genetic backgrounds of the sets of NILs or non-genetic effects.

4.2.12 Effect of year x QTL x allelic effects

In order to directly compare the effects of each year on the lipid composition of the NILs, CVA analysis was performed. In order to account for the heterogeneity of variance in the dataset, a logit (to the base *e*) transformation was applied. The transformation was $Z = \log(P/(100 - P))$, where P is the percentage value. The back-transformation is therefore $100*\exp(Z)/(1 + \exp(Z))$.

The use of CVA allowed discrimination between the combinations of year and alleles as well as QTL to be made in terms of new dimensions formed as a linear combination of the logit values of lipids. Therefore once again in two dimensional plots, the overall tentative significant biological, if not statistical, differences between the year x QTL x allele combinations could be assigned by non-overlapping 95% confidence circles, around the means of canonical variate (CV) scores in the retained CV dimensions. The analysis was done with the assumption of a multivariate normal distribution for the data, a reasonable assumption under the logit transformation.

Analysis was carried out on the 55 lipid species which were consistently determined in both years, which included 18 phospholipids (LPC, PI, PC and PE), 9 galactolipids (MGDG, DGDG) and 28 neutral lipids (FFA and TAG). CVA analysis was performed on the full lipid dataset as well as on the three lipid classes separately.

4.2.13 CVA of the full dataset over the two years

The first two CVs accounted for 91.53% of the variation and possible discrimination in the data, and it was sufficient to retain just these two.

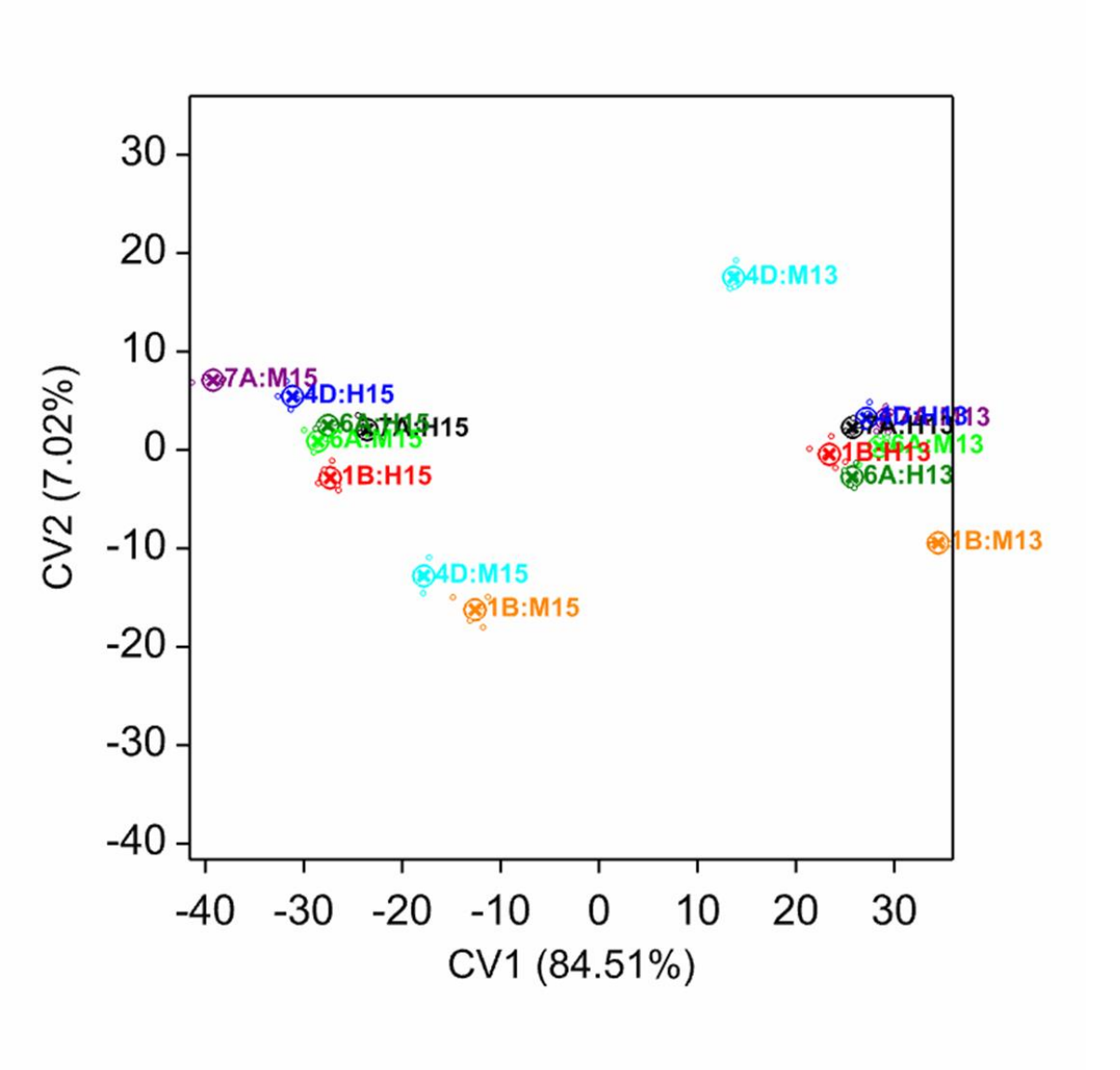


Figure 4.22 CVA analyses for the four QTL and their allelic combinations for the full lipid dataset across the two years. Included are QTL 1B, 4D, 6A and 7A with the alleles, M (Malacca) and H (Hereward). CV scores (**o**) and means of scores (**x**) in the three dimensions are plotted. 13 indicates 2013 the year 1 material, 15 indicates the 2015 year 2 material.

The CV dimensions show that CV1 (which accounts for 84.51% of the variance in the dataset) is responsible for the separation of the two years. CV2 on the other hand is responsible for the separation of the QTL x allele effects within those years.

This CVA analysis of the QTL x allele x year effects offers a good overview of the entire dataset. While there is some separation of the individual NIL sets, the differences are much smaller than the year differences which accounted for 84.51% of the variation in the dataset.

Greater variation was observed in the 2015 dataset compared to the 2013 dataset. When the datasets were combined the only separations observed were of the Malacca allele of the 1B and 4D QTLs. However, as discussed above, the Malacca alleles would be expected to cluster together while the Hereward alleles would be separated based on allelic effects. The consistent separation of the 1B and 4D Malacca alleles across the two years therefore indicates the lipid composition was not affected by allelic variation at the four QTLs.

In conclusion, this analysis indicates that across the full dataset, the overall lipid profile is mostly affected by year effects with no clear evidence for QTL or allelic effects.

To avoid the effects of minor components being masked by those of major lipids, CVA analysis was carried out on the PLs, NLs and GLs separately. Similar observations could be made from the CVA analysis of the PLs as well as the NLs across the two years where the year effects accounted for over 80% of the variation in the data. The figures for these can be found in appendix figures 4.9a and 4.9b.

However the CVA analysis for galactolipids revealed some interesting observations (Figure 4.23). The first three CVs accounted for 78.11% of the variation and possible discrimination, making it sufficient to retain these three. The main CVA plot shows separation by year in CV1 and separation by QTL and alleles in CV2. However, the effect of year accounted for a significantly lower proportion of the total variation. For example, CV1 accounted for 50.81% of the total variation as opposed to the 84.51% seen in figure 4.21.

The significantly lower year by year effects allowed separation of the Hereward alleles at the 7A and 6A QTLs to be observed. However the separations were weak and smaller than the year effects. Furthermore, the observation was not consistent with the separation observed in the CVA of the galactolipids in the individual years where neither 7A:H or 6A:H showed significant separation.

Despite this, the galactolipids are interesting as they display significantly lower year effects while showing a greater separation of the Hereward allele as opposed to the Malacca alleles seen elsewhere in the analysis. This possibly indicates stronger genetic control of GLs compared to other lipid classes.

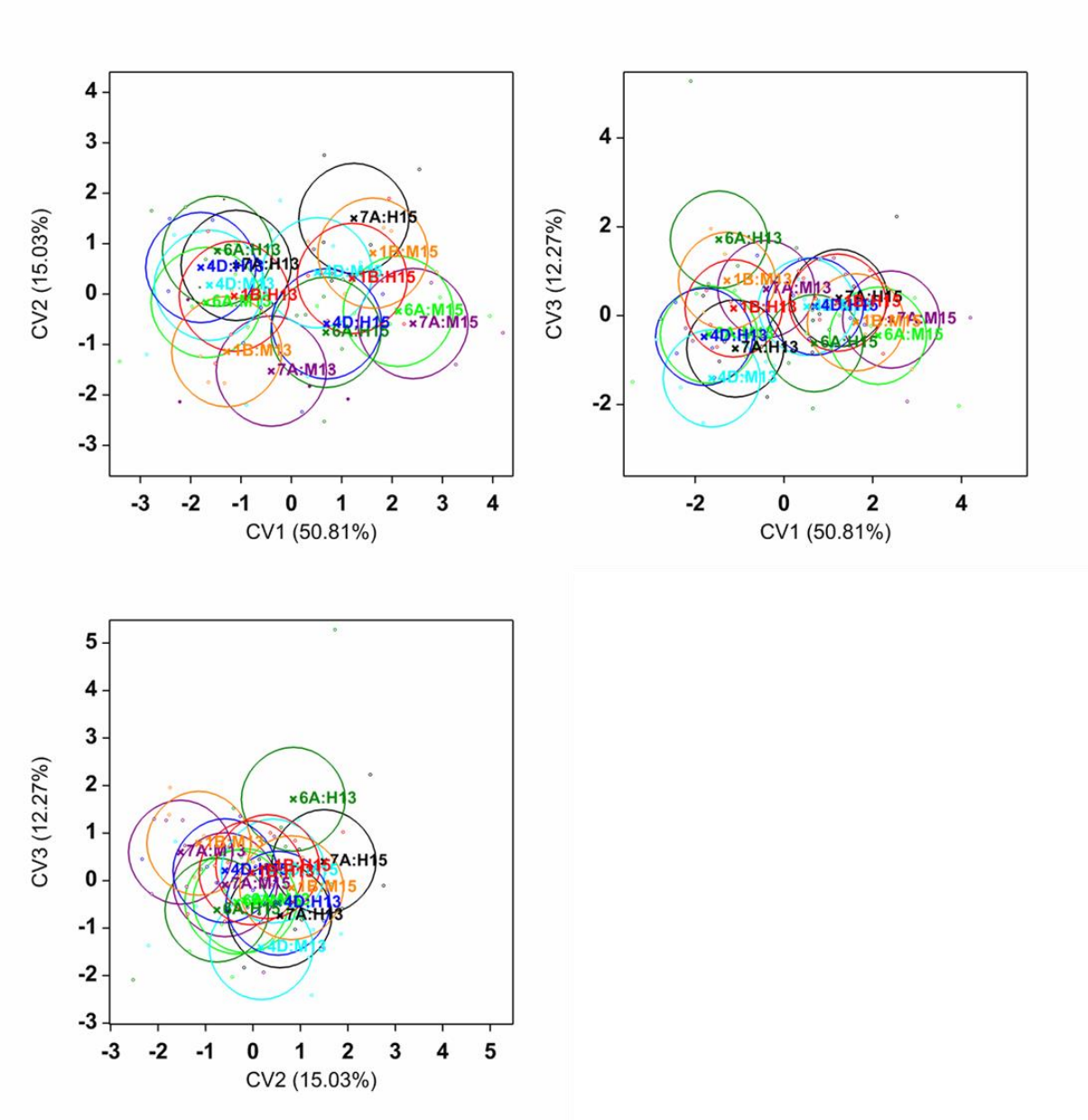


Figure 4.23 CVA analyses for the four QTL and their allelic combinations for the GL dataset across the two years. Included are QTL 1B, 4D, 6A and 7A with the alleles, M (Malacca) and H (Hereward). CV scores (**o**) and means of scores (**x**) in the three dimensions are plotted. 13 indicates 2013, year 1 material, 15 indicates the 2015 year 2 material.

4.2.14 Procrustes Analysis of the full dataset

CVA analysis provided an ordination for both the year 1 and year 2 data, as applied to the logit of the percentage scale data, and saving the canonical variate means of scores in the (three) retained dimensions. Two matrices where each of the 8 rows (indicating the QTL x allele combinations) and 3 columns (The retained three CV dimensions), were used as the input ordinations for Procrustes Analysis (PA).

PA analysis illustrates how the combinations differ relative to each other between the two years. The analysis takes the ordination for the first year, fixes it, and stretches, shrinks and rotates (as permitted) the ordination of the second year to give as good a fit to the fixed orientation as possible, using the sum of squared deviations between the ordinations as the minimisation criterion.

Therefore, while CVA allows us to observe the separation of the lipid data by year x QTL and alleles, Procrustes offers a useful tool to observe the consistency of the lipid data across the two years, as well as providing some indication of separation within the dataset.

The first two dimensions of the consensus configuration between the two years accounted for 70.17% of the total variation, making it sufficient to plot PA1 vs PA2 to tally up the two ordinations (Year 1 and Year 2). In this case PA3 would take up the remaining 29.83% of the variation as this Procrustes analysis used three retained CV dimensions.

The Procrustes analysis of the individual year and combined datasets displayed clear separation of the Malacca alleles at QTL 1B, 4D and 7A. This is again inconsistent with the predicted effects of allelic control, which would be expected to show separation of the Hereward rather than the Malacca alleles.

The length of the lines between the two ordination points for year 1 and year 2 for the QTL x allele combinations indicates the consistency of the data across the two years. Among the NIL sets, the greatest consistency was seen for the lipid profiles of the NILs with the Malacca alleles compared to the Hereward alleles. Therefore, the data possibly shows that the variation observed in the dataset was affected mostly by environmental and non-genetic effects.

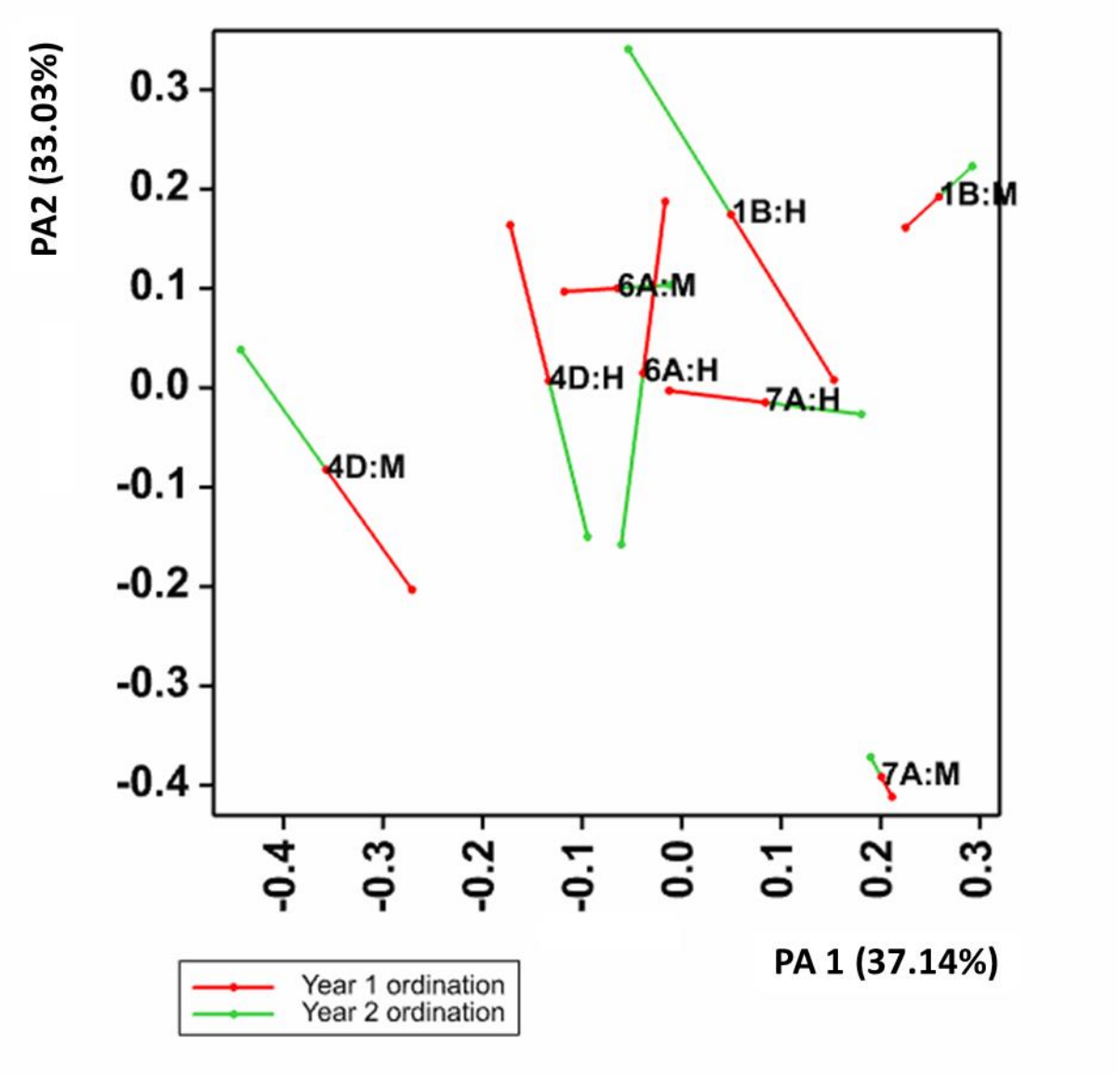


Figure 4.24 Procrustes Analysis for the full lipid dataset across the two years. The four QTLs, 1B, 4D, 6A and 7A are indicated as well as their allelic combinations. The points in red and green at the end of each line indicates the year 1 and year 2 ordination points for the QTL and allele combinations. The point in red at the centre of each line indicates the consensus point between the two ordinations.

4.3 Discussion

4.3.1 Degradation of PC-LPC

Compared to the other lipid classes, the difference between the levels of LPC and PC between the two years was particularly noticeable. In chapter three, analyses of the lipid species in cultivars identified significantly higher levels of LPC compared to literature sources. The proportion of LPC determined in the first year of study (25%) was about the same as that observed from the comparison of cultivars reported in Chapter 3 (27%).

However, the LPC levels observed in the second year data were more similar to that in literature. Other studies showed similar ratios of LPC: PC (Hargin & Morrison, 1980; Finnie et al., 2009). The high levels of LPC observed in year 1 may have resulted from the partial hydrolysis of PC by phospholipase A2 during storage. This may explain the varying ratio of LPC: PC observed across the two years in this study. When the proportions of LPC and PC across the two years are combined (Figure 4.4), the combined content was found to be consistent across the years. Hence, although other factors may be involved, it is reasonable to expect that the increased level of LPC is derived from the degradation and subsequent decrease in PC content.

The material from the 2 years differed in the length of time it was stored before analysis. While the second year material was milled and analysed within a month of milling, the first year material was milled and the flour stored at -20˚C for over a year before analysis. Although a separate experiment showed that no significant increase in LPC levels in white flour occurred during storage for three months at -20˚C conditions (author's unpublished results), longer storage periods were not investigated.

4.3.2 Impact of QTL x allele effects on the PL composition of the NIL population

For the vast majority of analysed lipids, there were no significant effects of alleles. However, some differences were observed for minor phospholipids as well as specific galactolipids which, as discussed in Chapter 1, may be relevant to breadmaking performance.

Among the phospholipid classes, only PI showed consistently significant effects across the two years. Among the QTL x allelic combinations, the 1B QTL showed the greatest differences between the levels of PI. This was particularly the case for PI 34:2, the PI present in the highest quantities. For QTL 1B, PI 34:2 showed consistent significant differences across the two years.

This effect on PI is of potential interest in relation to breadmaking quality, as PI is the only phospholipid that is active in low concentrations in the baking process, being active at levels between 0.02 - 0.1% of wheat flour (Helmerich and Koehler, 2005). In the past, PI was considered to be of minor importance to breadmaking quality (Pomeranz, 1970). However, Helmerich and Koehler (2005) used reconstituted phospholipids extracted from lecithins of various origins to investigate the effects of phospholipids on breadmaking quality. Lecithin is a generic term used to designate any group of yellow-brown fatty substances occurring in animal and plant tissues. The addition of low concentrations of PI to dough resulted in a substantial increase in loaf volume (around 13%). Furthermore, while PI resulted in increases in loaf volumes at low concentrations, at higher levels it reduced loaf volume.

In the study reported here the Hereward allele, which is associated with beneficial effects on gas cell number, was also associated with significantly higher levels of PI.

4.3.3 Impact of QTL x allele effects on the NL composition of the NIL population

Few effects on any neutral lipids were consistently observed across the two years. One potential interesting difference was observed for the levels of FFA 16:0 in year one, as the Malacca allele present in QTLs 1B and 7A showed significantly lower levels of FFA 16:0, the largest source of saturated fatty acids in flour. The levels of FFA 18:2 also differed significantly between the two years.

With regards to potential effects on breadmaking quality, neutral lipids destabilize gas cells and decrease loaf volume (MacRitchie & Gras, 1973; Sroan and MacRitchie, 2009). FFA and unsaturated FFA in particular reduce loaf volume (De Stefanis and Ponte, 1976; Sroan and MacRitchie, 2009). In this study the poor quality Malacca allele was associated with significantly lower levels of FFA 18:2 on QTL 4D (unsaturated) which contradicts expectations. However, the poor quality Malacca allele also reduced the levels of the saturated FFA 16:0, which helps form condensed monolayers around the liquid lamellae, stabilizing gas cells for breadmaking quality.

However, as discussed above, these effects were unlikely to be true effects of allelic variation, and the major effects on composition were environmental.

4.3.4 Impact of QTL x allele effects on the GL composition of the NIL population

The galactolipids showed the greatest differences between the lines with different QTLs and alleles compared to the other lipid groups. This was particularly the case for the first year data where the Hereward allele was associated with significantly higher levels of lipids for the two major galactolipids MGDG 34:2 and 36:4 for QTLs 1B and 7A. This was also consistent with the second year dataset where the Hereward allele was associated with higher levels of lipid for MGDG 36:4 on QTL 7A.

With regards to breadmaking quality, MGDG and DGDG are considered to play a crucial role on gas cell stability by helping form condensed monolayers, and by interacting with the glutenin proteins through hydrophobic and hydrogen interactions (Pareyt et al., 2011). Therefore, the significantly higher levels of galactolipids associated with the good quality Hereward allele at QTL 7A are of potential interest

However, the fact that CVA analysis showed separation of the Malacca alleles, rather than the Hereward alleles, indicates that these differences were not associated with the allelic variation. Year by year variation was also very high, showing that environmental factors and non-genetic effects are mainly responsible for the variation in the total lipid and total galactolipid profiles. However, the GL lipid profile was affected less by year to year differences than the profiles of the other lipid classes indicating that there is stronger genetic control for GL compared to the other lipid classes.

Galactolipids are crucial for photosynthetic tissues and therefore may be more stable to effects of the environment. In fact, it has been reported that MGDG becomes converted to DGDG and oligogalactolipids in order to prevent chloroplast fission in stress conditions such as freezing and drought (Dormann, 2013). In phosphate limited conditions, the amounts of DGDG in plant plastids increase, and DGDG is exported to the extraplastidial membranes to replace phospholipids (Kelly A et al., 2016).

4.3.5 Environmental effects

The differences in composition between the different sets of NILs were small compared to the enormous environmental effects. This is illustrated in Figure 4.22, which reveals the huge differences between the 2013 and 2015 material. Similarly large differences between years were also observed for the total PL and NL content, with effects on GL content being smaller.

These year effects could be caused by numerous factors, including but not limited to, differences in rainfall, temperature and site variation, considering the two years were grown in different fields. Also, as discussed above, the material from the two years differed in storage before analysis. The length of storage time was undeniably a large factor which is reflected in the differences seen in the levels of LPC and PC across the two years mentioned above. Furthermore with improvements in methodology with time, as mentioned in Chapter 3, this would further lead to differences in the lipid composition across the two years.

This large environmental effect would have played an important role in influencing the CVA analysis. The CVA did not show the clustering of the Malacca alleles, nor the expected separation of the Hereward alleles. In fact, while there was some separation of Hereward, it was the Malacca, and most notably 1B:M, 4D:M and 7A:M which showed the greatest separation. While this indicates the lack of genetic effects and the potential genetic unreliability of the material, it is important to note the difficulty of measuring a lipid phenotype. Easily quantifiable traits such as height, disease resistance can provide more robust data for genetic studies and the background could very well be clustered in the analysis. It may also clearly separate out the target allele if it is responsible for providing a desired phenotype. This however is not the case for biochemical data such as lipid analysis. The large quantity of lipid species being measured makes it unreasonable to expect no variability in the dataset. Also biochemical components are notorious for being significantly affected by GxE effects (Figure 3.9), and the significant environmental and experimental variation seen in the lipid data across Chapters 3 and 4 shows that lipids are no exception. Despite the bulking protocol, minimizing plot variation, and numerous repetition to account for machine variability, due to the limitation of lipidomic techniques, this was a significant challenge.

One factor which may have contributed to the failure to observe significant effects of the alleles is the choice of population. Hereward and Malacca were selected as being two good breadmaking cultivars whose quality was not due to effects of gluten protein composition. However, subsequent analyses showed that the parents did not differ greatly in lipid composition (Chapter 3). Although it is reasonable to expect transgressive segregation to be observed, as this resulted in greater differences between the progeny than between the parents, it may have been better in retrospect to have analysed a cross between parents differing in quality and with established differences in lipid composition.

4.3.6 Conclusions

Analysis of the sets of NILs grown in two years showed that the major effects on lipid composition were not genetic, relating to the environment and /or the storage and analysis of the samples.

Furthermore, whereas the backcrossing with Malacca should have resulted in clustering of the lines with the Malacca alleles and separation of those with the Hereward alleles, this was not the case. Hence, although some interesting differences in composition between the sets of lines were identified, particularly in the content and composition of the galactolipids, it was not possible to demonstrate that these were directly related to effects of the QTLs, or the allelic variants at these QTLs. Nevertheless, the strong evidence that galactolipids play a role in grain quality, including the demonstration that they are enriched in dough liquor (see Chapter 5 below), suggests that these lines are worthy of further study, to define the precise factors responsible for the differences in galactolipid content and composition, and to develop strategies to exploit the variation to develop improved cultivars.

Chapter 5 Investigating the Functionality of the MxH NILs

5.1 Introduction

Chapter 4 outlined how the MxH NILs showed significant lipid differences across the two years of study. The alleles of the NILs mainly affected the composition of the GLs, MGDG, DGDG as well as the phospholipid, PI. QTL 1B and 7A showed the greatest separation from the other QTLs, and the greatest lipid differences were observed for the combinations of QTL 7A:M, 4D:M and 1B:M. Among these, the combination 1B:M seemed mainly affected by PI differences, while 7A:M was more affected by GL differences. 7A:M in particular became an interesting candidate due to its separation, but also its consistency across the two years.

However, as interesting as these lipid differences are, a crucial question remains. Are these lipids providing any functional differences? With lipids being minor components of wheat flour, it is possible that these lipid differences, though significant, may not provide measurable functional differences.

The observed lipid differences between the NILs may be playing an important role facilitating gas cell stability, leading to the improved cell number and loaf volume traits associated with our candidate QTLs. For this, it is important to understand how gas cells/bubbles are stabilized in the dough, as well as the role of lipids on this system.

5.1.1 Bubble and Foam formation and stability

Gas bubbles in most systems are not stable structures and are prone to collapse. Pure liquids such as water are unable to support a foam structure. Although agitation and high energy mixing can create bubbles and form a temporary foam structure, the bubbles will soon coalesce and the foam will collapse. In order to form more stable foam structures, a protective, adsorbed layer of molecules are needed around the bubbles to prevent bubble coalescence. This layer allows the formation of longer-lasting foam whose stability is dependent on the surface properties of the adsorbed layers. The bubble structure within a system can be destabilized by three common mechanisms which must be minimised in order to retain a stable foam structure.

5.1.1.1 Drainage

Foam drainage is the amount or rate the continuous aqueous phase drains from the foam structure. This is particularly important in low viscosity fluids where the hydrodynamic forces allow for rapid flow of liquids between the bubbles. This liquid flow can be influenced by bubble size, bubble surface properties and the density and rheology of the aqueous phase (Haas and Johnson, 1967). As junctions (Plateau borders) between the bubbles contain most of the liquid in the foam, a smaller bubble size slows down liquid flow and provides a denser foam structure. Bubble surface properties also play an important role as an elastic surface will slow down liquid flow (drainage) while fluid surfaces will facilitate drainage (Wilde, 1996). The density and rheology of the aqueous phase is highly important as higher densities will facilitate rapid draining, while high viscosities are associated with slower draining (Haas & Johnson, 1967). Although the drainage of the aqueous phase is crucial for the aqueous phase of liquid foam structures, it is not such an issue for bread dough due to the highly viscoelastic nature of the dough that reduces drainage to a minimum.

5.1.1.2 Coalescence

Coalescence occurs when two gas bubbles are in close enough proximity for the thin liquid film that forms between the bubbles to rupture. Therefore strong repulsive electrostatic and steric forces are needed between the bubbles in order to keep them apart and maintain the stability of the thin film. However in foam, bubbles are usually forced into close proximity making these long range repulsive forces ineffective. In this case two main stability mechanisms for gas cells exist.

1) The visco-elastic mechanism requires the liquid lamellae to be stabilized by adsorbed layers of molecules with large interfacial elasticity. The components that form the adsorbed layer effectively forms a two dimensional gel at the surface of the gas cells with equivalent visco-elastic properties of a three dimensional polymer gel. This makes it so that the lamella remains intact as long as its deformation does not exceed the elasticity limit. If so the two adsorbed layers will return to their original positions.

2) The Gibbs-Marangoni mechanism is reliant on the fluidity of the adsorbed layer of surfactant. When the thin film gets deformed, the adsorbed layers naturally become thinner and the local surfactant concentration becomes depleted. In order to restore the concentration gradient, the molecules naturally migrate to the depleted areas. This flow of molecules drags the aqueous phase to the thinner regions of the lamella, restoring the original thickness. Molecules such as

detergents that can diffuse rapidly and associate strongly with the aqueous phase are the most effective at stabilising foams through this mechanism.

During breadmaking, coalescence is thought to be important during the later stages of proving and the early stages of baking (Hayman et al 1998) when the gas cells are in the closest proximity, before the gluten starch matrix becomes solid during baking.

5.1.1.3 Disproportionation

The mass transport of gas from small to large bubbles is called disproportionation. The Laplace pressure within a bubble is generated by the surface tension on the bubble surface, which acts as the driving force behind disproportionation. The surface tension acting on the bubble surface keeps the bubble spherical. The contraction of the bubble as greater numbers of bubbles are formed induces a small increase in pressure inside the bubble which is called the Laplace pressure which is defined as…

$\Delta P = 2y/r$

ΔP is the Laplace pressure difference between the inside and outside of the bubble, y is the surface tension and r is the bubble radius. Therefore ΔP will be greater in smaller bubbles. When two bubbles come into close contact, a net transfer of gas from smaller to larger bubbles occur. Eventually the smaller bubbles will completely disappear. The rate of disproportionation will also depend on the solubility of the gas in the aqueous phase. In breadmaking, the gas phase is largely CO_2, which is highly soluble in water, so the rate of transport could be high. Previous work has suggested that some small bubbles are lost to disproportionation at the end of proving and early baking (Shimiya and Nakamura 1997)

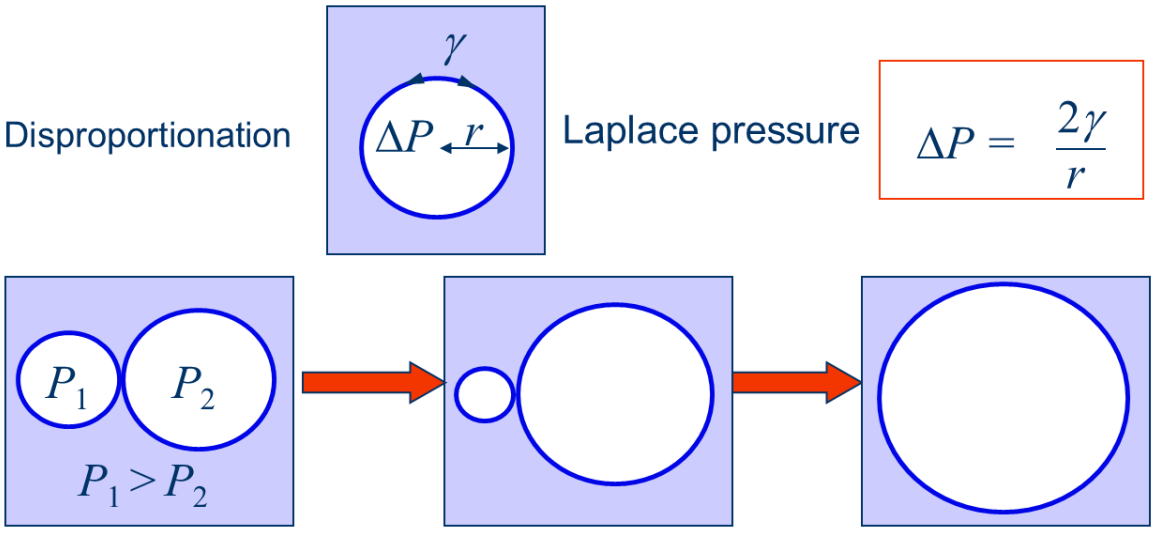


Figure 5.1 Schematic of bubble disproportionation. Where P is the Laplace pressure difference between the inside and outside of the bubble, y is the surface tension and r is the bubble radius. When two bubbles move to close proximity, there will be a net transfer of gas from smaller to larger bubbles.

5.1.2 Bubble formation and stability in breadmaking

Gases account for over 70% of the final volume of bread, and have a major influence on its textural and sensory attributes (Campbell and Mougeot, 1999). Therefore, the formation and stability of gas cells in dough is a crucial factor in the overall quality and sensory attributes of bread.

The bubble formation in dough is mainly affected by the energy input, surface tension and the viscosity of the dough. Bubbles are incorporated in the liquid phase through agitation, which occurs during the mixing stage of breadmaking. The amount of energy used during the mixing process can influence the size of the bubbles as well as their distribution within the dough (Hanselmann & Windhab, 1998). With larger energy inputs, the break-up of existing bubbles will be facilitated leading to a finer bubble structure and greater volumes of gas incorporated into the dough.

Dough viscosity is also important in this process, as higher viscosities require higher energies and mixing speeds. Therefore doughs with greater viscosities tend to provide smaller bubbles for the same mixing speeds. This has been observed in both foam (Koczo and Racz, 1991) and emulsion systems (Pandolfe, 1981). This is also related to the energy input of the system as more viscous doughs require higher energy levels to maintain a given mixing speed.

Surface tension is a highly important factor, especially for foam structures which are formed in low viscosity liquids. In order to create foam or an emulsion, a large increase in the interfacial area of the system is required. The surface tension is defined as the force per unit length acting on a surface, and acts as the energy barrier to surface expansion. Therefore a lower surface tension will require less energy to produce a specific increase in surface area. Therefore surface active molecules that lower the surface tension of the system in a rapid fashion are the most efficient at creating fine bubbles (Graham and Phillips, 1976; Kalischewski and Schugerl, 1979; Kim and Kinsella, 1985; Lorient et al., 1989; Nakai and Li-Chan, 1993).

However, gas cells developed during dough development can be destabilized by disproportionation (Vilet et al., 1992; Sahi, 1993) and coalescence (Vilet et al., 1992). This can be particularly prominent during the latter stages of dough development as the gas bubbles come in to closer proximity and may no longer be supported by the gluten network. As mentioned in Chapter 1, the gluten matrix acts as the primary stability mechanism for gas cell stability during the initial stages of dough development. However, as the bubbles expand during proving, they come into closer contact, and the risk of bubble coalescence is increased. The aqueous phase of dough plays a crucial role in preventing bubble coalescence due to their soluble surface active components that stabilizes the gas cells during dough development (Salt et al., 2006). Without the aqueous phase, bread dough loses its capacity for gas retention (Clark et al., 1991) suggesting that the aqueous phase of dough is important for breadmaking quality (Sahi, 1994).

The thin liquid lamellae between the gas cells act as the main structure in the aqueous phase preventing coalescence (Gan et al., 1990 and 1995). The gas cell stability is determined by the surface active components in dough which adsorbs to the surface of the liquid lamella. It is the composition of these components such as protein and lipids which determines whether the gas cells are stabilized by the visco-elastic mechanism or the Gibbs-Marangoni mechanism. Therefore, these surface active components that form the aqueous phase of dough are crucial in preventing gas cell rupture and dough collapse.

Lipids are one of the key components that adsorb to the surface of the liquid lamellae. Many endogenous wheat lipids are highly surface active and will compete with proteins to stabilize the bubble surface (Dubreil et al., 1997; Gan et al., 1995; Keller et al., 1997). Phospholipids in particular can positively impact breadmaking quality, although they show greater synergistic effects on loaf volume than individual effects (Helmerich and Koehler, 2005). Further details on the role of lipids in breadmaking quality can be found in Chapter 1.

To be active in foam, lipids and surfactants must diffuse rapidly at the interface and interact with the aqueous phase to stabilise the lamella via the Gibbs-Marangoni mechanism (Fruhner et al., 2000). While phospholipids are capable of this, neutral lipids have not shown positive effects in this process.

Depending on their solubility, lipids are more surface active than proteins. Lipid with greater chain length and those which are fully saturated will tend to form aggregates which will decrease their solubility, which takes longer to adsorb.

Lipid structure is also crucial to this process as the headgroup needs to interact with the aqueous phase in order to form stable foams (Fruhner et al., 2000). Therefore neutral lipids such as fatty acids, triglycerides and alcohols cannot stabilise via the Gibbs-Marangoni mechanism and thus cannot form stable foam structures. As FFAs are charged in a pH dependent fashion, it further restricts their ability to form stable foam strucutres. On the other hand, more amphipathic lipids such as PLs, LPLs and GLs with larger and greater numbers of head groups form more stable foams. Therefore the ratio of polar to neutral lipids can be crucial for foam integrity.

5.1.3 Studying the aqueous phase of dough

The aqueous phase of dough was first studied in 1945 in order to determine the composition of protein, sugar and salt (Baker et al., 1945). Since then, more recent studies have investigated the surface tension and surface rheology of the aqueous phase at the air/water interface (Sahi, 1993, 2003), and the stability and surface properties of the aqueous extracts of dough were related to baking quality (Sahi, 1994). Further studies were performed relating the aqueous phase of dough with regards to the protein composition of wheat flour (Dubreil et al., 1998), and also demonstrating that the surface properties of dough liquor were dominated by the polar lipid fraction (Salt et al., 2006).

It is possible to study the aqueous phase of dough and its properties through the extraction of an artificial preparation by ultracentrifugation ($1.2x10^5$ x g) for 30-60 minutes (Dubreil et al., 1998; Salt et al., 2006), which produces a straw-coloured viscous liquid (dough liquor). The surface properties of dough liquor can be correlated with lipid content and baking performance. While this method does not take into account the role of mixing and adsorption of insoluble components, and uses diffusive adsorption of the components to identify surface properties, it is the closest method currently available to investigate the components in the dough that play an active role at the surface of gas cells.

Isolation of dough liquor is an artificial preparation of the aqueous phase of dough. An artificial method was developed by Louise Salt at IFR (Institute of Food Research) based on previous studies

(Baker et al., 1946; MacRitchie et al., 1976; Sahi, 1994) which isolated dough liquor using conditions that would represent the mixing and proving stages of the Chorleywood Breadmaking process (CBP).

The components of dough liquor which include lipids, non-starch polysaccharides and proteins may all play an important role in determining the interfacial structure. Therefore, surface studies were pursued at the Institute of Food Research (now Quadram Institute Bioscience) in order to observe the potential biophysical differences existing within the MxH NILs. Short of doing actual baking trials, for which there was a significant lack of flour available, the surface analysis of the aqueous phase of dough provides an elegant method to observe the functional effects lipid can impart on a breadmaking system.

5.2 Development of a diluted DL extraction method

5.2.1 Diluted Dough liquor Preparation

Preparation of dough liquor also requires relatively large quantities of flour. Dough recipes require 61% flour, 37% water and 1.2% (w/w) NaCL (salt). Therefore, to produce enough dough liquor to study, each sample would require over 60 g of flour, meaning that counting replicates, this protocol would require over 180g of flour for each sample, a quantity of flour that was unavailable for the NILs of interest. Therefore it was necessary to develop a diluted dough liquor extraction method to compensate for the lack of flour material, while being able to determine robust consistent surface properties.

Flour was taken from two years' worth of Hereward flour material grown and harvested in 2012 and 2013. Dough recipes were developed based on the water absorption rates of the flours in both years. The biophysical properties of Hereward were studied previously, and the 2012 and 2013 material was selected on the basis of their functional differences (Salt et al., 2017).

The original dough recipe was diluted down with water, using 50, 40, 30, 20 and 10% of the flour used in the original dough recipe. The exact recipes for each concentration are summarized in table 5.1. For this study, we wished to observe whether the significant differences in the surface properties of the 2012 and 2013 Hereward dough liquor extracts could be replicated using these levels of dilutions.

2012 Hereward						
Dilutions	Original Recipe	50%	40%	30%	20%	10%
Flour (%)	61	30.5	24.4	18.3	12.2	6.1
Water (%)	37	68.3	74.4	81.5	86.6	92.7
Salt (%)	1.2	1.2	1.2	1.2	1.2	1.2

2013 Hereward						
Dilutions	Original Recipe (%)	50%	40%	30%	20%	10%
Flour (%)	63	31.5	25.2	18.9	12.6	6.3
Water (%)	35	67.3	73.6	79.9	86.2	92.5
Salt (%)	1.2	1.2	1.2	1.2	1.2	1.2

Table 5.1 Recipes for various diluted dough liquor extractions for the 2012 and 2013 material. Recipe for the water content between the two flour samples slightly differ due to the differences in water holding capacity between the two flours.

In order for the diluted dough liquor fractions to represent the aqueous phase of dough, the mixing and proving conditions needed to represent the conditions used in the Chorleywood Breadmaking Process (CBP). The dough ingredients were added to a glass beaker and were stirred with a magnetic stirrer at 500 rpm for approximately 4 minutes. Post mixing, the dough was weighed into polycarbonate ultracentrifuge bottles (38 x102 mm) with screw on titanium caps (Beckman, UK) and incubated at 30˚C for one hour.

The tubes were then centrifuged in a pre-warmed (30˚C) fixed angle rotor at 41,000 g for 30 minutes. This procedure maintained the dough at 30˚C for 90 minutes in total, in accordance with standard bakery practice.

The straw coloured viscous supernatant (the dough liquor) was collected and divided between ten 1.5ml micro tubes, and centrifuged using a Thermo Fresco 17 centrifuge for 20 minutes at 17,000 rpm. The resulting supernatant was collected while the TAG rich, creamy pellicle at the top of each micro tube was discarded. The TAG pellicle was discarded due to its destabilizing effects on the bubble surface.

5.2.2 Surface properties of diluted dough liquor

The surface tension and surface rheological properties of fresh dough liquor samples were determined for a range of dilutions, using the pendant drop technique (Ambwani and Fort, 1979; Husband et al., 1998).

Surface properties of the diluted dough liquor was determined using a FTA 200 pulsating drop tensiometer where a droplet hanging in air, was formed at the tip of a Teflon coated needle inside a glass cuvette. The needle was connected to a 50 µl glass syringe. Further details on the protocol can be found in the Materials and Methods (Chapter 2).

The surface tension (ST) of the dough liquor was determined by capturing images of a 10-13 µl droplet every second for a total of 600 seconds at room temperature. The droplet shape in every image was analysed by fitting the experimental drop profile to the Young-Laplace capillary equation to calculate the surface tension, volume and specific area. Each sample (e.g. 1B:H, 1B:M, 4D:H… etc) was measured in triplicate, with three batches of dough prepared and each measured using the pendant drop three times and plotted as a function of time.

The surface dilatational elasticity of the dough liquor surface was determined by pulsulating the droplet volume at a frequency of 0.1 Hz, and amplitude of 3 µl. By measuring the change in surface tension ($d\gamma$) as a function of the change in surface area (dA) of the droplet, due to the change in volume the surface elastic modulus G' could be calculated.

$$E' = A.\, d\gamma/dA$$

Samples were analysed in triplicate and the averaged G' values were plotted as a function of time.

As expected, increased dilution of the dough liquor fractions reduced the sensitivity of the analysis to the differences between the two samples. No differences could be observed between the surface tension of the Hereward 2012 and 2013 material for 10, 20 and 30% dilutions. While the 40% dilution provided some differences, the greatest separation of the surface tension for 2012 and 2013 was observed for the 50% dilution (Figure 5.1).

Similar observations could be made with the surface elastic modulus of the diluted dough liquor fractions. High levels of dilutions showed poor separation between the two years, as well as poor consistency, most likely due to the difficulty obtaining consistent levels of lipid in a highly diluted extract. Once again the 40-50% dilution was associated with the greatest differences in surface elasticity across the two years (Figure 5.2).

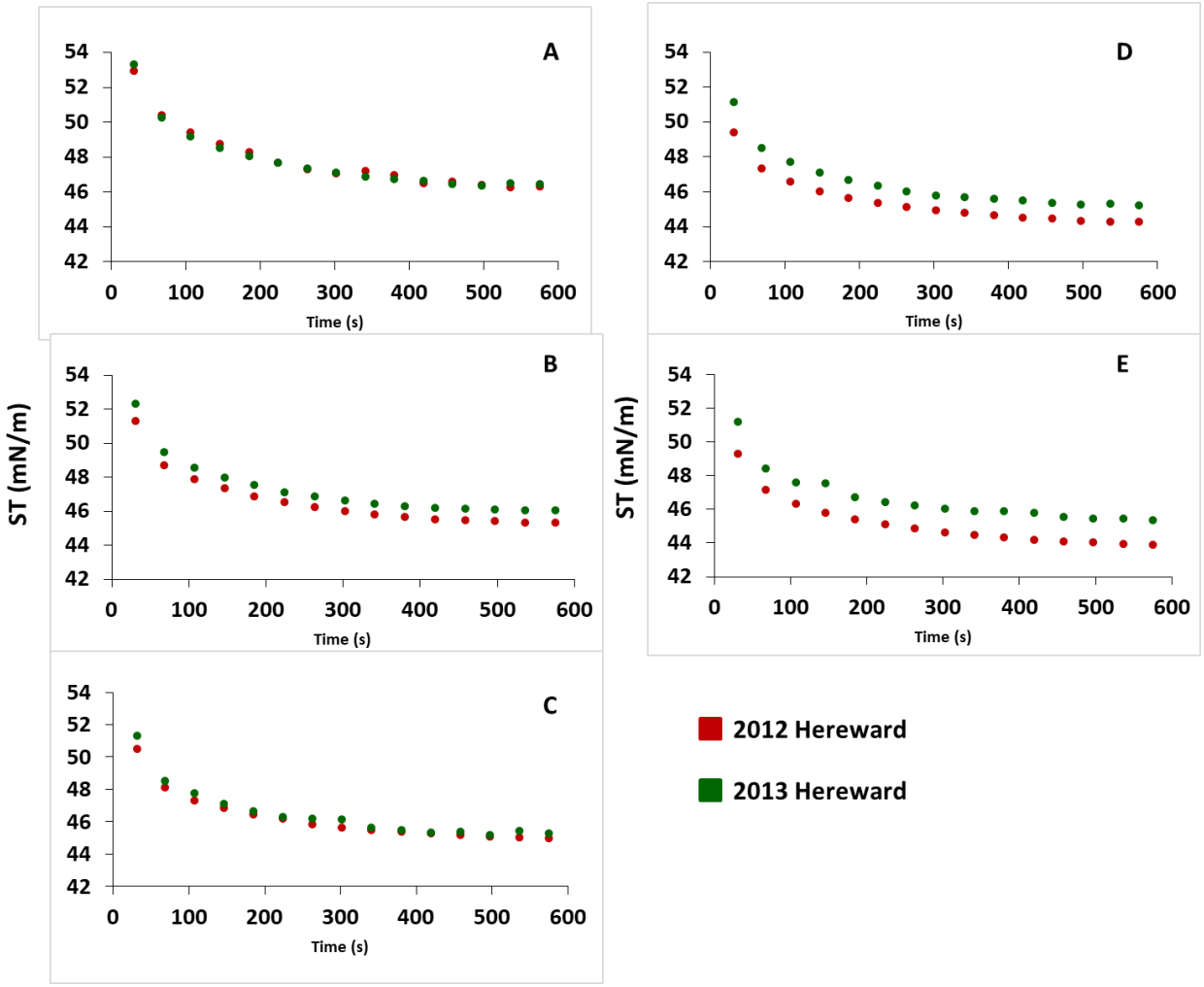


Figure 5.2 Comparison of the surface tension (ST) of various diluted dough liquor fractions in Hereward flour across two years. A- 10%, B-20%, C- 30%, D- 40%, E-50% dilution

Although the 50% dilution showed some inconsistencies for its elasticity modulus, overall considering the surface tension as well as the elasticity modulus, the 40 or 50% dilution stood out as primary candidates.

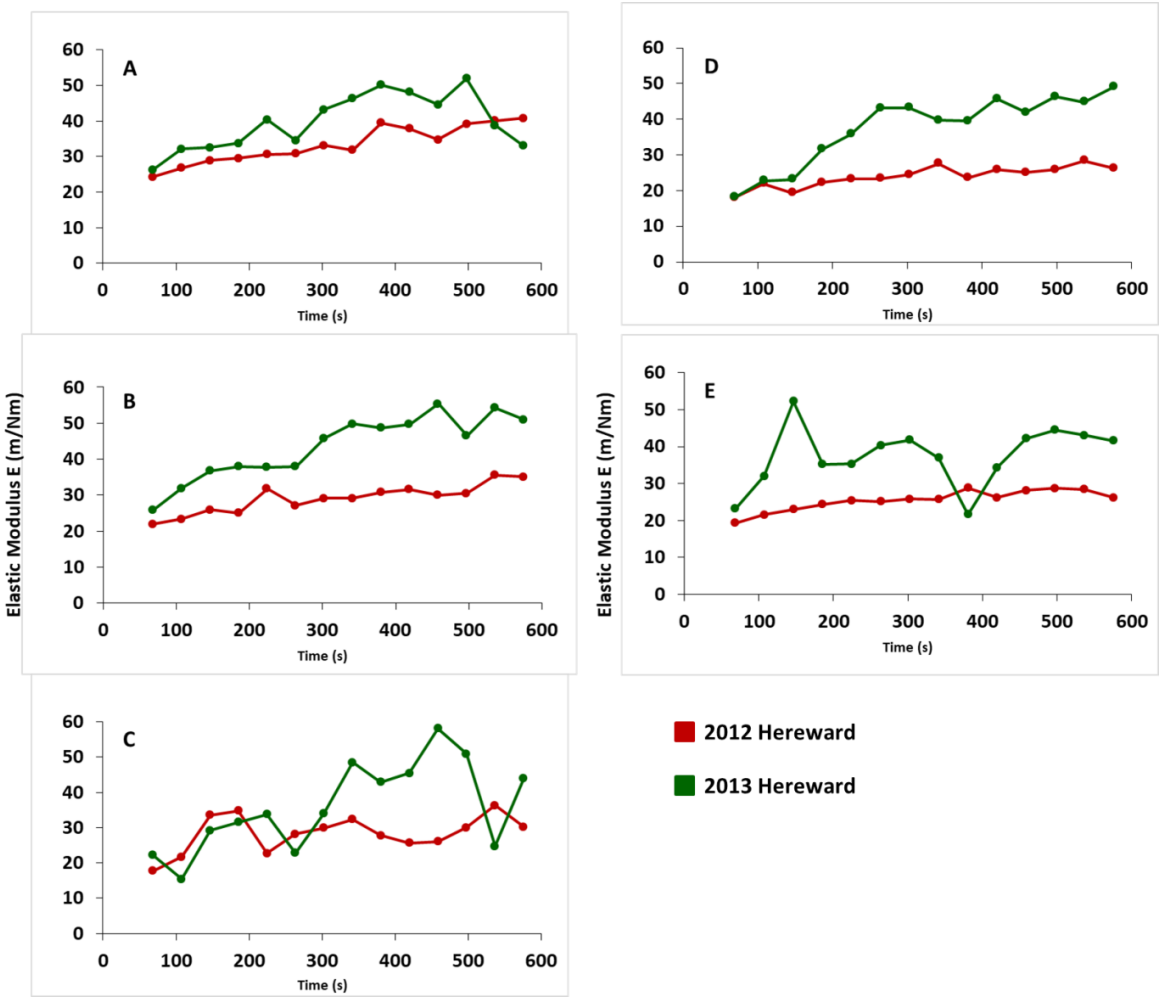


Figure 5.3 Surface elasticity of the various diluted dough liquor samples in Hereward flour across two years. A- 10%, B-20%, C- 30%, D- 40%, E-50% dilution

5.2.3 DL MS analysis of the various dilutions

In order to identify whether sufficient levels of lipids were present within the diluted dough liquor fractions, lipids were extracted, identified and quantified using ESI-MS/MS. The method for lipid extraction was based on the Bligh and Dyer method for lipid extraction from dough liquor, with some modifications, further details of which can be found in chapter 2.

MS analysis of the various diluted dough liquor samples showed clear lipid differences with increasing quantities of lipids occurring with increasing percentage dilutions. Increases in the quantity of lipids were observed as the dilutions decreased from 10-40%. However there was a significant increase in the lipid content at the 50% dilution (Figure 5.3).

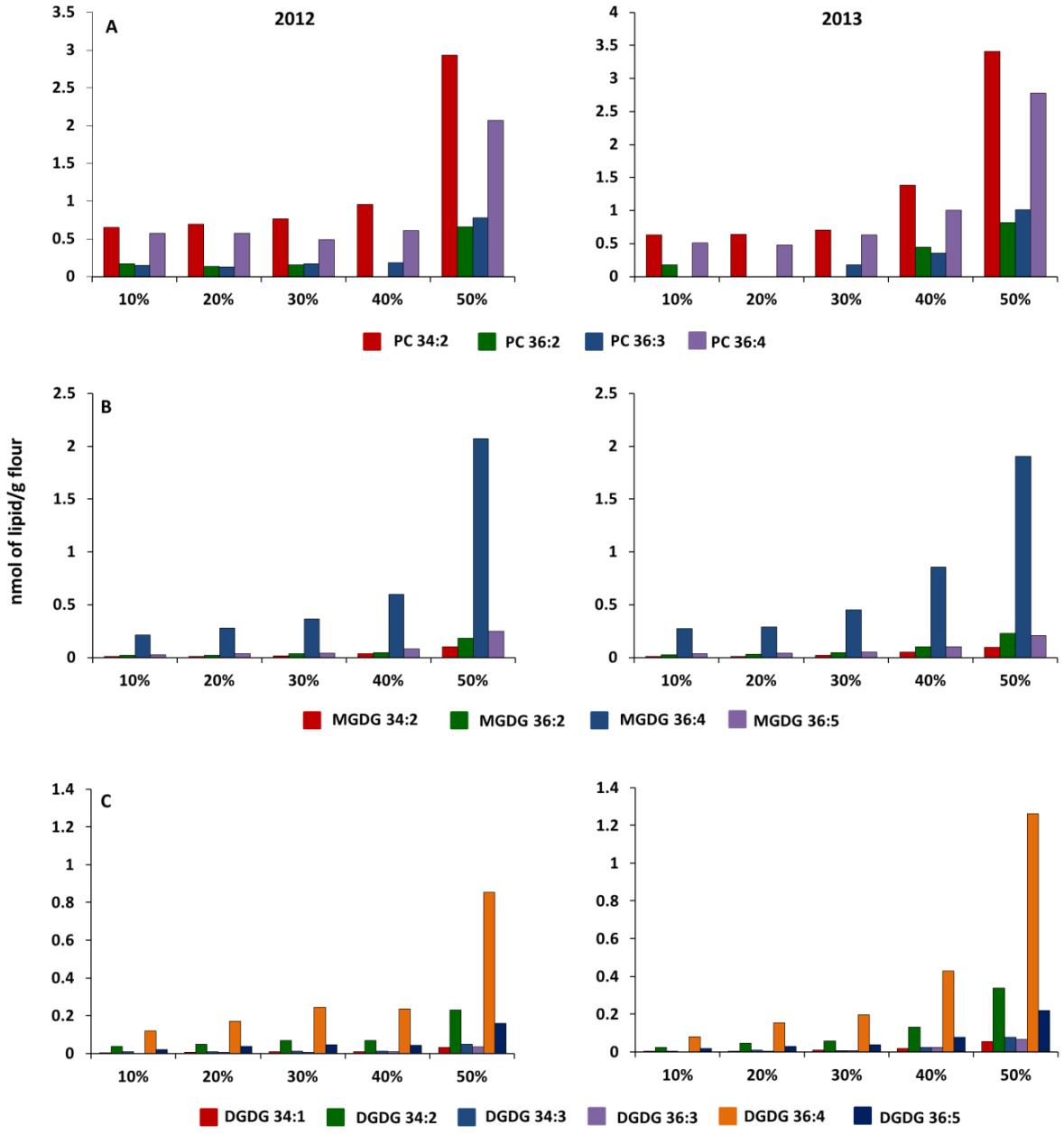


Figure 5.4 The profile of selected lipids in various dilutions of dough liquor from Hereward material grown in 2012 and 2013. The selected lipids were PI, MGDG and DGDG. A- PC profile, B- MGDG profile, C- DGDG profile. Data are mean values of ±SE of three independent samples analysed via ESI-MS/MS.

Considering this, although the 40% dilution showed sufficiently consistent surface tension and elasticity, the lack of lipids present compared to the 50% dilution made it unsuitable for this study. Therefore the 50% dilution was selected based on its interfacial properties as well as lipid content. The large difference between the lipid content of the 40% and 50% dilution was surprising, especially considering the steady, more predictable increase in lipid quantity between 10-40% dilutions. It may

be possible that the dilution range between 40-50% is the critical point upon which a sufficient amount of lipid can be captured using the dough liquor extraction technique.

Yet even with a 50% dilution, there were insufficient amounts of the flour sample to do the minimal three replicates. Therefore the method was scaled down to provide just 10 grams of dough liquor for the FTA 200 pulsating drop tensiometer as well as the ESI-MS/MS analysis. Therefore the final recipe came to 30.5% flour, 68.3% water and 1.2% salt in a 22 gram system.

5.3 Results

5.3.1 Dough Liquor surface properties of the MxH NILs in year 1

50% diluted dough liquor fractions were obtained for the MxH NILs using the method outlined above. Triplicate batches of diluted dough were developed for each NIL combination and dough liquor was extracted from each. Malacca x Hereward NILs with either an M or H allele for each of four QTLs on chromosomes 1B, 4D, 6A and 7A were considered as eight pools of material.

The analysis of surface tension curves are complex even for simple surfactant solutions. Dough liquor is a complex mixture, with no well-defined model to analyse surface tension behaviour. The surface tension data gave an exponentially decreasing trend (decay) over time. Rather than displaying the raw surface tension curves, the data was fitted to a simple exponential curve to determine the statistical differences between the samples. Although this approach does not describe the adsorption behaviour of the molecules at the diluted dough liquor interface, it is a useful statistical method to quantify differences between the NIL combinations. Example curves depicting the raw surface tension and elasticity dataset can be found in appendix figure 5.1.

The simple exponential decay model was as follows:

Surface tension $f(t) = \gamma + \alpha * \exp(-\beta * t)$

where parameter γ is the surface tension at time (t)=∞, α is the decrease in surface tension over the fullness of time from a theoretical time zero ($\gamma + \alpha$ being the surface tension at the theoretical time zero), and parameter β is the exponential rate of decrease with time (t). The gamma (γ) parameter keeps the value of surface tension above zero in the fullness of time as long as the value is positive. This is because α*exp(-β*infinity) =0 t theoretical time (t=∞). The α parameter is the surface tension value that is lost from time 0 until the fullness of time when gamma is reached. Therefore, at time 0 the surface tension is α+γ, as exp(-β*0)=1 in the model.

Due to the repeated measures over time on each sample, the model was fitted to each profile separately to estimate the three parameters using a non—linear least squares optimisation, rather than estimating the parameters from the data set as a whole. This formed the first stage of a two stage analysis approach to ensure analysis of independent (of time) estimates of the parameters. The second stage was to analyse each set of estimated parameters α, β and γ) according to the treatment structure of the experiment, to test the main effects and interaction between allelic (M or H) and QTL factors (1B, 4D, 6A or 7A). This was done via ANOVA to obtain the F-test results for these effects, the appropriate means, standard errors of differences (SED) between means and the least significant difference values at the 5% level of significance for the comparison of means.

A similar exercise was done for the surface elasticity data, for which the model was an asymptotic exponential. Once again, as with the surface tension data, the fitted curves for elasticity offers a statistical method to find differences between the elasticity of the NILs.

Elasticity $f(t) = \delta + \varepsilon(1 - \exp(-\beta * t))$

where parameter δ is the starting elasticity at a theoretical time zero, β is the exponential rate of increase with time and ε is the total increase in elasticity in the fullness of time above δ.

It was then possible to draw up predicted models, using those means of estimated parameters that were prescribed by the results (significance of main effects and interactions of alleles and QTL) of the ANOVA. In this asymptotic exponential model, ε ensures that the curve for elasticity plateaus to δ + ε, in the fullness of time. So at time 0 we start with elasticity = δ, as exp(-β*0)=1 in the model, giving δ + ε*(1-1) = δ. Then at a theoretical time = infinity, we get δ + ε*(1-0) = δ + ε, as exp(-β*t)=0 in the model.

All modelling and the subsequent analysis of estimated parameters was done using the GenStat (17th edition, © VSN International, Hemel Hempstead, UK) statistical package. Predicted models were plotted using SigmaPlot (version 13, © Systat Software Inc., Richmond, California, USA).

5.3.2 Surface Tension of Year 1 (2013) Material

The set of estimated parameters from the fitted model is given in Table 5.2. There was a problem with the data for the first 1B:H sample in that there was not a monotonic decrease in the surface tension data with time. It was therefore not possible to fit the model to these datasets and the sample was omitted for the further analysis of the parameters. All fits were based on 12 residual degrees of freedom.

A

QTL	Allele	α	β	γ	R^2
1B	M	7.381	0.009262	44.29	98
1B	M	7.673	0.009627	43.86	98.3
1B	M	7.861	0.009706	44.68	97.6
1B	H	*	*	*	*
1B	H	7.874	0.009967	44.56	98
1B	H	8.501	0.008105	43.86	86.9
4D	M	7.66	0.008504	43.92	98
4D	M	7.049	0.009385	42.88	98.4
4D	M	7.881	0.009203	43.4	98.5
4D	H	7.402	0.008994	44.03	98.8
4D	H	7.85	0.009796	44.03	98.8
4D	H	7.37	0.008894	44.06	98.6
6A	M	8.007	0.008432	43.78	98.2
6A	M	7.986	0.00844	42.83	98.2
6A	M	7.241	0.008848	43.2	98.1
6A	H	7.564	0.008636	43.67	98.2
6A	H	7.253	0.009433	42.53	97.9
6A	H	7.504	0.00922	42.91	98.2
7A	M	7.774	0.009631	44.97	98.2
7A	M	7.806	0.009776	43.4	98.2
7A	M	8.375	0.010809	44.27	98.2
7A	H	7.537	0.01026	44.2	97.4
7A	H	7.54	0.009617	44.32	98.3
7A	H	7.58	0.011306	43.34	97.5

B

	α	*β*	*γ*
Allele	0.736	0.589	0.930
QTL	0.191	0.007	0.011
Allele.QTL	0.071	0.513	0.422

Table 5.2 Data on the estimated parameters for the surface tension of the MxH NIL DDL for year 1. A- The set of estimated parameters from the surface tension fitted model for the first year MxH NIL material. R^2 is a statistical measure of how close the data is to the fitted line for the model. B- The p-values for the F-tests on the estimated parameters

There was a main effect of QTL for the exponential rate of decrease in surface tension over time (p=0.007, F-test) and for the surface tension attained in the fullness of time (p=0.011, F-test). There is therefore some evidence of a QTL effect regardless of alleles, on the surface tension of the dough.

No significant differences could be observed between the surface tensions of the NILs. The lack of an allelic effect indicates the low level or possibly non-existent effects of the Hereward allele on the DDL fraction. On the other hand, some differences were observed for the various QTLs. QTL 6A showed the slowest exponential rate and the lowest surface tension in the fullness of time. The samples of NILs for the other three QTLs were more similar, although QTL 7A showed the greatest decrease in surface tension which was significantly different from the rest ($p<0.05$, LSD). However, these significant QTL differences are not indicative of a specific genetic effect, as it is not showing the effect of the selected alleles. It may be an effect of one of the background genes, which have not been identified, or may possibly be an artefact of the environment or experimental procedure. Means tables and curve equations for all surface tension and elasticity measurements can be found in appendix figure 5.2.

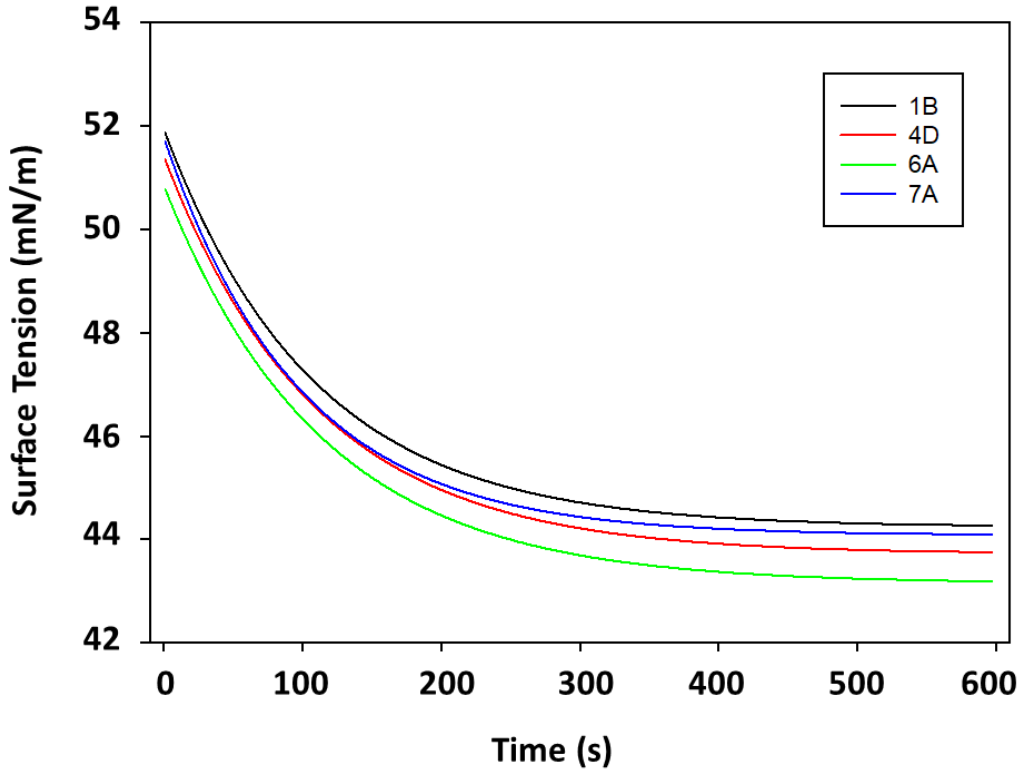


Figure 5.5 Predicted model for surface tension based on the results of the two-stage analysis, using the means of estimated parameter estimates according to the ANOVA on the sets of parameters. The form of the model was *Surface tension* = $\gamma + \alpha$*exp(-β**Time*) where a constant α is used as it needed to decay to value above zero in the fullness of time. Separate γ and β parameters for the QTLs according to the results of the ANOVAs. There was no significant ($p < 0.05$, F-test) effect of the alleles.

A

QTL	Allele	δ	ε	β	R2
1B	M	-14.29	60.57	0.00957	92.1
1B	M	-19.64	61.45	0.01103	92.3
1B	M	-14.46	44.28	0.00572	50.3
1B	H	-27.75	78.36	0.01312	95.7
1B	H	-19.67	63.69	0.01121	89.9
1B	H	-23.47	69.62	0.012	90.1
4D	M	-7.2	53.38	0.00583	66.8
4D	M	-7.2	53.38	0.00583	66.8
4D	M	-10.48	46.86	0.00861	54.7
4D	H	-3.91	48.95	0.00721	83.3
4D	H	-8.44	54.84	0.00772	89.9
4D	H	-22.06	66.99	0.01431	81.9
6A	M	-17.26	71.11	0.00949	87.6
6A	M	-36.3	80.31	0.01655	80.6
6A	M	-8.26	57.82	0.00752	83.1
6A	H	*	*	*	*
6A	H	-24.52	78.14	0.01174	93.1
6A	H	-19.32	59.93	0.01255	86.4
7A	M	-16.94	61.01	0.0103	92.8
7A	M	-29.5	55.5	0.01905	89.4
7A	M	-6.53	54.83	0.00355	82.2
7A	H	-45.98	64.27	0.02879	79.1
7A	H	-34.11	62.76	0.0211	96.6
7A	H	-80.6	98.1	0.04286	83.9

B

	δ	*ε*	*β*
Allele	**0.025**	**0.047**	**0.009**
QTL	**0.015**	0.125	**0.006**
Allele.QTL	0.075	0.439	**0.033**

Table 5.3 Data on the estimated parameters for the elasticity modulus of the MxH NIL DDL in year 1 (A)The set of estimated parameters for the elasticity modulus fitted model for the first year MxH NIL material. R^2 is a statistical measure of how close the data is to the fitted line for the model. (B) The p-values for the F-tests on the estimated parameters.

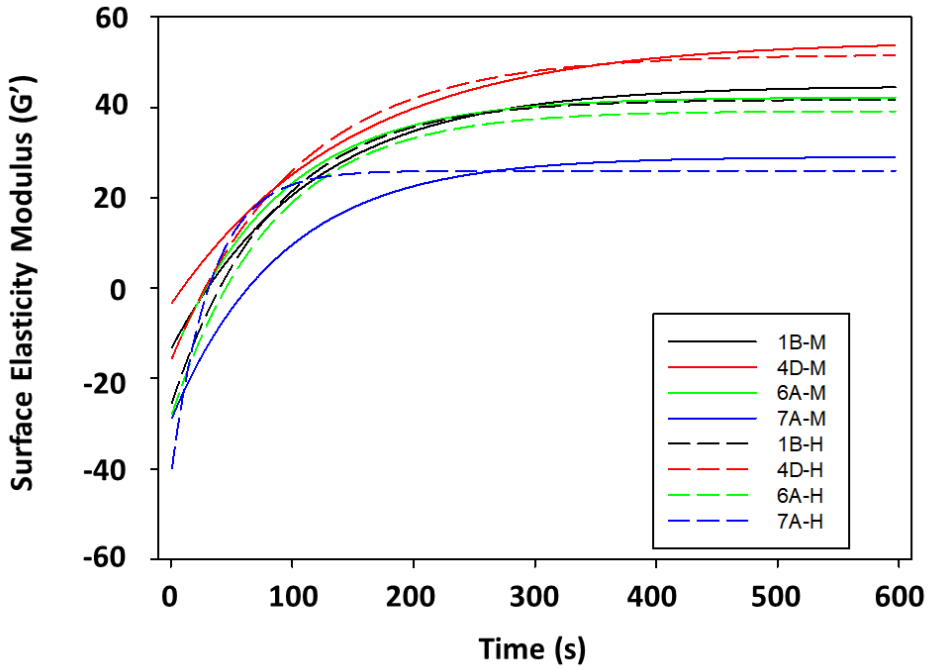


Figure 5.6 Predicted model for elasticity based on the results of the two-stage analysis, using the means of estimated parameter estimates according to the ANOVA on the sets of parameters. The form of the model was $Elasticity = \delta + \varepsilon(1 - \exp(-\beta * Time))$, where a common ε is used for each allele (M or H) as ε ensures that the curve for elasticity plateaus to δ + ε. Separate δ and β parameters are used for the QTLs and the alleles, according to the results of the ANOVAs. ε is held constant in order to keep

Clear differences in the elasticity of the dough liquor for the alleles of the MxH NILs could be observed, particularly for 7A:H which had a much greater exponential rate for elasticity over time. In comparison, the NILs with a Hereward allele for QTL 4D had a much lower exponential rate (dashed red curve), and this was also seen on the 4D:M combination (solid red curve). The combinations with 1B and 6A QTLs were more similar to each other, but overall it is clear that the Hereward allele gave rise to greater variation in elasticity than the Malacca allele (Figure 5.5). Although some specific allelic effects were seen, it is clear that greater effects were observed between the individual QTLs, with QTLs 1B and 7A in particular showing large elasticity differences to the QTLs 4D and 6A. This once again indicates the lack of a specific allelic effect, and implies other environmental or experimental effects are providing these differences in elasticity.

5.3.3 Surface tension of Year 2 (2015) material

The set of estimated parameters from the fitted models of the second year are shown in Table 5.4. All fits were based on 12 residual degrees of freedom.

A

QTL	Allele	α	β	γ	R^2
1B	M	6.745	0.005048	44.98	99.6
1B	M	6.898	0.005725	45.44	99.5
1B	M	*	*	*	*
1B	H	7.276	0.004254	45.4	98.7
1B	H	6.726	0.005202	45.53	99.3
1B	H	7.265	0.005769	46.19	99.7
4D	M	8.362	0.009044	43.54	98.8
4D	M	7.037	0.00735	43.27	97.8
4D	M	6.722	0.006292	43.19	99.8
4D	H	9.441	0.009854	42.94	97.9
4D	H	7.214	0.00643	42.77	99.8
4D	H	7.447	0.007329	43.68	99.6
6A	M	11.083	0.010755	44.23	97.1
6A	M	8.383	0.008266	43.67	98.9
6A	M	6.645	0.005689	43.36	99.3
6A	H	8.561	0.008122	43.85	99.4
6A	H	8.828	0.009281	43.51	98.9
6A	H	8.747	0.008736	43.21	98.9
7A	M	7.968	0.008478	45.18	98.9
7A	M	6.723	0.006646	44.41	99.7
7A	M	8.641	0.008537	44.13	99.1
7A	H	8.14	0.008726	45.14	99
7A	H	6.522	0.005044	44.77	98.3
7A	H	6.841	0.005732	45.33	99.4

B

	α	*β*	*γ*
Allele	0.858	0.720	0.402
QTL	0.084	**0.014**	<**0.001**
Allele.QTL	0.788	0.717	0.225

Table 5.4 Data on the estimated parameters for the surface tension of the MxH NIL DDL in year 2 (A) The set of estimated parameters for the surface tension fitted model for the second year MxH NIL material. R^2 is a statistical measure of how close the data is to the fitted line for the model. (B) The p-values for the F-tests on the estimated parameters.

There was a main effect of QTL for the exponential rate of decrease in surface tension over time ($p=0.014$, F-test) and for the surface tension attained in the fullness of time ($p<0.001$, F-test). This strong QTL effect indicates once again that some other factor such as another background gene or the environment is providing these differences rather than the specific alleles of the NILs. Furthermore, this effect was statistically stronger in the second year.

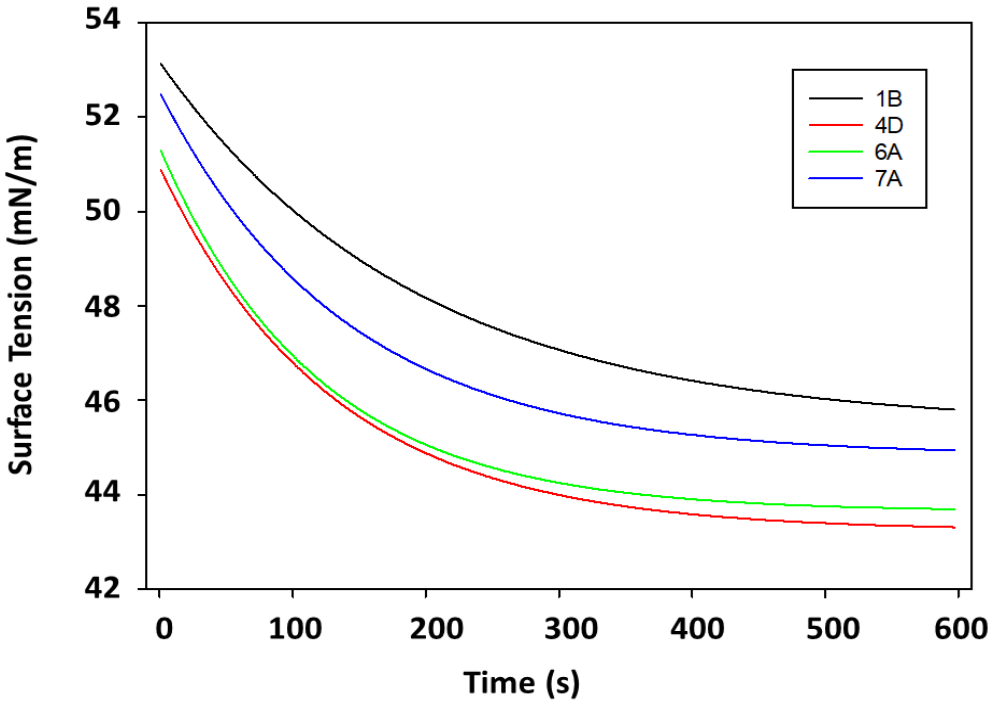


Figure 5.7 Predicted model for surface tension based on the results of the two-stage analysis, using the means of estimated parameter estimates according to the ANOVA on the sets of parameters. The form of the model was *Surface tension* = $\gamma + \alpha$*exp(-β**Time*) where a common α is used as it needs to decay to a value above zero in the fullness of time . Separate γ and β parameters used for the QTLs according to the results of the ANOVAs. There was no significant ($p < 0.05$, F-test) effect of the alleles.

There were greater differences between the QTLs on QTL 1B and 7A compared to the other two in the second year, but little differences between QTL 4D and 6A were observed. In this year, the alleles on QTL 1B showed the slowest exponential rate, significantly differing to the others and was associated with the highest surface tension attained in the fullness of time. The NILs on QTLs 4D and 6A had lower surface tensions in the fullness of time (Figure 5.6).

5.3.4 Elasticity Modulus of Year 2 (2015) material

Unfortunately the second year data showed considerable variability, fluctuating extensively rather than showing a consistent trend, making the model very difficult to fit, as seen by the lower R^2 values compared to year 1. This led to the model predicting a –ve elasticity modulus which is not possible. This is likely due to the high level of noise in the dataset which can be seen from the low R^2 values in Table 5.5 (A).

The set of estimated parameters from the fitted model is given in Table 5.5. All means were based on 36 residual degrees of freedom. The model was: $Elasticity = \delta + \varepsilon(1 - \exp(-\beta * Time))$.

A main effect of QTL (p=0.025, F-test) was observed for the elasticity at time zero, but even this was only a marginally significant effect at the 5% level. With the variability observed in the elasticity dataset, results should be treated with caution. Only the NILs present on the QTL 1B showed significantly different starting elasticities at a theoretical time zero. However, due to the highly varying data obtained in the second year, the elasticity measurements were very poor, with the fitted curve showing a –ve elasticity.

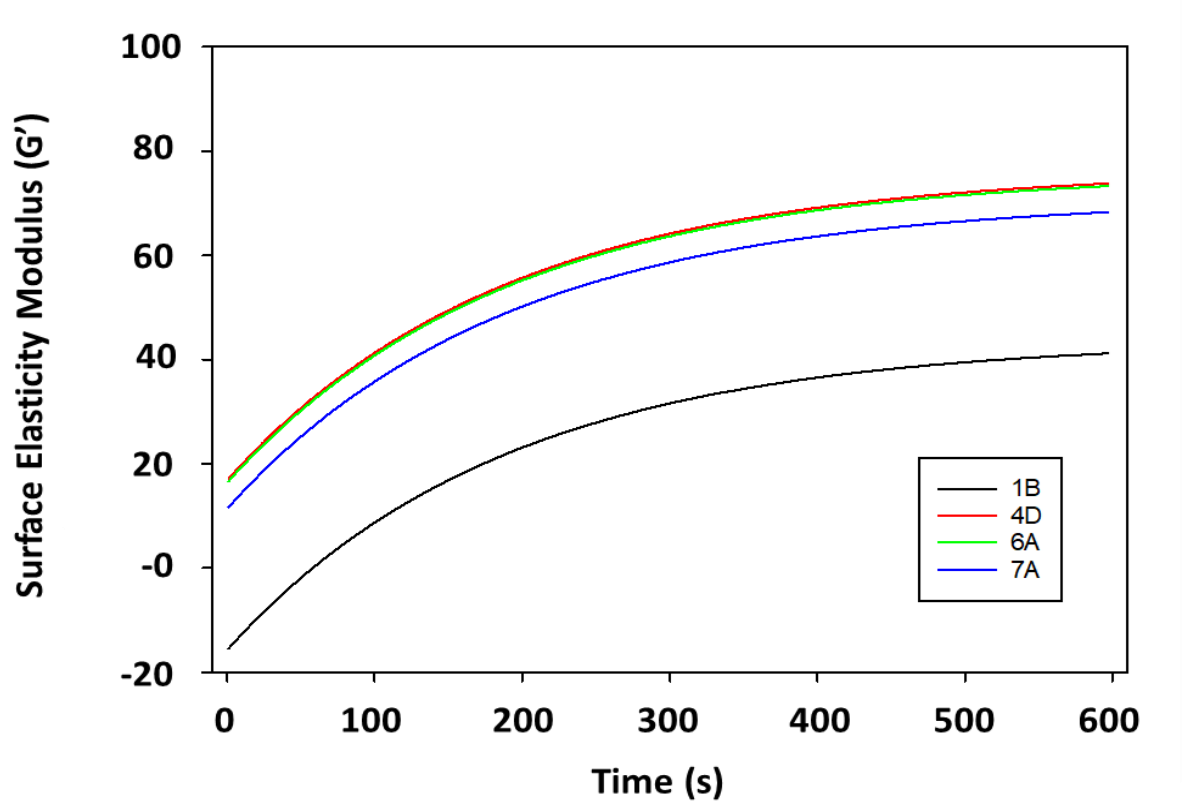


Figure 5.8 Predicted model for elasticity based on the results of the two-stage analysis, using the means of estimated parameter estimates according to the ANOVA on the sets of parameters. The form of the model was $Elasticity = \delta + \varepsilon(1 - \exp(-\beta * Time))$ where a common β and a common ε is used as β and ε ensures that the curve for elasticity plateaus to δ + ε. Separate δ parameters for the QTLs, according to the results of the ANOVAs.

A

QTL	Allele	δ	ε	β	R^2
1B	M	11.7	70.98	0.000218	18.5
1B	M	-29.8	70.36	0.017326	30.1
1B	M	*	*	*	
1B	H	-36.49	76.44	0.009899	58.1
1B	H	25.26	47.47	0.002226	87
1B	H	-56.2	94.81	0.014536	47.5
4D	M	2.91	50.98	0.007765	77.2
4D	M	*	*	*	
4D	M	26.98	51.15	0.001371	79.2
4D	H	13.23	36.37	0.005742	81.5
4D	H	21.03	38.17	0.002684	83.1
4D	H	22	78.91	0.000987	94.2
6A	M	18.87	37.01	0.003312	86.7
6A	M	11.99	38.72	0.004739	65.2
6A	M	19.73	89.88	0.000371	51.4
6A	H	17.7	36.32	0.004383	77.7
6A	H	13.82	33.85	0.005787	32
6A	H	*	*	*	
7A	M	10.37	34.08	0.008756	60.7
7A	M	21.28	212.63	0.000151	71
7A	M	18.13	19.45	0.008705	24.6
7A	H	-2.27	51.12	0.002031	89.4
7A	H	16.03	44.5	0.002701	80.2
7A	H	4.52	56.99	0.004166	79.4

B

	δ	*ε*	*β*
Allele	0.523	0.470	0.814
QTL	**0.041**	0.684	0.287
Allele.QTL	0.862	0.857	0.824

Table 5.5 Data on the estimated parameters for the elasticity modulusof the MxH NIL DDL in year 2 (**A**) The set of estimated parameters for the surface tension fitted model for the second year MxH NIL material. R^2 is a statistical measure of how close the data is to the fitted line for the model. (**B**)The p-values for the F-tests on the estimated parameters.

Although some interesting biophysical effects could be observed on individual years, especially the first year 2013 material, there was little consistency between the two years.

Interfacial tension (IFT) did not show any significant allelic effects. However there was a separation between QTL 1B and 7A compared to QTL 4D and 6A. This separation was observable across both years with QTL 1B in particular showing higher interfacial tension at time zero as well as higher levels of surface tension at the end. Similar effects could be seen for QTL 7A, although to a lesser extent. Once again, it must be noted that theses QTL effects are unlikely to show any genetic significance, as although these effects may be due to background genes, the alleles of the NILs do not contribute to these

On the other hand, elasticity showed some interesting differences in year 1 which was not replicated in year 2. QTL 7A showed some interesting differences in year 1, especially between the allelic combinations of QTL 7A, which was not observed in year 2. While QTL 1B showed significant separation in both years, having the highest surface tension, but the lowest elasticity, this is unlikely to be due to a genetic effect.

5.3.5 Lipidomic analysis of diluted dough liquor fractions in Year 1

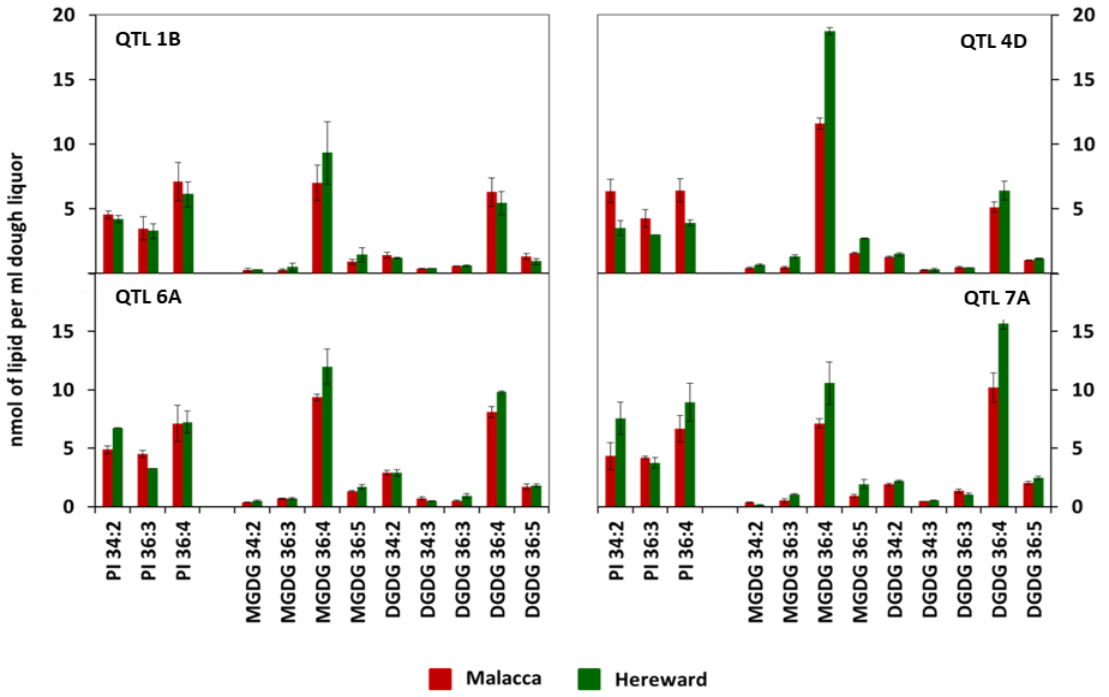


Figure 5.9 The lipid composition of selected lipids in the diluted dough liquor fractions of the first year MxH NIL material. Data are mean values of ±SE of three independent samples analysed via ESI-MS/MS.

The lipid composition of the diluted dough liquor fractions was determined using ESI-MS/MS. The lipids were extracted from the diluted fractions of dough liquor using a method based on Bligh and Dyer (1959) with some modifications.

Three phospholipid classes (LPC, PC, PI), two galactolipid classes (MGDG, DGDG) and two neutral lipid classes were determined (FFA and TAG). The phospholipids PE and PG were not determined due to their very low levels in the diluted dough liquor system. However, the galactolipids MGDG and DGDG gave consistent results between the replicates and samples, and is therefore the only lipid class presented. TAG is largely removed during the dough liquor extraction process, due to the detrimental effects it has on surface properties.

In the first year material, some significant differences in the lipid composition of the diluted dough liquor fractions could be observed. The alleles of QTL 1B showed no significant differences in the compositions of the galactolipids and PI. However, the alleles of the other three QTLs showed significantly greater differences with the Hereward allele showing higher levels of lipids for the major galactolipids MGDG 36:4 as well as DGDG 36:4 across the two years (Figure 5.8).

5.3.6 Lipidomic analysis of diluted dough liquor fractions in Year 2

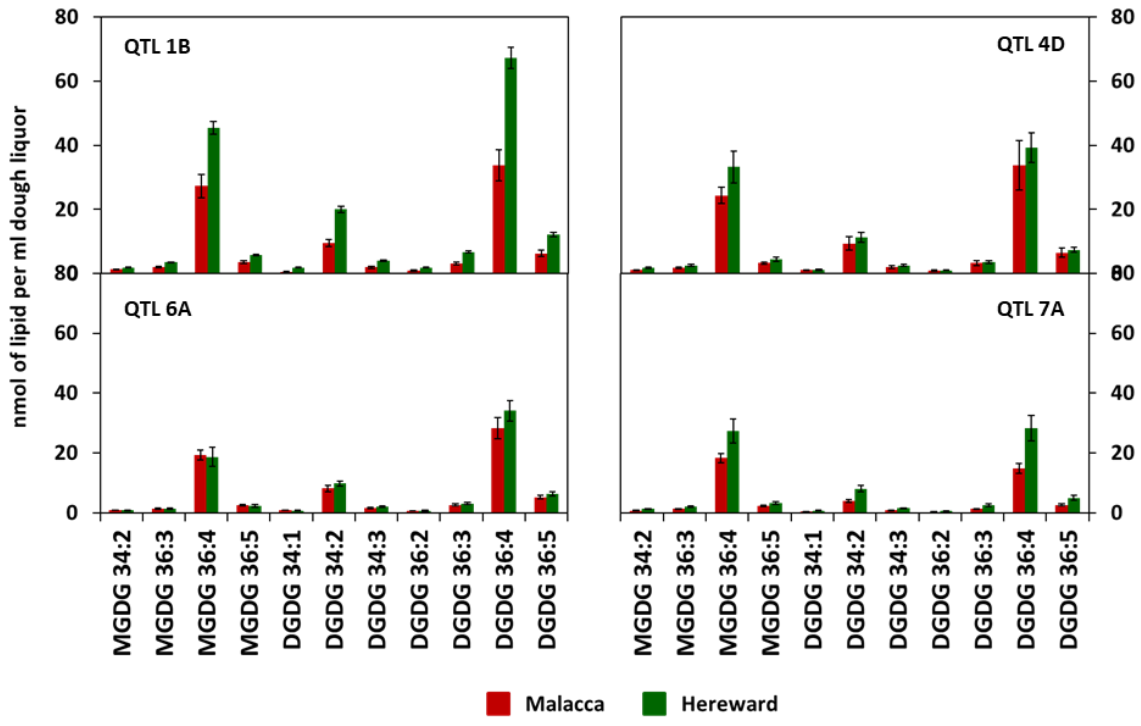


Figure 5.10 The lipid composition of selected lipids in the diluted dough liquor fractions of the second year MxH NIL material. Data are mean values of ±SE of three independent samples analysed via ESI-MS/MS.

The second year analysis showed less significant lipid differences between the QTL allelic regions. However, the QTL 1B alleles showed significant galactolipid differences, with the Hereward allele being associated with higher GL content. Similar effects were also observed for the 7A alleles while the alleles at the 4D and 6A QTLs showed no significant lipid profile differences (Figure 5.9).

However, there were very few consistencies in the data between the two years of material. While the galactolipid profile of QTL 1B alleles showed no significant differences in year 1, they showed significant differences in year 2. The alleles at the 4D and 7A QTLs showed significant lipid differences in year 1, but no differences in year 2. Only the alleles at the 7A QTL showed consistent lipid profile differences across the two years.

Furthermore, a significant difference in the quantities of the DDL could be observed across the two years, with the second year having significantly higher quantities of lipids. Although the reasons for this is unclear, this may be the result of better lipid extraction from the DDL fractions, as the extraction in the second year was performed with fresher material.

Similarly, it is difficult to relate these lipid profile differences to effects on surface rheology. Significant differences in the elasticity modulus were observed for the alleles on QTL 7A in year 1 which correlated with significant differences in the proportions of galactolipids in the diluted dough liquor. However, while similar differences in the lipid profile were observed in the second year, no functional differences were observed.

5.3.7 The enrichment of specific lipid classes in the diluted dough liquor fraction

Among the lipid classes, significant differences were observed consistently between the galactolipid profile of the QTL 7A NILs. These differences were consistent across two years, and two different systems, flour and diluted dough liquor. In dough liquor, lipids are present in lower quantities than flour (Salt et al., 2017), and even more so for diluted dough liquor. Also the lipid profile would differ significantly due to the removal of one of the major lipid classes in the flour fraction, TAG. With changes occurring in the lipid proportions, it would be interesting to see which lipid classes became enriched in the diluted dough liquor fraction compared to flour.

Due to the low levels of minor phospholipids such as PE, PI and PG which could not be measured consistently, these lipid classes were removed from the study.

LPC was present in lower proportions in DDL compared to flour in both years. However this difference was particularly prominent in year 1. PC was more enriched in the DDL compared to flour in both years. The ratio of LPC:PC in the diluted dough liquor (DDL) fractions reflect the

observations made in flour, with the levels of LPC being significantly higher than PC in year 1, while their levels remained relatively even in year 2 (Chapter 4, Figure 4.2, 4.3). The level of LPC and PC was reflective of what was observed in the flour samples. As with flour, significantly higher levels of LPC and lower levels of PC were observed in the first year, whereas the second year showed relatively similar amounts. This is again most likely due to the storage conditions the grain and flour were subject to prior to the analysis.

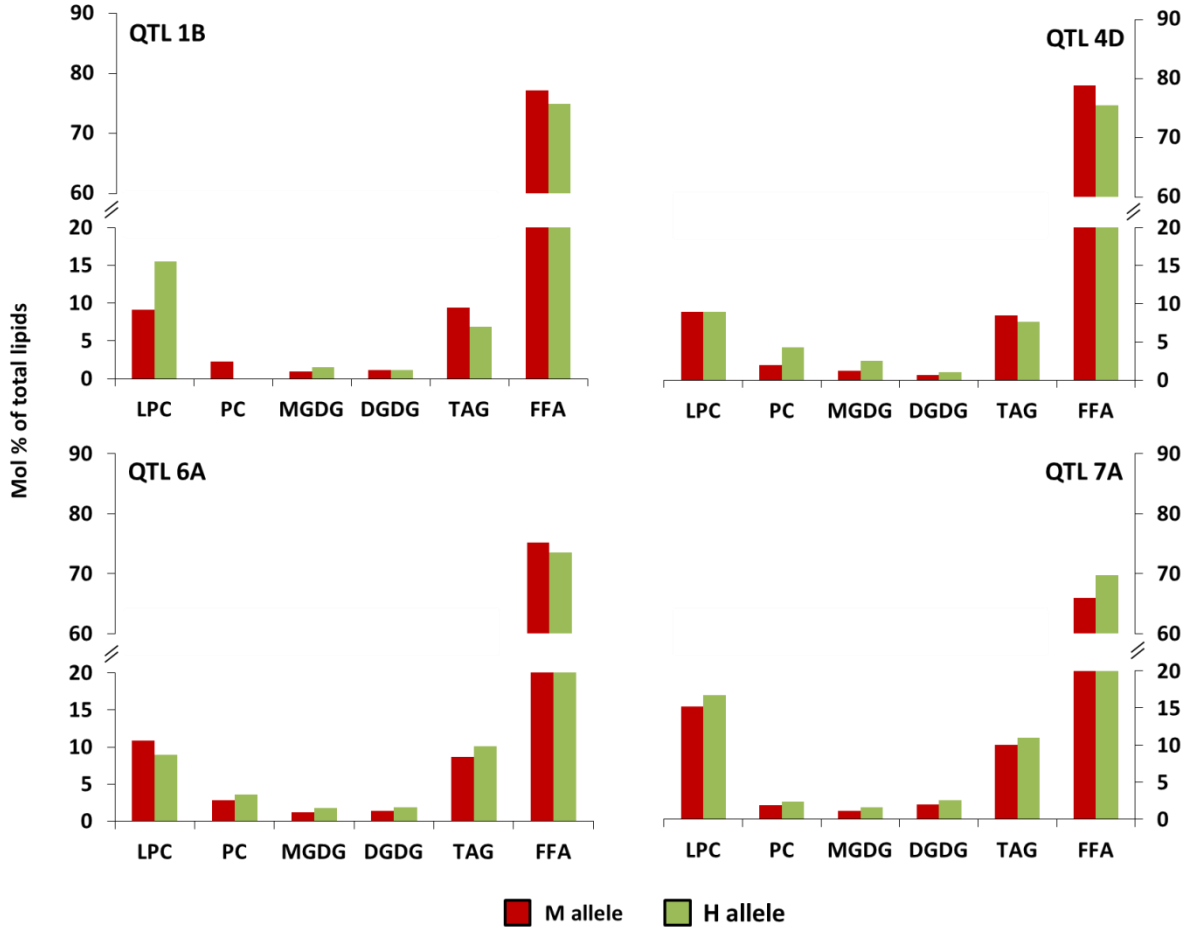


Figure 5.11 proportions of specific lipids present in the diluted dough liquor fraction of the MxH NILs in the year 1 (2013) dataset. The selected lipids were observed consistently across the two years of study. However it must be noted that these percentages are the mol% of total lipids measured in the diluted dough liquor, and is not directly comparable to the proportions of lipids present in flour.

The galactolipids MGDG and DGDG showed very small differences between the DDL and flour, being slightly enriched for most of the QTL x allele combinations across both years in the DDL fractions. Exceptions to this include 7A:M DGDG in year 1 and MGDG and DGDG for 7A:M and

7A:H in year 2. TAG was present in far lower levels in the DDL than flour. Although this was expected, there were higher than expected levels of TAG in the first year data which was surprising. FFA showed the greatest differences, being massively enriched in the DDL fraction, present in between 65-80% of the total measured lipids in both years. (Figure 5.10 & 11) This will partially be due to the significantly reduced amount of phospholipid and TAG measured in the study, but also may be an artefact of further lipid degradation during the DDL extraction process. This is feasible considering the DDL extraction protocol lacks the steps for reducing enzymatic activity, such as heat treating flour, or extraction with isopropanol compared to the lipid extraction of white flour.

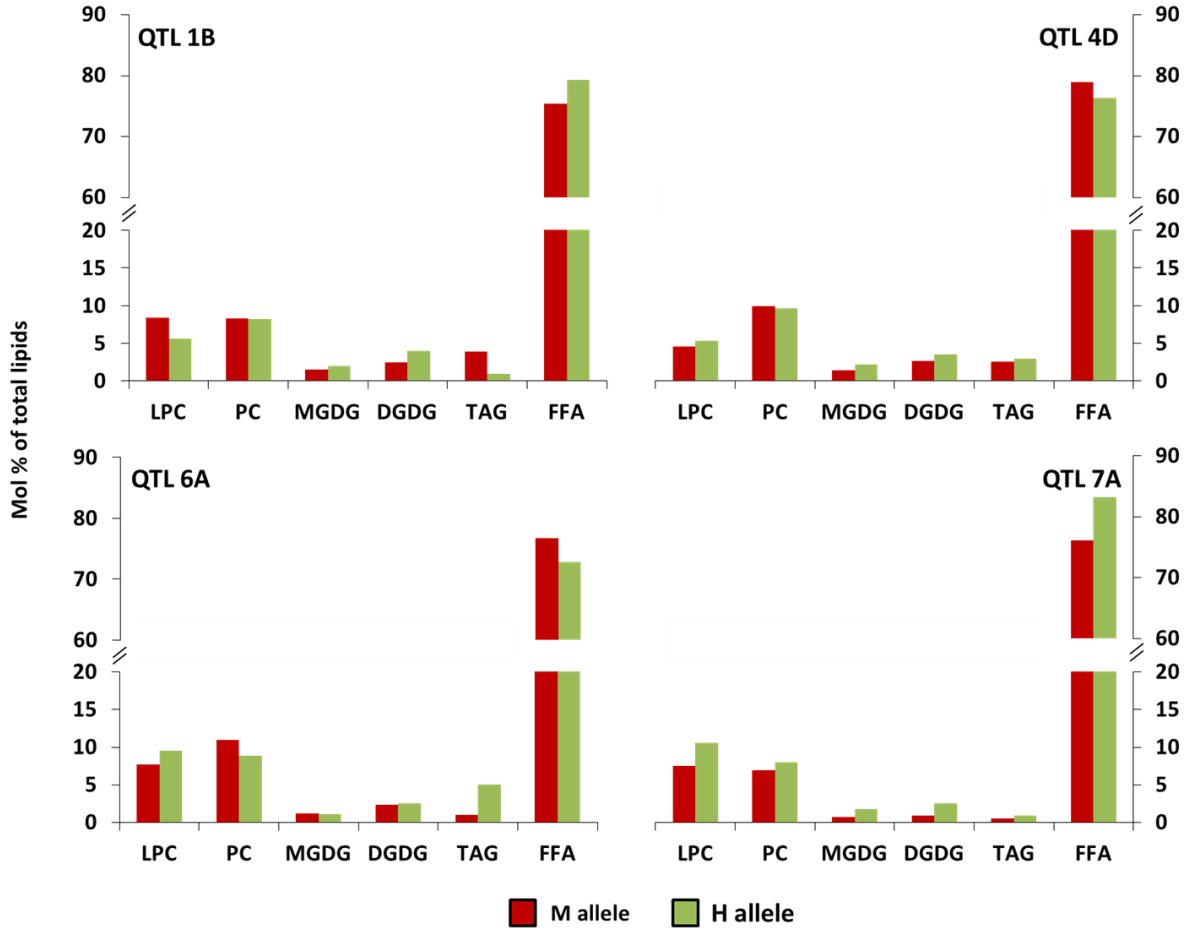


Figure 5.12 proportions of specific lipids present in the diluted dough liquor fraction of the MxH NILs in the year (2015) dataset. The selected lipids were observed consistently across the two years of study. However it must be noted that these percentages are the Mol% of total lipids measured in the diluted dough liquor, and is not directly comparable to the proportions of lipids present in flour.

5.4 Discussion

The first year material in particular showed some interesting rheological differences. The QTL 7A:M and 7A:H alleles showed significant differences in their elasticity modulus. However, no allelic differences were seen for surface tension. As the elastic modulus is sensitive to the interfacial composition of dough liquor, there may be a potential difference in the interfacial composition of 7A:M and 7A:H. However the lack of variation in surface tension indicates that the concentration of these components may not differ. This was not the case for the galactolipids in the QTL 7A dough liquor, as significant differences in the quantities of galactolipids could be seen between the QTL 7A alleles across the two years. The interpretation of the diluted dough liquor data was difficult as it cannot be directly compared to standard DL measurements as the preparation method is different. Also, being a diluted system, differentiating between the components potentially acting on the surface tension and elasticity of the diluted dough liquor is difficult.

Little consistency was observed across the two years. The differences in elasticity observed in the year 1 material for QTL 7A was not observed in year 2. There was a significant difference in the elasticity modulus for QTL 1B compared to the other QTLs which was not observed in year 1.

Despite the differences in lipid composition observed in the first year material, we were unable to observe significant and consistent functional differences between our NILs. No consistent elasticity or surface tension effects were seen for any of the QTLs or their alleles across the two years.

Furthermore, there were greater effects of QTLs observed compared to the specific alleles of the NILs, which was most noticeable for the surface tension of the DDL. These QTL effects are not genetic effects related to the NILs, and as mentioned in Chapter 4, ideally the NILs should show little to no separation between the surface tensions of any of the QTLs. Only the Hereward allele should show separation. These large differences between the surface tensions of the QTLs indicate that some other factor is contributing to this observation.

While the alleles of the NILs are not providing these surface tension differences, it may be caused by a background gene. However, considering the continual inconsistency of the surface tension effects this is unlikely. While year one showed the greatest separation of the QTL 6A, the second year showed the greatest separation of the QTLs 1B and 7A. Considering the nature of the analysis, these differences may be due to the experimental methodology. Due to the high level of dilution, any difference in the flour content, or even the room temperature during drop analysis could affect the surface tension. However, considering the consistency of the results and the fact that surface tension results are continually checked for contamination by measuring the surface tension of pure water, this is unlikely. Considering the large environmental effects that have been seen across the two years in

the flour fraction, this may account for the differences observed between the two years. Other biological components that have not been measured may significantly alter the surface properties of dough liquor and therefore may provide an explanation for some of the differences.

However while the genetic effects of the NILs on the surface properties of dough liquor are certainly weak, it is difficult to expect that these genetic differences would provide a biochemical phenotype accurate and consistent enough to provide little to no separation between the individual QTLs. As mentioned in Chapter 4, a lipid phenotype is difficult to measure effectively compared to simple quantifiable trait such as height or disease resistance, and is subject to enormous environmental and handling effects as can be seen from Chapters 3 and 4.

Overall, while there were some interesting lipid differences, these could not be correlated with rheological differences. The alleles at the 7A QTL showed significant differences in galactolipid composition, even in the diluted dough liquor fractions across the two years, the only QTL candidate to do so. However no differences were observed in the surface tension of the QTL 7A alleles across both years. Although there was no significant and consistent correlation between the lipid composition and surface properties, this may be due to the similarity of the lipid profiles of the MxH NILs in general. Also the DDL analysis may not be sensitive enough to pick up such small changes.

With G x E effects playing a major role in determining various components of wheat (Naryanan et al., 2014, Min et al., 2014) and year by year effects influencing the lipid composition of the NILs, (Chapter 4) the biophysical analysis of diluted dough liquor in this study was not sensitive enough to capture the already low lipid differences that occur between the NILs across the two years. Due to a lack of flour, the sensitivity of this study could not be improved through increased replication and avoiding the need to dilute the samples.

However, it is possibly too early to claim that the diluted dough liquor protocol was unsuccessful, as some positive results were seen. With further replication and some adaptation, this method may be used to identify functional differences in wheat flour on a small scale. Investigation into the surface properties of Hereward flour across the three years showed large differences between the years (Salt et al., 2017). Even with a 50% diluted preparation method, these differences were successfully picked up across two years (2012-2013). This indicates that the diluted dough liquor fraction was capable of replicating some of the sensitivity of the concentrated DL assay.

The QTL 7A for cell size/loaf volume is clearly the most interesting candidate for affecting gas bubble stability in dough, showing the most consistent lipid differences compared to the other QTL combinations. Significant differences in the galactolipid profile were observed between the two alleles

across two years in a diluted dough liquor system. This makes it a highly interesting candidate for future work.

While the availability of only small flour samples made it necessary to dilute the dough liquor system, therefore future work using undiluted dough liquor should provide more definitive results.

Chapter 6 FAMEs analysis of the MxH DH population

6.1 Introduction

The MxH NILs introduced in Chapter 4 were developed from the MxH Doubled Haploid (DH) population. These were developed as part of a HGCA funded project between 2001-2007, Investigating Wheat Functionality through Breeding and End Use. Three breadmaking populations were developed to map QTLs for milling and baking parameters (Millar et al., 2008).

6.1.1 DH production

The role of doubled haploids in genetics has a long history (Imperial Bureau of Plant Genetics, 1936) but was not implemented in wheat breeding until far more recently. Previously, DH generation worked by taking gametes from an F1, which are cultured to create haploid individuals. These are then treated to double up chromosome numbers and create diploid individuals which are completely homozygous. These are then multiplied and field tested.

However in wheat, wide crosses which crosses distantly related plant species together, have been used to produce haploids for crop improvement and genetic studies (Baum et al., 1992). Bread wheat DHs are produced by various intergenic crosses with maize (Suenaga and Nakajima 1989; Laurie and Benett, 1988; Inagaki and Tahir, 1990; Laurie and Reymondie, 1991). After fertilization, maize chromosomes are almost immediately eliminated (Laurie & Bennett, 1988). Due to the absence of adequate endosperm development, spikes are treated with plant growth regulators (PGRs) which stimulate embryo growth to a stage where they can be rescued onto a nutrient medium (12-16 days post pollination). The produced haploid seedlings are treated with colchicine to restore their diploid chromosome number. Due to the expense in creating DH lines, there is usually greater care taken when selecting parents and crosses are less likely to be created on a speculative basis.

DH production has two key advantages over other breeding methods. The lines produced are completely homozygous and the process is significantly quicker. With selfing, expected heterozygosity declines at a rate of ½ per generation. Therefore even at the F6 generation, 1/32 of the genome is heterozygous on average, and it takes many generations for breeders to be confident in the complete homozygosity of the line.

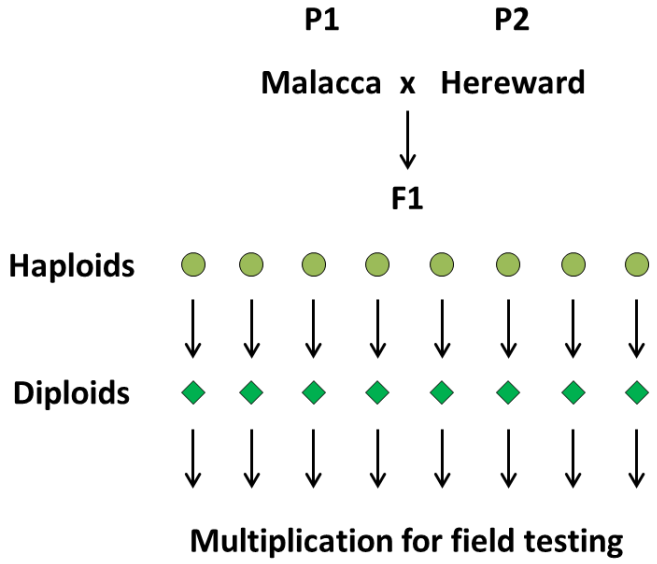


Figure 6.1 Schematic for the generation of the MxH DH populations

However, there are some disadvantages to DH production. As many breeders test lines in yield trials at the F3 and F4 generation, the time taken to create new lines is not greatly different between DH and other techniques such as single seed descent (SSD). In this case, the benefits of complete homozygosity do not outweigh the cost. Due to this, DHs are not favoured for all crops. In fact for barley, where DH production is well established, breeders still prefer to use SSD over DH for routine breeding. Also as DH progeny lines are produced through single meiosis, they are less recombined than SSD lines, which accumulate recombination over generations of selfing.

Therefore DH production is used for a small number of elite crosses where there is high expectation that high value progeny lines will be found, and there is a time limit to deliver a line.

6.1.2 The MxH doubled haploid population

The MxH Doubled Haploid population was produced using the inter-specific wheat x maize hybridization methodology first developed at The Plant Breeding Institute, modified by the John Innes Centre and in-house at Syngenta and RAGT (Millar et al., 2007). The robustness and integrity of the DH lines were assessed preliminarily by breeders using HMW glutenin subunit analysis.

The MxH population comprised 115 DH lines of which 15 were discarded for containing non-parental alleles. Therefore 100 lines were used to create the genetic map. A total of 580 Simple Sequence Repeat (SSR) markers were screened, of which 249 were polymorphic. In addition to these, a further

305 markers were provided from Diversity Arrays Technology (DArT) analysis, giving a total of 554 polymorphic markers. However among these, 203 co-segregated and 26 had no linkage. Therefore the final map consisted of 288 markers mapping into 35 linkage groups, with a total distance of 1021 cM.

	QTL 1B- Cell Number										QTL 4D- Cell Number			QTL 6A- Cell Number					QTL 7A- LV WT, CD		
	gwm264	wPt3465	wPt0328	wPt5801	wmc085	barc152	wPt5562	wPt8971	wmc500	barc008	barc098	psp3103	gdm129	gwm334	gdm036	wPt1290	wPt2573	barc003	psp3001	wPt6019	wPt1259
Sample No	1B	1B	1B	1B	1B	1B	1B	1B	1B	1B	4D	4D	4D	6A	6A	6A	6A	6A	7A	7A	7A
1	A	A	A	A	A	A	-	A	A	A	B	-	B	A	A	A	A	B	A	A	A
2	B	A	A	A	B	A	B	A	B	B	B	-	B	A	A	A	A	A	B	A	A
5	B	B	B	B	B	B	B	B	B	B	B	B	-	A	B	B	B	B	B	B	B
6	A	A	A	A	A	A	-	A	A	A	B	B	B	B	B	B	B	B	A	A	A
8	B	B	B	B	B	B	B	B	B	B	A	A	A	B	B	B	B	B	B	B	B
9	B	A	B	B	B	B	B	B	B	B	A	A	-	B	B	B	B	B	A	A	A
10	A	B	B	B	B	B	B	B	-	B	A	A	A	A	A	A	A	A	B	B	B
12	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
14	A	A	A	A	A	A	A	A	A	A	-	B	B	B	B	A	-	-	A	-	-
15	B	A	A	A	B	A	B	B	B	B	A	A	A	B	B	B	B	B	B	B	B
16	A	A	A	A	A	A	A	A	A	A	A	A	-	B	B	B	B	B	B	B	B
17	B	A	A	A	B	A	A	A	A	A	A	A	-	B	B	B	B	B	B	B	B
18	A	A	A	A	A	A	-	A	A	A	A	A	-	B	B	B	B	B	A	A	A
19	B	B	B	B	B	B	B	B	B	B	A	A	A	A	B	B	B	B	A	A	A
20	B	B	B	-	B	B	-	B	B	B	B	B	-	B	B	B	B	B	B	-	-
21	A	A	A	A	A	A	A	A	A	A	B	B	-	B	A	-	A	A	B	-	-
22	B	-	-	-	B	B	-	-	B	B	A	A	A	A	A	-	-	B	A	-	-
23	A	A	-	A	A	A	A	A	A	A	B	B	B	A	A	-	B	A	A	A	B
24	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	-	A	A	B	B	B
25	B	B	B	B	B	B	B	B	B	B	A	A	A	A	B	B	B	B	A	A	-
28	A	B	B	B	B	B	-	B	B	B	B	B	B	A	A	B	B	B	A	B	B
32	B	B	B	B	B	B	-	B	-	B	B	B	B	A	A	A	A	A	A	A	A
33	A	A	A	A	A	A	-	A	B	B	A	A	A	A	A	-	A	A	B	B	B
37	A	A	A	A	A	A	-	A	B	B	B	B	B	B	B	B	B	B	B	B	B
39	A	A	-	A	-	A	A	-	A	A	A	A	A	A	A	-	-	A	A	A	-
43	A	B	A	A	A	A	A	A	B	A	B	B	B	A	A	A	A	A	B	B	B
45	B	A	A	B	B	B	B	B	B	B	A	A	B	B	B	B	B	B	A	-	-
46	A	-	-	-	A	A	-	-	A	A	A	A	A	A	A	-	-	A	B	-	-
47	A	B	B	B	B	B	B	B	B	B	B	B	B	A	A	A	-	A	B	B	B
48	A	A	-	A	A	A	A	A	A	A	A	A	A	B	B	-	B	B	A	A	A
49	A	A	-	A	B	A	A	A	B	B	A	A	-	A	A	A	A	A	A	A	A
50	A	B	A	A	B	A	A	A	A	A	B	B	-	B	B	-	B	B	B	A	A
52	A	A	A	A	A	A	A	A	A	A	A	A	A	B	A	B	A	A	A	A	A
54	B	A	A	B	B	B	-	B	B	B	A	A	A	B	A	B	A	A	B	B	B
55	A	A	-	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	-
56	A	A	-	A	A	A	A	A	A	-	A	A	A	A	A	A	A	A	A	A	A
57	A	A	A	B	A	A	A	A	A	A	B	B	-	B	A	-	A	A	A	B	B
58	A	B	B	B	B	B	-	B	-	B	B	B	B	A	B	B	B	B	A	B	B
59	B	B	B	B	B	B	A	A	A	A	A	A	B	B	B	B	B	B	B	B	B
60	A	A	A	A	A	A	A	A	A	A	B	B	B	A	A	B	A	A	B	B	B
61	B	B	B	B	B	B	-	B	B	B	B	B	-	B	B	B	B	B	A	A	A
62	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	-
63	B	B	B	B	B	B	B	B	B	B	B	B	B	A	-	A	-	A	B	B	B
64	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B	A	A
65	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	B	A	A	A
66	A	B	B	-	B	B	-	B	B	B	A	A	A	B	A	A	A	A	B	B	B
67	A	A	A	A	A	A	A	A	A	A	B	B	B	A	A	A	A	A	-	B	B
68	A	A	A	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	A	A	A
69	A	A	A	A	A	A	A	A	A	A	B	-	B	B	B	B	B	B	A	A	B
70	A	A	A	-	A	B	B	B	B	B	-	B	B	B	B	-	B	B	A	A	A
71	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	B	A	A	A
72	A	A	-	A	A	A	A	A	A	A	A	A	A	A	A	B	B	B	A	A	A
74	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
75	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B
76	A	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
77	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
78	A	B	B	B	B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	-	A
79	A	A	A	A	A	B	B	B	B	B	B	B	B	A	A	A	A	A	B	A	A
80	B	B	B	B	B	B	B	A	B	B	B	B	-	B	B	B	B	B	B	A	A
82	A	-	A	A	A	A	A	A	A	A	A	A	B	A	A	A	A	A	B	-	-
83	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	B	A	A	A
84	B	B	B	B	B	B	B	B	B	B	B	B	-	B	B	B	B	B	B	B	B
96s	A	B	B	A	A	A	A	A	A	A	B	B	-	A	B	B	B	B	A	A	A
86	A	-	A	A	A	A	-	A	A	A	A	A	A	A	-	A	A	A	A	A	-
87	A	A	A	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	A	B	B
88	B	B	B	B	B	B	B	B	B	B	A	A	A	B	A	-	A	A	A	A	-
89	B	B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	B	B	B
91	A	B	B	B	B	B	B	B	B	B	B	B	-	B	B	B	B	B	B	B	B
92	A	B	B	B	-	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
93	A	B	B	B	B	B	B	B	B	B	B	B	B	A	A	A	A	A	B	A	-
94	B	B	B	B	B	A	-	A	A	A	B	B	B	A	B	B	B	B	A	B	B
95	B	B	B	B	B	-	B	B	-	-	-	B	B	B	-	B	B	B	B	B	B
96 t	A	A	B	A	A	-	A	A	-	-	-	A	A	A	-	A	A	A	A	A	A
97	A	-	-	-	B	B	-	-	B	B	A	A	A	B	B	-	-	B	-	-	-
98	A	B	B	B	B	B	B	B	B	B	B	B	B	A	A	A	A	A	-	B	B
99	B	B	B	B	B	A	A	A	A	-	B	B	B	A	-	A	B	B	-	-	B
100	B	B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	-	A	B
101	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	B	-	-	A
102	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	-	-	A
103	B	B	B	B	B	B	-	A	A	A	B	B	B	A	A	A	-	A	-	-	A
106	B	-	-	-	B	B	-	-	B	B	B	B	B	B	B	-	-	B	-	-	-
107	A	-	-	-	A	B	-	-	A	A	B	B	B	B	-	-	-	B	-	-	-
108	B	-	-	-	B	B	-	-	B	B	A	A	A	A	B	-	-	B	-	-	-
110	B	-	-	-	B	B	-	-	B	B	A	A	A	A	B	-	-	B	-	-	-
111	A	-	-	-	A	A	-	-	A	A	B	B	B	B	B	-	-	B	-	-	-
112	B	-	-	-	B	B	-	-	B	B	B	B	B	B	B	-	-	B	-	-	-
115	B	-	-	-	B	B	-	-	B	B	B	B	B	A	A	-	-	A	-	-	-

Figure 6.2 Genotype Scores for the MxH DH mapping population. Areas highlighted in light green indicate markers with the Genotype score: Malacca indicated by (**A**). Areas highlighted in dark green indicate markers with the genotype score: Hereward, indicated by (**B**).

6.1.2.1 Simple Sequence Repeats (SSR)

SSR, sometimes called microsatellites, are a tract of repetitive DNA where certain DNA motifs (ranging from 2-5 base pairs) are repeated. It is a type of Variable Number Tandem Repeat. SSRs are co dominant molecular markers that distinguish homozygotic and heterozygotic individuals that possess a large number of alleles.

6.1.2.2 Diversity Arrays Technology (DArT)

DArT employs a hybridization based approach to type thousands of genomic loci in parallel. The DNA of each individual line is hybridized to a chip containing around 1000 DNA fragments. Polymorphisms can be identified through the presence or absence of hybridization of genomic DNA from each individual line. DArT can classify germplasm without any prior knowledge of genomic sequences and can identify several hundred polymorphisms simultaneously.

Due to the significant GxE effects in the MxH DH population across two years (2005, 2006), false positive QTLs could be detected. Therefore the QTLs identified must be consistent across the two years. The QTLs were required to show consistencies in their location and their effects across products. Products here included various types of breadmaking quality trials such as CBP, Spiral White and Wholemeal. Details on CBP and Spiral white can be found in chapter 1.

A total of 179 QTLs were identified from the MxH DH population for a wide range of breadmaking quality traits. Among these, QTLs present on the chromosomes 1B, 4D, 6A and 7A were selected on the basis of their characteristics, as they were shown to influence important quality traits such as gas cell number, loaf volume and gas cell wall thickness. These QTLs were selected for the development of NILs used for the work reported in chapters 4 and 5.

Using the QTL maps, it was possible to identify the markers for good and poor alleles at the four QTL regions of interest, the QTLs on chromosomes 1B, 4D, 6A and 7A. The markers identified in the four QTL regions can be found in the second row of figure 6.2. To these markers, a genotype score could be applied, with which it was possible to identify the homozygous and recombinant individuals within the DH population for each of the QTLs (Figure 6.2).

The previous chapters of this thesis have outlined how genotype can affect the lipid composition of wheat flour. Using the MxH NILs, some significant differences could be observed in the lipid composition of specific lipid classes, associated with the Hereward or Malacca allele for a given QTL. It was therefore feasible to consider that the lipid composition of the MxH DH population may be affected as well.

Due to time constraints, it was not possible to perform detailed lipidomic analysis using ESI-MS/MS on the whole MxH DH population with the necessary replications. Therefore Fatty Acid Methyl Ester-Gas Chromatography Flame Ionisation Detection (GC-FID) analysis was performed in order to observe the potential effects these QTLs have on the fatty acid composition of white flour. FAMEs are derived from acyl lipids and FFA, obtained by the transesterification of fatty acids with methanol. The FAMEs GC-FID method used in this study follows the method described in the Materials and Methods (Chapter 2).

This study used the MxH DH population grown and harvested in 2012. However, due to missing samples over the years, primarily due to harvesting issues, 88 of the 100 line mapping population remained for the analysis. Samples were milled using a Chopin CD1 mill to produce white flour in the same way as described in chapters 3, 4 and 5.

6.1.3 SDS-PAGE analysis

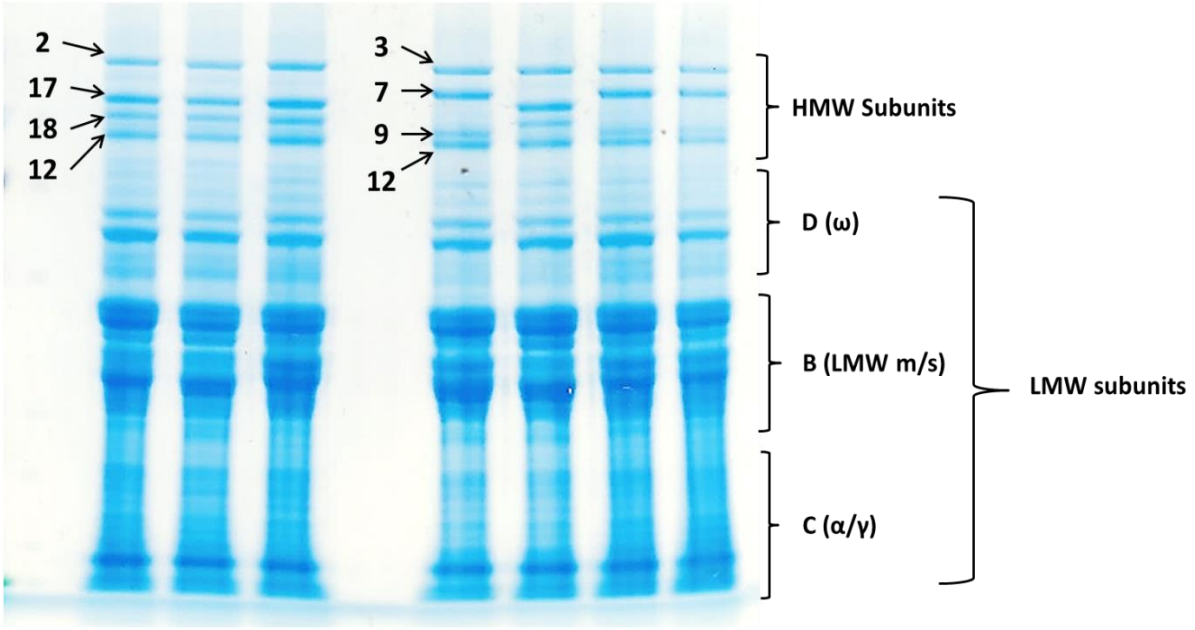


Figure 6.3 SDS-PAGE of HMW glutenin subunits. The numbering on the HMW glutenin subunits is according to Payne and Lawrence (1983) with subsequent modifications by other workers. **2**- 1Dx2, **3**- 1Dx3, **7**- 1Bx7, **9**-1By9, **12**- 1Dy12, **17**-1Bx17, **18**- 1By18.

Over the years, the MxH DH population was used separately by groups between the John Innes Centre (JIC) and Rothamsted Research. This resulted in the use of a different numbering scheme for the population and it was therefore necessary to confirm the identities of the lines. Lines within the population were identified by the analysis of HMW glutenin subunits by SDS-PAGE (Sodium

Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis) analysis. This also allowed the recombinants within the population to be identified.

The HMW subunits are controlled by genes located on the long arm of the homeologous group 1 chromosomes of hexaploid bread wheat at loci designated as *Glu-A1*, *Glu-B1* and *Glu-D1*. Each Glu-1 locus contains two tightly linked genes encoding subunits of high and low Mr, which are termed x- and y- types respectively. A numbering system was developed for allelic variants with three major alleles being identified at the Glu-A1 locus, eleven at the Glu-B1 locus and five at the Glu-D1 locus (Payne & Lawrence, 1983). Using this system, the HMW glutenin subunits were identified and scored in the DH lines (Figure 6.3). The scores of the full MxH DH population can be found in appendix figure 6.1.

6.2 Results

6.2.1 FAMEs-GC analysis

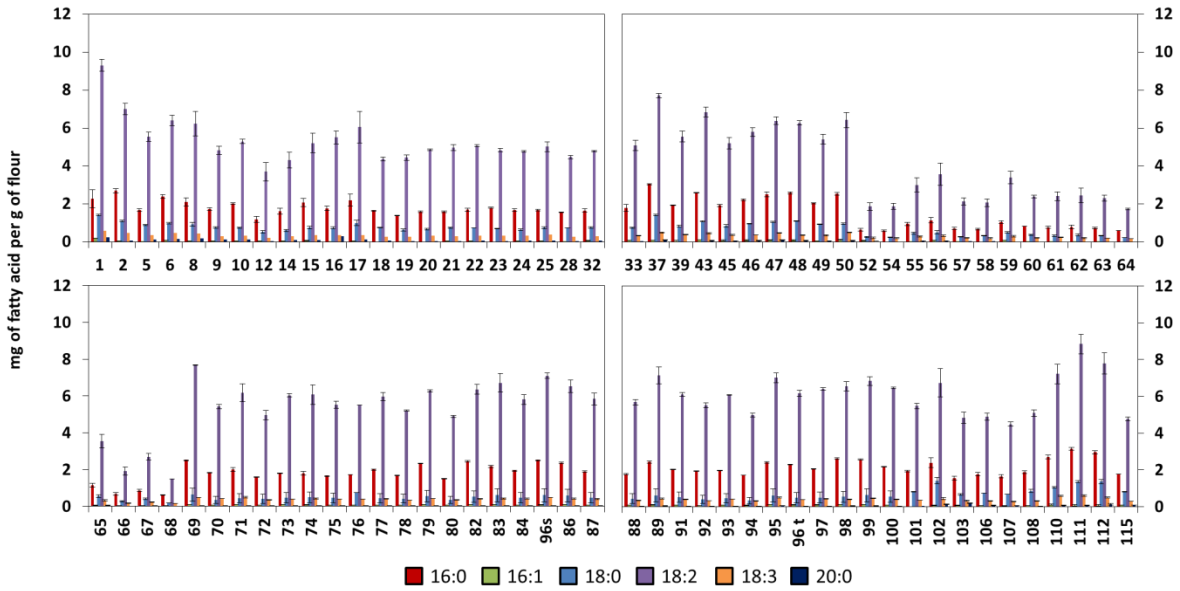


Figure 6.4 FAMEs analysis of the MxH DH population. 6 fatty acids were identified, FA 16:0, 16:1, 18:0, 18:2, 18:3 and 20:0 (acyl carbons:number of desaturations). Data are mean values ±SE of three replicates analysed via GC-FID.

A genotype score was applied to the marker regions for the four QTL regions of interest, with which it was possible to identify the homozygous and recombinant individuals within the DH population for

each of the QTLs (Figure 6.2). The fatty acid profiles of the MxH DH population could be related to members of the DH population homozygous for the Malacca allele or the Hereward allele. As the MxH NILs showed interesting lipid differences, it was possible that genotypic differences would affect the fatty acid profile of the MxH DH population.

Data for the FAMEs 16:0, 16:1, 18:0, 18:2, 18:3 and 20:0 were obtained for the 88 lines in the MxH DH population. Some clear differences in the fatty acid composition could be observed between the lines but the majority of the population showed similar levels of total fatty acids, ranging from 6-8 mg of fatty acid per gram of flour. Lines 52 and 54 in particular showed significantly lower levels of fatty acid content compared to the other samples while lines 1, 69 and 111 showed significantly higher levels, notably for FAME 18:2 (Figure 6.4). These latter lines contained 11-14 mg of fatty acid in total per gram of flour, significantly higher than the average, while samples 52 and 54 contained approximately 3-4 mg of fatty acid per gram of flour (Figure 6.5).

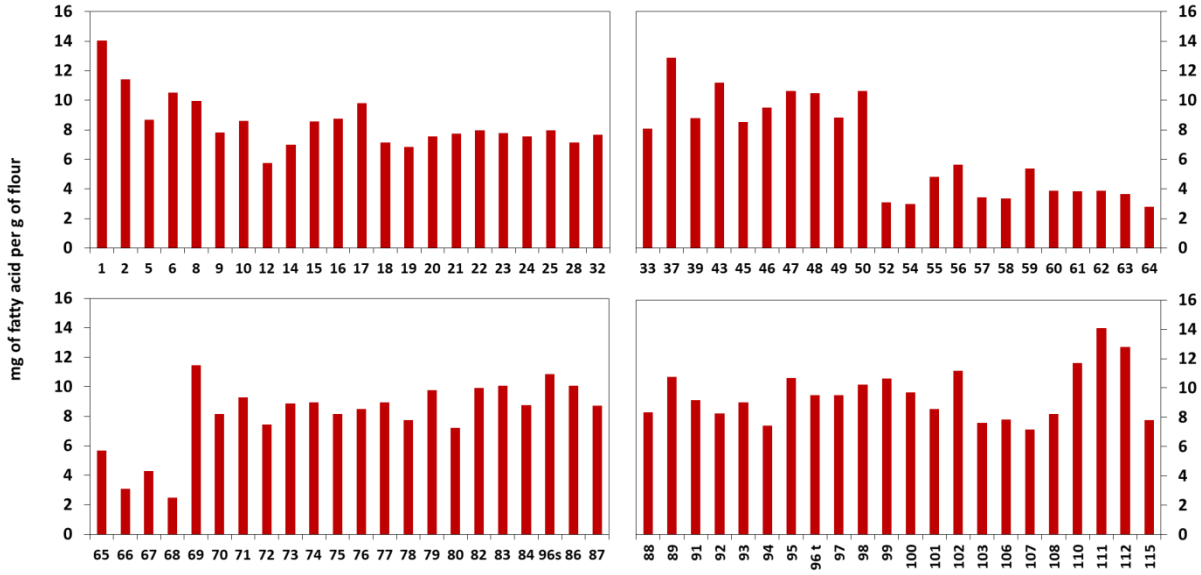


Figure 6.5 FAMEs analysis of the total fatty acids present in the MxH DH population. Data are mean values ±SE of three replicates analysed via GC-FID.

6.2.2 QTL mapping of the fatty acids in wheat flour

Through collaborations with the John Innes Centre, conventional QTL analysis was performed in order to provide an unbiased means to identify loci associated with the traits of interest. Traits of

interest for the analysis included the individual fatty acids such as FA 16:0, 16:1, 18:0, 18:2, 18:3 and 20:0, as well as the total FA values and the total saturated and unsaturated fatty acids.

Unfortunately no QTLs were identified for any of the traits at the standard level. Therefore the significance threshold of the analysis was artificially set to 1.5 LOD, as opposed to 3, in order to find a 'near QTL.' Through this, some effects on the QTL 6B and one effect on the QTL 7A was detected, primarily for FA 20:0.Tables for the QTL analysis can be found in Appendix Figure ().

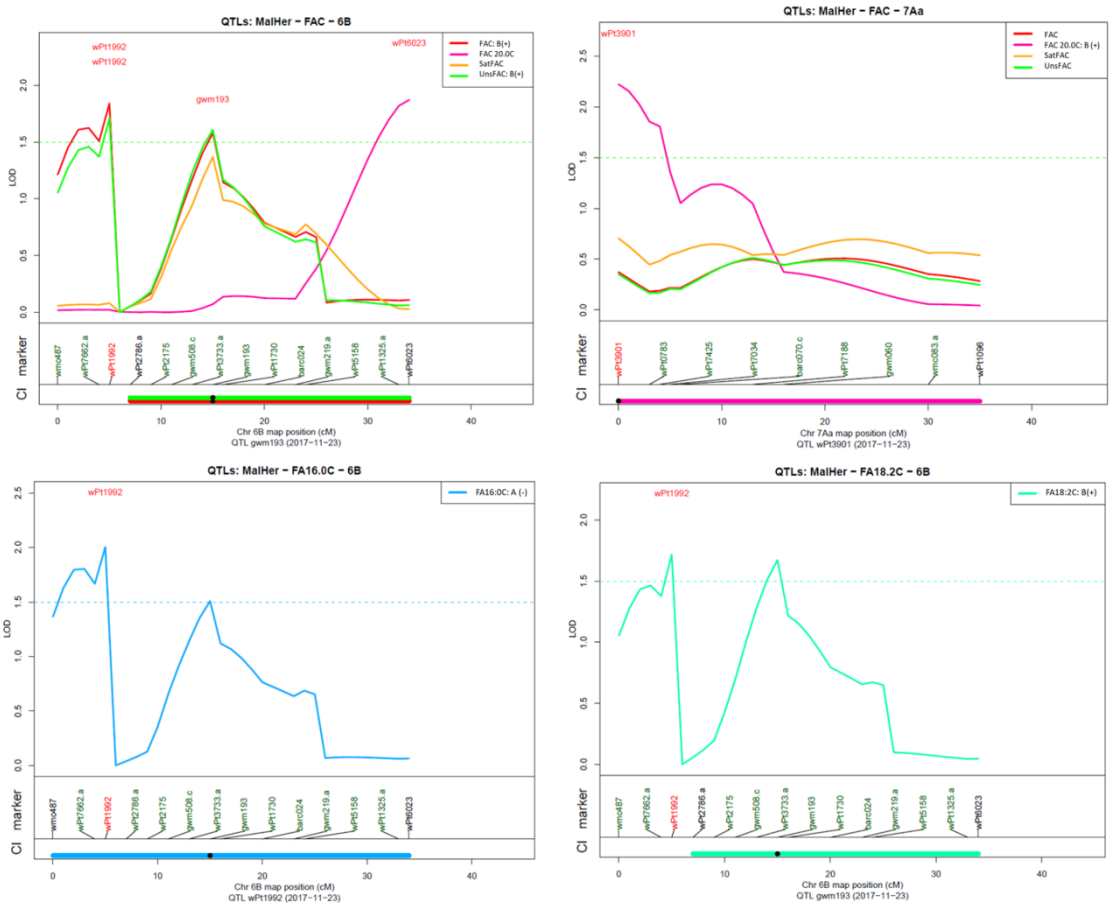


Figure 6.6 QTL plots with a significance threshold set as a LOD score of 1.5.

6.3 Summary and Discussion

Unfortunately the QTL analysis of the FAMEs analysis did not provide any significant results, with no QTLs detected for any of the fatty acid traits. However, at a lowered significance threshold, some interesting QTLs for FA 20:0 were observed. This was mainly seen on chromosome 6B and on chromosomes 7A in one instance. While this may be considered an interesting observation, unfortunately there are too many mitigating factors.

With the QTLs being at merely half the significance, and FA 20:C being the lowets level of fatty acid, it is highly unlikely that these QTLs are robust, nor that they will play a role on breadmaking quality. This is where the limitations of this approach become apparent. While this approach may reveal the genetic control of lipids in wheat flour, it is unable to provide any important information on the lipid headgroups which are more important in revealing breadmaking quality traits.

On the other hand, some differences were observed between individual lines of the DH population, with quantities being 3-fold greater in some lines than in others (for example, line 1 compared to line 52).

The lack of differences and QTL effects probably reflects on the lacking sensitivity of this broad analytical approach. Although the analyses of the MxH NILs described in Chapter 4 revealed some interesting lipid differences, these were limited to the galactolipids and phosphatidylinositol. These lipid classes together make up less than 5% of the lipid composition of wheat flour while the composition of the FAMEs will be dominated by those of major lipid classes (notably PC, LPC, FFAs and TAG). The lack of effects may also indicate that the allelic differences at QTLs relate to the effects of lipid head groups rather than the acyl chains.

While ESI-MS/MS would have been a method better suited to investigate the relationship between lipids and the QTLs, this was not possible due to time constraints. However, a more detailed study using MS analysis may provide more interesting results. Not only may this identify QTL effects on specific lipid classes, which FAMEs analysis was not sensitive enough to detect, but it would also identify the origin of the differences in FAME amount.

Chapter 7 Overall Discussion and Future Work

7.1 Discussion and Conclusions

Lipids are minor components of the wheat grain, making up only 1.5-2.0% of white flour. Despite this, intrinsic wheat grain lipids play a crucial role in breadmaking quality, stabilizing the gas cells and providing the number and size of gas cells which determine the loaf volume and crumb structure associated with UK white bread. However, wheat grain lipids had not been studied extensively and their genetic control has not been determined.

Analyses of six UK wheat cultivars grown in three nitrogen conditions showed significant effects of genotype on the lipid composition of white flour (Chapter 3). Two cultivars, Cadenza (a hard breadmaking spring wheat) and Istabraq (a soft winter feed wheat) showed significantly different lipid profiles compared to four other hard breadmaking winter wheat varieties (Figure 3.6).Comparison of samples grown with three nitrogen inputs also showed some interesting differences, especially for TAG, though these effects were weaker than that of genotype (Table 3.1).

Analysis of NILs developed from a cross between Hereward and Malacca and differing in QTLs for traits contributing to breadmaking quality, such as loaf volume and gas cell number, showed that alleles with positive and negative effects on breadmaking quality were associated with lipid profile differences. In particular, a QTL for loaf volume/wall thickness/cell diameter on chromosome 7A showed consistent differences in the profile of galactolipids differences between the good (Hereward) and poor (Malacca) breadmaking quality alleles across two years of study (Figure 4.10 & 4.11). As galactolipids have been reported to be beneficial for breadmaking quality (Pareyt et al., 2011), these differences are particularly interesting. Furthermore, the sets of NILs differing at this QTL (7A:M and 7A:H) showed the most stable lipid profiles across the two years (Figure 4.24). Since the composition of the galactolipids showed the least effects of year compared to PLs and NLs, and potentially the greatest levels of genetic control (Figure 4.23), the allelic regions on QTL 7A may warrant further study.

However, analysis of the surface property and rheology of dough liquor fractions from the NILs failed to identify consistent differences between the liquor from sets of lines with the good and poor quality alleles. In the first year, lines differing in alleles at the QTL 7A showed significant differences in the elasticity modulus of dough liquor (Figure 5.5). However, this effect was not observed in the second year. By contrast, MS analysis of the diluted dough liquor fractions showed consistent differences in

the galactolipid profiles between lines with the good and poor quality alleles at the QTL 7A across the two years (Figure 5.8 & 5.9). With consistent lipid differences being observed across two years, in two types of analysis systems, the QTL 7A remains an interesting candidate.

Analysis of the MxH DH population showed some differences between individual lines, although no significant effects of the Malacca or Hereward alleles could be found for any of the QTLs (Figure 6.4 and 6.7). QTL analysis of the FAMEs data also provided no significant results. This may have been due to the lack of sensitivity of the GC-FID approach

Although some interesting differences were observed, the results must be interpreted with care. A great deal of variation was seen in the dataset for the MxH NIL which raises concern about the material used in the project. In particular, the high levels of separation observed for the Malacca alleles in the CVA analysis, raises concerns due to the identity of the NILs (Figure 4.22 & 4.24). Since all lines were backcrossed to the Malacca parent the Malacca alleles should be clustered together, while the Hereward alleles should separate out. The lack of separation of the Hereward alleles and the separation of the Malacca alleles together with the significant differences observed by ANOVA of the independent QTLs indicates the lack of a genetic effect associated with the NILs.

This was also reflected in the analysis of dough liquor elasticity which showed little effect of alleles, but large and unexpected differences between QTLs. These differences in the lipid composition, surface tension and elasticity of QTLs, rather than alleles, may be occurring due to environmental effects, experimental procedure or background genes.

Although the failure to observe clear differences associated with the alleles was disappointing, it is in fact not surprising in view of the highly complex phenotype which was studied.. Unlike height, disease resistance or other simple and quantifiable traits, lipid analysis was difficult and highly intensive and lipid composition was highly affected by environment and GxE effects. Variability in the dataset was therefore inevitable, and a perfect world scenario of having overlapping lipid profiles for the Malacca alleles and clearly separating Hereward alleles was never likely to occur.

Despite these challenges, lipids were considered a crucial component for breadmaking quality, due to their effects on gas cell stability. While in hindsight, the hypothesis of linking the existing breadmaking quality QTLs on chromosomes 1B, 4D, 6A and 7A to lipid profile may seem over-ambitious, it was a reasonable target at the time, and some interesting results were nevertheless generated. Furthermore, it should be remembered that the aim of this project was not to map, or identify new QTLs associated with lipids, nor was it to validate the existing QTLs in this project. The primary aim of the project was to determine whether the composition of wheat flour lipids are significantly affected by genotype, environment or GxE effects, (which has been achieved), with the

analysis of the NILs being carried out as an aid to identify lipids associated with differences in gas cell stability (which has been partially achieved).

The M x H DH population, and the NILs derived from this, were selected in order to focus on differences in quality between these two cultivars which have been shown to differ in a number of QTLs for traits related to breadmaking quality. Furthermore, although both lines have good quality, neither has HMW glutenin subunits associated with breadmaking quality, suggesting that other factors, such as lipids, may contribute.. Although this strategy carried a higher risk of failure,than a conventional approach of crossing good x poor lines, particularly if the lipid effect proved to be the same in both cultivars, it was selected due the focus of the BBSRC CIRC (who funded the project) on topics of relevance to practical crop improvement. This focus was also underpinned by Chapter 3, which reported the first detailed study of effects of genotype and environment on flour lipid composition.

7.2 Improvements and Future Work

All of the studies carried out identified strong environmental and experimental effects on flour lipid composition. This was probably the reason for the wide variation seen between the analyses of the MxH NIL grown in 2 years (Figu5.7 & 5.8) as well as the failure to identify consistent differences in surface tension. There are several possible explanations for the large environmental effects.. Storage is a key issue and the results in Chapters 3 and 4 show how storage of grain and flour have affected the lipid composition, despite efforts being made to minimise lipase activity. While these methods, such as boiling the flour before lipid extraction and the use of isopropanol during extraction are clearly necessary, more care must be taken to prevent lipid degradation during storage. Ideally, flour should not be stored for a long time, even at -20˚C, as we have observed effects on lipids as well as protein degradation. .

Care should be taken from grain harvesting. Post-harvest, grain may be best stored at cold conditions of (4˚C) and lipids should be extracted from flour within a month at most of milling. A previous study of the conversion of PC to LPC during storage was carried out at Rothamsted over three months, which unfortunately showed little differences. However it is clear that more detailed studies are required, comparing material stored at different temperatures (20, 4, -20 degrees) for periods up to a year. Large differences in the lipid composition were observed across the two years, likely due to storage conditions. These changes could have significant effects on dough liquor surface properties and subsequently affect gas cell stability. .

Sets of lines with the same alleles at the different QTLs were bulked in order to provide sufficient material for biochemical and biophysical analyses, which may also have reduced environmental differences between plots. Single plots normally gave only 50-100 grams of grain giving only 25-50 grams of white flour. Bulking 4-5 lines of the NILs with the same alleles was therefore required to provide around 100-200 grams of flour for analysis. Even when this was done it was still necessary to modify the method for analysis of surface activity of dough liquor by dilution.

In retrospect, it would probably been more realistic to focus the study on lipid analysis in flour rather than also attempt to carry out dough liquor surface activity analysis. Simply treating each individual line from the NILs as a single batch from which three replicates could come from would provide more robust lipid analyses, as it would offer 3 biological replicates, from which 3-5 technical replicates of flour could be taken. However, this would require a massive increase in the number of samples for analysis and may not have been feasible within the time constraints of the project. As discussed above, the project provided the most detailed study of variation in lipid composition between wheat cultivars which has so far been carried out.. The results, which are reported in in Chapter 3, showed wide variation in the lipid profiles of six UK wheat cultivars and analysis of a wider range of lines, including genotypes differing in their age, quality and geographical origin would clearly be interesting. This would also certainly reveal a much wider range of variation in composition, and identify more divergent cultivars for further studies of the role of lipids in grain quality.

Due to time and resource constraints further experiments to relate the elasticity and surface tension of dough liquor to lipid composition were not pursued. However, published work (Gerits et al., 2012) have shown that it is possible to improve dough quality by treatment with lipases and the same approach could be taken to identify and increase the surface active lipids in dough liquor. An alternative strategy, adding exogenous lipids to delipidated flour is less attractive as it is difficult to incorporate lipid into an aqueous dough system and the removal of lipids from the flour may remove other components as well as alter interactions between other components of the system.

The ultimate test of whether components, including lipids, affect breadmaking quality is baking tests. However, even micro-baking requires large amounts of flour, which was unavailable for the NIL material, and is costly. More robust material and better data analysis will be required before such analysis becomes worthwhile.

One reason for studying the H x M population and NILs was to cast light on the “Hereward conundrum”. Hereward itself showed no significant lipid differences compared to the other cultivars, nor was the superior breadmaking quality associated with the Hereward alleles associated with consistently significant differences in lipid composition compared to the poor quality Malacca alleles, with the exception of the QTL 7A allele and galactolipid content. However, as high quantities of

lipids are not responsible for breadmaking quality, it may be too soon to conclude that the lipid profile of the Hereward lipid profile is not associated with quality. As other studies have shown, phospholipids confer improved breadmaking quality, but by working in a synergistic fashion rather than alone (Helmerich and Koheler, 2005). The balance of lipids present may therefore be more important than the amount. The addition of lipids to defatted flours is not a solution, and as far as the author is aware lipases are not specific enough to alter a single type of lipid alone to allow testing of what lipids are beneficial or not. Overall this has made, the rheological analysis of a dough liquor system the most feasible and reproducible method to work with so far.

Overall, this study has revealed evidence for genetic effects on the lipid composition of wheat flour. While further work is required to achieve the goal of establishing the lipid components contributing to breadmaking quality and relating these to allelic variation at mapped QTLs, this study has identified a potentially interesting candidate QTL for future work.

Chapter 8 Appendix

Chapter 2

Class of lipid		TAG	DAG	FFA
Scan type		Neutral loss	Neutral loss	Q1 ESI-MS
NL Mass	Fatty acid	Fatty acids mass		
	FA 15:0 standard	NL259.0		
	FA16:0	NL273.2	NL273.2	
	FA18:0	NL301.2	NL301.2	
	FA18:1	NL299.2	NL299.2	
	FA18:2	NL297.2	NL297.2	
	FA18:3	NL295.2	NL295.2	
	FA20:0	NL329.2	NL329.2	
	FA20:4 standard		NL321.2	
Mode		Positive	Positive	Negative
Curtain Gas (arbitrary units)		10	20	10
Ion Source 1 (arbitrary units)		12	45	12
Ion Source 2 (arbitrary units)		0	45	off
Declustering Potential		+120V	+100V	-125V
Entrance Potential		+15V	+10V	-10V
Collision energy		+37V	+40V	-
Collision cell exit potential		+6.5V	+10V	-
Ion spray Voltage		+5500V	+5500V	-4500V
Interface heater		ON	ON	ON
Number of cycles		40	100	25
Delay time (sec)		0	0	200

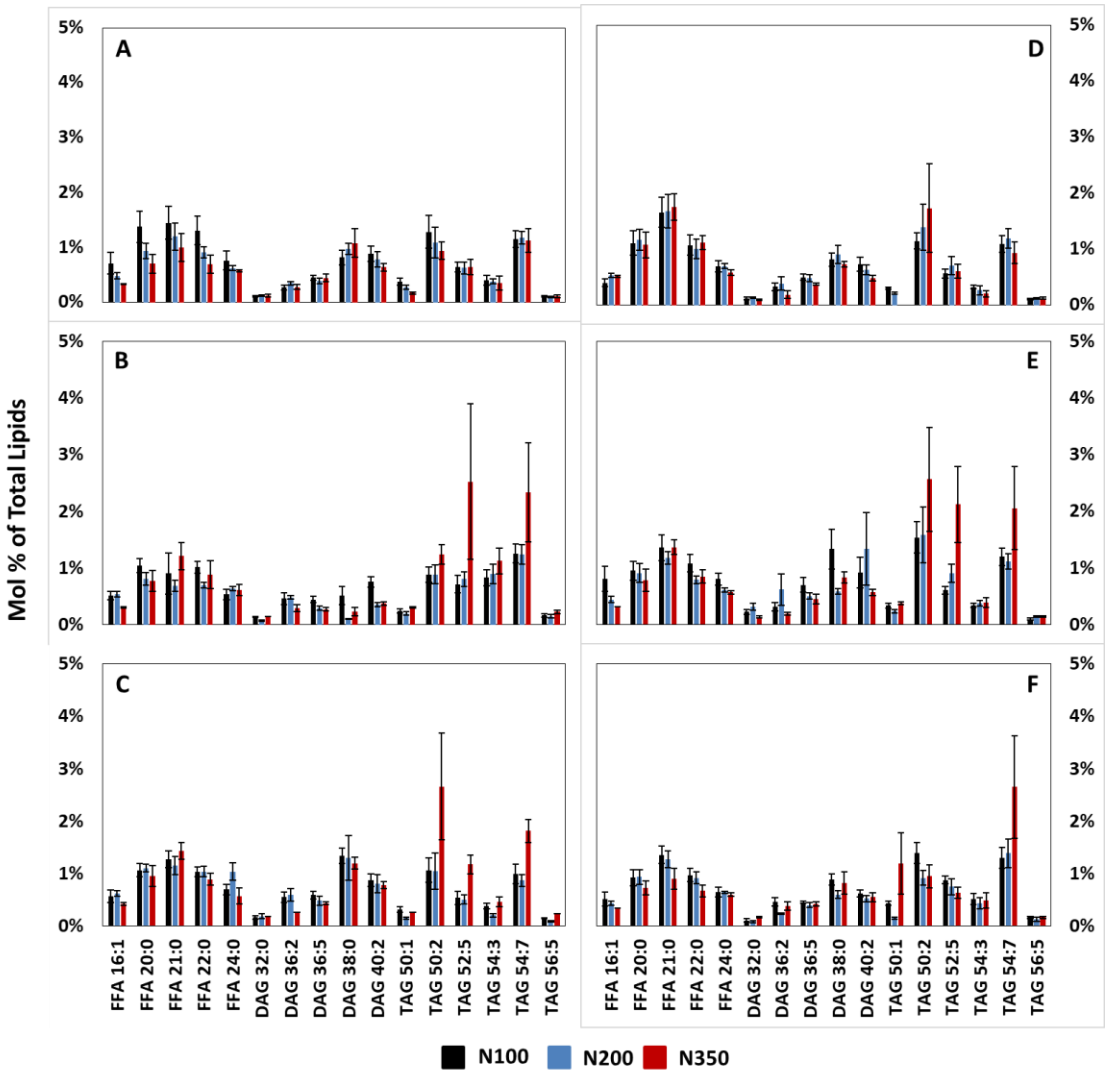
Appendix Figure 2.1 ESI-MS/MS methods and parameters for TAG, DAG and FFA molecular species identification. Specific acyl groups present in TAG and DAG were identified by neutral loss masses as indicated in the table. The mass range used in Q1 was 500-1100, 500-800 and 100-500 for TAG, DAG and FFA, respectively. The 4000 Qtrap System, AB Sciex (Framingham, MA, U.S.A.) was used for the lipidomic analysis.

Chapter 3

Term	Cultivar	N	Cultivar.N
LPC_16_0	0.3885405	0.25056	0.954221893
LPC_18_0	0.0361586	0.080203	0.018013238
LPC_18_1	0.2179006	0.108227	0.991993416
LPC_18_2	0.3930545	0.503396	0.96134238
LPC_18_3	0.0678433	0.448656	0.912590787
PC_32_0	<0.001	0.762219	0.203734502
PC_34_1	0.0141714	0.327753	0.536481777
PC_34_2	<0.001	0.20164	0.454600525
PC_34_3	<0.001	0.331235	0.00862601
PC_36_2	0.0077685	0.642013	0.22997265
PC_36_3	0.0025025	0.530251	0.356733309
PC_36_4	<0.001	0.665536	0.432686001
PC_36_5	<0.001	0.55144	0.041277598
PC_36_8	<0.001	<0.001	<0.001
PE_34_2	<0.001	0.161359	0.043827468
PE_36_3	<0.001	0.091712	<0.001
PE_36_4	<0.001	0.010399	0.013631264
DAG_32_0	<0.001	0.622409	<0.001
DAG_34_1	0.0095237	0.057711	0.413670852
DAG_34_2	0.9011123	0.631353	0.452229562
DAG_36_2	0.1360818	0.023807	0.058254174
DAG_36_3	0.5853317	0.204898	0.515012849
DAG_36_4	0.0956175	0.467496	0.806734015
DAG_36_5	0.0042274	0.003481	0.722262182
DAG_38_0	<0.001	0.019355	0.067223693
DAG_40_2	0.0025617	0.035266	0.575111116
TAG_50_1	0.0179616	<0.001	0.014220097
TAG_50_2	0.148813	0.235567	0.560340378
TAG_52_2	0.001332	0.053227	0.21429281
TAG_52_3	0.2298979	0.674224	0.288218354
TAG_52_4	0.676528	0.918665	0.183704764
TAG_52_5	0.023442	0.00217	0.040158285
TAG_54_3	<0.001	0.598836	0.320546056
TAG_54_4	0.0841536	0.381636	0.826577704
TAG_54_5	0.8705689	0.285697	0.627418813
TAG_54_6	0.3633121	0.420527	0.515550966
TAG_54_7	0.2925274	0.053145	0.688137393
TAG_56_5	0.0030076	0.00306	0.002248171
FFA_16_0	0.2241776	0.064504	0.089694761
FFA_16_1	0.4926499	0.005328	0.069000269
FFA_18_0	0.5567381	0.344071	0.899851775

FFA_18_1	0.170416	0.472381	0.46392888
FFA_18_2	0.126885	0.007664	0.129600442
FFA_18_3	0.109274	0.004337	0.232217731
FFA_20_0	0.6707817	0.071941	0.986329012
FFA_21_0	0.078663	0.404548	0.543749713
FFA_22_0	0.6776908	0.064257	0.737119652
FFA_24_0	0.6493473	0.029244	0.221194239

Appendix Figure 3.1 The full list of the p-values and F-test of cultivar, nitrogen and cultivar by nitrogen interaction for all the lipid species analysed in this study. Lipids significantly affected ($p<0.05$, F-test) by genotype, environment or GxE are highlighted in bold.



Appendix Figure 3.2 Lipid compositions of remaining neutral lipids between the three nitrogen conditions. A, Avalon; B, Cadenza; C, Crusoe; D, Hereward; E, Istabraq; F, Malacca. Data are mean values of ±SE of five independent samples analysed via ESI-MS/MS.

Percentage variation			
CV1	**CV2**	**CV3**	
33.48	21.36	11.33	
Latent Vectors (Loadings)			
	CV1	**CV2**	**CV3**
LPC_16_0	-2.906	3.515	5.688
LPC_18_0	1.871	-0.758	0.539
LPC_18_1	0.934	-0.799	0.727
LPC_18_2	-2.007	-2.343	-5.515
LPC_18_3	3.431	0.145	0.553
PC_32_0	-0.175	-2.873	2.211
PC_34_1	-4.23	2.988	-4.486
PC_34_2	-1.025	-1.167	1.454
PC_34_3	-0.801	-2.328	-2.905
PC_36_2	2.94	2.611	3.312
PC_36_3	-0.858	0.218	0.742
PC_36_4	1.589	0.251	-1.997
PC_36_5	2.492	-0.446	2.263
PC_36_8	2.42	0.821	-0.201
PE_34_2	-0.95	-2.605	1.092
PE_36_3	3.18	2.059	2.191
PE_36_4	2.255	1.875	-2.462
DAG_32_0	-0.886	0.255	3.498
DAG_34_1	1.862	2.366	-2.268
DAG_34_2	1.69	-1.807	-1.845
DAG_36_2	0.43	0.414	-0.614
DAG_36_3	5.076	-1.107	5.291
DAG_36_4	-10.016	3.428	1.611
DAG_36_5	-1.366	-3.063	-3.995
DAG_38_0	1.825	-1.167	-0.014
DAG_40_2	1.546	-1.126	-2.876
TAG_50_1	1.342	0.162	-0.11
TAG_50_2	0.004	-3.702	-1.055
TAG_52_2	-1.372	-0.161	0.275
TAG_52_3	-0.714	1.252	0.016
TAG_52_4	-0.019	0.286	0.299
TAG_52_5	1.449	4.459	4.467
TAG_54_3	0.32	3.88	-1.748
TAG_54_4	1.038	-0.354	0.374
TAG_54_5	0.211	1.311	1.558
TAG_54_6	-0.389	-1.137	-0.404
TAG_54_7	-0.605	-4.083	-0.329
TAG_56_5	-3.265	-2.119	-0.779

FFA_16_0	2.706	0.336	2.755
FFA_16_1	-4.31	0.216	-2.064
FFA_18_0	-0.624	2.524	1.851
FFA_18_1	1.972	-1.919	-3.017
FFA_18_2	-0.428	4.095	3.715
FFA_18_3	0.873	0.829	-1.195
FFA_20_0	-1.385	-1.174	-1.699
FFA_21_0	-2.585	-2.679	2.03
FFA_22_0	3.659	2.854	1.508
FFA_24_0	-3.165	-3.87	-3.704

Appendix Figure 3.3 CVA Loading scores for the WGIN lipids

LPC total

Mean	SE
7.024	0.055

PC total

Cultivar	
Avalon	6.178
Cadenza	5.304
Crusoe	5.455
Hereward	5.583
Istabraq	5.232
Malacca	6.063

SED = 0.1879 on 72 df; LSD (5%) = 0.3746

PE total

Cultivar	100	200	350
Avalon	2.599	3.276	1.938
Cadenza	2.088	2.159	1.784
Crusoe	1.939	1.614	1.853
Hereward	1.550	1.874	1.733
Istabraq	0.665	0.857	0.685
Malacca	2.068	2.176	2.086

SED = 0.2811 on 72 df; LSD (5%) = 0.5603

DAG total

Mean	SE
6.186	0.033

TAG total

Cultivar	
Avalon	6.885
Cadenza	7.295
Crusoe	6.792
Hereward	6.943
Istabraq	6.643
Malacca	7.180

SED = 0.1123 on 72 df; LSD (5%) = 0.2240

N	
100	6.803
200	6.943
350	7.124

SED = 0.0794 on 72 df; LSD (5%) = 0.1584

FFA total

Cultivar	100	200	350
Avalon	7.198	7.116	7.236
Cadenza	6.798	7.589	7.412
Crusoe	7.096	7.435	6.594
Hereward	7.158	7.409	7.174
Istabraq	7.192	7.207	7.090
Malacca	7.307	7.434	7.355

SED = 0.2339 on 72 df; LSD (5%) = 0.4662

Polar lipids total

Mean	SD
7.302	0.043

Neutral lipids total

Cultivar	100	200	350
Avalon	7.920	7.941	8.072
Cadenza	7.770	8.359	8.365
Crusoe	7.879	8.005	7.774
Hereward	7.869	8.174	8.022
Istabraq	7.884	7.874	7.759
Malacca	8.117	8.146	8.231

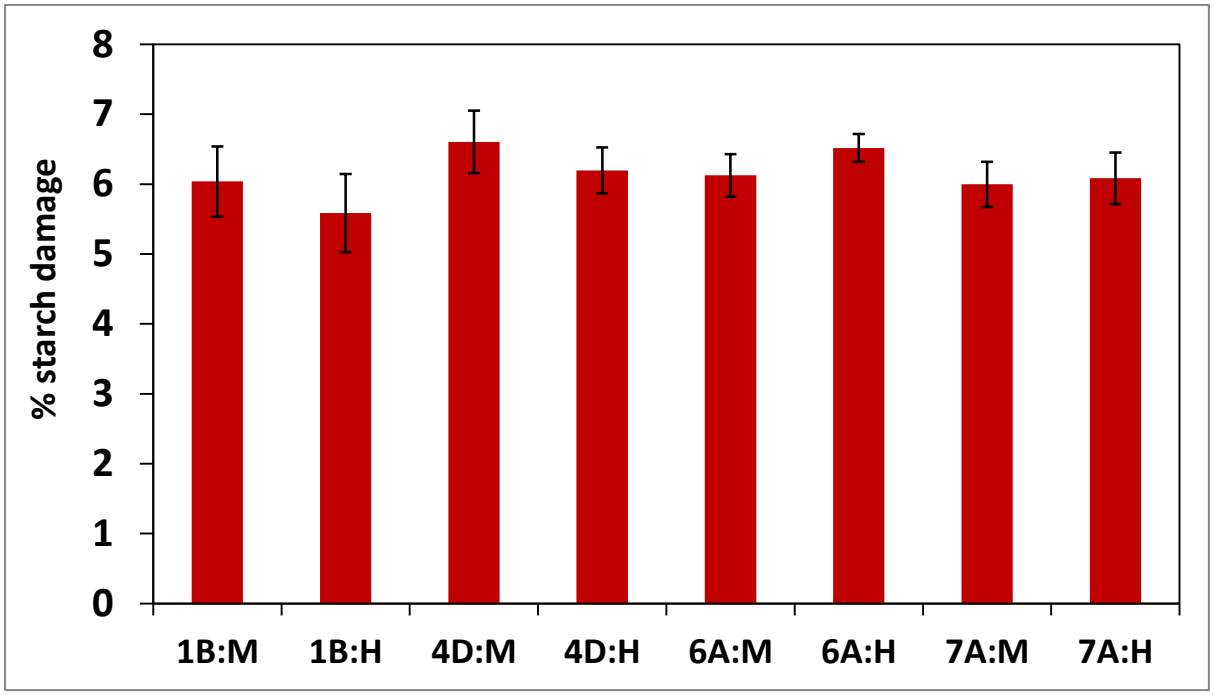
SED = 0.1359 on 72 df; Max LSD (5%) = 0.2709

Appendix Figure 3.4 Relevant means on the log scale for the WGIN lipid totals. The p-values for F-test of cultivar, N and cultivar by N interactions are shown.

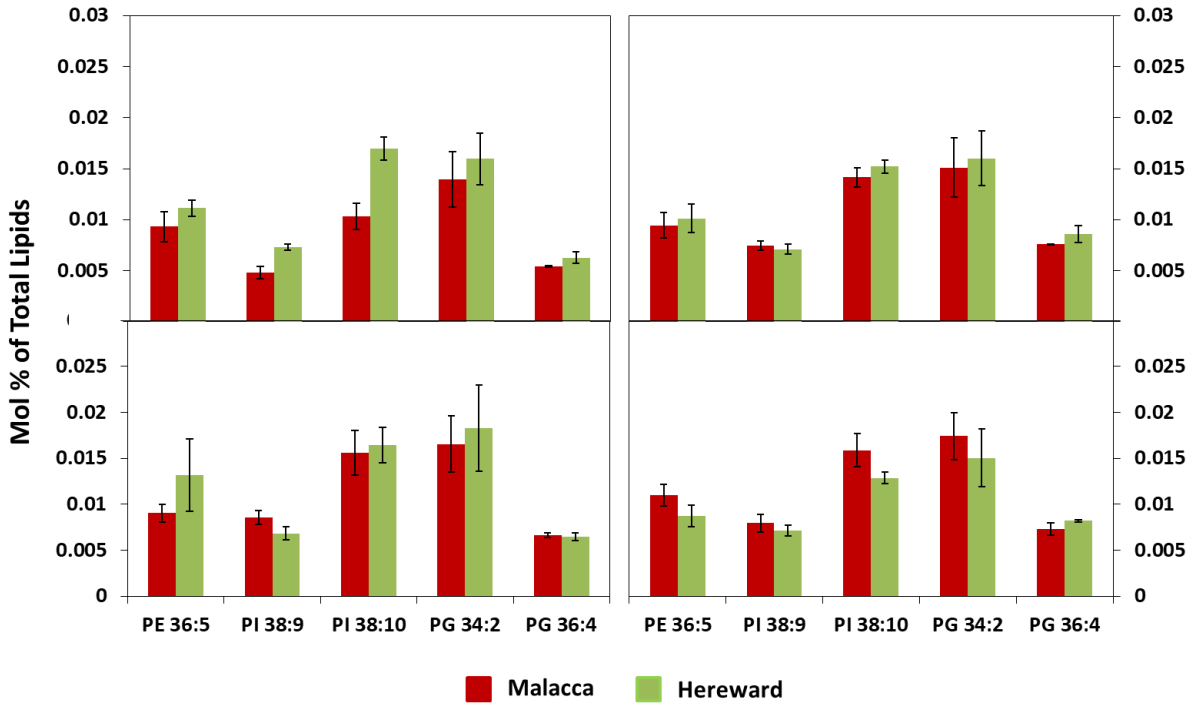
Chapter 4

Malacca x Hereward breadmaking functionality NILs			BC3F3 sown 2012-13	Bagged Her W11-12		
			Rookery	Bagged Mal S12		
			Harvested 20/8/13	Malacca = a		
				Hereward = b		
Rack	**Bed**	**Genotype**	**Chromosome & trait**	**Hom at QTL**	**EE**	**Notes**
M1	5	**MH100/Mal4 188-3 9**	**1B Number of cells**	a	20	
M1	9	MH100/Mal4 188-4 4	1B Number of cells	b	20	
M1	13	MH100/Mal4 188-4 17	1B Number of cells	a	20	
M1	16	MH100/Mal4 188-6 1	1B Number of cells	a	17	
M1	19	MH100/Mal4 188-6 10	1B Number of cells	b	16	
M1	20	MH100/Mal4 188-6-11	1B Number of cells	b	17	
M2	3	MH100/Mal4 188-6 14	1B Number of cells	b	18	
M2	6	MH100/Mal4 188-6 22	1B Number of cells	b	20	
M2	12	MH100/Mal4 188-9 14	1B Number of cells	a	19	
M3	5	MH100/Mal4 188-14 12	1B Number of cells	a	20	
M3	16	**MH1/Mal4 161-1 1**	**4D Cell number, L***	a	18	
M3	17	MH1/Mal4 161-1 3	4D Cell number, L*	a	17	
M3	20	MH1/Mal4 161-1 15	4D Cell number, L*	a		
M4	3	MH1/Mal4 161-1 22	4D Cell number, L*	b	17	
M4	4	MH1/Mal4 161-2 16	4D Cell number, L*	b		
M4	8	MH1/Mal4 161-2 21	4D Cell number, L*	a		
M4	10	MH1/Mal4 161-5 3	4D Cell number, L*	b	19	
M4	12	MH1/Mal4 161-5 13	4D Cell number, L*	a	19	
M4	19	MH1/Mal4 161-5 24	4D Cell number, L*	b	20	
M4	20	MH1/Mal4 161-7 7	4D Cell number, L*	b	21	
M5	10	**MH70/Mal4 168-1 15**	**6A Cell number**	a	17	Single marker
M5	11	MH70/Mal4 168-1 16	6A Cell number	a	18	Single marker
M5	13	MH70/Mal4 168-3 2	6A Cell number	b	18	Single marker
M5	14	MH70/Mal4 168-3 3	6A Cell number	b		Single marker
M5	16	MH70/Mal4 168-3 4	6A Cell number	a	18	Single marker
M5	18	MH70/Mal4 168-3 6	6A Cell number	b	19	Single marker
M5	20	MH70/Mal4 168-3 8	6A Cell number	a	16	Single marker
M6	6	MH70/Mal4 168-3 18	6A Cell number	a	16	Single marker
M6	13	MH70/Mal4 168-9 2	6A Cell number	b	21	Single marker
M6	16	MH70/Mal4 168-9 4	6A Cell number	b	18	Single marker
M8	20	**MH60/Mal4 186-5 1**	**7A Wall thick, Cell diam/vol, Loaf vol**	a	21	Single marker
M9	1	MH60/Mal4 186-5 3	7A Wall thick, Cell diam/vol, Loaf vol	b	20	Single marker
M9	2	MH60/Mal4 186-5 7	7A Wall thick, Cell diam/vol, Loaf vol	b	19	Single marker
M9	3	MH60/Mal4 186-5 10	7A Wall thick, Cell diam/vol, Loaf vol	b	20	Single marker
M9	9	MH60/Mal4 186-5 14	7A Wall thick, Cell diam/vol, Loaf vol	b	19	Single marker
M9	10	MH60/Mal4 186-5 15	7A Wall thick, Cell diam/vol, Loaf vol	b	19	Single marker
M10	3	MH60/Mal4 186-8 11	7A Wall thick, Cell diam/vol, Loaf vol	a	19	Single marker
M11	7	MH60/Mal4 186-11 24	7A Wall thick, Cell diam/vol, Loaf vol	a	20	Single marker
M11	12	MH60/Mal4 186-12 17	7A Wall thick, Cell diam/vol, Loaf vol	a	19	Single marker

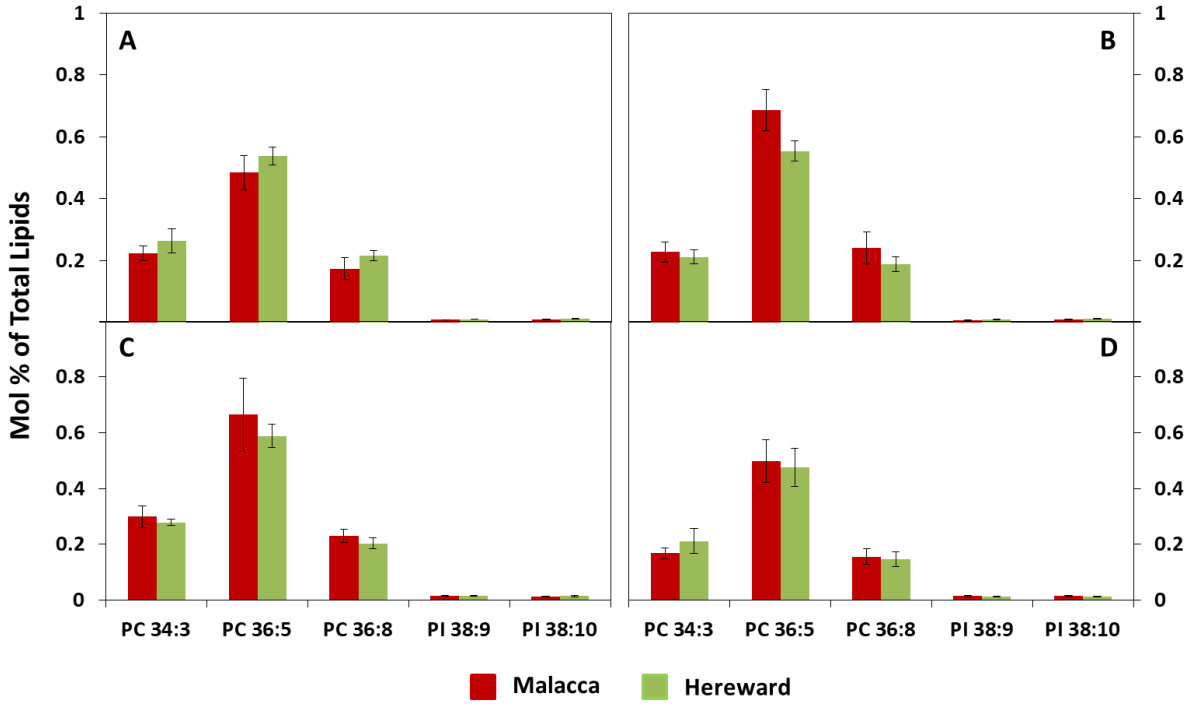
Appendix Figure 4.1 List of the MxH NILs for each of the 4 QTLs and their alleles. Highlighted in green are NILs with the Malacca allele and highlighted in yellow are the NILs with the Hereward allele at the QTL of interest.



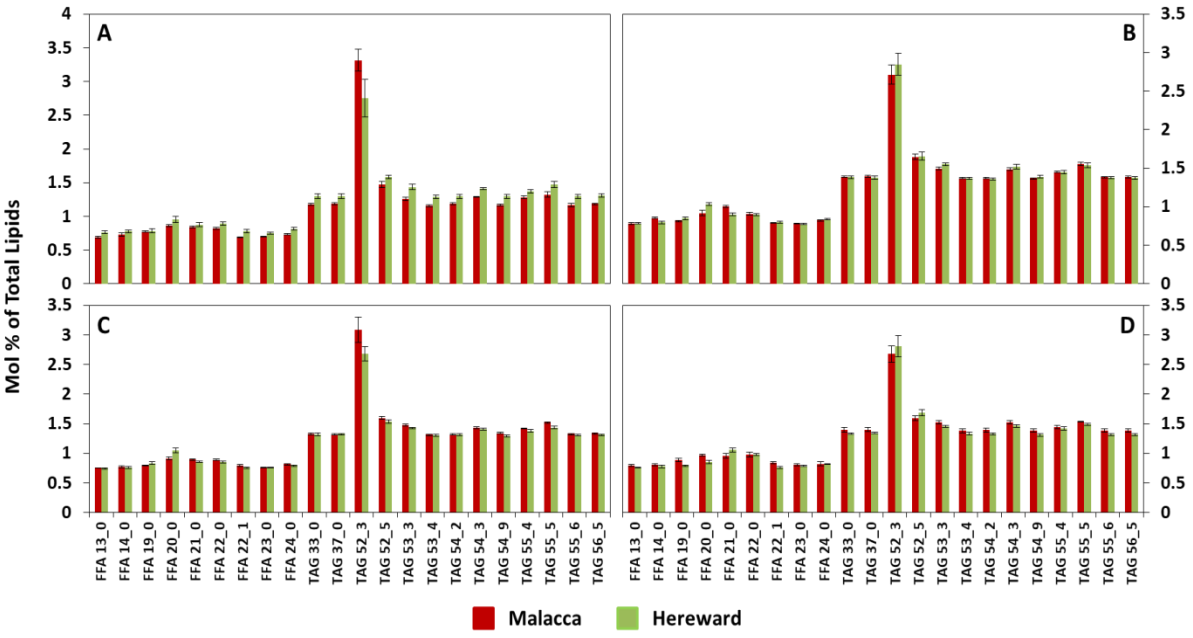
Appendix Figure 4.2 Starch Damage in the MxH NILs. Starch damage levels were low being below 7% in all cases. Data are mean values of ±SE of three independent samples analysed using the Megazyme starch damage kit (Leinster, Ireland).



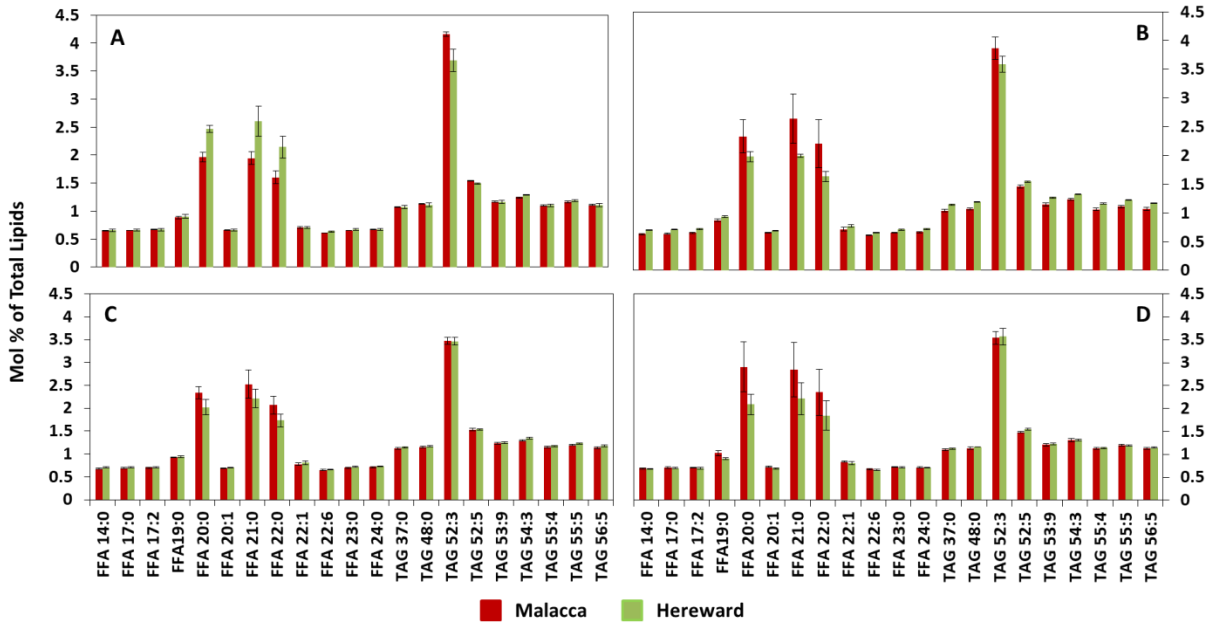
Appendix Figure 4.3a Comparison of minor phospholipids (mol% of total lipids) between M and H NILs among the four QTLs in Year 1. Data are mean values of ±SE of five independent samples analysed via ESI-MS/MS



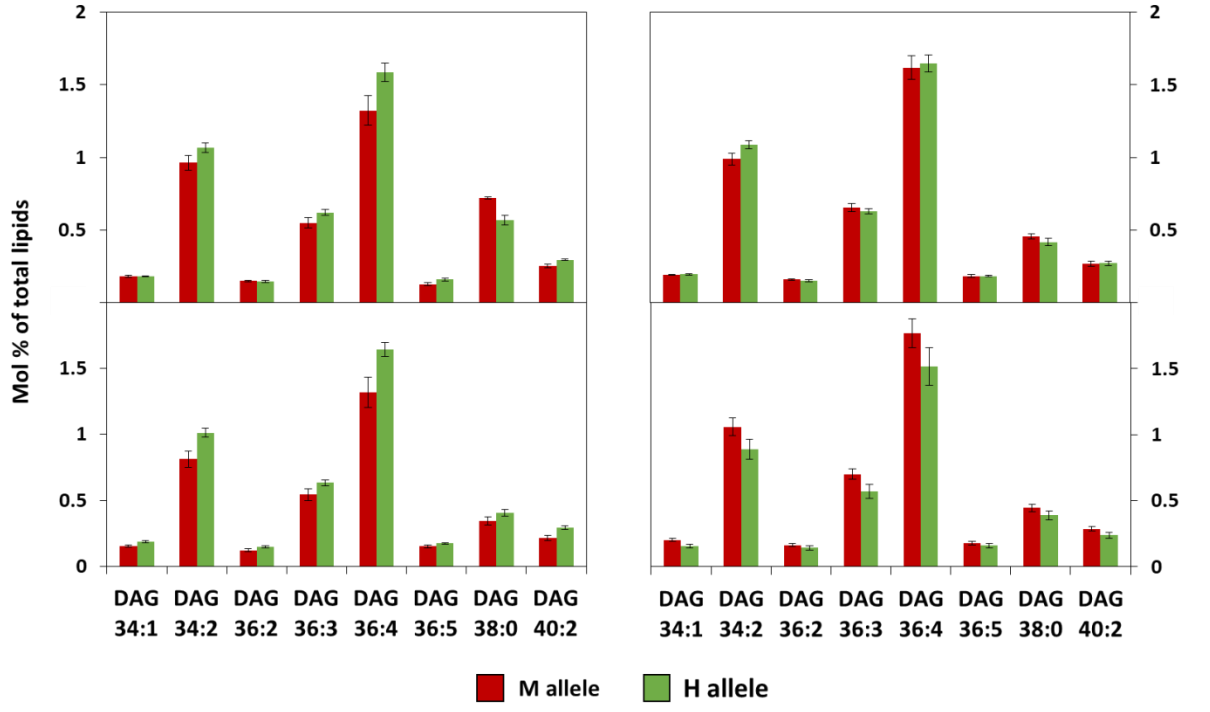
Appendix Figure 4.3b Comparison of minor phospholipids (mol% of total lipids) between M and H NILs among the four QTLs in Year 2. Data are mean values of ±SE of five independent samples analysed via ESI-MS/MS



Appendix Figure 4.4a Comparison of selected minor neutral lipid species across the four QTLs and their allelic combinations in year 1. Data are mean values of ±SE of five independent samples analysed via ESI-MS/MS.



Appendix Figure 4.4b Comparison of selected minor neutral lipid species across the four QTLs and their allelic combinations in year 1. Data are mean values of ±SE of five independent samples analysed via ESI-MS/MS.



Appendix Figure 4.5 Comparison of selected major DAG species across the four QTLs and their allelic combinations in year 2. Data are mean values of ±SE of five independent samples analysed via ESI-MS/MS.

Percentage variation			

CV1			
31.25	20.99	18.11	
Latent vectors (loadings)			

Column1			
LPC 16:0	0.4334	-0.0709	0.0424
LPC 18:0	-0.1829	0.4094	0.3782
LPC 18:1	0.5214	0.2522	-0.0971
LPC 18:2	0.0955	0.0196	-0.204
LPC 18:3	0.4656	-0.5593	0.4843
PC 34:1	0.6821	-0.3544	-0.1366
PC 34:2	0.4658	0.5218	0.245
PC 36:3	0.0586	-0.5611	-0.0551
PC 36:4	0.3327	0.078	-0.0617
PE 34:2	0.3343	0.1707	-0.3505
PE 36:3	0.0991	-0.0524	0.2527
PE 36:4	-0.3245	0.1177	0.5582
PE 36:5	0.0501	0.0718	0.2164
PI 34:1	-0.048	-0.0706	-0.6097
PI 34:2	-0.7081	0.2911	-0.1219
PI 34:3	-0.0123	0.32	-0.4938
PI 36:2	0.0208	0.077	-0.0432
PI 36:3	0.0452	0.417	-0.1716
PI 36:4	-0.1389	0.8672	0.3451
PI 36:5	0.0257	-0.2229	-0.8017
PI 37:2	0.6279	-0.0981	0.0253
PI 37:3	0.0738	-0.3553	0.3423
PG 34:2	-0.1271	-0.052	0.1638
PG 36:4	-0.1995	-0.6551	0.2846
MGDG 34:2	0.3194	-0.4113	-0.0918
MGDG 36:3	-0.8664	0.4347	-0.0824
MGDG 36:4	0.455	-0.5726	-0.3721
MGDG 36:5	-0.4276	0.0506	0.3287
DGDG 34:2	-0.0149	-0.1358	-0.1496
DGDG 34:3	0.1592	0.1781	0.4007
DGDG 36:3	-0.5163	-0.8404	0.0104
DGDG 36:4	-0.124	-0.0184	-0.2215
DGDG 36:5	-0.3016	0.2685	-0.073
FFA 13:0	0.2781	-0.1974	-0.1045

FFA 14:0	-0.0624	0.0517	0.1919
FFA 16:0	-0.0158	0.6849	0.0319
FFA 18:0	-0.1033	0.8802	0.3472
FFA 18:1	0.1426	0.4809	-0.1165
FFA 18:2	0.0953	0.1889	-0.0928
FFA 18:3	-0.0728	-0.7385	-0.0092
FFA 19:0	0.3958	0.4174	0.2186
FFA 20:0	-0.974	-0.1048	0.4738
FFA 21:0	0.5018	0.0139	-0.6126
FFA 22:0	0.2695	-0.0271	0.4535
FFA 22:1	0.309	0.2406	0.2682
FFA 23:0	0.2515	-0.3871	0.0761
FFA 24:0	0.0124	-0.0507	0.266
TAG 33:0	0.1664	-0.0882	0.0998
TAG 37:0	0.0652	0.0892	0.1367
TAG 50:1	-0.0841	-0.5117	-0.2228
TAG 50:2	0.0091	0.2525	0.3726
TAG 52:2	-0.0292	-0.0356	0.2455
TAG 52:3	0.1874	-0.0784	0.0012
TAG 52:4	-0.3167	-0.1567	-0.0382
TAG 52:5	0.0133	0.1487	-0.3061
TAG 53:3	0.1087	0.1417	0.1175
TAG 53:4	0.0605	-0.1134	0.0339
TAG 54:2	0.2551	0.0183	0.0094
TAG 54:3	0.2043	0.0774	-0.0894
TAG 54:4	-0.369	-0.4404	0.2403
TAG 54:5	-0.55	0.4039	0.592
TAG 54:6	0.6557	0.2119	-0.1805
TAG 54:7	-0.8435	0.1214	0.162
TAG 54:9	0.0634	-0.057	-0.0882
TAG 55:4	0.2384	0.0243	-0.0634
TAG 55:5	-0.0224	-0.5309	0.0051
TAG 55:6	0.1609	-0.1098	0.0716
TAG 56:5	0.0516	0.0263	-0.0224

Percentage variation			
	CV1	**CV2**	**CV3**
	31.25	20.99	18.11
Latent vectors (loadings)			
	CV1	**CV2**	**CV3**
LPC 16:0	0.4334	-0.0709	0.0424
LPC 18:0	-0.1829	0.4094	0.3782
LPC 18:1	0.5214	0.2522	-0.0971
LPC 18:2	0.0955	0.0196	-0.204
LPC 18:3	0.4656	-0.5593	0.4843
PC 34:1	0.6821	-0.3544	-0.1366
PC 34:2	0.4658	0.5218	0.245
PC 36:3	0.0586	-0.5611	-0.0551
PC 36:4	0.3327	0.078	-0.0617
PE 34:2	0.3343	0.1707	-0.3505
PE 36:3	0.0991	-0.0524	0.2527
PE 36:4	-0.3245	0.1177	0.5582
PE 36:5	0.0501	0.0718	0.2164
PI 34:1	-0.048	-0.0706	-0.6097
PI 34:2	-0.7081	0.2911	-0.1219
PI 34:3	-0.0123	0.32	-0.4938
PI 36:2	0.0208	0.077	-0.0432
PI 36:3	0.0452	0.417	-0.1716
PI 36:4	-0.1389	0.8672	0.3451
PI 36:5	0.0257	-0.2229	-0.8017
PI 37:2	0.6279	-0.0981	0.0253
PI 37:3	0.0738	-0.3553	0.3423
PG 34:2	-0.1271	-0.052	0.1638
PG 36:4	-0.1995	-0.6551	0.2846
MGDG 34:2	0.3194	-0.4113	-0.0918
MGDG 36:3	-0.8664	0.4347	-0.0824
MGDG 36:4	0.455	-0.5726	-0.3721
MGDG 36:5	-0.4276	0.0506	0.3287
DGDG 34:2	-0.0149	-0.1358	-0.1496
DGDG 34:3	0.1592	0.1781	0.4007
DGDG 36:3	-0.5163	-0.8404	0.0104
DGDG 36:4	-0.124	-0.0184	-0.2215
DGDG 36:5	-0.3016	0.2685	-0.073
FFA 13:0	0.2781	-0.1974	-0.1045
FFA 14:0	-0.0624	0.0517	0.1919
FFA 16:0	-0.0158	0.6849	0.0319
FFA 18:0	-0.1033	0.8802	0.3472
FFA 18:1	0.1426	0.4809	-0.1165

FFA 18:2	0.0953	0.1889	-0.0928
FFA 18:3	-0.0728	-0.7385	-0.0092
FFA 19:0	0.3958	0.4174	0.2186
FFA 20:0	-0.974	-0.1048	0.4738
FFA 21:0	0.5018	0.0139	-0.6126
FFA 22:0	0.2695	-0.0271	0.4535
FFA 22:1	0.309	0.2406	0.2682
FFA 23:0	0.2515	-0.3871	0.0761
FFA 24:0	0.0124	-0.0507	0.266
TAG 33:0	0.1664	-0.0882	0.0998
TAG 37:0	0.0652	0.0892	0.1367
TAG 50:1	-0.0841	-0.5117	-0.2228
TAG 50:2	0.0091	0.2525	0.3726
TAG 52:2	-0.0292	-0.0356	0.2455
TAG 52:3	0.1874	-0.0784	0.0012
TAG 52:4	-0.3167	-0.1567	-0.0382
TAG 52:5	0.0133	0.1487	-0.3061
TAG 53:3	0.1087	0.1417	0.1175
TAG 53:4	0.0605	-0.1134	0.0339
TAG 54:2	0.2551	0.0183	0.0094
TAG 54:3	0.2043	0.0774	-0.0894
TAG 54:4	-0.369	-0.4404	0.2403
TAG 54:5	-0.55	0.4039	0.592
TAG 54:6	0.6557	0.2119	-0.1805
TAG 54:7	-0.8435	0.1214	0.162
TAG 54:9	0.0634	-0.057	-0.0882
TAG 55:4	0.2384	0.0243	-0.0634
TAG 55:5	-0.0224	-0.5309	0.0051
TAG 55:6	0.1609	-0.1098	0.0716
TAG 56:5	0.0516	0.0263	-0.0224

Appendix Figure 4.6a The magnitude of the CV loadings on the percentage lipid variables of total lipids in Year 1

Percentage variation			
	CV1	**CV2**	**CV3**
	40.71	21.66	16.69
Latent vectors (loadings)			
	CV1	**CV2**	**CV3**
LPC 16:0	0.2564	0.8143	-0.1043
LPC 18:0	-0.2345	0.5728	-0.235
LPC 18:1	-0.1417	0.075	0.0891
LPC 18:2	-0.0854	-0.1777	0.3072
LPC 18:3	0.485	0.4425	-0.2166
PC 34:1	-0.5066	-0.5625	-0.279
PC 34:2	-0.2002	0.0299	0.3178
PC 34:3	0.0158	-0.1107	-0.23
PC 36:2	-0.3387	0.1456	-0.1824
PC 36:3	-0.0215	0.092	-0.4116
PC 36:4	0.0055	-0.2998	0.169
PC 36:5	0.0128	0.0592	0.4563
PC 36:8	0.0839	0.0441	-0.315
PE 34:3	-0.2804	0.2373	-0.0934
PE 36:3	0.2728	0.4325	-0.3794
PE 36:4	0.2131	-0.2262	-0.6465
PI 34:1	0.2988	-0.232	0.0957
PI 34:2	0.3013	0.0275	-0.3675
PI 34:3	0.6631	0.1763	0.5524
PI 36:2	0.0042	0.0931	-0.643
PI 36:3	0.4484	0.6764	-0.1381
PI 36:4	-0.081	0.3672	-0.4727
PI 36:5	0.0303	0.2512	-0.2638
PI 38:9	-0.3819	-0.2223	0.0457
PI 38:10	0.3368	0.0655	0.3054
MGDG 34:2	0.3421	0.3115	-0.2577
MGDG 36:3	-0.0478	-0.3262	0.0537
MGDG 36:4	-0.4411	-0.3583	-0.2425
MGDG 36:5	0.1811	0.1441	0.3114
DGDG 34:2	-0.3411	-0.7674	-0.0436
DGDG 34:3	0.3198	0.1643	0.2639
DGDG 36:3	0.1173	0.4817	0.588
DGDG 36:4	-0.4299	0.2885	-0.5077
DGDG 36:5	0.4428	0.3808	0.0296
FFA 14:0	0.0801	0.2283	0.0894
FFA 16:0	0.2735	-0.2216	0.5858
FFA 17:0	0.1496	-0.0486	0.1015
FFA 17:2	0.1602	-0.0305	0.1635

FFA 18:0	-0.132	-0.2452	0.5272
FFA 18:1	-0.0875	-0.2543	0.4118
FFA 18:2	-0.0046	0.0462	0.0383
FFA 18:3	-0.3467	-0.5759	-0.0069
FFA 19:0	0.1847	0.2494	-0.1443
FFA 20:0	0.4222	-0.26	-0.0277
FFA 20:1	0.191	-0.0347	0.0465
FFA 21:0	-0.327	-0.0315	-0.1662
FFA 22:0	-0.1498	0.1678	0.2067
FFA 22:1	0.5246	-0.4504	0.6216
FFA 22:6	0.014	-0.0462	0.0479
FFA 23:0	0.0111	-0.2117	0.0327
FFA 24:0	0.3363	0.1392	0.1402
TAG 37:0	-0.0183	-0.0322	-0.0268
TAG 48:0	-0.1033	0.0901	-0.2424
TAG 50:1	0.1255	0.1026	0.0749
TAG 50:2	0.0069	0.1161	-0.7952
TAG 52:2	-0.1922	-0.0786	-0.424
TAG 52:3	-0.634	-0.1814	-0.0767
TAG 52:4	0.4052	0.662	-0.009
TAG 52:5	-0.1185	0.0621	0.0565
TAG 53:9	-0.1252	-0.1118	0.1273
TAG 54:3	-0.0536	-0.2694	0.0492
TAG 54:4	-0.7036	-0.4403	-0.196
TAG 54:5	-0.3363	-0.5065	0.1529
TAG 54:6	0.1557	-0.0016	0.0298
TAG 54:7	-0.2409	-0.3941	0.2311
TAG 55:4	0.1162	-0.0755	0.0685
TAG 55:5	0.2414	0.0524	-0.0108
TAG 56:5	0.1932	0.1781	0.0474
DAG 32:0	-0.3394	0.3921	0.26
DAG 34:1	0.3257	-0.0663	0.4669
DAG 34:2	-0.0979	0.0356	0.0864
DAG 34:3	0.7362	-0.2333	0.4431
DAG 36:2	-0.4441	-0.2147	0.3038
DAG 36:3	-0.22	-0.1829	-0.0153
DAG 36:4	-0.1207	-0.0948	-0.2216
DAG 36:5	0.3196	-0.2715	-0.0322
DAG 38:0	-0.2372	0.8181	-0.0283
DAG 38:1	0.6597	0.3681	0.2484
DAG 38:3	0.0361	-0.0961	-0.5373
DAG 38:6	-0.2196	-0.3496	-0.0815
DAG 40:0	-0.1138	0.2809	0.1634
DAG 40:1	-0.4903	-0.2901	0.3408

DAG 40:2	0.1568	0.3743	-0.1087
DAG 40:3	0.0924	0.0651	-0.35
DAG 42:0	-0.3856	-0.0688	-0.1896

Appendix Figure 4.6b The magnitude of the CV loadings on the percentage lipid variables of total lipids in Year 2

Percentage variation			
	CV1	**CV2**	**CV3**
	35.53	30.43	14.41
Latent vectors (loadings)			
	CV1	**CV2**	**CV3**
LPC 16:0	4.916	1.887	2.787
LPC 18:0	0.226	3.659	-1.633
LPC 18:1	-4.51	-5.083	-2.338
LPC 18:2	1.827	15.934	6.879
LPC 18:3	11.215	-6.843	2.285
PC 34:1	3.756	2.749	6.823
PC 34:2	3.602	9.006	2.305
PC 36:3	-2.431	-2.544	3.137
PC 36:4	1.224	-2.838	-5.869
PE 34:2	-4.918	-5.514	-1.487
PE 36:3	5.57	1.759	0.499
PE 36:4	0.361	3.493	-1.485
PE 36:5	-0.616	1.597	-1.186
PI 34:1	12.285	-17.003	-1.609
PI 34:2	2.307	0.365	-21.585
PI 34:3	-11.316	-5.347	11.545
PI 36:2	0.418	-1.55	-3.87
PI 36:3	0.367	2.797	1.848
PI 36:4	-9.301	12.104	15.295
PI 36:5	0.21	-8.816	-1.073
PI 38:9	2.179	0.235	1.757
PI 38:10	3.492	0.349	-1.01
PG 34:2	2.14	2.302	0.922
PG 36:4	5.35	10.219	-0.904

Appendix Figure 4.7a The magnitude of the CV loadings on the percentage lipid variables of phospholipids in Year 1

Percentage variation			
	CV1	**CV2**	**CV3**
	41.66	36.01	12.46
Latent vectors (loadings)			
	CV1	**CV2**	**CV3**
LPC 16:0	-1.412	4.557	32.932
LPC 18:0	2.666	-16.94	-3.56
LPC 18:1	-5.592	1.541	6.977
LPC 18:2	21.818	-20.154	-20.828
LPC 18:3	-20.247	59.675	-20.857
PC 34:1	-6.229	-6.722	11.541
PC 34:2	-6.16	-14.016	11.674
PC 34:3	3.687	-3.812	2.7
PC 36:2	-2.942	15.506	-3.341
PC 36:3	5.483	-1.433	-1.498
PC 36:4	-0.788	6.417	-17.393
PC 36:5	3.299	1.328	-1.426
PC 36:8	-0.464	-2.65	2.274
PE 34:3	-7.572	17.459	-12.816
PE 36:3	6.602	-2.471	-3.31
PE 36:4	4.662	-26.105	7.473
PI 34:1	1.762	1.543	0.497
PI 34:2	7.163	-10.463	4.353
PI 34:3	3.042	1.933	0.476
PI 36:2	2.506	2.364	0.772
PI 36:3	3.652	5.121	9.585
PI 36:4	0.888	-14.969	-11.577
PI 36:5	0.862	-3.9	4.982
PI 38:9	-0.738	8.124	0.725
PI 38:10	1.81	3.961	-1.71

Appendix Figure 4.7b The magnitude of the CV loadings on the percentage lipid variables of phospholipids in Year 2

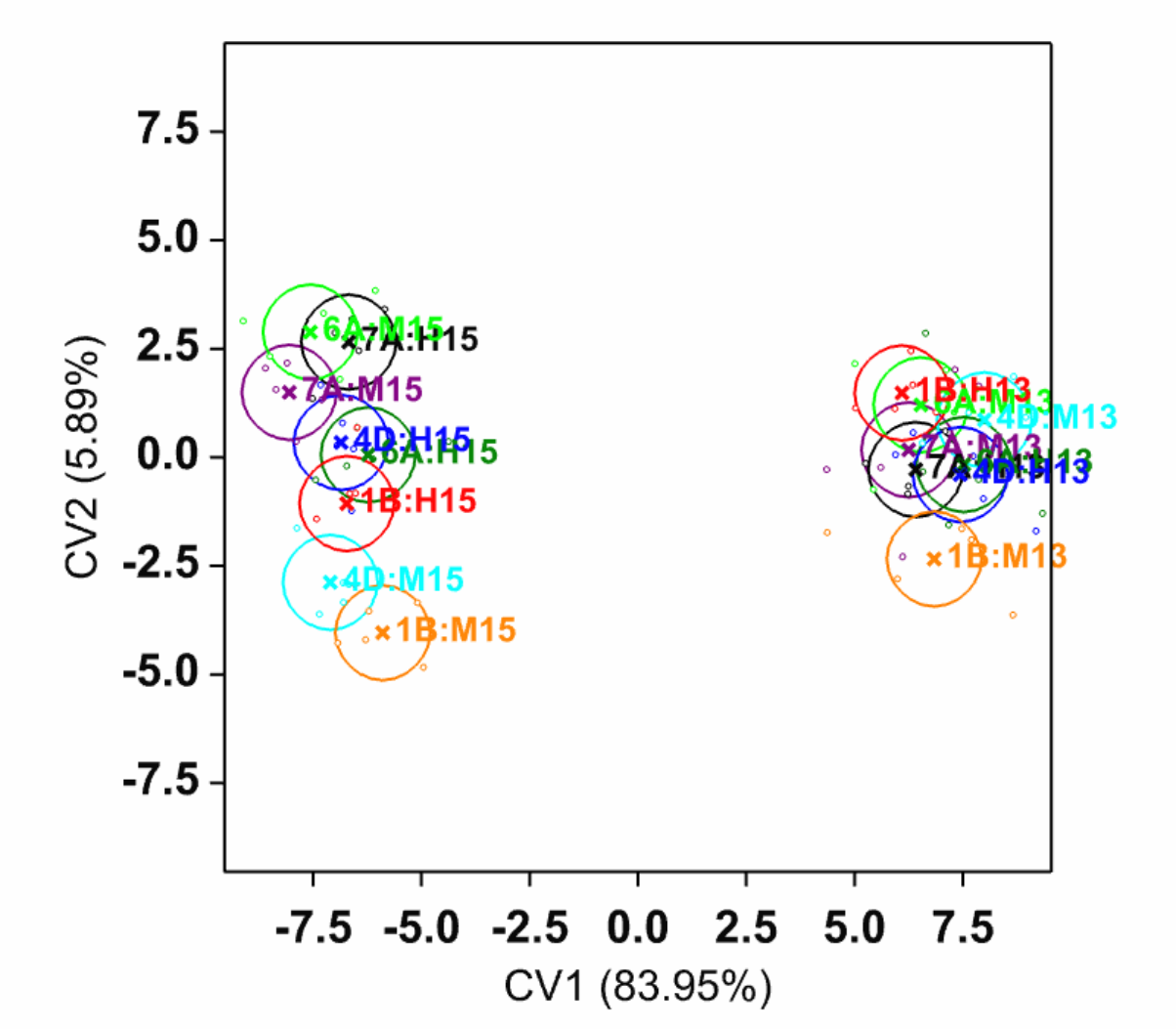
Percentage variation			
	CV1	**CV2**	**CV3**
	50.66	32.21	9.3
Latent vectors (loadings)			
	CV1	**CV2**	**CV3**
FFA 13:0	11.475	14.716	-13.714
FFA 14:0	10.337	9.041	-16.711
FFA 16:0	0.254	2.962	1.844
FFA 18:0	0	0.97	0.817
FFA 18:1	1.986	-1.078	-1.38
FFA 18:2	-0.729	1.197	1.127
FFA 18:3	-0.885	2.6	1.839
FFA 19:0	-1.116	18.451	6.497
FFA 20:0	5.271	0.371	-2.742
FFA 21:0	4.051	-8.708	-1.481
FFA 22:0	6.881	3.586	0.093
FFA 22:1	-0.236	2.56	2.752
FFA 23:0	11.424	0.719	2.075
FFA 24:0	-1.131	-18.13	1.579
TAG 33:0	7.164	-24.393	4.675
TAG 37:0	-2.412	-12.965	8.183
TAG 50:1	-11.662	16.182	4.599
TAG 50:2	-1.727	-2.756	0.488
TAG 52:2	3.025	0.184	-0.18
TAG 52:3	-0.695	-1.618	-2.909
TAG 52:4	0.159	1.29	-0.423
TAG 52:5	10.11	-0.774	9.081
TAG 53:3	-13.137	9.246	6.804
TAG 53:4	-0.686	-3.324	5.662
TAG 54:2	-0.73	-16.029	-12.758
TAG 54:3	4.695	2.487	-5.678
TAG 54:4	-2.893	-9.518	1.454
TAG 54:5	1.974	2.47	1.129
TAG 54:6	-1.795	2.126	1.422
TAG 54:7	0.604	-4.586	-2.913
TAG 55:4	-7.938	-4.074	0.956
TAG 55:5	4.274	12.961	2.222
TAG 55:6	-16.389	-7.049	-5.73
TAG 56:5	-17.596	11.963	-5.77

Appendix Figure 4.8a The magnitude of the CV loadings on the percentage lipid variables of neutral lipids in Year 1

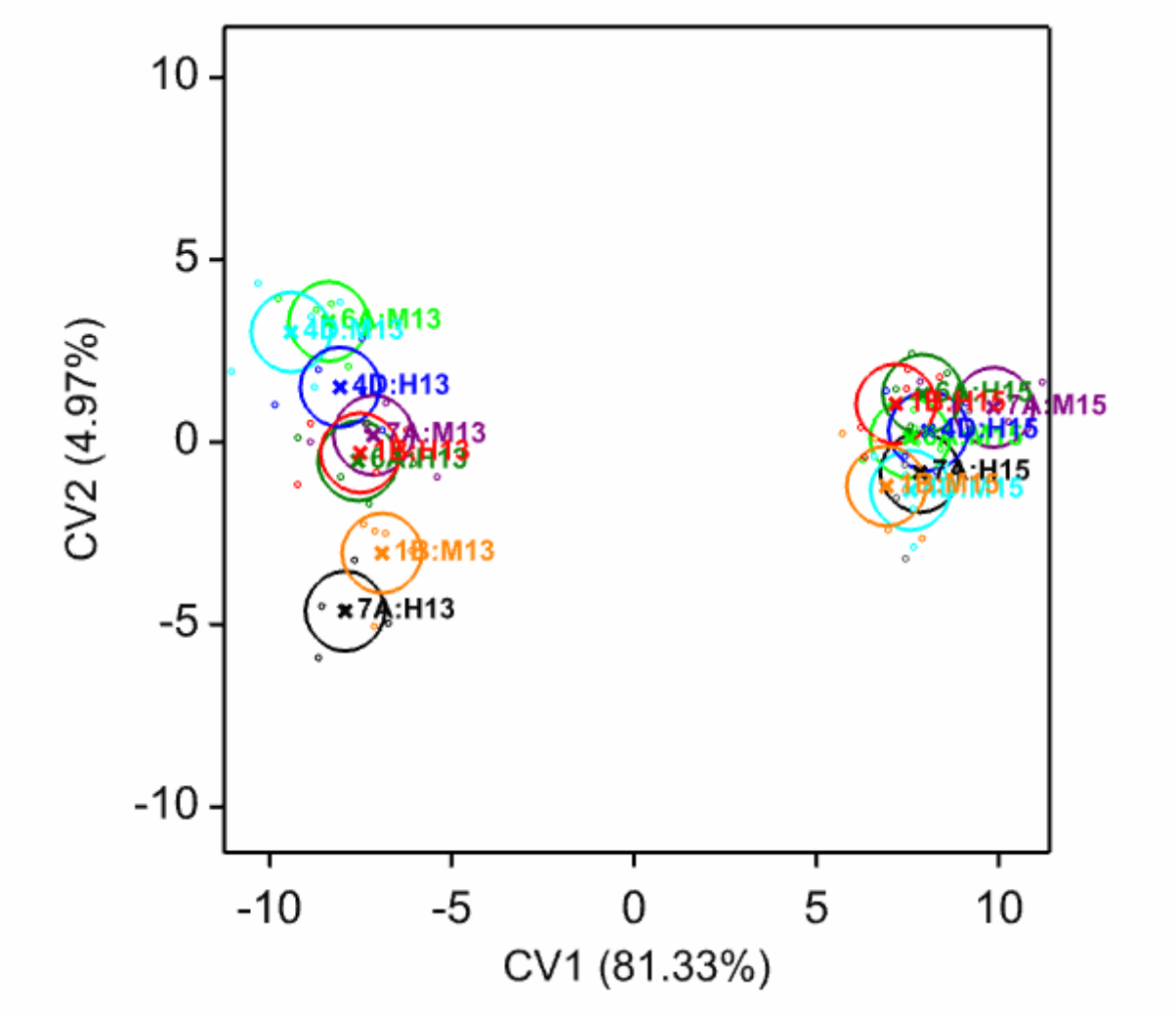
Percentage variation			
	CV1	**CV2**	**CV3**
	52.75	24.04	8.75
Latent vectors (loadings)			
	CV1	**CV2**	**CV3**
FFA 14:0	0.961	-1.175	-5.384
FFA 16:0	-1.719	1.761	1.094
FFA 17:0	2.46	-1.661	-1.172
FFA 17:2	3.055	0.073	-1.324
FFA 18:0	-0.906	-0.242	-0.052
FFA 18:1	3.78	1.577	0.348
FFA 18:2	0.133	0.812	0.21
FFA 18:3	0.105	-2.908	0.56
FFA 19:0	3.985	1.271	1.828
FFA 20:0	-3.153	0.375	-1.393
FFA 20:1	1.216	0.157	-1.288
FFA 21:0	0.359	0.741	0.531
FFA 22:0	1.457	-1.158	-0.292
FFA 22:1	-3.215	1.913	0.142
FFA 22:6	-0.911	-1.172	-4.266
FFA 23:0	-3.401	1.038	2.055
FFA 24:0	4.475	1.597	-0.008
TAG 37:0	1.966	-0.537	-1.337
TAG 48:0	3.64	1.8	-0.773
TAG 50:1	-1.842	1.186	1.59
TAG 50:2	-2.977	2.542	-0.965
TAG 52:2	2.156	-1.548	-0.969
TAG 52:3	-0.401	-1.503	0.041
TAG 52:4	-2.753	2.469	0.694
TAG 52:5	1.523	0.371	0.307
TAG 53:9	-1.489	0.157	3.632
TAG 54:3	-1.64	1.822	1.97
TAG 54:4	1.856	0.927	0.77
TAG 54:5	0.455	0.83	-0.872
TAG 54:6	1.397	-0.801	-3.958
TAG 54:7	0.108	-0.183	0.655
TAG 55:4	-1.351	-1.391	1.293
TAG 55:5	-2.848	-1.962	0.864
TAG 56:5	-0.131	0.57	0.447
DAG 32:0	1.203	-2.028	0.516
DAG 34:1	-2.079	0.548	-0.225
DAG 34:2	0.052	-0.141	0.203
DAG 34:3	2.518	-4.57	-1.126

DAG 36:2	-0.017	0.512	0.957
DAG 36:3	2.698	-0.178	-1.587
DAG 36:4	1.732	1.842	0.68
DAG 36:5	2.563	-0.634	-0.605
DAG 38:0	-3.304	1.271	-1.965
DAG 38:1	-0.843	0.996	-1.347
DAG 38:3	-1.101	-0.196	1.061
DAG 38:6	-0.787	-0.156	0.677
DAG 40:0	1.95	-1.362	0.105
DAG 40:1	-5.42	1.673	0.965
DAG 40:2	1.471	-0.962	1.769
DAG 40:3	-1.62	3.062	-0.315
DAG 42:0	-0.385	0.034	0.702

Appendix Figure 4.8b The magnitude of the CV loadings on the percentage lipid variables of neutral lipids in Year 2

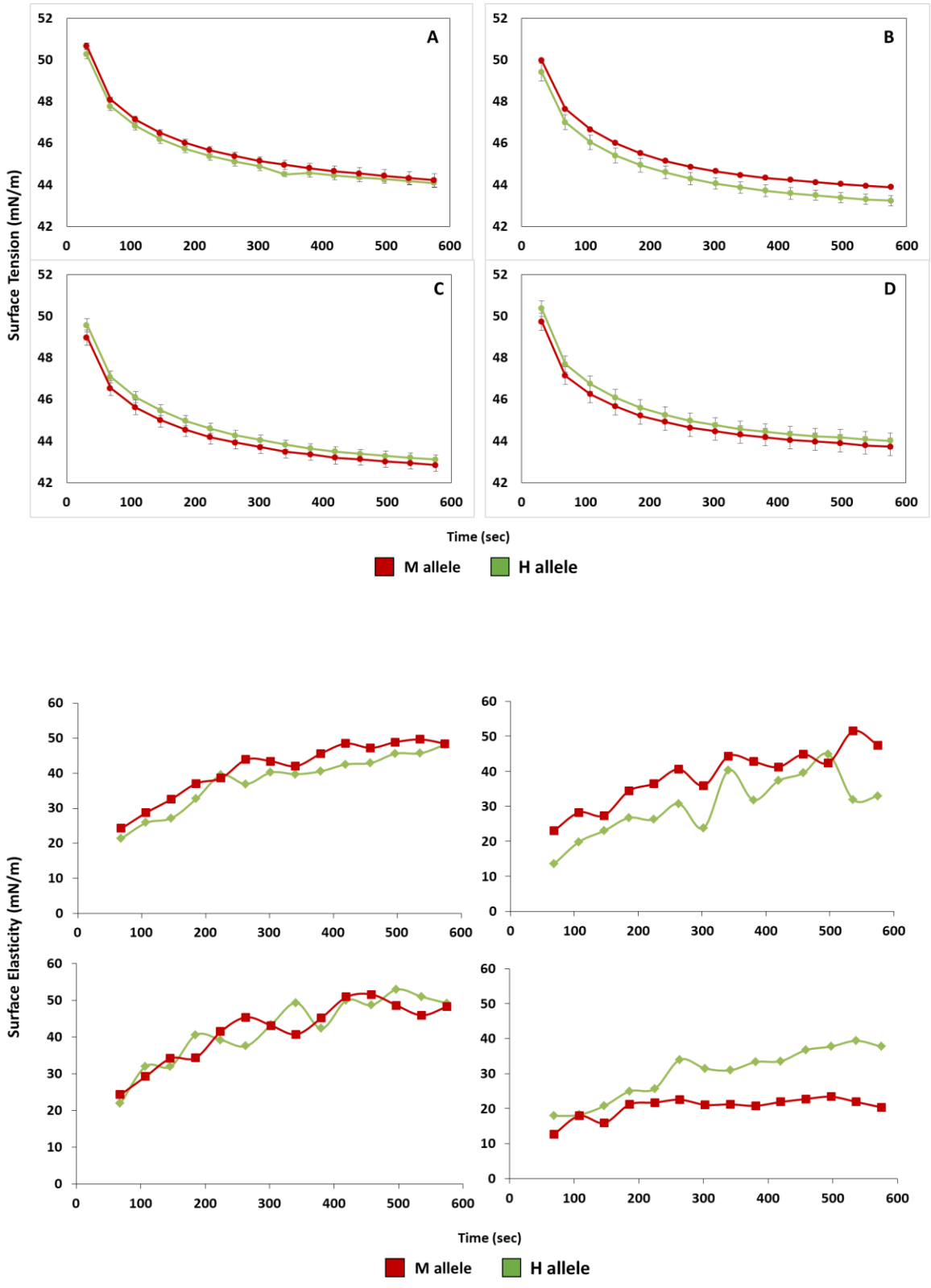


Appendix Figure 4.9a CVA analyses for the four QTL and their allelic combinations for the phospholipid dataset across the two years. Included are QTL 1B, 4D, 6A and 7A with the alleles, M (Malacca) and H (Hereward). CV scores (**o**) and means of scores (**x**) in the three dimensions are plotted. 13 indicates 2013 the year 1 material, 15 indicates the 2015 year 2 material.

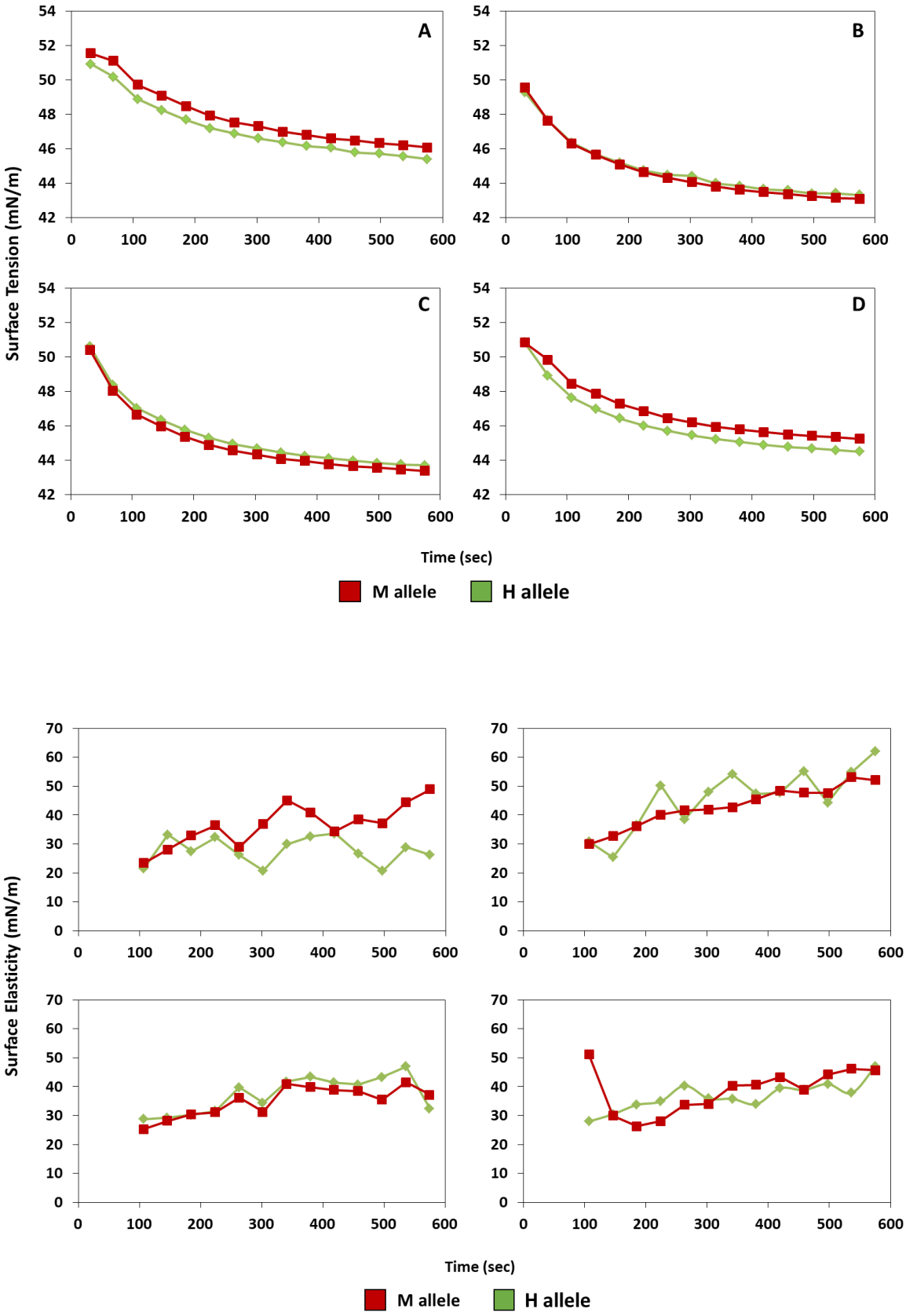


Appendix Figure 4.9b CVA analyses for the four QTL and their allelic combinations for the neutral lipid dataset across the two years. Included are QTL 1B, 4D, 6A and 7A with the alleles, M (Malacca) and H (Hereward). CV scores (**o**) and means of scores (**x**) in the three dimensions are plotted. 13 indicates 2013 the year 1 material, 15 indicates the 2015 year 2 material.

Chapter 5



Appendix Figure 5.1 The surface tension and elasticity raw 1st year dataset of the diluted dough liquor fractions for the MxH NILs



Appendix Figure 5.2 The surface tension and elasticity raw 2nd year dataset of the diluted dough liquor fractions for the MxH NILs

Year 1									
	QTL								
Curve (γ + α*exp(-β*Time))	*β*	*γ*							
Surface tension (1B) = 44.24 + 7.702*exp(-0.00928**Time*)	0.00928	44.24							
Surface tension (4D) = 43.72 + 7.702*exp(-0.00913**Time*)	0.00913	43.72							
Surface tension (6A) = 43.15 + 7.702*exp(-0.00883**Time*)	0.00883	43.15							
Surface tension (7A) = 44.08 + 7.702*exp(-0.01023**Time*)	0.01023	44.08							
SED (15 df)	**0.00035**	**0.3**							
LSD (5%)	**0.000745**	**0.639**							
	QTL x allele			**QTL**			**allele only**		
Curve (δ + ε(1 - exp(-β*Time))	*β*			δ			δ	*ε*	
Elasticity (1B-M) = -13.6 + 58.4*(1 - exp(-0.0088**Time*))	0.0088		**1B**	-19.9		**Malacca**	-15.7	58.4	
Elasticity (1B-H) = -26.1 + 67.9*(1 - exp(-0.0121* *Time*))	0.0121								
Elasticity (4D_M) = -3.6 + 58.4*(1 - exp(-0.0068* *Time*))	0.0068		**4D**	-9.9					
Elasticity (4D_H) = -16.2 + 67.9*(1 - exp(-0.0097* *Time*))	0.0097								
Elasticity (6A_M) = -16.1 + 58.4*(1 - exp(-0.0112* *Time*))	0.0112		**6A**	-21.3		**Hereward**	-27.6	67.9	
Elasticity (6A_H) = -28.7 + 67.9*(1 - exp(-0.0121* *Time*))	0.0121								
Elasticity (7A_M) = -29.3 + 58.4*(1 - exp(-0.0110* *Time*))	0.011		**7A**	-35.6					
Elasticity (7A_H) = -41.9 + 67.9*(1 - exp(-0.0309* *Time*))	0.0309								
SED (15 df)	**0.00454**			**6.82**			**4.82**	**4.39**	
LSD (5%)	**0.00967**			**14.53**					
Year 2									
	QTL								
Curve (γ + α*exp(-β*Time))	*β*	*γ*							
Surface tension (1B) = 45.46 + 7.71*exp(-0.00523**Time*)	0.00523	45.46							
Surface tension (4D) = 43.23 + 7.71*exp(-0.00772**Time*)	0.00772	43.23							
Surface tension (6A) = 43.64 + 7.71*exp(-0.00847**Time*)	0.00847	43.64							
Surface tension (7A) = 44.83 + 7.71*exp(-0.00719**Time*)	0.00719	44.83							
SED (15 df)	**0.000882**	**0.229**							
LSD (5%)	**0.000188**	**0.488**							
	QTL								
Curve (δ + ε(1 - exp(-β*Time))	δ								
Elasticity (1B) = -15.8 + 59.5*(1 - exp(-0.0053**Time*))	-15.8								
Elasticity (4D) = 16.8 + 59.5*(1 - exp(-0.0053* *Time*))	16.8								
Elasticity (6A) = 16.3 + 59.5*(1 - exp(-0.0053* *Time*))	16.3								
Elasticity (7A) = 11.3 + 59.5*(1 - exp(-0.0053* *Time*))	11.3								
SED (15 df)	**11.42**								
LSD (5%)	**24.67**								

Appendix Figure 5.3 The curve equations for the surface tension and elasticity measurements of the MxH NIL diluted dough liquor fractions.

Chapter 6

	1B					1D		
	x7	y9	x17	y18		x2	x3	y12
1								
2								
3								
4								
5								
6								
8								
9								
10								
11								
12								
13								
14								
15								
16								
17								
18								
19								
20								
21								
22								
23								
24								
25								
27								
28								
30								
32								
33								
34								
35								
36								
37								
39								
40								
41								
43								
45								
46								
47								
48								
49								
50								
52								
53								
54								
55								

56								
57								
58								
59								
60								
61								
62								
63								
64								
65								
66								
67								
68								
69								
70								
71								
72								
73								
74								
75								
76								
77								
78								
79								
80								
82								
83								
86								
87								
88								
89								
91								
92								
93								
94								
95								
96								
97								
98								
99								
100								
101								
102								
103								
104								
105								
106								
107								
108								
109								
110								
111								

Appendix Figure 6.1 The HMW glutenin subunit scores of the MxH DH population. The red blocks indicate the presence of the HMW glutenin subunit in question separated through SDS-PAGE. Yellow blocks indicate faint bands.

	chr	pos	LOD	%var	mean	add eff	sim marker	nearest marker	pos nearest marker	CIstart	CIend
6B@15.0_FA16.0C	6B	15	1.501	7.992	1.801	-0.079	wPt1992	wPt1992	5	0	34
6B@15.0_FA18.2C	6B	15	1.652	8.758	5.149	0.993	gwm193	gwm193	15	7	34
6B@15.0_FAC	6B	15	1.546	8.218	8.09	1.517	gwm193	gwm193	15	7	34
7Aa@0.0_FAC19.2C	7Aa	0	1.047	5.646	0.062	0.021	wPt3901	wPt3901	0	0	35
6B@15.0_UnsFAC	6B	15	1.582	8.402	5.635	1.036	gwm193	gwm193	15	7	34

	start marker	end marker	Pvalue(F)	trait	env	population
6B@15.0_FA16.0C	wmc487	wPt6023	0.01	FA16.0C	MalHer_FAMEs	MalHer
6B@15.0_FA18.2C	wPt2786.a	wPt6023	0.007	FA18.2C	MalHer_FAMEs	MalHer
6B@15.0_FAC	wPt2786.a	wPt6023	0.009	FAC	MalHer_FAMEs	MalHer
7Aa@0.0_FAC19.2C	wPt3901	wPt1096	0.031	FAC19.2C	MalHer_FAMEs	MalHer
6B@15.0_UnsFAC	wPt2786.a	wPt6023	0.008	UnsFAC	MalHer_FAMEs	MalHer

	CI markers
6B@15.0_FA16.0C	wPt7662.a|wPt1992|wPt2786.a|wPt2175|gwm508.c|wPt3733.a|gwm193|wPt1730|barc024|gwm219.a|wPt5158|wPt1325.a
6B@15.0_FA18.2C	wPt2175|gwm508.c|wPt3733.a|gwm193|wPt1730|barc024|gwm219.a|wPt5158|wPt1325.a
6B@15.0_FAC	wPt2175|gwm508.c|wPt3733.a|gwm193|wPt1730|barc024|gwm219.a|wPt5158|wPt1325.a
7Aa@0.0_FAC19.2C	wPt0783|wPt7425|wPt7034|barc070.c|wPt7188|gwm060|wmc083.a
6B@15.0_UnsFAC	wPt2175|gwm508.c|wPt3733.a|gwm193|wPt1730|barc024|gwm219.a|wPt5158|wPt1325.a

Appendix Figure 6.2 QTL table for the Malacca x Hereward FAMEs data. Chr-Chromosome, pos-Postion, LOD- Logarithm of Difference, % var- % variation, CI-confidence interval.

Chapter 9 References

Alvarez, M., Guelman, S., Halford, N., Lustig, S., Reggiardo, M., Ryabushkina, N., Schewry, P., Stein, J. and Vallejos, R. (2000) 'Silencing of HMW glutenins in transgenic wheat expressing extra HMW subunits', *TAG Theoretical and Applied Genetics*, 100(2), pp. 319-327.

Ambwani, D. S. and Fort Jr, T. (1979) 'Pendant drop technique for measuring liquid boundary tensions', *Surface and Colloid Science*: Springer, pp. 93-119.

Aravindan, R., Anbumathi, P. and Viruthagiri, T. (2007) 'Lipase applications in food industry'.

Athenstaedt, K. and Daum, G. (2006) 'The life cycle of neutral lipids: synthesis, storage and degradation', *Cellular and Molecular Life Sciences*, 63(12), pp. 1355-1369.

Bacic, A. and Stone, B. (1981) 'Chemistry and organization of aleurone cell wall components from wheat and barley', *Functional Plant Biology*, 8(5), pp. 475-495.

Baker, J., Parker, H. and Mize, M. (1946) 'Supercentrifugates from dough', *Cereal Chemistry*, 23, pp. 16-30.

Baldwin, P., Adler, J., Davies, M. and Melia, C. (1998) 'High resolution imaging of starch granule surfaces by atomic force microscopy', *Journal of Cereal Science*, 27(3), pp. 255-265.

Baldwin, P. M. (2001) 'Starch Granule-Associated Proteins and Polypeptides: A Review', *Starch-Stärke*, 53(10), pp. 475-503.

Barron, C., Surget, A. and Rouau, X. (2007) 'Relative amounts of tissues in mature wheat (Triticum aestivum L.) grain and their carbohydrate and phenolic acid composition', *Journal of Cereal Science*, 45(1), pp. 88-96.

Baum, M., Lagudah, E. S. and Appels, R. (1992) 'Wide crosses in cereals', *Annual Review of Plant Biology*, 43(1), pp. 117-143.

Becarri, Jacopo and Bartolomeo (1745) 'Frumento. De Bononiensi Scientarium et Artium.', *Instituto atque Academia Commentarii 2 (Part 1)* 2(1), pp. 122-127.

Beleggia, R., Platani, C., Nigro, F., De Vita, P., Cattivelli, L. and Papa, R. (2013) 'Effect of genotype, environment and genotype-by-environment interaction on metabolite profiling in durum wheat (Triticum durum Desf.) grain', *Journal of cereal science*, 57(2), pp. 183-192.

Bennett, M. K. (1935) 'British wheat yield per acre for seven centuries', *Economic History*, 3(10), pp. 12-29.

Blain, J. and Todd, J. (1955) 'The lipoxidase activity of wheat', *Journal of the Science of Food and Agriculture*, 6(8), pp. 471-479.

Bligh, E. G. and Dyer, W. J. (1959) 'A rapid method of total lipid extraction and purification', *Canadian journal of biochemistry and physiology*, 37(8), pp. 911-917.

Bobrow-Strain, A. (2012) *White bread: a social history of the store-bought loaf*. Beacon Press.

Branlard, G., Dardevet, M., Saccomano, R., Lagoutte, F. and Gourdon, J. (2001) 'Genetic diversity of wheat storage proteins and bread wheat quality', *Wheat in a global environment*: Springer, pp. 157-169.

Browse, J. and Somerville, C. (1991) 'Glycerolipid synthesis: biochemistry and regulation', *Annual review of plant biology*, 42(1), pp. 467-506.

Brügger, B., Erben, G., Sandhoff, R., Wieland, F. T. and Lehmann, W. D. (1997) 'Quantitative analysis of biological membrane lipids at the low picomole level by nano-electrospray ionization tandem mass spectrometry', *Proceedings of the National Academy of Sciences*, 94(6), pp. 2339-2344.

Buléon, A., Colonna, P., Planchot, V. and Ball, S. (1998) 'Starch granules: structure and biosynthesis', *International journal of biological macromolecules*, 23(2), pp. 85-112.

Campbell, G. M. and Mougeot, E. (1999) 'Creation and characterisation of aerated food products', *Trends in Food Science & Technology*, 10(9), pp. 283-296.

Carr, N. O., Daniels, N. W. and Frazier, P. J. (1992) 'Lipid interactions in breadmaking', *Critical Reviews in Food Science & Nutrition*, 31(3), pp. 237-258.

Castello, P., Jollet, S., Potus, J., Baret, J.-L. and Nicolas, J. (1998) 'Effect of exogenous lipase on dough lipids during mixing of wheat flours', *Cereal Chemistry*, 75(5), pp. 595-601.

Cauvain, S. P. (2012) *Breadmaking: improving quality*. Elsevier.

Cauvain, S. P. and Young, L. S. (2007) 'Technology of breadmaking'.Elsevier.

Cauvain, S. P. and Young, L. S. (2009) *More baking problems solved.* Elsevier.

Chichti, E., George, M., Delenne, J.-Y. and Lullien-Pellerin, V. (2015) 'Changes in the starch-protein interface depending on common wheat grain hardness revealed using atomic force microscopy', *Plant Science*, 239, pp. 1-8.

Chung, O., Ohm, J., Ram, M., Park, S. H. and Howitt, C. (2009) 'Wheat lipids', *Wheat: Chemistry and technology*, (Ed. 4), pp. 363-399.

Clarke, B., Hobbs, M., Skylas, D. and Appels, R. (2000) 'Genes active in developing wheat endosperm', *Functional & integrative genomics*, 1(1), pp. 44-55.

Consortium, I. W. G. S. 2014. A chromosome-based draft sequence of the hexaploid bread wheat (Triticum aestivum) genome. Science.

Damania, A. and Payne, P. (1984) 'Wheat Storage Proteins: Their Genetics and Their Potential for Manipulation by Plant Breeding: Discussion', *Philosophical Transactions of the Royal Society of London Series B*, 304, pp. 371.

Day, L., Bhandari, D. G., Greenwell, P., Leonard, S. A. and Schofield, J. D. (2006) 'Characterization of wheat puroindoline proteins', *The FEBS journal*, 273(23), pp. 5358-5373.

De Maria, L., Vind, J., Oxenbøll, K., Svendsen, A. and Patkar, S. (2007) 'Phospholipases and their industrial applications', *Applied Microbiology and Biotechnology*, 74(2), pp. 290-300.

De Stefanis, V. and Ponte Jr, J. (1976) 'Studies on the breadmaking properties of wheat-flour nonpolar lipids', *Cereal Chemistry*.

DEFRA *New data reveals the nations changing tastes*. Available at: https://www.gov.uk/government/news/new-data-reveals-the-nations-changing-tastes.

Digby, P. 'KEMPTON. RA 1987. Multivariate analysis of ecological communities', *Population Control of short-term variability*.

Douliez, J.-P., Michon, T., Elmorjani, K. and Marion, D. (2000) 'Mini review: structure, biological and technological functions of lipid transfer proteins and indolines, the major lipid binding proteins from cereal kernels', *Journal of cereal science*, 32(1), pp. 1-20.

Dubcovsky, J. and Dvorak, J. (2007) 'Genome plasticity a key factor in the success of polyploid wheat under domestication', *Science*, 316(5833), pp. 1862-1866.

Dubreil, L., Compoint, J.-P. and Marion, D. (1997) 'Interaction of puroindolines with wheat flour polar lipids determines their foaming properties', *Journal of Agricultural and Food Chemistry*, 45(1), pp. 108-116.

Dubreil, L., Méliande, S., Chiron, H., Compoint, J.-P., Quillien, L., Branlard, G. and Marion, D. (1998) 'Effect of puroindolines on the breadmaking properties of wheat flour', *Cereal Chemistry*, 75(2), pp. 222-229.

Dvorak, J., Luo, M.-C., Yang, Z.-L. and Zhang, H.-B. (1998) 'The structure of the Aegilops tauschii genepool and the evolution of hexaploid wheat', *TAG Theoretical and Applied Genetics*, 97(4), pp. 657-670.

Dörmann, P. (2013) 'Galactolipids in plant membranes', *eLS*.

D'Appolonia, B. and Gilles, K. (1971) 'Effect of various starches in baking1', *Cereal Chemistry*, 48, pp. 625-636.

Ellis, R. P., Cochrane, M. P., Dale, M. F. B., Duffus, C. M., Lynn, A., Morrison, I. M., Prentice, R. D. M., Swanston, J. S. and Tiller, S. A. (1998) 'Starch production and industrial use', *Journal of the Science of Food and Agriculture*, 77(3), pp. 289-311.

Fabflour. Available at: http://fabflour.co.uk/fab-bread/misconceptions-about-bread/.

Fabflour. Available at: https://www.fob.uk.com/about-the-bread-industry/.

FAOSTAT. Available at: http://www.fao.org/faostat/en/#data/QC.

Fawcett, T., Copse, C. L., Simon, J. W. and Slabas, A. R. (2000) 'Kinetic mechanism of NADH-enoyl-ACP reductase from Brassica napus', *FEBS letters*, 484(2), pp. 65-68.

Feiz, L., Wanjugi, H., Melnyk, C., Altosaar, I., Martin, J. and Giroux, M. (2009) 'Puroindolines co-localize to the starch granule surface and increase seed bound polar lipid content', *Journal of cereal science*, 50(1), pp. 91-98.

Field, J., Shewry, P., Burgess, S., Forde, J., Parmar, S. and Miflin, B. (1983) 'The presence of high molecular weight aggregates in the protein bodies of developing endosperms of wheat and other cereals', *Journal of Cereal Science*, 1(1), pp. 33-41.

Finnie, S., Jeannotte, R. and Faubion, J. (2009) 'Quantitative characterization of polar lipids from wheat whole meal, flour, and starch', *Cereal Chemistry*, 86(6), pp. 637-645.

Finnie, S., Jeannotte, R., Morris, C. and Faubion, J. (2010) 'Variation in polar lipid composition among near-isogenic wheat lines possessing different puroindoline haplotypes', *Journal of cereal science*, 51(1), pp. 66-72.

French, D. (1984) 'Organization of starch granules', *Starch chemistry and technology*, pp. 183-247.

Fruhner, H., Wantke, K.-D. and Lunkenheimer, K. (2000) 'Relationship between surface dilational properties and foam stability', *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 162(1), pp. 193-202.

Fu, D., Szűcs, P., Yan, L., Helguera, M., Skinner, J. S., Von Zitzewitz, J., Hayes, P. M. and Dubcovsky, J. (2005) 'Large deletions within the first intron in VRN-1 are associated with spring growth habit in barley and wheat', *Molecular Genetics and Genomics*, 273(1), pp. 54-65.

Fulcher, R., O'brien, T. and Simmonds, D. (1972) 'Localization of arginine-rich proteins in mature seeds of some members of the Gramineae', *Australian Journal of Biological Sciences*, 25(3), pp. 487-498.

Gallant, D. and Sterling, C. (1976) 'Electron microscopy of starch and starch products', *Examination and Analysis of Starch and Starch Products*: Springer, pp. 33-59.

Gan, Z., Angold, R., Williams, M., Ellis, P., Vaughan, J. and Galliard, T. (1990) 'The microstructure and gas retention of bread dough', *Journal of Cereal Science*, 12(1), pp. 15-24.

Gan, Z., Ellis, P. and Schofield, J. (1995) 'Gas cell stabilisation and gas retention in wheat bread dough', *Journal of Cereal Science*, 21(3), pp. 215-230.

Gautier, M.-F., Aleman, M.-E., Guirao, A., Marion, D. and Joudrier, P. (1994) 'Triticum aestivum puroindolines, two basic cystine-rich seed proteins: cDNA sequence analysis and developmental gene expression', *Plant molecular biology*, 25(1), pp. 43-57.

Gerits, L. R., Pareyt, B. and Delcour, J. A. (2014) 'A lipase based approach for studying the role of wheat lipids in bread making', *Food chemistry*, 156, pp. 190-196.

Giroux, M. J. and Morris, C. F. (1998) 'Wheat grain hardness results from highly conserved mutations in the friabilin components puroindoline a and b', *Proceedings of the National Academy of Sciences*, 95(11), pp. 6262-6266.

Goesaert, H., Brijs, K., Veraverbeke, W., Courtin, C., Gebruers, K. and Delcour, J. (2005) 'Wheat flour constituents: how they impact bread quality, and how to impact their functionality', *Trends in food science & technology*, 16(1), pp. 12-30.

Goff, S. A., Ricke, D., Lan, T.-H., Presting, G., Wang, R., Dunn, M., Glazebrook, J., Sessions, A., Oeller, P. and Varma, H. (2002) 'A draft sequence of the rice genome (Oryza sativa L. ssp. japonica)', *Science*, 296(5565), pp. 92-100.

Gonzalez-Thuillier, I., Salt, L., Chope, G., Penson, S., Skeggs, P., Tosi, P., Powers, S. J., Ward, J. L., Wilde, P. and Shewry, P. R. (2015) 'Distribution of lipids in the grain of wheat (cv. Hereward) determined by lipidomic analysis of milling and pearling fractions', *Journal of agricultural and food chemistry*, 63(49), pp. 10705-10716.

Graham, D. and Phillips, M. (1976) 'The conformation of proteins at the air-water interface and their role in stabilizing foams', *Foams*, pp. 237-255.

Greenblatt, G., Bettge, A. and Morris, C. (1995) 'Relationship between endosperm texture and the occurrence of friabilin and bound polar lipids on wheat starch', *Cereal Chemistry*, 72(2), pp. 172-175.

Groos, C., Bervas, E., Chanliaud, E. and Charmet, G. (2007) 'Genetic analysis of bread-making quality scores in bread wheat using a recombinant inbred line population', *Theoretical and Applied Genetics*, 115(3), pp. 313-323.

Grundas, S. (2003) 'Wheat| grain structure of wheat and wheat-based products', *Encyclopedia of Food Science and Nutrition*, pp. 6130-6137.

Hagio, M., Sakurai, I., Sato, S., Kato, T., Tabata, S. and Wada, H. (2002) 'Phosphatidylglycerol is essential for the development of thylakoid membranes in Arabidopsis thaliana', *Plant and cell physiology*, 43(12), pp. 1456-1464.

Halford, N., Field, J., Blair, H., Urwin, P., Moore, K., Robert, L., Thompson, R., Flavell, R., Tatham, A. and Shewry, P. (1992) 'Analysis of HMW glutenin subunits encoded by chromosome 1A of bread wheat (Triticum aestivum L.) indicates quantitative effects on grain quality', *TAG Theoretical and Applied Genetics*, 83(3), pp. 373-378.

Hammer, K. (1984) 'Das domestikations syndrom', *Die Kulturpflanze*, 32(1), pp. 11-34.

Hanselmann, W. and Windhab, E. (1998) 'Flow characteristics and modelling of foam generation in a continuous rotor/stator mixer', *Journal of Food Engineering*, 38(4), pp. 393-405.

Hargin, K. D. and Morrison, W. R. (1980) 'The distribution of acyl lipids in the germ, aleurone, starch and non-starch endosperm of four wheat varieties', *Journal of the Science of Food and Agriculture*, 31(9), pp. 877-888.

Harwood, J. L. (2010) 'Plant fatty acid synthesis', *The AOCS Lipid Library" Electronic resource http://lipidlibrary. aocs. org*.

Hayman, D. A., Sipes, K., Hoseney, R. and Faubion, J. (1998) 'Factors controlling gas cell failure in bread dough', *Cereal Chemistry*, 75(5), pp. 585-589.

Hebeda, R. (1996) *Baked goods freshness: Technology, evaluation, and inhibition of staling.* CRC Press.

Heilmann, I. (2009) 'Using genetic tools to understand plant phosphoinositide signalling', *Trends in plant science*, 14(3), pp. 171-179.

Helmerich, G. and Koehler, P. (2005) 'Functional properties of individual classes of phospholipids in breadmaking', *Journal of cereal science*, 42(2), pp. 233-241.

Heun, M., Schäfer-Pregl, R., Klawan, D., Castagna, R., Accerbi, M., Borghi, B. and Salamini, F. (1997) 'Site of einkorn wheat domestication identified by DNA fingerprinting', *Science,* 278(5341), pp. 1312-1314.

Hildebrand, D. (2012) 'Production of unusual fatty acids in plants', *The AOCS Lipid Library" Electronic resource*. http://lipidlibrary.aocs.org/plantbio/ unusualfa/index.html.

Horn, R., Wingen, L., Snape, J. and Dolan, L. (2016) 'Mapping of quantitative trait loci for root hair length in wheat identifies loci that co-locate with loci for yield components', *Journal of experimental botany,* 67(15), pp. 4535-4543.

Hoseney, R. C., Finney, K., Pomeranz, Y. and Shogren, M. (1971) 'Functional (breadmaking) and biochemical properties of wheat flour components. VIII. Starch', *Cereal chemistry*.

Hsein-Chih, H. W. and Sarko, A. (1978) 'The double-helical molecular structure of crystalline B-amylose', *Carbohydrate Research,* 61(1), pp. 7-25.

Huebner, F. and Wall, J. (1976) 'Fractionation and quantitative differences of glutenin from wheat varieties varying in baking quality', *Cereal Chem,* 53(2), pp. 258-269.

Husband, F., Sarney, D., Barnard, M. and Wilde, P. (1998) 'Comparison of foaming and interfacial properties of pure sucrose monolaurates, dilaurate and commercial preparations', *Food Hydrocolloids,* 12(2), pp. 237-244.

Imberty, A., Buléon, A., Tran, V. and Péerez, S. (1991) 'Recent advances in knowledge of starch structure', *Starch-Stärke,* 43(10), pp. 375-384.

Inagaki, M. and Tahir, M. (1990) 'Comparison of haploid production frequencies in wheat varieties crossed with Hordeum bulbosum L. and maize', *Japanese Journal of Breeding,* 40(2), pp. 209-216.

Jantasuriyarat, C., Vales, M., Watson, C. and Riera-Lizarazu, O. (2004) 'Identification and mapping of genetic loci affecting the free-threshing habit and spike compactness in wheat (Triticum aestivum L.)', *Theoretical and Applied Genetics,*108(2), pp. 261-273.

Joyard, J., Maréchal, E., Miège, C., Block, M. A., Dorne, A.-J. and Douce, R. (1998) 'Structure, distribution and biosynthesis of glycerolipids from higher plant chloroplasts', *Lipids in photosynthesis: Structure, function and genetics*: Springer, pp. 21-52.

Kalischewski, K. and Schügerl, K. (1979) 'Investigation of protein foams obtained by bubbling', *Colloid & Polymer Science,* 257(10), pp. 1099-1110.

Keller, R., Orsel, R. and Hamer, R. (1997) 'Competitive adsorption behaviour of wheat flour components and emulsifiers at an air–water interface', *Journal of Cereal Science,* 25(2), pp. 175-183.

Kelly, A. A., Kalisch, B., Hölzl, G., Schulze, S., Thiele, J., Melzer, M., Roston, R. L., Benning, C. and Dörmann, P. (2016) 'Synthesis and transfer of galactolipids in the chloroplast envelope membranes of Arabidopsis thaliana', *Proceedings of the National Academy of Sciences,* 113(38), pp. 10714-10719.

Kim, S. and Kinsella, J. (1985) 'Surface activity of food proteins: relationships between surface pressure development, viscoelasticity of interfacial films and foam stability of bovine serum albumin', *Journal of Food Science,* 50(6), pp. 1526-1530.

Kim, W. and Seib, P. (1993) 'Lipids in some commercial wheat flours 1', *Cereal 451 Chem*, 70, pp. 367-372.

Koczo, K. and Racz, G. (1991) 'Foaming properties of surfactant solutions', *Colloids and surfaces*, 56, pp. 59-82.

Konopka, I., Rotkiewicz, D. and Tańska, M. (2005) 'Wheat endosperm hardness. Part II. Relationships to content and composition of flour lipids', *European Food Research and Technology*, 220(1), pp. 20-24.

Krog, N. (1981) 'Theoretical aspects of surfactants in relation to their use in breadmaking', *Cereal Chemistry*.

Kruger, J. E. (1972) 'Changes in the amylases of hard red spring wheat during grwoth and maturation', *Cereal chemistry*.

Kruger, J. and Preston, K. (1976) 'The nature and role of proteolytic enzymes during early germination', *Cereal research communications*, pp. 213-219.

Kuchel, H., Langridge, P., Mosionek, L., Williams, K. and Jefferies, S. (2006) 'The genetic control of milling yield, dough rheology and baking quality of wheat', *Theoretical and Applied Genetics*, 112(8), pp. 1487.

Kusunose, C., Fujii, T. and Matsumoto, H. (1999) 'Role of starch granules in controlling expansion of dough during baking', *Cereal chemistry*, 76(6), pp. 920-924.

Larré, C., Nicolas, Y., Desserme, C., Courcoux, P. and Popineau, Y. (1997) 'Preparative separation of high and low molecular weight subunits of glutenin from wheat', *Journal of cereal science*, 25(2), pp. 143-150.

Laurie, D. and Reymondie, S. (1991) 'High frequencies of fertilization and haploid seedling production in crosses between commercial hexaploid wheat varieties and maize', *Plant Breeding*, 106(3), pp. 182-189.

Laurie, D. A. and Bennett, M. (1988) 'The production of haploid wheat plants from wheat x maize crosses', *TAG Theoretical and Applied Genetics*, 76(3), pp. 393-397.

Levanony, H., Rubin, R., Altschuler, Y. and Galili, G. (1992) 'Evidence for a novel route of wheat storage proteins to vacuoles', *The Journal of Cell Biology*, 119(5), pp. 1117-1128.

Levy, A. and Feldman, M. (1988) 'Ecogeographical distribution of HMW glutenin alleles in populations of the wild tetraploid wheat Triticum turgidum var. dicoccoides', *Theoretical and Applied Genetics*, 75(4), pp. 651-658.

Li, M., Baughman, E., Roth, M. R., Han, X., Welti, R. and Wang, X. (2014) 'Quantitative profiling and pattern analysis of triacylglycerol species in Arabidopsis seeds by electrospray ionization mass spectrometry', *The Plant Journal*, 77(1), pp. 160-172.

Li-Beisson, Y., Shorrosh, B., Beisson, F., Andersson, M. X., Arondel, V., Bates, P. D., Baud, S., Bird, D., DeBono, A. and Durrett, T. P. (2013) 'Acyl-lipid metabolism', *The Arabidopsis Book*, 11, pp. e0161.

Liu, L., Guo, Q., He, Z., Xia, X., Waters, D. L., Raymond, C. A. and King, G. J. (2017) 'Genotypic Variation in Wheat Flour Lysophospholipids', *Molecules*, 22(6), pp. 909.

Liu, L., Tong, C., Bao, J., Waters, D. L., Rose, T. J. and King, G. J. (2014) 'Determination of starch lysophospholipids in rice using liquid chromatography–mass spectrometry (LC-MS)', *Journal of agricultural and food chemistry*, 62(28), pp. 6600-6607.

Lorient, D., Closs, B. and Courthaudon, J. L. (1989) 'Surface properties of the bovine casein components: relationships between structure and foaming properties', *Journal of Dairy Research*, 56(3), pp. 495-502.

Luo, M.-C., Yang, Z.-L., You, F., Kawahara, T., Waines, J. and Dvorak, J. (2007) 'The structure of wild and domesticated emmer wheat populations, gene flow between them, and the site of emmer domestication', *Theoretical and Applied Genetics*, 114(6), pp. 947-959.

MacRitchie, F. (1976) 'Liquid phase of dough and its role in baking', *Cereal Chemistry*.

MacRitchie, F. (1983) 'Role of lipids in baking', *Lipids in cereal technology*, pp. 165-188.

MacRitchie, F. (1992) 'Physicochemical properties of wheat proteins in relation to functionality', *Advances in food and nutrition research*, 36, pp. 1-87.

MacRitchie, F. and Fletcher, P. (1990) Chemistry at interfaces: Academic Press, San Diego, CA, 1990 (ISBN 0-12-464785-5). Elsevier. pp 277

Margiotta, B., Urbano, M., Colaprico, G., Turchetta, T. and Lafiandra, D. (1998) 'Variation of high molecular weight glutenin subunits in tetraploid wheats of genomic formula AAGG'. *Proceedings of the 9th International wheat Genetics Symposium''(AE Slinkard, ed.)*, 195-197.

Matsoukas, N. P. and Morrison, I. R. (1991) 'Breadmaking quality of ten greek breadwheats. II. Relationships of protein, lipid and starch components to baking quality', *Journal of the Science of Food and Agriculture*, 55(1), pp. 87-101.

McCann, T. H., Small, D. M., Batey, I. L., Wrigley, C. W. and Day, L. (2009) 'Protein–lipid interactions in gluten elucidated using acetic acid fractionation', *Food Chemistry*, 115(1), pp. 105-112.

Millar, S., Snape, J., Ward, J., Shewry, P., Belton, P., Boniface, K. and Summers, R. (2008) 'Investigating wheat functionality through breeding and end use', *CCFRA Report on LINK Project FQS*, 23.

Mills, E., Wilde, P., Salt, L. and Skeggs, P. (2003) 'Bubble formation and stabilization in bread dough', *Food and bioproducts processing*, 81(3), pp. 189-193.

Min, B., Gonzalez-Thuillier, I., Powers, S. J., Wilde, P., Shewry, P. R. and Haslam, R. P. (2017) 'Effects of Cultivar and Nitrogen Nutrition on the Lipid Composition of Wheat Flour', *Journal of Agricultural and Food Chemistry*.

Morris, C., Greenblatt, G., Bettge, A. and Malkawi, H. (1994) 'Isolation and characterization of multiple forms of friabilin', *Journal of Cereal Science*, 20(2), pp. 167-174.

Morrison, W., Milligan, T. and Azudin, M. (1984) 'A relationship between the amylose and lipid contents of starches from diploid cereals', *Journal of Cereal Science*, 2(4), pp. 257-271.

Morrison, W. R., Law, C. N., Wylie, L. J., Coventry, A. M. and Seekings, J. (1989) 'The effect of group 5 chromosomes on the free polar lipids and breadmaking quality of wheat', *Journal of Cereal Science,* 9(1), pp. 41-51.

Murray, J. (1970) 'The first European agriculture; a study of the osteological and botanical evidence until two thousand BC'.

Nakai, S. and Li-Chan, E. (1993) 'Recent advances in structure and function of food proteins: QSAR approach', *Critical Reviews in Food Science & Nutrition,* 33(6), pp. 477-499.

Nalam, V. J., Vales, M. I., Watson, C. J., Kianian, S. F. and Riera-Lizarazu, O. (2006) 'Map-based analysis of genes affecting the brittle rachis character in tetraploid wheat (Triticum turgidum L.)', *Theoretical and Applied Genetics,* 112(2), pp. 373-381.

Narayanan, S., Prasad, P. and Welti, R. (2016a) 'Wheat leaf lipids during heat stress: II. Lipids experiencing coordinated metabolism are detected by analysis of lipid co-occurrence', *Plant, cell & environment,* 39(3), pp. 608-617.

Narayanan, S., Tamura, P. J., Roth, M. R., Prasad, P. and Welti, R. (2016b) 'Wheat leaf lipids during heat stress: I. High day and night temperatures result in major lipid alterations', *Plant, cell & environment,* 39(4), pp. 787-803.

Oda, S. and Schofield, J. (1997) 'Characterisation of friabilin polypeptides', *Journal of Cereal Science,* 26(1), pp. 29-36.

Ö rnebro, J., Nylander, T. and Eliasson, A.-C. (2000) 'Critical review-interfacial behaviour of wheat proteins', *Journal of Cereal Science,* 31(2), pp. 195-221.

Osborne, T. and Mendel, L. (1924) 'Continuation and extension of work on vegetable proteins', *Exp Stn Rec,* 53, pp. 364.

Ozkan, H., Brandolini, A., Pozzi, C., Effgen, S., Wunder, J. and Salamini, F. (2005) 'A reconsideration of the domestication geography of tetraploid wheats', *Theoretical and Applied Genetics,* 110(6), pp. 1052-1060.

Pandolfe, W. D. (1981) 'Effect of dispersed and continuous phase viscosity on droplet size of emulsions generated by homogenization', *Journal of Dispersion Science and Technology,* 2(4), pp. 459-474.

Panozzo, J., O'Brien, L., MacRitchie, F. and Bekes, F. (1990) 'Baking quality of Australian wheat cultivars varying in their free lipid composition', *Journal of Cereal Science,* 11(1), pp. 51-57.

Pareyt, B., Finnie, S. M., Putseys, J. A. and Delcour, J. A. (2011) 'Lipids in bread making: Sources, interactions, and impact on bread quality', *Journal of Cereal Science,* 54(3), pp. 266-279.

Pauly, A., Pareyt, B., Lambrecht, M. A., Fierens, E. and Delcour, J. A. (2013) 'Flour from wheat cultivars of varying hardness produces semi-sweet biscuits with varying textural and structural properties', *LWT-Food Science and Technology,*53(2), pp. 452-457.

Payne, P., Holt, L., Harinder, K., Macartney, D. and Lawrence, G. (1987a) 'use of near-isogenic lines with different HMW glutenin subunits in studying bread-making quality and glutenin

structure'. *Proceedings of the 3rd International Workshop on Gluten Proteins, Budapest, Hungary, May 9-12, 1987/editors, R. Lasztity, F. Bekes*: Singapore: World Scientific, c1987.

Payne, P. I. (1987) 'Genetics of wheat storage proteins and the effect of allelic variation on bread-making quality', *Annual Review of Plant Physiology*, 38(1), pp. 141-153.

Payne, P. I., Corfield, K. G., Holt, L. M. and Blackman, J. A. (1981) 'Correlations between the inheritance of certain high-molecular weight subunits of glutenin and bread-making quality in progenies of six crosses of bread wheat', *Journal of the Science of Food and Agriculture*, 32(1), pp. 51-60.

Payne, P. I. and Lawrence, G. J. (1983) 'Catalogue of alleles for the complex gene loci, Glu-A1, Glu-B1, and Glu-D1 which code for high-molecular-weight subunits of glutenin in hexaploid wheat', *Cereal Research Communications*, pp. 29-35.

Payne, P. I., Nightingale, M. A., Krattiger, A. F. and Holt, L. M. (1987b) 'The relationship between HMW glutenin subunit composition and the bread-making quality of British-grown wheat varieties', *Journal of the Science of Food and Agriculture*, 40(1), pp. 51-65.

Peers, F. (1953) 'The phytase of wheat', *Biochemical Journal*, 53(1), pp. 102.

Pomeranz, Y., Carvajal, M., Shogren, M., Hoseney, R. and Finney, K. (1970) 'Wheat germ in breadmaking. II. Improving breadmaking properties by physical and chemical methods', *Cereal chemistry*.

Pomeranz, Y., Shogren, M. and Finney, K. (1968) 'Natural and modified phospholipids effects on bread quality', *Food Technology*, 22(7), pp. 1897-&.

Ragupathy, R., Naeem, H. A., Reimer, E., Lukow, O. M., Sapirstein, H. D. and Cloutier, S. (2008) 'Evolutionary origin of the segmental duplication encompassing the wheat GLU-B1 locus encoding the overexpressed Bx7 (Bx7OE) high molecular weight glutenin subunit', *Theoretical and Applied Genetics*, 116(2), pp. 283-296.

Riedelsheimer, C., Brotman, Y., Méret, M., Melchinger, A. E. and Willmitzer, L. (2013) 'The maize leaf lipidome shows multilevel genetic control and high predictive value for agronomic traits', *Scientific reports*, 3.

Rocha, J. M., Kalo, P. J. and Malcata, F. X. (2012) 'Composition of neutral lipid classes and content of fatty acids throughout sourdough breadmaking', *European journal of lipid science and technology*, 114(3), pp. 294-305.

Roche, I. A. d. l., Andrews, C., Pomeroy, M., Weinberger, P. and Kates, M. (1972) 'Lipid changes in winter wheat seedlings (Triticum aestivum) at temperatures inducing cold hardiness', *Canadian Journal of Botany*, 50(12), pp. 2401-2409.

Roughan, P. G. (1985) 'Phosphatidylglycerol and chilling sensitivity in plants', *Plant physiology*, 77(3), pp. 740-746.

Ruiz-Lopez, N., Haslam, R. P., Usher, S., Napier, J. A. and Sayanova, O. (2015) 'An alternative pathway for the effective production of the omega-3 long-chain polyunsaturates EPA and ETA in transgenic oilseeds', *Plant biotechnology journal*,13(9), pp. 1264-1275.

Sahi, S. (1994) 'Interfacial properties of the aqueous phases of wheat flour doughs', *Journal of Cereal Science*, 20(2), pp. 119-127.

Sahi, S. S. and Alava, J. M. (2003) 'Functionality of emulsifiers in sponge cake production', *Journal of the Science of Food and Agriculture*, 83(14), pp. 1419-1429.

Sahlstrom, S., Boevre, A. and Graybosch, R. (2006) 'Impact of Waxy, Partial Waxy, and Wildtype Wheat Starch Fraction Properties on Hearth Bread Characteristics', *Cereal chemistry*, 83(6), pp. 647-654.

Salt, L., Wilde, P., Georget, D., Wellner, N., Skeggs, P. and Mills, E. (2006) 'Composition and surface properties of dough liquor', *Journal of Cereal Science*, 43(3), pp. 284-292.

Salt, L. J., González-Thuillier, I., Chope, G., Penson, S., Tosi, P., Haslam, R. P., Skeggs, P. K., Shewry, P. R. and Wilde, P. J. (2017) 'Intrinsic wheat lipid composition effects the interfacial and foaming properties of dough liquor', *Food Hydrocolloids*.

Seilmeier, W., Belitz, H.-D. and Wieser, H. (1991) 'Separation and quantitative determination of high-molecular-weight subunits of glutenin from different wheat varieties and genetic variants of the variety Sicco', *Zeitschrift für Lebensmitteluntersuchung und-Forschung A*, 192(2), pp. 124-129.

Shewry, P. R., Halford, N. G., Belton, P. S. and Tatham, A. S. (2002) 'The structure and properties of gluten: an elastic protein from wheat grain', *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 357(1418), pp. 133-142.

Shewry, P. R. and Lookhart, G. L. (2003) *Wheat gluten protein analysis.* American Association of Cereal Chemists.

Shewry, P. R., Tatham, A. S., Forde, J., Kreis, M. and Miflin, B. J. (1986) 'The classification and nomenclature of wheat gluten proteins: a reassessment', *Journal of Cereal Science*, 4(2), pp. 97-106.

Shimiya, Y. and Nakamura, K. (1997) 'Changes in size of gas cells in dough and bread during breadmaking and calculation of critical size of gas cells that expand', *Journal of texture studies*, 28(3), pp. 273-288.

Simons, K. J., Fellers, J. P., Trick, H. N., Zhang, Z., Tai, Y.-S., Gill, B. S. and Faris, J. D. (2006) 'Molecular characterization of the major wheat domestication gene Q', *Genetics*, 172(1), pp. 547-555.

Skylas, D., Mackintosh, J., Cordwell, S., Basseal, D., Walsh, B., Harry, J., Blumenthal, C., Copeland, L., Wrigley, C. and Rathmell, W. (2000) 'Proteome approach to the characterisation of protein composition in the developing and mature wheat-grain endosperm', *Journal of Cereal Science*, 32(2), pp. 169-188.

Slabas, A., Chase, D., Nishida, I., Murata, N., Sidebottom, C., Safford, R., Sheldon, P., Kekwick, R., Hardie, D. and Mackintosh, R. (1992) 'Molecular cloning of higher-plant 3-oxoacyl-(acyl carrier protein) reductase. Sequence identities with the nodG-gene product of the nitrogen-fixing soil bacterium Rhizobium meliloti', *Biochemical journal*, 283(2), pp. 321-326.

Sroan, B. S. and MacRitchie, F. (2009) 'Mechanism of gas cell stabilization in breadmaking. II. The secondary liquid lamellae', *Journal of Cereal Science*, 49(1), pp. 41-46.

Statista. Available at: https://www.statista.com/statistics/299765/wheat-uk-imports-from-eu-countries-united-kingdom/.

Suenaga, K. and Nakajima, K. (1989) 'Efficient production of haploid wheat (Triticum aestivum) through crosses between Japanese wheat and maize (Zea mays)', *Plant Cell Reports,* 8(5), pp. 263-266.

Takeda, Y., Hizukuri, S., Takeda, C. and Suzuki, A. (1987) 'Structures of branched molecules of amyloses of various origins, and molar fractions of branched and unbranched molecules', *Carbohydrate Research,* 165(1), pp. 139-145.

Toepfer, E., Polansky, M., Eheart, J., Slover, H., Morris, E., Hepburn, F. and Quackenbush, F. (1972) 'Nutrient composition of selected wheats and wheat products. XI. Summary', *Cereal chemistry*.

Tong, C., Chen, Y., Tang, F., Xu, F., Huang, Y., Chen, H. and Bao, J. (2014) 'Genetic diversity of amylose content and RVA pasting parameters in 20 rice accessions grown in Hainan, China', *Food chemistry,* 161, pp. 239-245.

Uauy, C., Distelfeld, A., Fahima, T., Blechl, A. and Dubcovsky, J. (2006) 'A NAC gene regulating senescence improves grain protein, zinc, and iron content in wheat', *Science,* 314(5803), pp. 1298-1301.

Vawser, M. and Cornish, G. B. (2004) 'Over-expression of HMW glutenin subunit Glu-B1 7x in hexaploid wheat varieties (Triticum aestivum)', *Australian Journal of Agricultural Research,* 55(5), pp. 577-588.

Veraverbeke, W. S. and Delcour, J. A. (2002) 'Wheat protein composition and properties of wheat glutenin in relation to breadmaking functionality', *Critical Reviews in Food Science and Nutrition,* 42(3), pp. 179-208.

Vigh, L., Horvàth, I., van Hasselt, P. R. and Kuiper, P. J. (1985) 'Effect of frost hardening on lipid and fatty acid composition of chloroplast thylakoid membranes in two wheat varieties of contrasting hardiness', *Plant physiology,* 79(3), pp. 756-759.

Vliet, T. v., Janssen, A., Bloksma, A. and Walstra, P. (1992) 'Strain hardening of dough as a requirement for gas retention', *Journal of Texture Studies,* 23(4), pp. 439-460.

Waines, J. and Payne, P. (1987) 'Electrophoretic analysis of the high-molecular-weight glutenin subunits of Triticum monococcum, T. urartu, and the A genome of bread wheat (T. aestivum)', *TAG Theoretical and Applied Genetics,* 74(1), pp. 71-76.

Weber, K. and Osborn, M. (1969) 'The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis', *Journal of Biological Chemistry,* 244(16), pp. 4406-4412.

Whattam, J. and Cornell, H. (1991) 'Distribution and composition of the lipids in starch fractions from wheat flour', *Starch-Stärke,* 43(4), pp. 152-156.

Wieser, H. (2007) 'Chemistry of gluten proteins', *Food microbiology,* 24(2), pp. 115-119.

Wilde, P. J. (1996) 'Foam measurement by the microconductivity technique: An assessment of its sensitivity to interfacial and environmental factors', *Journal of colloid and interface science,* 178(2), pp. 733-739.

Wilde, P. J., Clark, D. C. and Marion, D. (1993) 'Influence of competitive adsorption of a lysopalmitoylphosphatidylcholine on the functional properties of puroindoline, a lipid-binding protein isolated from wheat flour', *Journal of Agricultural and Food Chemistry*, 41(10), pp. 1570-1576.

William, A., Alain, B. and Maarten, V. G. (2011) *The world wheat book: a history of wheat breeding.* Lavoisier.

Williams, M., Shewry, P. and Harwood, J. (1994) 'The influence of the 'greenhouse effect'on wheat (Triticum aestivum L.) grain lipids', *Journal of experimental botany*, 45(10), pp. 1379-1385.

Yan, L., Helguera, M., Kato, K., Fukuyama, S., Sherman, J. and Dubcovsky, J. (2004) 'Allelic variation at the VRN-1 promoter region in polyploid wheat', *Theoretical and applied genetics*, 109(8), pp. 1677-1686.

Young, L. S., Cauvain, S. P. and Davies, P. R. (2002) 'Rise again, fair knowledge', *Applications and Innovations in Intelligent Systems IX*: Springer, pp. 89-99.

Zghal, M., Scanlon, M. and Sapirstein, H. (2002) 'Cellular structure of bread crumb and its influence on mechanical properties', *Journal of Cereal Science*, 36(2), pp. 167-176.

Chapter 10 Publication

Min, B., Gonzalez-Thuillier, I., Powers, S. J., Wilde, P., Shewry, P. R. and Haslam, R. P. (2017) 'Effects of Cultivar and Nitrogen Nutrition on the Lipid Composition of Wheat Flour', *Journal of Agricultural and Food Chemistry*.