Characterization of old and modern durum wheat genotypes in relation to gluten protein and dietary fibre composition

Contents

Abstract

Old wheat varieties have been suggested to have greater health benefits compared with modern cultivars in relation to both bioactive components and gluten composition. However limited data are available supporting this hypothesis, in particular for durum wheat. So the purpose of this thesis was to improve our understanding of the influence of Italian 20th century breeding on the main grain quality characters. To this aim phenotyping of an old and a modern durum wheat group of genotypes was performed in relation to gluten and dietary fibre composition. The better gluten index observed in the modern group of genotypes was related to higher contents of glutenin and B- type LMW-GS which were, on average, two times higher in the modern group of durum wheat genotypes. Instead, a drastic reduction of the content of ω- 5 gliadins, also known as Tri a 19 a major allergen in food wheat allergy (WDEIA), was observed in the modern genotypes. The immunological and proteomic approaches adopted allowed these differences to be related not only to global down-expression, but also differences in specific isoforms.

In relation to environmental influence on gluten protein composition, a higher glia/glu ratio, and contents of omega gliadins and type B LMW-GS content were observed when water deficit occurred during grain filling in 2013 crop season.

Cell wall dietary fibre were determined with arabinoxylan (AX) and β-glucan (MLG) composition being determined by enzymatic fingerprinting in wholemeal and semolina flour.

Although no significant variations were observed in the total amount of AX, a higher proportion of water soluble AX was observed in the modern varieties in wholemeal flour.

The water soluble AX extracted from semolina flour showed a lower arabinose : xylose ratio in the old genotypes while a higher MLG content in semolina was observed in modern varieties. No differences were observed between the viscosities of aqueous extracts of soluble DF in old and recent varieties but considerable variability was observed between the different durum wheat genotypes. Similarly, no significant differences were observed between the contents of bound phenolic acids in the old and the modern genotypes.

In relation to environmental influence on dietary fibre composition, increases in %WE-AX, relative viscosity and G3/G4 β-glucan ratio were observed when higher rainfall occurred during grain filling in 2014.

In conclusion the 20th century breeding seems to have improved both technological and healthy quality of Italian durum wheat genotypes.

Key words: durum wheat, breeding, gluten, grain quality, health, dietary fibre

1. INTRODUCTION

1.1.Durum wheat: ***economical, botanical and agronomical aspects***

Cereals are the most cultivated plants in the world. According to the Food and Agriculture Organization of the United States (FAO), wheat is the first cultivated crop interesting about 18% of the world cultivated area (Figure 1) and is included in the "big three" cereal crops with over 700 millions of tons produced together with corn and rice (http://faostat.fao.org/).

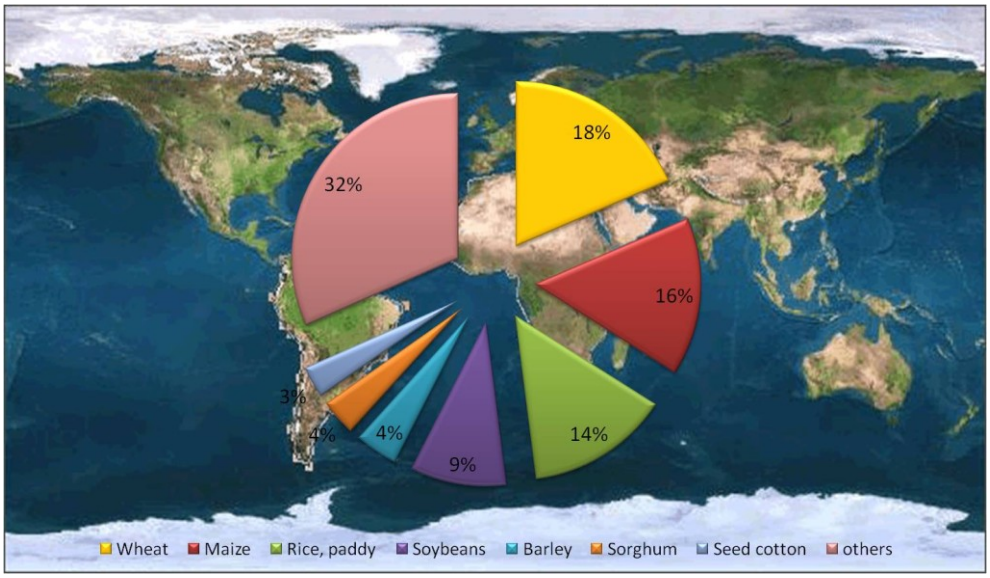


Figure 1 List of the top 7 crops produced in the world (FAO, 2015)

Among cultivated wheat species, durum wheat (*Triticum turgidum* L., subsp. *durum* Desf.), often called pasta wheat (Shewry, 2009), represents about 5% of the total wheat production (Shewry, 2009) and it is mainly cultivated in the temperate world areas, in particular in north America and Mediterranean basin (International Grain Council, 2015). Italy, together with Canada, represents one of the top world producer (Table 1), with the national production concentrated in the Southern and in the central regions (De Vita *et al.*, 2006). Semolina flour obtained by the milling process is used for pasta production, but also for semolina bread, cous cous and other bakery products (Flagella, 2006; Sissons, 2008).

Table 1 Worldwide durum wheat production (expressed as millions of tons) – International Grain Council 2015

Country	2011/12	2012/13	2013/14	2014/15	Variation
EU-28	8.2	7.9	7.9	7.7	-2.5%
France	2.1	2.4	1.8	1.6	-11.1%
Greece	0.9	0.7	0.8	0.9	12.5%
Italy	3.9	4.2	4.0	4.0	0%
Spain	0.9	0.4	0.9	0.9	0%
Kazakhstan	3.0	1.4	2.0	2.1	5.0%
Canada	4.2	4.6	6.5	4.8	-26.2%
Mexico	2.2	2.1	2.3	2.3	0.1%
USA	1.4	2.2	1.7	2.5	47.1%
Argentina	0.2	0.2	0.3	0.3	0.0%
Syria	1.7	1.5	1.5	1.5	0.0%
Turkey	3.0	3.0	3.0	2.8	-6.7%
India	1.1	1.2	1.2	1.3	8.3%
Algeria	2.5	3.0	2.5	2.5	0.0%
Morocco	1.7	1.3	1.9	1.6	-15.8%
Tunisia	1.2	1.3	1.2	1.2	0.0%
Australia	0.6	0.5	0.5	0.5	0.0%
others	5.8	8.2	5.7	5.7	0.0%
World total	**36.7**	**35.2**	**38.0**	**36.6**	**-3.7%**

Durum wheat is a tetraploid (AABB) species belonging to the Poaceae family and *Triticum* genus. The first cultivation of wheats, in particular diploid einkorn (AA) and tetraploid emmer (AABB), occurred in the Neolithic revolution (about 10000 years ago) as part of the transition from hunting and gathering of food to settled agriculture in the south-eastern part of Turkey (Heun *et al.*, 1997; Nesbitt, 1998; Dubcovsky and Dvorak, 2007; Shewry, 2009). The spread of domesticated landraces from its site of origin into Europe was via Anatolia to Greece (8000 before present - BP) and then both northwards through the Balkans to the Danube (7000 BP) and across to Italy, France and Spain (7000 BP), finally reaching the UK and Scandinavia by about (5000 BP). Similarly, wheat spread via Iran into central Asia reaching China by about (3000 BP) and to Africa, initially via Egypt. It was introduced by the Spaniards to Mexico in 1529 and to Australia in 1788 (Feldman, 2001; Shewry, 2009).

The A genomes of tetraploid and hexaploid wheats are clearly related to the A genomes of wild and cultivated einkorn (Table 2). By contrast, the B genome of tetraploid and hexaploid wheats is probably derived from the S genome present in the Sitopsis section of Aegilops, with *Ae. speltoides* being the closest extant species. The S genome of *Ae. speltoides* is also closest to the G genome of *T. timopheevi*, a tetraploid species with the A and G genomes (Shewry, 2009).

Table 2 Summary of the major cultivated and wild species of wheat (from Shewry and Tatham, 2016)

Ploidy genome	**Wild species**	**Cultivated species**	
diploid			
D	*T. tauschii*		
A	*T. urartu*		
A	*T. monococcum* var. *boeticum*	*T. monococcum* var. *monococcum*	einkorn
tetraploid			
AB	*T. turgidum* var. *dicoccoides*	*T. turgidum* var. *dicoccum*	emmer
		T. turgidum var. *durum*	durum
hexaploid			
ABD		*T. aestivum* var. *aestivum*	bread wheat
		T. aestivum var. *spelta*	spelt

In Mediterranean basin durum wheat is a winter crop, usually sown in autumn-winter and harvested from June (exceptionally late May) to July. Phenological growth stages are shown in Figure 2, described by Zadoks *et al.* (1974). Briefly, from germinated seed first leaf emerge from the soil (emergence) and first root appear; during plant development a number of leaves are produced (5-8) and main stem keeps a reduced size; in this stage a number of secondary stems are produced, in relation to the seed density and environmental conditions (tillering); subsequently stem elongation and spike formation occurs (booting) until plant reaches its maximum size and spike emerge (heading). Self-fertilization occurs within the spikelets present into each spike, with the formation and the development of grains (grain filling) and a subsequent final dehydration stage (ripening) until grain maturation is reached. Yield and its component (number of kernels per unit area and kernel weight) are mainly affected by thermo-pluviometrical conditions that can occur, in particular from advanced booting (stage 49 of Zadoks growth scale) to maturity.

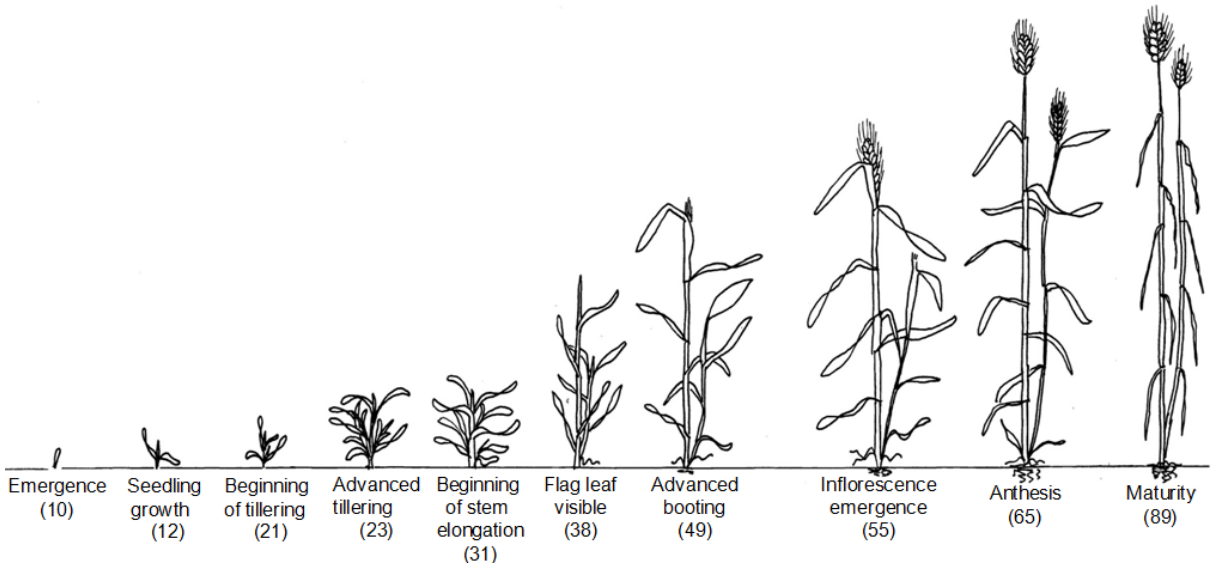


Figure 2 Wheat phenological growth stages. Different numbers refer to different growth stages, according to Zadoks *et al*, (1974)

In Mediterranean area drought and heat stress are critical events that can influence spike and kernel formation and development (Rharrabti *et al.*, 2003; Guzman *et al.*, 2016). In cereals, variations in grain yield are more associated with changes in grain number than to mean grain weight (Fischer, 2011). Nutrient availability, in particular nitrogen, can affect yield and grain protein content (Shewry *et al.*, 2009) and the rate and duration of wheat grain development (Dupont and Altenbach, 2003, Altenbach, 2012). Soil management can affect the water, nitrogen and CO_2 availability during plant life cycle (Tellez-Rio *et al.*, 2015); the influence of deep, conventional or no tillage may vary in order to temperature and rainfall conditions (De Vita *et al.*, 2006).

At maturation, kernels are harvested from the spike, stored and milled for production of flour. Grain composition mainly consists on about 65-80% of starch and 8-20% of proteins, with a lower content of lipids (1.9%) localized in the germ fraction, minerals (1.5%), non starch-polysaccharides (1.6%) and other chemical compounds (vitamins, phenols). Botanically the grain is the fruit of wheat, formed after self-fertilization into the spikelet. Grain morphological aspects are shown in Figure 3. Briefly, grain consists on external layers (pericarp and aleurone) rich in cellulose, hemicelluloses, lignin and minerals; these layers form the bran fraction after milling process that represents about 13-17% of grain dry weight. The main part of the grain is represented by endosperm, with abundance of starch and storage proteins (Pomeranz, 1982). Germ or embryo is the botanical seed of the grain and it is rich of lipids and minerals, and it represents about 2-3% of grain dry weight.

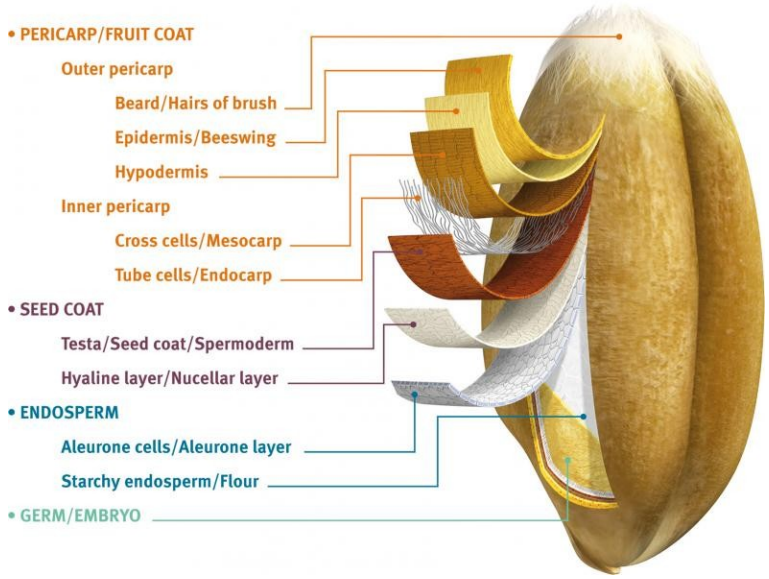


Figure 3 Wheat kernel morphology

1.2.Storage proteins: gliadins and glutenins

Grain storage proteins are soluble in alcohol and they are subdivided in monomeric and polymeric fractions as represented in Figure 4a, respectively classified on the basis of the molecular weight (Figure 4b) in gliadins (α-, γ- and ω-subunits) and glutenins (high and low molecular weight glutenin subunits, HMW-GS and LMW-GS),.

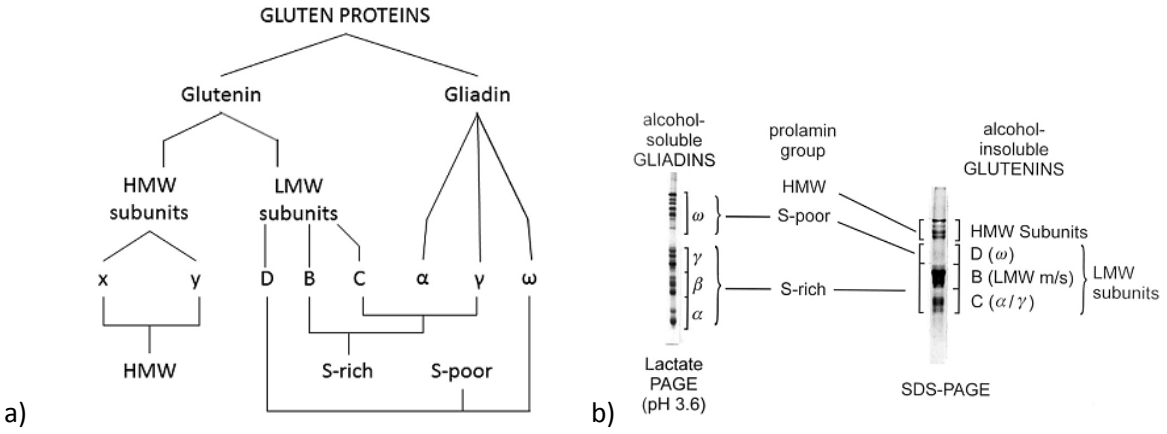


Figure 4 The group of gliadin and glutenin proteins separeted by SDS-PAGE (a) and schematic summary of the classification of gluten proteins (b), from Shewry and Tatham (2016)

Glutenins and gliadins constitute a continuous visco-elastic network in dough called gluten (Shewry, 2009), as represented in Figure 5.

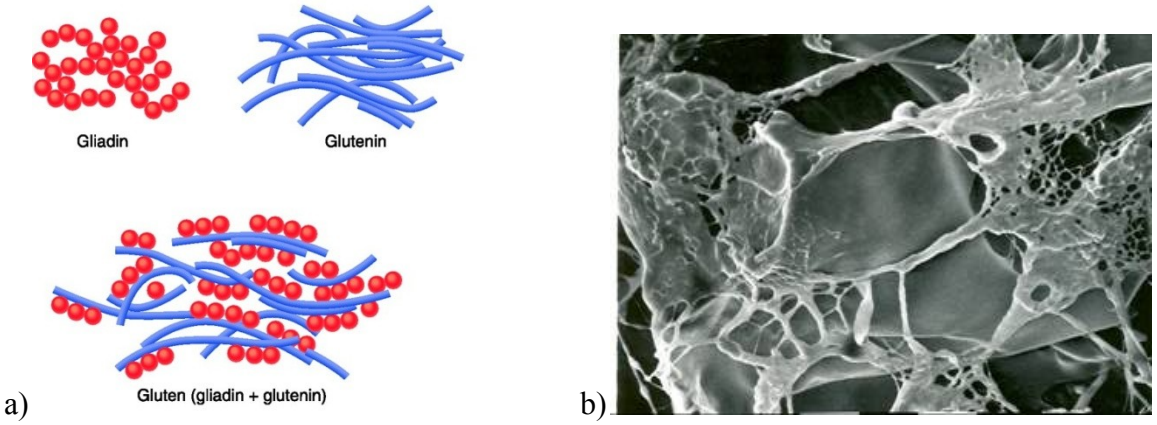


Figure 5 Representation of monomeric gliadin and polimeric glutenin (a) and enlarged image of gluten network (from Shewry, 2009)

In tetraploid wheat, HMW-GSs are subdivided into x- and y-types, both encoded on the long arm of chromosomes 1A and 1B, at the Glu-A1 and Glu-B1 loci.

Some of the most important HMW-GS allelic forms are shown in Figure 6. LMW-GSs are classified in B-, C-, and D-subunits, according to their structural and functional properties; they are encoded on the short arms of chromosomes 1 (1A and 1B) at the Glu-A3 and Glu-B3

loci, and also by loci tightly linked to the Gli-1 and Gli-2 loci on chromosomes 6, depending on the LMW-GS subgroup.

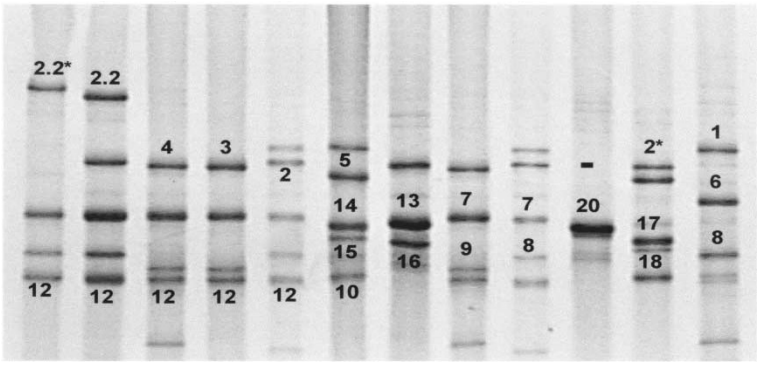


Figure 6 Different allelic forms of HMW-GS by SDS-PAGE on bread wheat

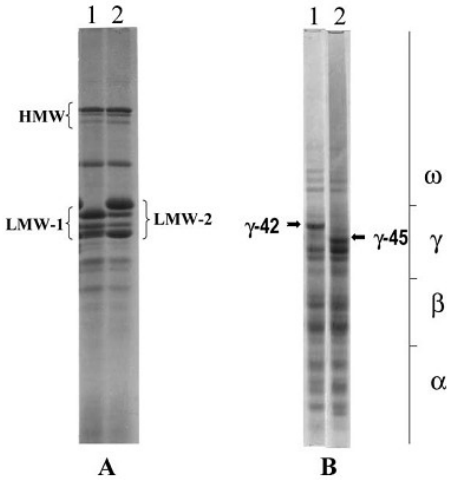


Figure 7 SDS-PAGE of glutenins (A) and acid polyacrylamide gel electrophoresis (A-PAGE) of gliadins (B) in gamma 42 and gamma 45 durum wheat biotypes (from D'Ovidio and Masci, 2004)

The B-subunits are typical LMW-GS with a peculiar structure encoded by genes on chromosomes 1; instead, the C- and D- subunits are gliadin-like LMW-GS encoded by genes on chromosomes 6, structurally similar to gliadins but functionally acting as glutenins, due to their ability to form intermolecular disulfide bonds by means of unpaired cysteine residues. γ- and ω- gliadin subunits are coded on the short arms of group 1 chromosomes by genes at Gli-1 loci, meanwhile α- and β- subunits, are coded by genes at the Gli-2 loci on the short arms of group 6 chromosomes. The LMW-GS are encoded by genes at the Glu-3 loci on the short arms of chromosomes 1A and 1B and are also important in the determination of the final use of wheat. It has been reported that LMW-GS, especially subunits encoded by loci on chromosome 1B (Glu B3), are involved in the end-use quality of durum wheat; in particular the specific group LMW-2 (Figure 7) was related with the best pasta-making properties while

LMW-1 was associated with poor pasta-making characteristics (D'Ovidio *et al.*, 1999). The typical LMW-GS are divided into three groups according to the first amino acid residue of the mature protein. They are LMW-m, LMW-s and LMW-i types, starting with Met, Ser and Ile, respectively.

The role of gliadins in viscosity and extensibility is well documented (Wieser *et al.*, 2007; Sissons *et al.*, 2008), even if not particularly relevant in pasta-making quality. However, an increasing interest on the characterization of gluten proteins is due to the immune stimulating effects of certain sub-fractions (mostly gliadins) on susceptible human population.

The introduction of wheat storage (gluten) proteins in diet is responsible of disorders to susceptible human patients. A nomenclature of gluten related disorders has been suggested, as reported in Figure 8. Wheat allergy (WA) and celiac disease (CD) are currently considered the most important immune mediated disorders associated to the ingestion of gluten in diet (Sapone *et al.*, 2012).

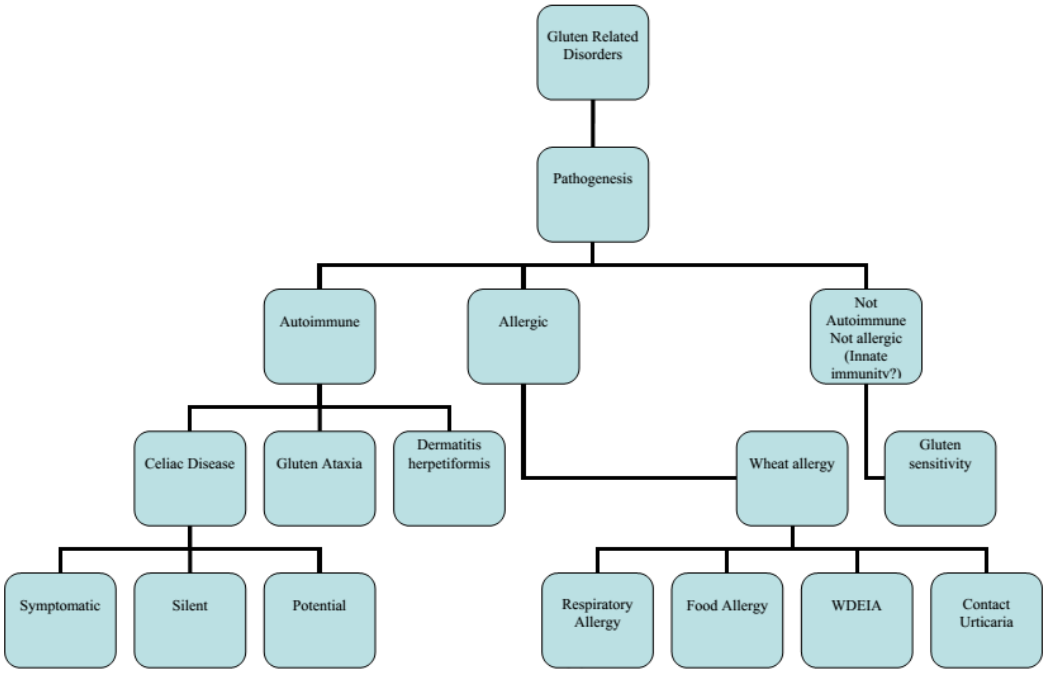


Figure 8 Proposed nomenclature of gluten related disorders, from Sapone *et al.* (2012)

The list of the known wheat allergens (Table 3a) includes either soluble or storage proteins (Matsuo *et al.*, 2015), while T- cell epitopes related to CD are present only in storage proteins (Table 3b).

Table 3 List of the main known wheat allergen (a) - from Matsuo *et al.* (2015), and list of coeliac disease relevant T-cell epitopes from wheat, barley and rye. Glutamine residues deamidated by tissue transglutaminase

are shown in bold, additional glutamine residues targeted by transglutaminase are underlined (from Shewry and Tatham, 2016)

Wheat allergens.

Protein name		IUIS name	MW (kDa)
Water/salt-soluble protein			
Profilin		Tri a 12	14
Non-specific lipid transfer protein 1		Tri a 14	9
α-Amylase inhibitors			
Monomeric	0.28	Tri a 15	12
Dimeric	0.19	Tri a 28	13
Tertameric	CM1/CM2	Tri a 29	13
	CM3	Tri a 30	16
	CM16/CM17	–	17
Agglutinin		Tri a 18	17
Thioredoxin		Tri a 25	13
Thiol reductase homologue		Tri a 27	27
Triosephosphate isomerase		Tri a 31	26
1-Cys-peroxiredoxin		Tri a 32	24
Serpin		Tri a 33	40
Glyceraldehyde-3-phosphate-dehydrogenase		Tri a 34	40–42
Dehydrin		Tri a 35	12
α-Purothionin		Tri a 37	12
Serine protease inhibitor-like protein		Tri a 39	9
Glutathione transferase		–	25
Thaumatin like protein		–	18
Peroxidase		–	36
Water/salt-insoluble protein			
α/β-Gliadin		Tri a 21	28–35
γ-Gliadin		Tri a 20	28–35
ω1,2-Gliadin		–	40
ω5-Gliadin		Tri a 19	65
High molecular weight glutenin subunits		Tri a 26	88
Low molecular weight glutenin subunits		Tri a 36	32–40

a)

Epitope	Sequence
DQ2.5 restricted epitopes	
Wheat	
DQ2.5-glia-α1a	PFPQP**Q**LPY
DQ2.5-glia-α1b	PYPQP**Q**LPY
DQ2.5-glia-α2	PQP**Q**LPYPQ
DQ2.5-glia-α3	FRP**Q**QPYPQ
DQ2.5-glia-γ1	PQQSFP**Q**QQ
DQ2.5-glia-γ2	IQP**Q**QPAQL
DQ2.5-glia-γ3	QQP**Q**QPYPQ
DQ2.5-glia-γ4a	SQP**Q**QQFPQ
DQ2.5-glia-γ4b	PQP**Q**QQFPQ
DQ2.5-glia-γ4c	QQP**Q**QPFPQ
DQ2.5-glia-γ4d	PQP**Q**QPFCQ
DQ2.5-glia-γ5	QQPFP**Q**QPQ
DQ2.5-glia-ω1	PFPQP**Q**QPF
DQ2.5-glia-ω2	PQP**Q**QPFPW
DQ2.5-glut-L1	PFS**Q**QQQPV
DQ2.5-glut-L2	FSQQQ**Q**SPF
Barley	
DQ2.5-hor-1	PFPFP**Q**QPF
DQ2.5-hor-2	PQP**Q**QPFPQ
DQ2.5-hor-3	PIP**Q**QPQPY
Rye	
DQ2.5-sec-1	PFPQP**Q**QPF
DQ2.5-sec-2	PQP**Q**QPFPQ
DQ2.2 restricted epitopes	
Wheat	
DQ2.2-glut-L1	PFS**Q**QQQPV
DQ8 restricted epitopes	
Wheat	
DQ8-glia-α1	**Q**GSFQPSQ**Q**
DQ8-glia-γ1a	**Q**QPQQPFPQ
DQ8-glia-γ1b	**Q**QPQQPYP**Q**
DQ8-glut-H1	QGYYPTSPQ
DQ8.5 restricted epitopes	
Wheat	
DQ8.5-glia-α1	**Q**GSFQPSQ**Q**
DQ8.5-glia-γ1	PQQSFP**Q**QQ
DQ8.5-glut-H1	QGYYPTSPQ

b)

1.3.Environmental changes in storage protein composition

An increasing number of investigations by proteomic approach on wheat protein composition has occurred in the latest years. Most of the studies were focused on individuating genetic and environmental differences, in particular on bread wheat (Dupont *et al.*, 2006; Zorb *et al.*, 2009; Hurkman *et al.*, 2013). Few studies are available on durum wheat. An initial group of studies on durum identified and characterized gliadin and glutenin proteins, as basis for further experiments (Mamone *et al.*, 2005; Mamone *et al.*, 2009; Muccilli *et al.*, 2010; Muccilli *et al.*, 2011, Pompa *et al.*, 2013). Recently the implications of environmental stress conditions on storage protein expression and composition during grain development were investigated on bread (Skylas *et al.*, 2000; Shewry *et al.*, 2009; Liu *et al.*, 2012; Koga *et al.*, 2015) and durum wheat (Giuliani *et al.*, 2014, Giuliani *et al.*, 2015). Drought and high temperatures during grain filling are the major abiotic stressors, causing significant alterations

to both yield and quality in many crops worldwide (Flagella *et al.*, 2010). To date, a number of studies used two-dimensional electrophoresis (2-DE) to investigate gluten protein responses to high temperature, fertilizers, and drought (Dupont *et al.*, 2006; Zorb *et al.*, 2009). Concerning the effect of high temperatures on gluten proteins, Dupont *et al.* (2006) reported increases in HMW-GS and α-gliadins and a decrease in LMW-GS in response to thermal stress. Moreover, Majoul *et al.* (2003) identified three α-gliadins that increased in response to high temperature, and Yang *et al.* (2011) reported changes in the relative amounts of some α-gliadins, an increase in γ-gliadins and in some LMW-GS, and a decrease in some ω-gliadins when high temperatures occurred in post anthesis. The same authors observed a decrease in α- and γ-gliadins and changes in the relative amounts of some LMW-GS as a consequence of drought.

The different developmental profiles reported in the literature demonstrate that gluten protein accumulation is a complex process that is subject to spatial and temporal regulation as well as to environmental signaling. During grain filling, environmental factors change protein composition influencing also the final quality (Naeem *et al.*, 2012; Sherwry *et al.*, 2009; Hurkman *et al.*, 2013; Giuliani *et al.*, 2015). High temperature (and also water deficit) shortens the duration of grain filling and reduces the time taken to reach apoptosis and harvest maturity (Altenbach *et al.*, 2012). Consistent with these events, transcripts for α-, γ-, and ω-gliadins, HMW-GS, and LMW-GS accumulate and disappear earlier. Yang *et al.* (2011) applied both heat and water stress to a *T. aestivum* cultivar and found the highest gliadin contents at 19 DAA under heat treatment and at maturity under water stress conditions. Moreover, the authors found that high-temperature treatment resulted in higher glutenin and gliadin contents at maturity; this contrasts with the findings of other authors, who found that high temperature can induce synthesis of gliadins in wheat at the expense of glutenins (Daniel and Triboi, 2000). In contrast to the gliadin fractions, multiple high temperature or water-deficit events applied at both terminal spikelet and anthesis resulted in significantly higher glutenin content at maturity than did the single-stress treatments (Yang *et al.*,2011). Moreover, the same authors reported that some storage proteins, including α- and γ-gliadins, LMW-GS, and globulins, decreased and that one globulin and one specific LMW-GS increased in response to water deficit. It is fundamentally important to understand how environmental treatments affect the timing of grain developmental processes in order to pursue wheat quality, because protein synthesis of storage proteins occurs during this stage.

1.4.Carbohydrates

Carbohydrates account for the vast majority of the wheat mature grain dry weight, exact amounts vary between different studies but; they are monosaccharide (glucose, fructose) and disaccharides (sucrose and maltose), oligosaccharides (raffinose, fructans) starch and up to about 10% cell wall polysaccharides (mainly cellulose, arabinoxylan and mixed linkage-glucan), the latter forming the major DF components (Stone and Morell, 2009; Andersson *et al.*, 2013). However, there are large differences between the compositions of the different grain tissues. In particular, the aleurone and outer layer (pericarp and testa), which form the bran fraction on milling of wheat, contain little starch but up to half of the dry weight is cell wall polysaccharides, while the starchy endosperm (the major storage tissue of the grain) comprises about 85% starch and only 2-3% cell wall polysaccharides (Lafiandra *et al.*, 2015). Wheat starch consists on α-$_D$-glucose units which make up the polymers amylose and amylopectin in a ratio of 1:3. The former is a linear α- glucan polymer ((α- 1→4) linked glucose units, while the latter has a more branched structure with some ((α- 1→6) linkages. Starch properties are affected by the complexity of the branching architecture, in particular chain length distribution and clustering, which can influence the ability to form double helical conformations and the crystalline features (Jeon *et al.*, 2010). The influence of starch in pasta cooking is well documented (Soh *et al.*, 2006). In some cases starch or starch fragments may escape digestion in the small intestine and are termed resistant starch (RS). Resistant starch is classified into five types. High amylose starch has a higher RS content than normal starch (Birt *et al.*, 2013, Lafiandra *et al.*, 2015). Recently TILLING (Targeting Induced Local Lesions IN Genomes) has been used to obtain high amylose starch in durum wheat (Sestili *et al.*, 2015). Plant species with lower amylose starch also exist, such as the pseudo cereals amaranth and quinoa (Qian and Kuhn, 1999).

1.5.Cereal dietary fibre in human diet

Non-starch polysaccharides (NSP) comprise polymers of hexose sugars (glucose, mannose, deoxy-hexoses rhamnose and fucose, glucuronic and galacturonic acids) and pentose sugars (arabinose and xylose). They form the major cell wall polysaccharides with the major components in wheat being cellulose, mixed-linkage β- glucan and arabino-xylan. (Lafiandra *et al.*, 2014; Lovegrove *et al.* 2015). Dietary fibre is one of the major food constituents that influence the rate and extent to which blood glucose increases after ingestion of a carbohydrate food. DF is not digested and absorbed in the small intestine but passes to the colon where it undergoes bacterial degradation (fermentation). The principal types of DF are

NSP, resistant starch (RS), oligosaccharides (short chain carbohydrates) and some polyols and modified starches. The daily intake of NSP in the diet in European countries varies between 11 and 33 g, all of which will reach the colon (Saris *et al.*, 1998).

An important health benefit of DF is the capacity to lower the glycaemic response of the foods in which it is present: foods rich in DF release glucose more slowly which is absorbed into the blood which is relevant to the prevention of disorders such as obesity and type II diabetes (Nugent, 2005). Food viscosity is a potential mechanism by which DF slows down the rate of digestion of starch and the absorption of sugars. The effect is considered to be related to viscosity, which inhibits mixing and diffusion in the intestinal tract and delays gastric emptying. Moreover, high viscosity may also decrease enzyme diffusion and result in the formation of an unstirred water layer which decreases glucose transport to enterocytes. It has been demonstrated that reducing the viscosity of DF, after pre- acid hydrolysis, results in concurrent loss of its clinical efficacy (Riccardi *et al.*, 2004).

A significant proportion of DF that enters the large bowel will be fermented by the commensal bacteria that live in the colon. Fermentation is an anaerobic process which produces the short chain fatty acids (SCFA) acetate, propionate and butyrate as the principal end-products, together with methane, hydrogen, carbon dioxide and lactate. The SCFA's are believed to maintain healthy colonocyte function and are to be effective in the prevention of serious diseases such as colorectal cancer (Lafiandra *et al.*, 2014).

1.5.1. Arabinoxylans

Arabinoxylan (AX) polymers represent about 70% of total grain wheat cell wall NSPs (Mares et Stone, 1973). AX consists on a backbone of xylose (β-$_D$-xylopyranosyl) linked by (1 → 4) glycosidic linkages, which may be substituted with arabinose (α-$_L$-arabinopyranosyl) at either the 3 or the 2 and 3 positions, as shown in Figure 9. AX is present as water-extractable (WE-AX) and water unextractable (WU-AX) fractions. In white flours which correspond broadly to the starchy endosperm tissue, the average contents of WE-AX and WU-AX are 0,5 and 1,7 g/100g flour, respectively (Dervilly-Pinel *et al.*, 2001a; Ordaz-Ortiz and Saulnier, 2005; Saulnier *et al.*, 1995, 2007). Thus, the total AX content in wheat flour/endosperm is about 2.2% and approximately 1/4 of total AX are WEAX (Table 1). The structure of WU-AX, which represents the major part of AX in cell walls of the endosperm, is very similar to that of WE-AX but the average molecular weight and the ratio of arabinose to xylose (A/X ratio) are slightly higher for WU-AX (Izydorczyk and Biliaderis, 1995). The higher A/X ratio corresponds to a higher proportion of mXyl and a lower proportion of uXyl in WU-AX

compared to WE-AX whereas the proportions of dXyl are similar in WE-AX and WU-AX. The amount of ferulic acid linked to AX is low and represents 0.2-0.4% of WE-AX (w/w) and 0.6-0.9% of WU-AX in wheat (Bonnin *et al.*, 1999). This corresponds to about 2-4 ferulic acid residues for 1000 xylose residues in WE-AX (6-10 for WU-AX). Dehydrodiferulic acids were also detected in very low amounts (10-15 times less than ferulic acid) in WE-AX from wheat (Dervilly-Pinel *et al.*, 2001a). In the endosperm, the water solubility of AX is mainly determined by chain length and arabinose substitution, and also by the proportion of chain-chain cross-linking through covalent "diferulic bridges" which is higher in WU-AX than in WE-AX (from Saulnier *et al.* 2012).

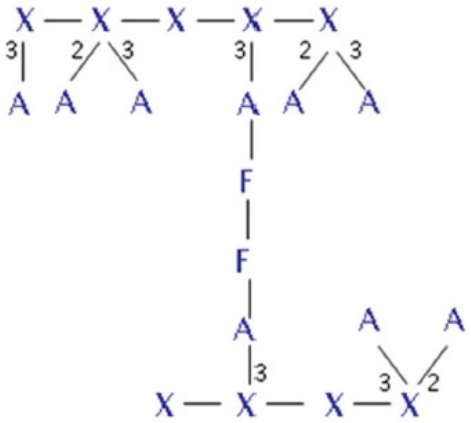


Figure 9 Schematic (top) structures of wheat starchy endosperm arabinoxylan, from Lafiandra *et al.* (2014)

1.5.2. Mixed-Linkage Glucans

Mixed-linked β-glucans are glucose units linked (1→4)-β (as in cellulose) but interspersed with (1→3)-β-linkages (Figure 10). The (1→3)-β-linkages generally occur after three or four β-(1→4) linkages, but more extensive cellulose-like stretches of up to 20 (1→4)-β-linked residues have been reported in wheat bran (Li *et al.*, 2006). The irregular linkage structure prevents the formation of an ordered crystalline structure, leading to the β -glucans being partially water-soluble. The (1→3)(1→4)-β-D-glucans are characteristic of the cell walls of cereals while β-glucans with (1→3)(1→6) linkages occur in fungi. Water-soluble mixed-linked β-glucans of barley (where they constitute ~70% of endosperm cell wall polysaccharides (Fincher and Stone, 1986) and oats are able to form viscous solutions and dispersions.

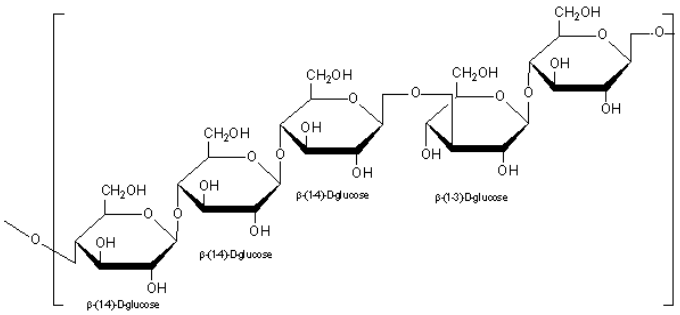


Figure 10 Representation of chemical structures of mixed linkage glucan

1.6.Phenolic compounds

Phenolic acids represent the most common form of phenolic compounds found in whole grains. The most abundant phenolics acids in whole grain are ferulic (FA), vanillic (VA), p-coumaric (CA), and syringic acids which derive from hydroxycinnamic acid (Figure 11). These acids are present in three forms: as soluble free acids, soluble conjugates that are esterified to sugars and other low molecular mass components, and insoluble bound forms (Li *et al.*, 2008). The bound form is the most abundant, linking cell wall structural components such as cellulose, lignin, and proteins and through ester bonds crosslinking polymers, particularly arabinoxylans in cereal cell walls (Saulnier *et al.*, 2007). They are concentrated in the bran fraction and are present at lower levels in white flour. A role of FA and other phenolic compounds in antioxidant activity with anti-inflammatory, anti-ageing, anti-bacterial related effect is suggested. As well as the scavenging activity of these antioxidants, their affinity with lipid substrates, might be important factors in protecting lipids from oxidising events (Kikuzaki *et al.*, 2002).

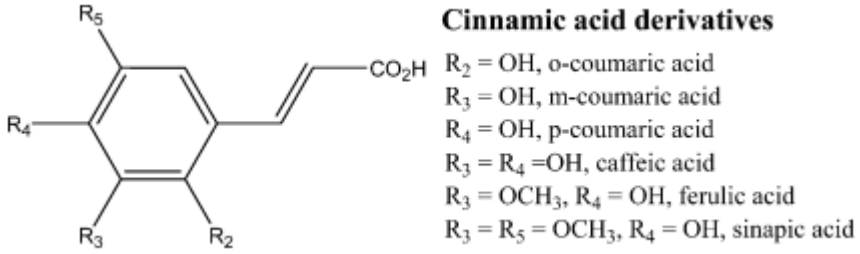


Figure 11 Structure of cinnamic acid derivatives (Li *et al.*, 2008)

1.7.Influence of breeding activity on durum wheat yield and quality

During the 20th century breeding programs were aimed to obtain genotypes according to the Donald's ideotype with higher grain yield, shorter size and early maturation to better fit Mediterranean conditions where drought and high temperature can occur during ripening (De Vita *et al.*, 2007). The Italian breeding program started with the activity of the breeder Nazareno Strampelli (1866–1942) who selected North African landraces to obtain durum wheat with earlier maturity, ability to avoid drought and heat stress conditions typical of the Mediterranean crop area. The result was the introduction of the cultivar Senatore Cappelli (Bozzini, 1970). Further, breeding activity was concentrated on the selection of well adapted genotypes, with the introduction of Syropalestinian durum wheats (i.e. Eiti), representing the backbone of crop cultivation until the 1960s. During this period the aim to reduce the plant size was promoted by Strampelli who crossed the Japanese wheat variety Akakomugi, the source variety for Rht8 (reduced height) and PpD1 genes with the cross of Wilhelmina Tare x Rite to shorten the straw and achieve resistance to lodging, as well as early ripening, obtaining several bread wheat varieties (1918): Villa Gloria, Ardito, Mentana and Damiano in 1918 (Borojevic and Borojevic, 2005). However, the introgression of the dwarfing genes Rht1 and Rht2 by Norman Borlaug (1970 Nobel Peace Prize winner) from Norin 10 (Triticum aestivum, L.) was carried out in 1968 in CYMMIT short recombinant lines and transferred to durum wheat varieties. A generation of semi- dwarf varieties (Figure 12) with higher harvest index (harvestable mass on total plant mass ratio) from the 1970s were cultivated and crossed for high yield, better stability and environmental adaptability, according to the ideotype postulated by Donald (1968).

A secondary aim of the breeding was to obtain durum wheat with storage proteins suitable for better pasta making quality (Raciti *et al.*, 2003). Storage proteins are defined as proteins that accumulate during the grain-filling period and are used as nitrogen (N) sources during seed germination (Shewry and Halford, 2002). Despite higher yield, generally lower grain protein content occurs in modern genotypes, which is explained by dilution due to higher numbers of grains per unit area, and increased starch content (Triboi *et al.*, 2003). Triboi (2000) found in particular that N availability is a key factor influencing protein content and composition. However, the technological properties of gluten proteins are mainly related to specific allelic variants of high molecular weight and low molecular weight glutenin subunits (Rao *et al.*, 2001; Shewry and Halford, 2002; Sissons, 2008,). Several studies on durum wheat heritage varieties demonstrated that an indirect selection according to the gluten strength and flour mixograph properties occurred (Raciti *et al.*, 2003; De Vita *et al.*, 2007; Nazco *et al.*, 2014,

Subira *et al.*, 2014); this activity occurred in an early stage of the breeding with the substitution of the allelic LMW-1 glutenin subunit with the LMW-2 and in the latter part the substitution of the allelic Glu B1 (Bx + By) associated with poor quality for pasta making (20, 6+15, 13+16) with the better quality allelic Glu B1 forms (6+8, 7+8). The higher grain protein content did not significantly affect pasta making quality in low temperature pasta drying (< 40°C); however, in high temperature pasta drying (> 70°C) grain protein content is more important (Sissons, 2008). In the latest decades breeding activity has also focused on the efficient use of natural resources (water, minerals, N) and to enhance health properties of the grains (Shewry *et al.*, 2015).

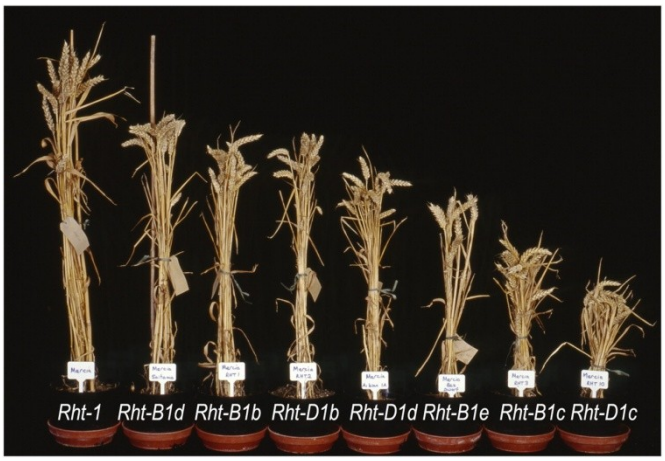


Figure 12 Effect of allelic dwarfing Rht gene on wheat size

Investigations using genetic, proteomic and immunological approach (especially on bread wheat) have been carried out on storage proteins involved in celiac disease (De Vita *et al.*, 2013, Suligoj *et al.*, 2013, van den Broeck 2010, Shewry, 2015) and wheat allergy (Denery-Papini *et al.*, 2007; Denery-Papini *et al.*, 2011; Lupi *et al.*, 2013; Altenbach *et al.*, 2015). A study focused on relevant immune-stimulant α- gliadins on old and modern wheat genotypes showed a variability in α- 9 and α- 20 gliadin, individuating a lower amount in a group of old landraces (van den Broeck *et al.*, 2010); however this study on only 2 epitopes on 28 is not conclusive. Gregorini *et al.* (2009) carried out an investigation on two α- gliadins involved in CD on a small group of durum wheat varieties, including some old genotype without finding significant differences. Those results were further confirmed by Suligoj *et al.* (2013) on small intestinal T cell lines generated from celiac patients, including ancient wild species (T. *monococcum* and *speltoides*).

The health properties of wheat grain are related to the contents of several class of components, including high-amylose starch, non-starch polysaccharides (arabinoxylan, mixed-linkage β- glucan, fructans) and phenolic compounds. In the HEALTH-GRAIN integrated project, differences in dietary fibre and phenolic compounds on 150 bread wheats,

10 durum wheat genotypes, and lines of emmer, einkorn, barley, oat and rye were observed. The heritability of the AX was found to be about 70% in white flour, with about 30% effect of environment (Shewry *et al.*, 2010) and a relatively low effect of the genotype x environment interaction (Gebruers *et al.*, 2010).. The AX content was measured in white flour and bran, showing a lower relative content of water extractable AX (%WE-AX) in ancient species (emmer), and a higher range of values in bread wheat species; however a larger number of bread wheats were analysed. Recently on a population of 104 tetraploid accessions (including durum wheat of different years of registration), Marcotuli *et al.* (2015) found a range 1.5 – 5.5% of AX (dry weight) , individual significant Marker-trait associations (MTA), identifying 19 QTL associated with AX content, in particular on chromosome 5A. In the same investigation, a variability in mean and range of AX content between tetraploid species was observed, with lower content in some wild species (wild emmer, *Triticum turgidum* subsp *dicoccoides*). Studies on DF includes resistant starch (RS) in wheat with high amylose starch (Raksegi *et al.*, 2014), also in durum (Sestili *et al.*, 2015).

Concerning phenolic compounds, Dinelli *et al.* (2011) identified several phenolic acids in old and durum wheat genotypes, reporting also the relative proportions of bound, free and conjugated form, without finding significant differences between old and modern types (Dinelli *et al.*, 2009). Furthermore, an indirect evaluation of phenolic acid contents based on the antioxidant activity was adopted to explore differences in a collection of old and modern durum wheat genotypes; however, no statistically significant differences between the two groups were found (Laus *et al.*, 2015).

Phenotyping for morphological and molecular quality traits, in association with selection based on molecular markers, should allow genetic diversity to be studied under different environmental and agronomical conditions (Lopes *et al.*, 2014), including collections of old types (Royo *et al.*, 2014) both for improving technological quality and health benefits and for adaptation to climate change (Lopes *et al.*, 2015).

1.8.Aim

Old wheat varieties have been suggested to have healthier benefits compared with modern cultivars in relation to both bioactive components and gluten composition. However limited data are available supporting this hypothesis, in particular for durum wheat. So the purpose of this thesis was to give a contribution to the comprehension of the influence of Italian 20th century breeding on the main grain quality characters. To this aim a characterization of an old and a modern durum wheat group of genotypes was performed in relation to storage protein and dietary fibre composition.

2. MATERIALS AND METHODS

2.1.Genotypes

Durum wheat genotypes were selected based on the year of release from 1900 to 2005, as showed in Table 4. Genetic relationship with genotypes is shown in Figure 13.

Table 4 List of the investigated genotypes with relative pedigree and year of release

Genotype	Pedigree	Year of release
Dauno III	old apulian landraces	1900
old Saragolla	old apulian landraces	1900
Cappelli	Jean Retifah north african cultivar	1915
Russello	old sicilian landraces	1910
Garigliano	Tripolino x Cappelli	1927
Timilia RB	local sicilian landraces	1910
Grifoni 235	Cappelli x Aziziah	1949
Adamello	Valforte x turkish line 7116	1985
Simeto	Capeiti 8 x Valnova	1988
Preco	Edmore/WPB881//Selected line 3	1995
Svevo	Cimmyt line x Zenit	1996
Iride	Altar 84 x Ionio	1996
Claudio	(Sel. Cimmyt x Durango) x (IS1938 x Grazia)	1998
Saragolla	Iride x PSB 014 line	2004
PR22D89	(Ofanto x Duilio) x Ixos	2005

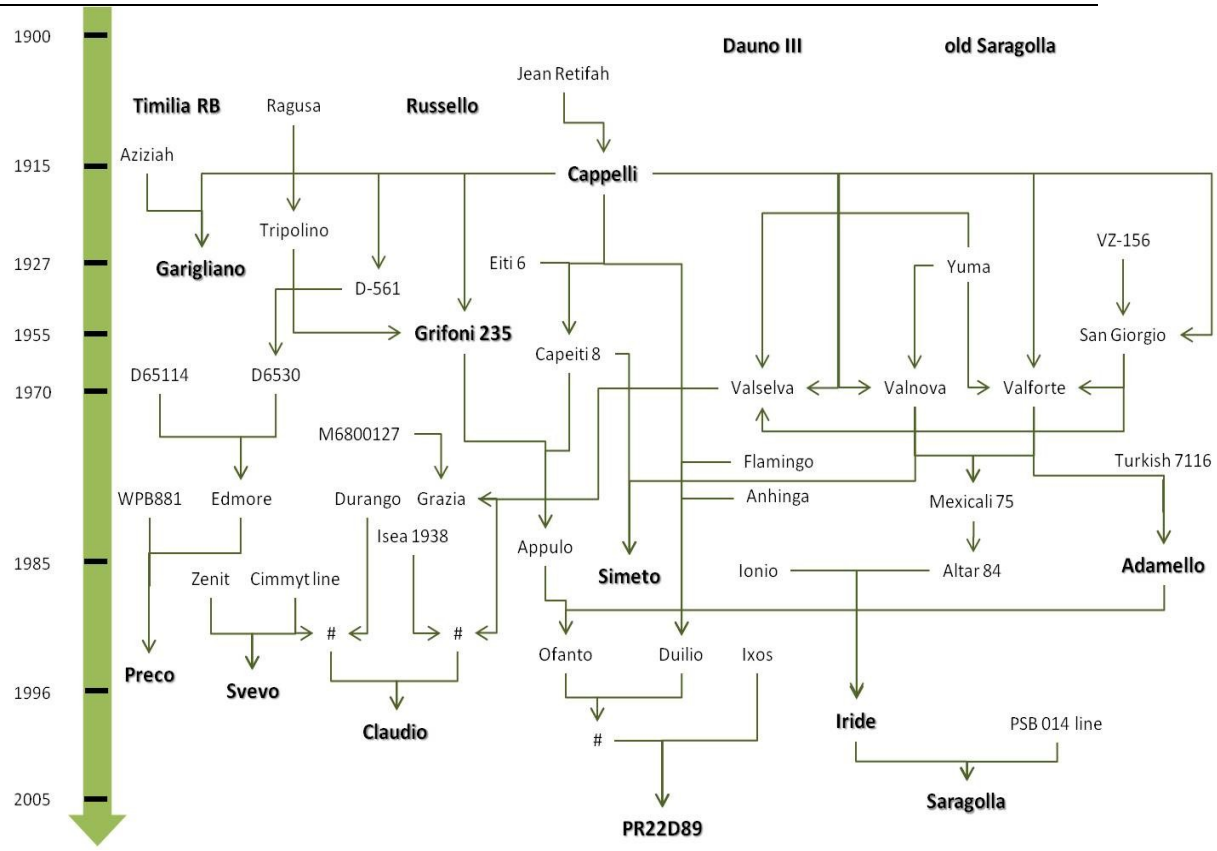


Figure 13 Genetic tree of the investigated durum wheat genotypes

Four genotypes are Italian old local landraces from Apulia (Dauno III, old Saragolla) and Sicily (Russello, Timilia R.B. "*reste bianche*"). Cappelli is the first Italian durum wheat cultivar, obtained from introduction of north African landraces by Nazareno Strampelli (1866-

1942). Garigliano and Grifoni 235 were obtained by selection further selection from Cappelli. Modern genotypes were selected after the introduction of the Rht gene. Iride, Simeto and Saragolla represent the top cultivated cultivars in Italy, as shown in Table 5.

Table 5 List of the top cultivated durum wheat cultivars in Italy (ENSE 2012).

Ranking	Genotype	hectares	%
1	Iride	8324	12%
2	Simeto	7402	10%
3	Saragolla	6184	9%
4	Core	5035	7%
5	Quadrato	3306	5%
6	Claudio	3103	4%
7	Duilio	2432	3%
8	Pietrafitta	2097	3%
9	Levante	2050	3%
10	Anco Marzio	1722	2%
11	Rusticano	1354	2%
12	Arcangelo	1331	2%
13	Achille	1227	2%
14	San Carlo	1209	2%
15	Svevo	1086	2%
16	Creso	1065	1%
17	Aureo	1051	1%
18	Orobel	1032	1%
1-18		51010	71%
	total	71813	

2.2. Field trials

Plants have been grown in field on clay–loam soil at Foggia (Italy, 41° 28' N, 15° 32' E and 75 m a.s.l.), in two growing season (2013 and 2014). Field trials were conducted by at Centro di Ricerca per la Cerealicoltura (Crea-Cer) that provided grain samples.

In both years fertilization plan consisted on an application of 80 kg ha^{-1} of nitrogen and 70 kg ha^{-1} of phosphorous. A reduced N input was adopted in according to the ordinary agronomic practises adopted in Mediterranean area, in particular to reduce lodging in high-size old genotypes. N fertilizer was applied in two rates: 2/3 at sowing date (150 kg ha^{-1} of diammonium phosphate N 18%) and 1/3 at tillering stage (200 kg ha^{-1} of ammonium nitrate N 26-27%). Sowing time was at the first decade of December in both crop seasons. Heading date was recorded when about half of the culms showed emerging spikes (growth stage 55; Zadoks *et al.*, 1974). Varietal earliness was expressed as heading date after April 1st, as shown in Figure 14. Plots were harvested mechanically in June each year.

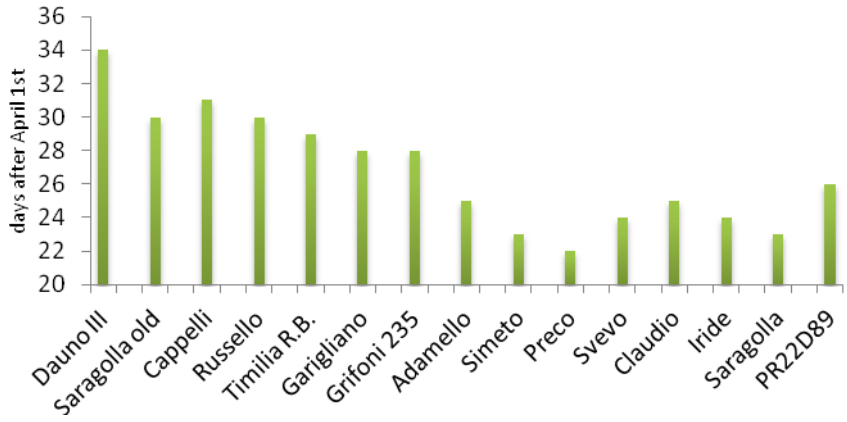


Figure 14 Heading date expressed as days after April 1st

2.2.1 Weather condition

In Figure 15 the rainfall distribution and maximum and minimum decadal mean temperatures of the 2013 (a) and 2014 (b) crop seasons are reported. With the exception of December and January, higher and better distributed rainfall occurred in the second crop season with respect to the first one. This trend was more evident after flowering when the rainfall occurred from the 2nd decade of April (flowering) to the 2nd decade of June (harvest) was three times higher in the second crop season (Table 6). On the contrary, the max and min temperature trend was similar between the two years.

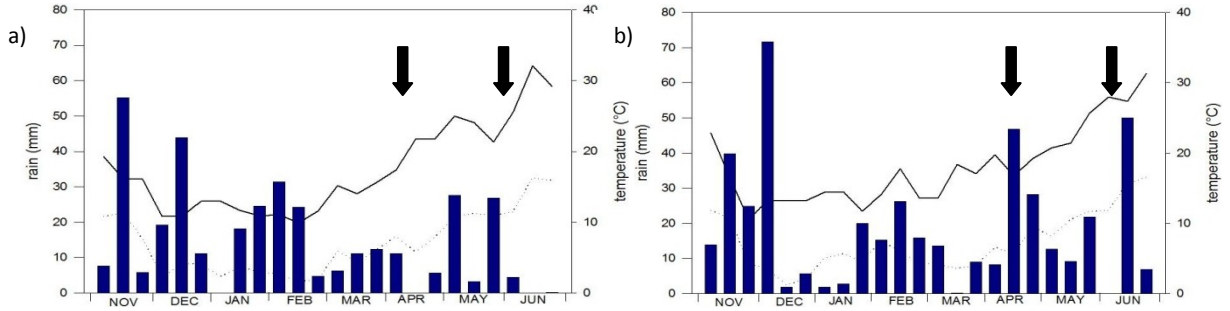


Figure 15 Rainfall distribution and maximum and minimum decadal mean temperatures of the 2013 (a) and 2014 (b) crop seasons

Table 6 Focus on grain filling weather condition (from 2nd decade of April to 2nd decade of June)

	T max (°C)	mean T min (°C)	mean T (°C)	evaporated (mm)	rainfall (mm)
2013	24.9	11.1	18.0	284	53.8
2014	23.1	11.1	17.0	241	152.8

2.2.2 Yield and quality parameters

At harvest, grain yield (kg m^{-2}) and its main components, 1000 kernel weight (TKW) and number of kernels m^{-2}, were determined. Test weight, moisture, grain protein content and ash have been determined by NIR System Infratec 1241 Analyzer (Foss, Hillerod, Denmark) at Department of SAFE (Unifg) (Figure 16a).

Seed dimensions were analysed by Marvin Seed Analyser (Gta Sensors Gmbh-Germany).

a)  b) 

Figure 16 Foss Infratec 1241 (a) and Cyclotec Tecator 1093

Wholemeal and semolina flours have been obtained from kernels respectively milled by Cyclotec Tecator 1093 (Figure 16b) sample mill (sieve 1mm) and Bona mill 4 cylinders (sieve 180µm). Samples for DF analysis have been re-milled by Ball mill (sieve 150µm). Total N content of wholemeal and semolina flour was measured by the Dumas method (1831) and expressed as percent dry matter. Protein content of flour was then calculated by multiplying the N content by 5.7. Grain protein content (GPG) and semolina protein content (SPC) was calculated on a dry weight basis and expressed as percentage.

2.3.Storage proteins

2.3.1. Gluten index

The gluten index (G.I.), an indicator of the gluten strength, was determined using the Glutomatic system according to ICC standard 155 (ICC, 1986). In particular, gluten was separated from semolina by centrifugation to force wet gluten through a specially constructed sieve under standardized conditions. The percentage of wet gluten remaining on the sieve after centrifugation is defined as the gluten index. If the gluten is very weak all of the gluten may pass through the sieve, the gluten index is 0. When nothing passes through the sieve, the index is 100.

2.3.2. Extraction of gliadins and glutenins.

Endosperm storage proteins were extracted according to a modified protocol according to Hurkman and Tanaka (2004) and Giuliani *et al.* (2015). Briefly 100 mg of flour (semolina and wholemeal) have been suspended with 0.4mL of KCl buffer (pH 7.8) and centrifuged to remove soluble proteins. The KCl insoluble fraction has been suspended with a 1-propanol solution (50% v/v) and centrifuged for 10 minutes at 4,500g (repeated twice) to separate gliadins from glutenins and following suspended in SDS buffer. Extracted protein content (gliadin and glutenin) were measured by Biuret method. (Giuliani *et al.*, 2015).

2.3.3. SDS-PAGE

Extracted gliadins and glutenins were run in 12% acrylamide gels at 20mA per gel for 4 hours, by SE 600 apparatus (Hoefer, Inc., Holliston, MA, USA - Figure 17a). Gels were stained with Comassie Brilliant Blue G250 (20% MetOH), distained with tap water and following digitally acquired by scanner (Epson Perfection V750pro – Figure 17b) and analysed by ImageQuant Tl (GE-Healthcare). Two biological and three technical replicates were performed. Band expression have been quantified as total and relative volumes, after removing background by rolling ball method (Figure 18). On the basis of the molecular weight gliadins have been subdivided into three classes: ω-, γ-, α/β-; also glutenins have been subdivided into HMW-GS (type Bx and By) and LMW-GS (D-, B- and C-).

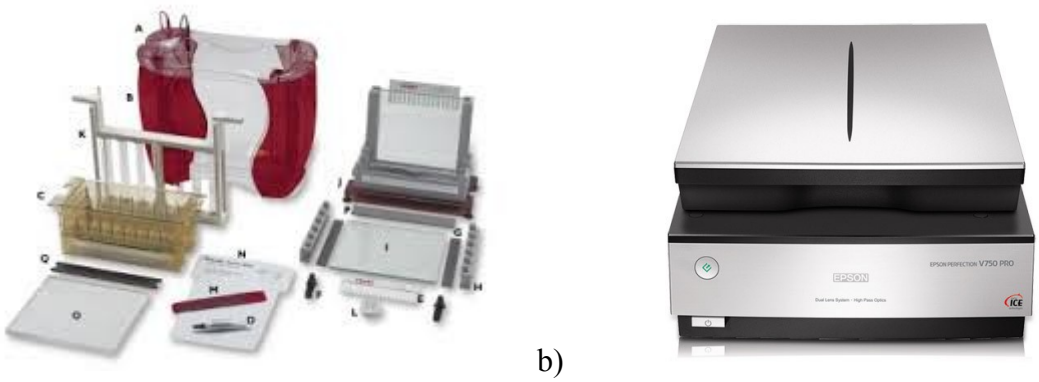


Figure 17 SE 600 apparatus for electrophoresis (a) and scanner adopted to digitally acquire electrophoretic gels (b)

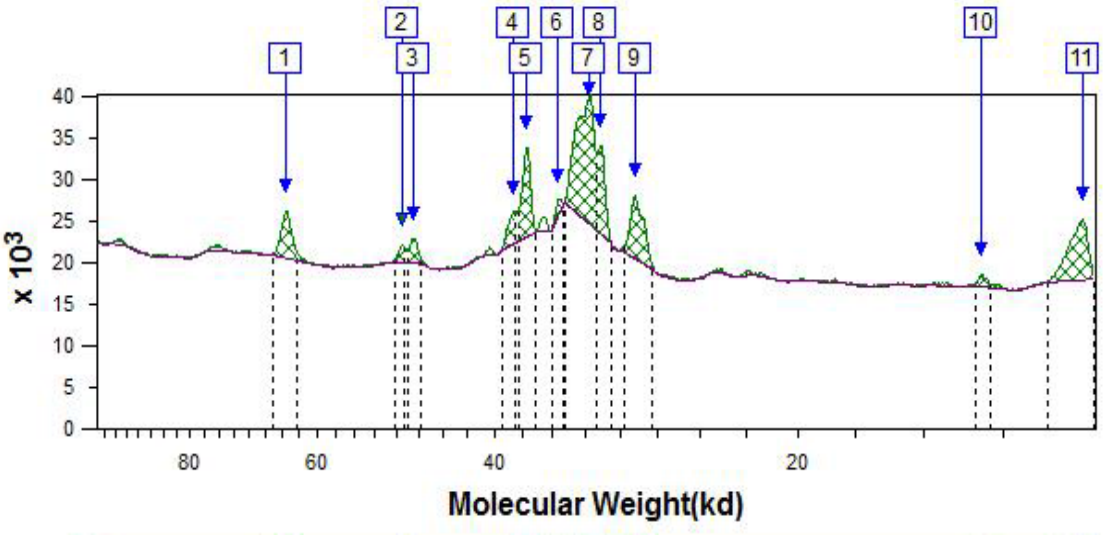


Figure 18 Example of band expression of gliadins elaborated by software ImageQuant TL

2.3.4. 2DE SDS-PAGE

One old (Cappelli) and one modern (Simeto) genotype from two crop seasons have been characterized by 2DE SDS-PAGE according to Giuliani *et al.* (2015). Gliadins and glutenins were separated into each class in relation to their isoelectric point and molecular weight (Figure 19). Overall spot gel volume were individuated, quantified and analysed by using Image Master 2D Platinum on three laboratory replicates (Figure 20).

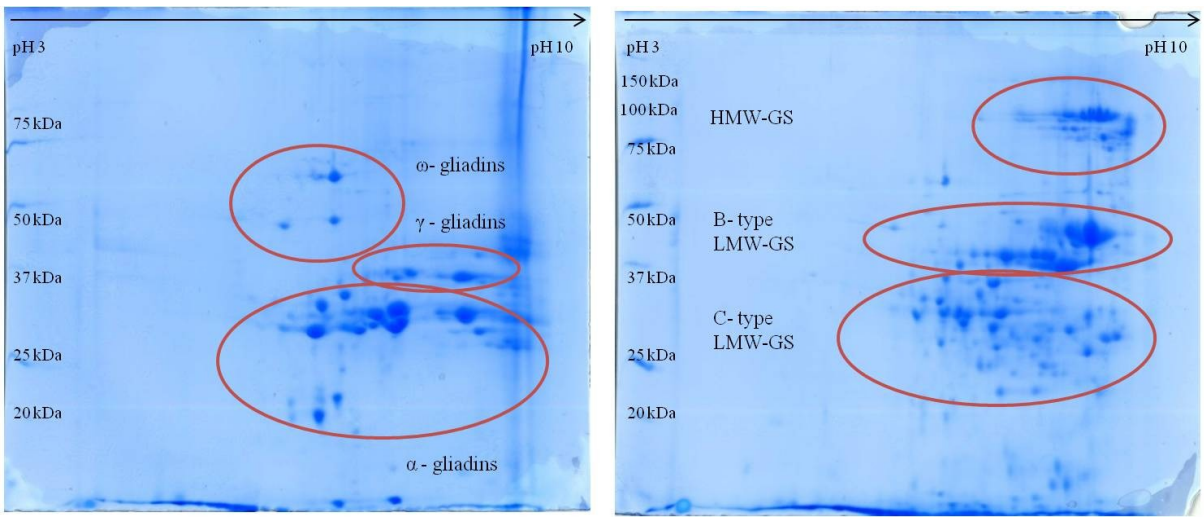


Figure 19 Example of 2DE SDS-PAGE of gliadin (left) and glutenin (right) from durum wheat

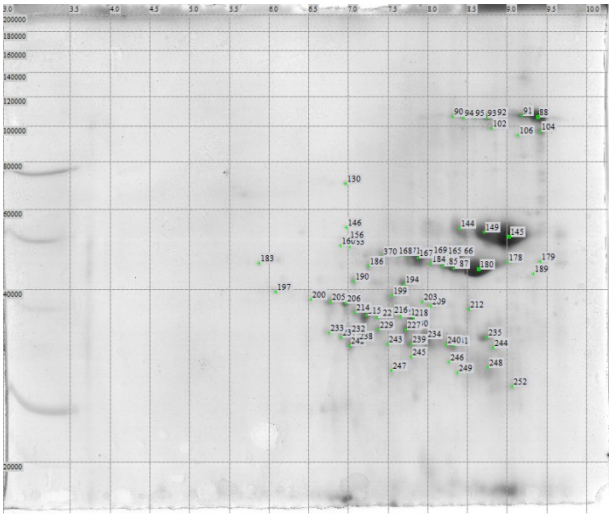


Figure 20 Example of 2DE image analysis on durum wheat glutenin sample (Simeto). Each spot is individuated by a code number

2.3.5. Western blot

Extracted storage proteins (gliadins) were analysed by western blotting by monoclonal antibody specific to ω-5 gliadin (kindly provided by Prof. Peter Shewry), as described in Wan *et al.* (2013).

2.4.Dietary fibre

2.4.1. Determination of total and water extractable pentosan (AX)

The whole population was analysed for AX content. Total (Tot-AX) and water extractable (WE-AX) pentosans were determined using a colorimetric method as described by Douglas (1981) and Finnie *et al.* (2006). Both wholemeal and semolina flour were analysed; two biological and three technical replicates were adopted. The % WE-AX was obtained by the ratio of the WE-AX content divided by the total AX multiplied by 100. Water unextractable (WU-AX) was obtained by difference, Tot-AX minus WE-AX.

2.4.2. Enzymatic fingerprinting of AX and MLG

Samples from wholemeal and semolina were prepared according to the protocol for enzymatic fingerprinting of AX and mixed beta-glucan in wheat grains, adapted from Ordaz-Ortiz, J.J. *et al.*, (2005). Semolina and wholemeal flour were digested by enzymatic treatment with (endo 1,4 β-xylanase (E.C.3.2.1.8), a xylanase of the GH11 group) for AX, endo 1,3(4) glucanase ('lichenase') (E.C.3.2.1.73) for MLG) and the products of digestion separated and resolved by HP-AEC-PAD.

Briefly, 1 ml of 80% (v/v) ethanol was added to 100 mg of flour and heated in a 95°C water bath for 10 min to inactivate endogenous enzymes present in the samples. After centrifugation (10,000 g x 5 mins RT), the residue was washed with 80% (v/v) ethanol and then with 95% (v/v) ethanol to remove and free sugars, and dried using a Speedvac centrifugal evaporator. The dried powder was resuspended in 1 ml of water containing 16 U of endoxylanase and 2 U of lichenase and incubated at 40°C for 16 h with continuous rotation. After centrifugation, 0.6 ml of the supernatant was heated for 10 min in a 95°C hot water bath to inactivate the enzymes.

2.4.3. HPAEC system (high-performance anion-exchange chromatography)

Supernatant obtained after enzyme treatment (above) was filtered through 0.45μm filters (PVDF) (Millipore), diluted 1 in 5 in water and a 20 μL aliquot was injected onto the Carbopac PA1 analytical column (4 × 250 mm) according to Ordaz-Ortiz *et al.* (2005). Duplicate analyses were carried out on each sample. Oligosaccharides (AXOS) released by enzyme digestion were grouped accordingly: 4 unsubstituted (US: x, xx, xxx, xxxxx), 3 mono-substituted (MS: xa3xx, xa3a3xx, xa3xa3xx) and 3 di-substituted (DS: xa2+3xx, xa3a2+3xx, xa3xa2+3xx). Two main peaks obtained from enzymatic digestion of MLG by lichenase were obtained: G3 and G4 which is a measure of oligosaccharides of 3 glucose

residues linked together (β-1,4) or 4 glucose residues linked together (β-1,4). These peaks had previously been identified in the lab of Luc Saulnier (Ortiz-Ortaz *et al.*, 2005) and the method for enzymatic fingerprinting replicated at Rothamsted (Figure 21).

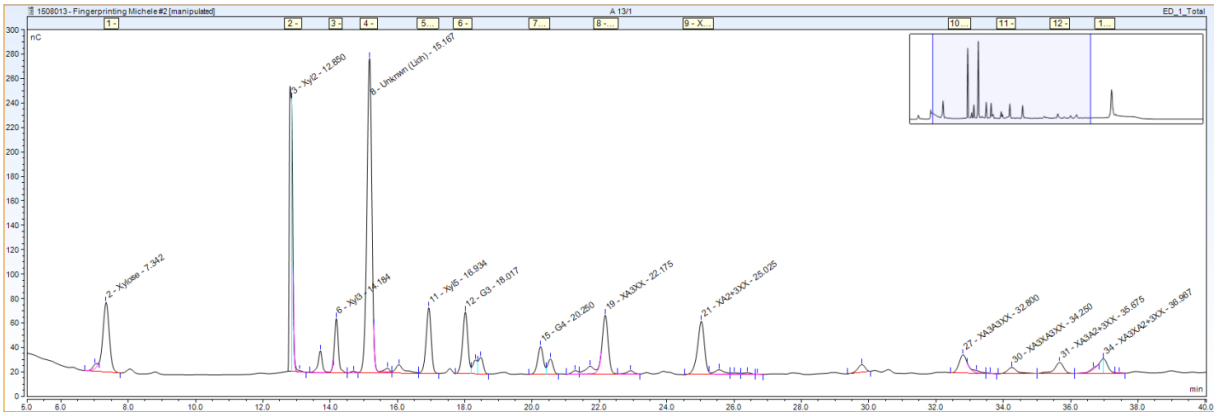


Figure 21 Example of peak area of digested AX and MLG obtained by HPAEC

2.4.4. PACE

Polysaccharide analysis by carbohydrate gel electrophoresis (PACE) was performed on wholemeal and semolina flour from old and modern genotypes of both crop seasons, according to Kosik *et al.* (2012) of the enzymatic digests generated as described above.

2.4.5. Determination of relative viscosity of aqueous extract

The viscosity of the aqueous extract, according to Saulnier *et al.* (1995) was measured at 30°C using an automated viscometer (AVS 310, Schott Gerate, Germany) fitted with an Ostwald capillary tube (2 ml, diameter 0.4mm). Relative viscosities ηrel=t/t0 were determined (t: flow time of distilled water, 72-74s). Measured values are means of two extractions. The flow time of each extract was measured 5 times.

2.4.6. High-performance size-exclusion chromatography (HPSEC)

Aqueous extracts obtained from relative viscosity measurements were injected (50 µL) on the high-performance size exclusion chromatography (HPSEC) system according to Lovegrove *et al.* (2013). Intrinsic viscosity, size of the polymers, molecular size and concentrations of AX and BG polysaccharides were measured.

2.4.7. Ferulic and coumaric acid content

Based on the WU-AX content in the two crop seasons, four genotypes were selected and ferulic acid (FA) and coumaric acid (CA) content determined. in both wholemeal or semolina flour. Bound and free + conjugated FA and CA were extracted and analysed by HPLC, according to Li *et al.* (2008).

2.5.Statistical analysis

Mean and standard deviation was perfomed by MS Excel (Microsoft software). Test T student was performed by JMP (SAS Institute) software. After testing variance homogeneity in the studied characters by Bartlett's test, data of the two years were analysed together, using the analysis of variance (ANOVA). The significant differences among the mean values were calculated following Tukey's test, by JMP (SAS Institute) software.

Due to high correlations observed among the different variables, principal component analysis (PCA) was performed on the protein and dietary fibre correlation matrix separately.

Both for gluten protein and dietary fibre analysis, the data set consisted of 90 samples tested with regard to nine variables. Before performing PCA, the values of each variable were properly standardised. Then, a factorial analysis was performed on the PCA values; the varimax method was chosen to obtain the best orthogonal factor rotation. The PCA results were graphically represented in two-dimensional plots, using JMP (SAS Institute) software.

All the experiments on gluten proteins were performed at the Herbaceous Crop Quality lab - Department of Agriculture, Food and Environmental Sciences (SAFE), University of Foggia – with the supervision of Prof. Zina Flagella and Prof. Marcella Michela Giuliani.

All the experiments relating to dietary fibre, phenolic compounds and the western blot were carried out at the Department of Plant Biology and Crop Science (PBCS), Rothamsted Research (Harpenden, UK), during a 6 months internship as visiting worker with the supervision of Prof. Peter Shewry and Dr. Alison Lovegrove.

3. RESULTS

3.1. Yield and quality parameters

Results relative to yield and its components, grain protein content (GPC), semolina protein content (SPC), yellow index and ash content are reported in Table 7, for old and modern genotypes, grown in 2013 and 2014.

Grain yield ranged from 0.26 (Timilia RB, in 2014) to 0.66 kg m^{-2} (Saragolla, in 2014), showing significant statistically differences between old and modern durum wheat genotypes grown in the two crop seasons. Modern group of genotypes (1985-2005) showed significant higher yield than old group (1900-1949). As for the effect of the crop season a general higher yield in 2014 was observed, especially for the modern genotypes Claudio, Iride, Saragolla and PR22D89. In Timilia RB a significant higher yield was found in 2013. The effect of the G x Y interaction relative to thousand-kernel weight (TKW) was highly significant ($P < 0.001$). Values ranged from 31.5 (Preco, in 2014) to 64.3 g (Garigliano, in 2014). A high significant ($P < 0.001$) reduction of the TKW was observed in the modern group of genotypes, except for Saragolla.

Number of kernel per unit area was significantly higher in the modern group of genotypes with respect to the old one, in particular in 2014, when a general higher number was found ($P < 0.01$). It ranged from 5261 (Garigliano, in 2014) to 15797 (Iride, in 2014). Old genotypes showed a higher stability between the two crop seasons, with a significant reduction in 2014 only for Timilia RB. As for the test weight, no significant differences between old and modern group were evident, and values ranged from 66.9 (Preco, in 2014) to 84.3 kg hl^{-1} (Claudio, in 2013). Evident was the effect of the crop season, with a general high significant ($P < 0.001$) reduction in 2014, except for Dauno III, Russello, and Simeto that showed higher values in 2014. GPC and SPC showed a similar behaviour, with a correlation of 0.976 ($P < 0.001$) and for this reason protein content is discussed in terms of GPC. Values ranged from 11.6 in Iride in 2014 to 16.9% in Cappelli in 2013. Significant higher protein content was observed in the old genotypes in both crop seasons. A significant reduction occurred in 2014, except for old Saragolla, Adamello, Preco and PR22D89 that showed a higher protein content in 2014 ($P < 0.001$). Yellow index measured in semolina was significantly higher in the modern group of genotypes ($P < 0.001$) and ranged from 7.6 (Russello in 2014 and Timilia R.B. in 2013) to 13.2 (Preco, in 2013). Significant higher values were observed in 2013 only in Russello. No significant differences in ash content in semolina between old and modern group were observed. The effect of the crop season showed a significant higher content in 2014 in modern group. The observed values ranged from 0.6 (Simeto, in 2013) to 1.0 % (Preco, in 2014).

Table 7 Yield and quality parameters in old and modern genotypes.

	Crop season	Yield (kg m^{-2})	TKW (g/1000)	n. kernel m^{-2}	TW (kg hl^{-1})	GPC	SPC	Yellow Index	Ash (%)
1900-1949									
Dauno III	2013	0.31^{ijk}	47.9^{ef}	6389^{j-m}	77.2^{l}	15.8^{e}	14.1^{de}	10.0^{no}	0.77^{ghi}
	2014	0.31^{ijk}	45.8^{fg}	6842^{h-m}	78.4^{k}	14.2^{i}	13.2^{gh}	10.2^{mn}	0.8^{efg}
old Saragolla	2013	0.28 ^{jk}	49.3^{ef}	5765^{klm}	80.5^{c}	15.3^{g}	13.7^{ef}	8.4^{r}	0.8^{efg}
	2014	0.28 ^{jk}	49.2^{ef}	5791^{klm}	76.6^{m}	16.3^{c}	15.4^{a}	8.7^{r}	0.76^{hij}
Cappelli	2013	0.33^{g-k}	54.5^{bcd}	6174^{j-m}	81.1^{g}	16.9^{a}	15.4^{a}	9.8^{op}	0.82^{def}
	2014	0.28^{g-k}	48.5^{ef}	5686^{klm}	79.0^{j}	14.6^{h}	13.6^{fg}	10.5^{kl}	0.8^{efg}
Russello	2013	0.29^{ijk}	48.8^{ef}	6041^{klm}	78.8^{jk}	16.6^{b}	14.6^{bc}	11.4^{fg}	0.85^{bcd}
	2014	0.30^{ijk}	54.8 ^{bc}	5484^{lm}	79.0^{j}	13.7^{k}	12.4^{jk}	7.6^{ij}	0.88^{b}
Timilia R.B.	2013	0.37^{f-i}	36.0 ^{jk}	10246^{ef}	80.4^{h}	16.5^{b}	14.9^{b}	7.6^{s}	0.7^{k}
	2014	0.26^{k}	34.7^{jkl}	7602^{g-k}	79.0^{j}	14.0^{j}	12.9^{hi}	10.6^{s}	0.88^{gh}
Garigliano	2013	0.33^{g-k}	55.9 ^{bc}	5976^{klm}	79.0^{j}	15.3^{fg}	13.9^{ef}	9.6^{jk}	0.83^{cde}
	2014	0.34^{g-k}	64.3 a	5261^{1m}	76.0^{n}	14.8^{f}	14.4^{opq}	10.7^{p}	0.8^{efg}
Grifoni 235	2013	0.39^{d-g}	53.5^{cd}	7381^{g-m}	80.5^{h}	15.8^{e}	12.0^{cd}	9.0^{jk}	0.79^{fgh}
	2014	0.34^{f-i}	54.8^{bc}	6292^{j-m}	75.5^{o}	13.0^{m}	11.0^{kl}	9.8^{q}	0.86^{bc}
1985-2005									
Adamello	2013	0.39^{e-h}	55.4^{bc}	6990^{g-m}	79.8^{i}	14.1^{ij}	12.6^{ij}	10.5^{op}	0.83^{cde}
	2014	0.41^{c-g}	37.5^{j}	11044de	67.4^{s}	16.1^{cd}	14.9^{b}	10.4^{klm}	0.87^{b}
Simeto	2013	0.39^{d-h}	47.5^{ef}	8287^{ghi}	69.4^{i}	15.5f	14.0^{de}	12.1^{klm}	0.6^{m}
	2014	0.44^{c-f}	38.0^{ij}	11702de	81.6^{r}	13.5^{l}	12.0^{kl}	12.7^{c}	0.82^{def}
Preco	2013	0.37^{f-i}	57.3^{b}	6392^{i-m}	79.7^{ef}	13.1^{m}	11.7^{lm}	12.7^{b}	0.87^{b}
	2014	0.40^{c-g}	31.5^{l}	12819^{bcd}	66.9^{t}	16.0^{de}	14.7^{bc}	13.1^{a}	0.96 ^{a}
Svevo	2013	0.47^{cd}	45.8^{fg}	10327^{ef}	82.1^{de}	16.2^{cd}	14.9^{b}	12.1 ^{c}	0.82^{def}
	2014	0.48^{c}	33.6^{kl}	14176^{ab}	73.5^{q}	15.1^{g}	13.9^{ef}	11.9^{cde}	0.73^{jk}
Claudio	2013	0.45^{cde}	51.0^{de}	8833^{fg}	84.3 ^{a}	12.5^{o}	10.7^{q}	10.5^{jkl}	0.83^{cde}
	2014	0.60^{ab}	41.5^{hi}	14358^{ab}	81.6^{fg}	11.8^{q}	10.9^{opq}	10no	0.6^{l}
Iride	2013	0.46^{cde}	54.2^{bcd}	8568^{fgh}	82.3^{d}	13.1^{m}	11.4^{mn}	10.2^{lmn}	0.6^{l}
	2014	0.57^{b}	35.9^{jk}	15797^{a}	80.4^{h}	11.6^{q}	10.9^{pq}	11.2^{gh}	0.86^{bc}
Saragolla	2013	0.46^{cde}	37.9^{ij}	12086^{cde}	82.9^{c}	12.8^{n}	11.3^{m-p}	11.7^{de}	0.74^{ij}
	2014	0.66^{a}	43.7 ^{gh}	15077 ^{a}	80.1^{hi}	12.1^{p}	11.3^{m-p}	11.6^{ef}	0.85^{bc}
PR22D89	2013	0.46^{cde}	56.8^{bc}	8091^{g-j}	83.7^{b}	12.5 ^{o}	10.8^{q}	11^{hi}	0.8^{efg}
	2014	0.57^{b}	41.2 ^{hi}	13928^{abc}	74.6^{p}	13.0^{m}	12.3^{jk}	12^{cd}	0.87^{b}
1900-1949	2013	0.33^{c}	49.4 ^{a}	6853 ^{c}	79.6 ^{ab}	16.0 ^{a}	14.4 ^{a}	9.8 ^{b}	0.79 ^{ab}
	2014	0.30^{c}	50.3 ^{a}	6137 ^{c}	77.6 ^{b}	14.1 ^{b}	12.9 ^{b}	9.5 ^{b}	0.81 ^{ab}
1985-2005	2013	0.43^{b}	50.7 ^{a}	8697 ^{b}	82.0 ^{a}	13.7 ^{b}	12.2 ^{b}	11.1 ^{a}	0.76 ^{b}
	2014	0.52^{a}	37.9 ^{b}	13613 ^{a}	74.2 ^{c}	13.6 ^{b}	12.6 ^{b}	11.6 ^{a}	0.83 ^{a}

TKW = thousand kernel weight; TW = Test weight; GPC = grain protein content; SPC = semolina protein content. Different letters are significantly different at $P < 0.05$ according to Tukey's test

Dimension parameters (length, width and thickness) were measured in all samples (data not shown). A high significant correlation between the kernel size measures and the TKW (0.34 * with length, 0.54 ** with width and thickness, * $P < 0.05$; ** $P < 0.01$) was observed.

A not significant different trend, in terms of the response of the thickness in the two growing seasons, was observed between the two groups of durum wheat genotypes. In particular, old genotypes showed a not significant increase of the thickness in 2014, while a not significant reduction was observed in the same crop season in the modern genotypes (except for cultivar Saragolla).

3.2.Storage protein

3.2.1. Gluten index

Analysis of variance showed a significant effect of the G x Y (genotype x year) interaction on the gluten index, as shown in Figure 22.

Gluten index was significantly higher in the modern group of genotypes ($P < 0.001$). The old genotypes (from 1900 to 1949) showed a very low gluten index (min 5, max 13) without significant differences, while in the modern group of durum wheat genotypes G.I ranged from 30 to 80. Among these genotypes Claudio and Saragolla showed the highest values and Preco the lowest. The effect of the crop season was significant only in three modern cultivars (Adamello, Preco, PR22D89), resulting in higher values in 2013 crop year, characterized by water deficit during grain maturation. The highest G.I. values were measured in modern cv Saragolla in both crop seasons, while the lowest in Timilia RB and Garigliano G.I. (5).

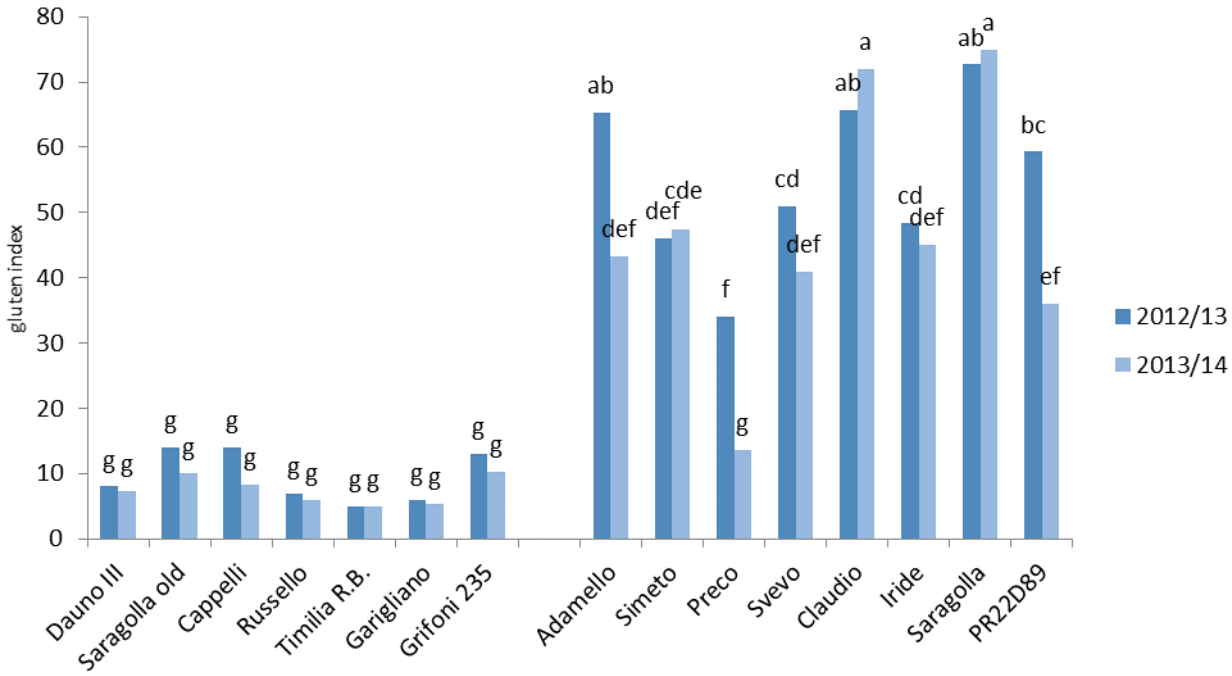


Figure 22Gluten index in old and modern durum wheat genotypes. Different letters are significantly different at $P < 0.05$ according to Tukey's test

3.2.2. Gliadin : glutenin ratio

Differences in gliadin: glutenin ratio (*glia : glut*) were observed between the old and the modern group of genotypes (2.8 [a] vs 1.7 [b], T Student $p < 0{,}001$) with the latter group statistically lower than the former (Figure 23). The highest values were observed in old genotype Garigliano (5.2) followed by Timilia RB (3.7) while the lowest in modern genotypes Saragolla (1.0) and Svevo (1.2). Differences between crop seasons were found in only three old (Dauno III, Timilia and Garigliano) and three modern (Preco, Iride and PR22D89) genotypes that showed higher values in 2014.

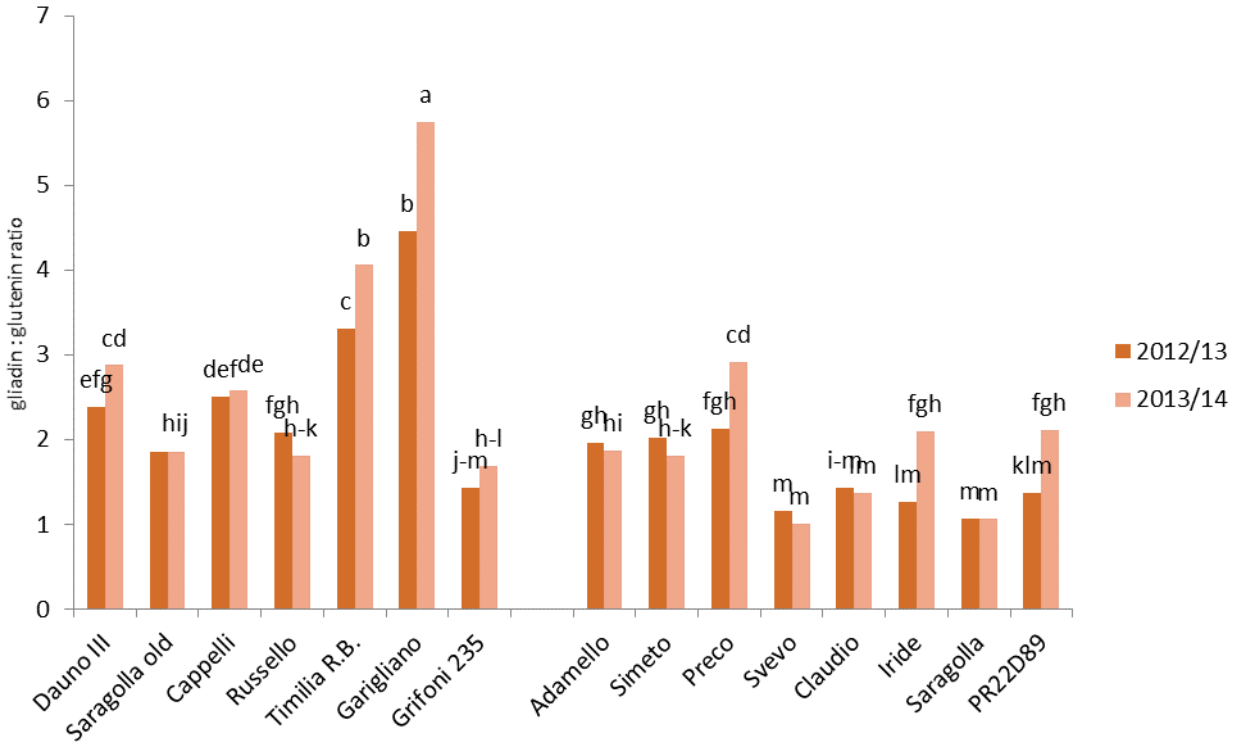


Figure 23 Gliadin : glutenin ratio in old and modern durum wheat genotypes. Different letters are significantly different at $P < 0.05$ according to Tukey's test

3.2.3. SDS-PAGE

Analysis of gluten proteins and of their relative expression by SDS-PAGE was performed separately on gliadin and glutenin sub fractions. Preliminary electrophoretic gels of gliadins (Figure 24) and glutenins (Figure 25) were analysed for each year.

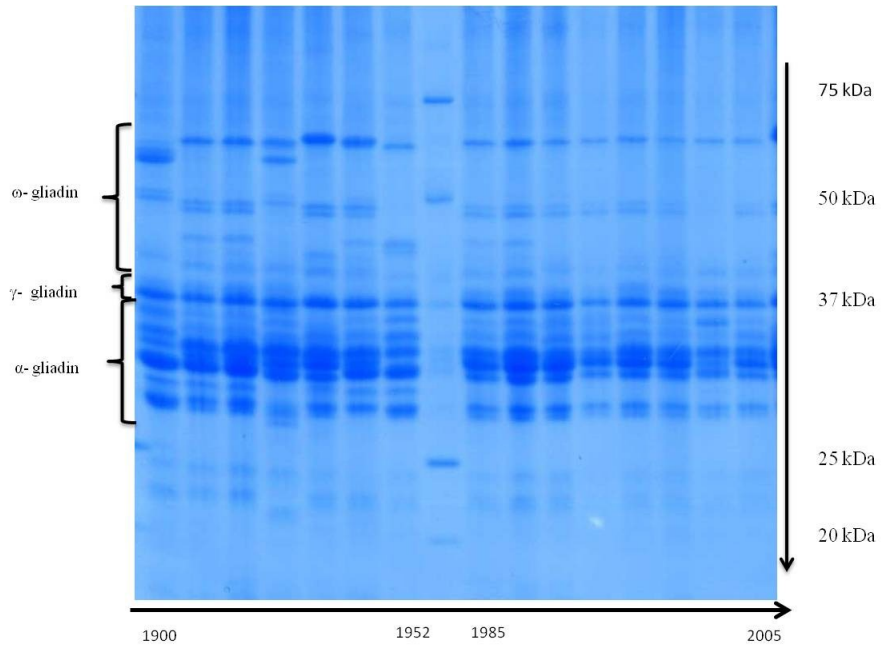


Figure 24 SDS-PAGE of gliadins. Samples were ordered according to the year of release (from left to right)

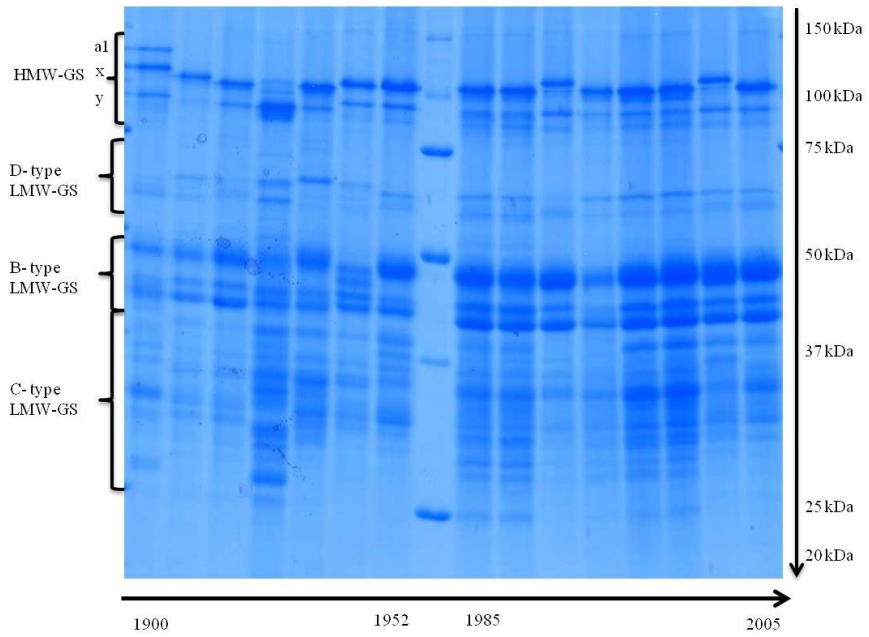


Figure 25 SDS-PAGE of glutenins. Samples were ordered according to the year of release (from left to right)

Allelic differences in glutenins (HMW and LMW) are reported in Figure 25 and Table 8. The investigated genotypes consisted mostly in 7+8 Glu B1 and null Glu A1 allelic combination (8/15).

Table 8 Allelic composition of HMW and LMW glutenin subunits in investigated durum wheat genotypes. *Attribution on the basis of the MW position is proposed when no information was available

Genotype	Pedigree	Year of release	Glu A1 allele	Glu B1 allele	Gli-B1 LMW
Dauno III	landraces from south Italy	1900	2*	6 + 8 *	LMW-1*
old Saragolla	landraces from south Italy	1900	null	7	LMW-2
Russello	landraces from Sicily, Italy	1910	null	13 + 16	LMW-1
Timilia RB	landraces from Sicily, Italy	1910	null	20	LMW-2
Cappelli	selection from Tunisian population Jean Retifah	1915	null	20	LMW-2
Garigliano	Tripolino x Cappelli	1927	null	7 + 8	LMW-2
Grifoni 235	Cappelli x *Triticum aestivum*	1949	null	7 + 8	LMW-2
Adamello	Valforte x turkish line 7116	1985	null	7 + 8	LMW-2
Simeto	Capeiti 8 x Valnova	1988	null	7 + 8	LMW-2
Preco	(Edmore x WPB881) x Selected line 3	1995	null		LMW-2
Svevo	Cimmyt line x Zenit	1996	null	7 + 8	LMW-2
Iride	(Cimmyt selection x Durango) x (IS1938 x Grazia)	1996	null	7 + 8	LMW-2
Claudio	Altar 84 x Ionio	1998	null	7 + 8	LMW-2
Saragolla	Iride x PSB 014 line	2004	null	6 + 8	LMW-2
PR22D89	(Ofanto x Duilio) x Ixos	2005	null	7 + 8	LMW-2

Two biological and three technical replicates were adopted. In order to evaluate the storage protein composition, the relative ω– gliadin, γ– gliadin, α–gliadin, HMW-GS and B- and C-type LMW-GS content on the total storage proteins extracted was determined. In Table 9, the effect of the interaction G x Y on storage protein composition in the 15 genotypes under study is reported. Also, a comparison between the old and the modern durum wheat groups was performed on relative storage protein expression normalised per semolina protein unit (Figure 27).

Table 9 Effect of the interaction G x Y on storage protein composition in the 15 genotypes under study. Different letters are significantly different at P < 0.05 according to Tukey's test

Genotype	% ω– gliadin		% γ- gliadin		% α– gliadin		% HMW-GS		% B- LMW-GS		% C- LMW–GS	
	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014	2013	2014
1900-1949												
Dauno III	14.1^{ab}	11.2^{cde}	11.1^{c-i}	11.1^{c-i}	45.2^{c-h}	51.7^{a-d}	9.7^{c-g}	9.2 $^{d-i}$	11.3^{hij}	8.1^{j}	7.4^{b-j}	7.3^{b-j}
old	10.3^{efg}	10.8^{def}	12.7^{b-h}	7.6^{d-i}	41.7^{e-h}	46.2^{c-h}	9.3^{d-h}	7^{g-k}	19.4^{c-f}	17.9^{d-g}	4.3^{e-k}	8.6^{b-g}
Saragolla												
Cappelli	9.2^{efg}	7.7^{ghi}	14.1^{a-e}	15.5^{b-h}	47.8^{c-f}	51.8^{a-d}	7.3^{g-k}	6.1^{ijk}	16.8^{efg}	16.7^{efg}	3.5^{h-k}	4.4^{e-k}
Russello	10.7^{d-g}	7.9^{fgh}	11.1^{b-I}	6.0^{j}	44^{d-h}	49.7^{b-e}	9.5^{c-g}	9.7^{b-g}	7.0^{j}	13.3^{ghi}	14.8^{a}	10.8^{abc}
Timilia	18.2^{a}	17.1^{ab}	17.8^{a}	18.0^{a}	40.6^{fgh}	44.9^{c-h}	7.4^{f-k}	5.6^{jk}	8.8^{ij}	9.7^{ij}	5.7^{d-k}	3.7^{h-k}
R.B.												
Garigliano	13.7^{cd}	11.7^{cde}	15.2^{ab}	14.3^{a-d}	52.4^{abc}	59^{a}	6.2^{h-k}	4.9^{k}	7.5^{j}	6.64^{j}	4.2^{f-k}	2.7^{jk}
Grifoni	5.8^{hij}	4.8^{h-k}	9.1^{g-j}	8.7^{g-j}	43.7^{d-h}	49.1^{b-e}	12.8^{ab}	8.6^{e-j}	17.3^{efg}	19.7^{c-f}	9.2^{b-f}	8^{jk}
1985-2005												
Adamello	4.5^{hij}	4.5^{jkl}	13.3^{b-g}	13.3^{b-g}	46.9^{c-h}	47.2^{c-g}	8^{f-j}	6.9^{g-k}	17.8^{d-g}	14.7^{fgh}	5.7^{c-k}	10.7^{abc}
Simeto	5.8^{hij}	5.2^{h-k}	11.9^{b-h}	12.8^{b-h}	49.1^{b-e}	46.2^{c-h}	8.5^{e-j}	9^{d-i}	15.4^{fgh}	16.7^{efg}	7.7^{b-j}	7.8^{b-j}
Preco	5.1^{h-k}	4.6^{i-l}	15.1^{abc}	12.9^{b-g}	47.7^{c-f}	56.4^{ab}	9.3^{d-j}	8.7^{d-j}	19.7^{c-f}	13.7^{ghi}	1.8^{k}	2.9^{ijk}
Svevo	4.8^{ijk}	3.3^{jkl}	7.6^{ij}	7.3^{ij}	40.7^{fgh}	38.9^{h}	14.5^{a}	15^{a}	22.3^{bcd}	25.9^{b}	8.8^{b-f}	7.2^{b-j}
Claudio	4^{ijk}	3.1^{jkl}	12.7^{b-h}	11.8^{b-h}	42^{e-h}	42.8^{e-h}	11.3^{b-e}	12.1^{a-d}	17.3^{efg}	19.8^{d-g}	11^{ab}	9.4^{b-e}
Iride	3.7^{jkl}	2.4^{kl}	11.3^{b-i}	13^{b-g}	40.7^{fgh}	52.3^{abc}	9.7^{c-g}	7^{g-k}	23^{bc}	17.7^{d-g}	9.8^{a-d}	6.7^{b-k}
Saragolla	2.2^{kl}	1.5^{l}	9.2^{f-j}	10.5^{d-i}	40.2^{fgh}	39.1^{gh}	10.5^{b-f}	12.6^{abc}	31^{a}	26.4^{ab}	5.4^{d-k}	7.6^{b-j}
PR22D89	3.7^{jkl}	3.5^{jkl}	10.1^{e-j}	11.2^{b-i}	43.8^{d-h}	53^{abc}	9.3^{d-h}	7^{g-k}	24.9^{b}	19.2^{c-f}	6.2^{b-k}	4.9^{d-k}

ω– gliadin. The SDS-PAGE showed differences among the genotypes under study in bands number and molecular weight (MW) in the 50-70 kDa region (Figure 24) highlighting the presence of different allelic forms, most of them previously genetically characterized (De Vita *et al.*, 2007). A large variability of the relative expression of the ω– gliadins (from 1.5 to 18.2% of the total storage proteins) was observed. The old genotypes, with the exception of Grifoni 235, showed a significant higher relative content than the modern ones in both years. The highest ω– gliadins relative content was observed in genotype Timilia RB (18.2% and 17.1%, in the first and second year, respectively) and the lowest in modern Saragolla (2.2% and 1.5% in the first and second year, respectively). Between the two years only the old genotype Dauno III showed significant differences with the higher expression in the more stressed crop season (2013). Further, the ω– gliadin, normalised per protein unit, confirmed the genetic differences between the old and modern durum wheat genotypes, with a three times lower expression in the modern ones, in particular in 2013 (Figure 27).

γ– gliadin. The γ- gliadins were expressed in the 37-42 kDa gliadin gel region in all investigated genotypes. According to the bands MW and to the literature (De Vita *et al.*, 2007) all genotypes are characterized by the γ-45 gliadin allele form (Figure 24). For Dauno III no genetic information is available. No significant statistical differences were observed between the 1900-1945 and the 1985-2005 investigated durum wheat groups; however, differences of expression within the two groups were found (Table 9). The γ- gliadin relative

content on total storage proteins ranged from 6.0% (Russello in the second year) to 18.0% (Timilia RB in the second year). A general higher expression in 2013 was observed for the old genotypes even if significant differences were evident only for old Saragolla and Russello. The normalized expression per protein showed a significant higher expression in the old group only in 2013 (Figure 27).

α– gliadin. The gliadin gel region from 20 to 37 kDa, belonging to the α– gliadin, resulted the most expressed fraction in all samples. Different allelic forms were observed according to the number of the bands and their MW (Figure 24) and to the genetic information available (De Vita *et al.*, 2007, Subira *et al.*, 2014). The overall expression ranged from 38.9% (Svevo in the second year) to 59% (Garigliano in the second year) of total storage proteins, as shown in Table 9. As far as the γ- gliadin, no statistical differences were observed in general between the old and the modern group (46% vs 45%, $P < 0.05$). A higher expression was observed in the second season (2014) with significant differences only for the modern genotypes Preco, Iride and PR22D89. The relative expression of α– gliadin normalized per protein unit showed differences in the response to the crop season between old and modern groups (Figure 27). In particular, the old genotypes showed a global higher expression in 2014, while the modern durum genotypes showed a higher relative stability.

HMW-GS. All the investigated genotypes presented the gene *Glu A1* allele null, with the exception of Dauno III. The allelic configuration of the Glu B1 gene was determined on the basis of the literature (De Vita *et al.*, 2007) and reported in Table 8. No information was found in the literature for Dauno III; on the basis of the bands MW the most probably allele configuration is a Glu A1 2 and 6 + 8 or 6 + 15 (Figure 4 a and b). The HMW-GS expression ranged from about 4,9% (Garigliano, in the second year) up to 15% (Svevo, in the second year), as shown in Table 9, with a general higher expression in the 1985-2005 group. A statistically significant higher expression in 2013 was found only in Grifoni 235. HMW-GS content normalized per protein unit showed a significant higher expression in the modern genotypes in both crop seasons (Figure 27).

B- type LMW-GS. On the basis of the MW (Figure 25) and of the information from literature (De Vita *et al.*, 2007; Subira *et al.*, 2014) the LMW-GS type II resulted present in all the investigated genotypes, associated to the γ- 45 gliadin allele. However, De Vita *et al.* (2007) reported that Russello presents a rare intralocus recombination with γ- 45 gliadin and LMW-1. No data from literature were found for Dauno III; on the basis of the MW position of the bands in the gel, as for HMW-GS, the most probable allelic form is LMW-1. The B-type LMW-GS expression ranged from 6.6% (Garigliano in the second year) to 31% (Saragolla in

the first year) of the total storage proteins (Table 29). In general, the values were higher for the 1985-2005 durum wheat genotypes with respect to the oldest ones in both year; moreover this trend was more evident in the second year. Among the oldest genotypes only the old landrace Russello showed a value significantly higher in the second year; on the contrary among the modern genotypes Preco, Iride and PR22D89 showed values significant higher in 2013. The B- type LMW-GS normalized per protein unit showed a high statistically significant higher expression in the 1985-2005 durum wheat genotypes, as shown in Figure 27 (21% vs 13%).

C-type LMW-GS. The glutenin region below 37 kDa showed a different composition in protein bands (Figure 25), according to the genetic differences reported in literature (De Vita *et al.*, 2007). Differences in expression within old and modern genotypes were observed (Table 29), with a range from 1.8% (Preco, in the first year) to 14.8% (Russello, in the first year) of the total gluten proteins. No evidence of a trend in relative expression with the year of release, was found. No statistically significant differences between the crop seasons were found. The relative expression normalized per protein unit showed no differences between 1900-1949 and 1985-2005 groups in both crop seasons (Figure 27).

HMW-GS : B- type LMW-GS ratio. Large differences in the proportion of HMW-GS with respect to the B- type A was found, as shown in Figure 26. Analysis of variance on this ratio showed a significant higher proportion in the old genotypes (0.70) than in modern ones (0.50). Within the 1900-1949 group, Cappelli showed the lower ratio (0.4), while the higher ratio was observed in the landraces Dauno III and Russello (1.0). In the modern group it ranged from 0.4 (PR22D89 and Saragolla) to 0.6 (Claudio and Svevo). A significant higher ratio was observed in all genotypes in 2013 (0.63 vs 0.56, $P < 0.05$) when water deficit occurred during grain filling; within the genotypes the crop season effect was significant only in Russello, Timilia RB and Grifoni 235; for Dauno III a significant higher ratio was observed in the better watered crop season (2014).

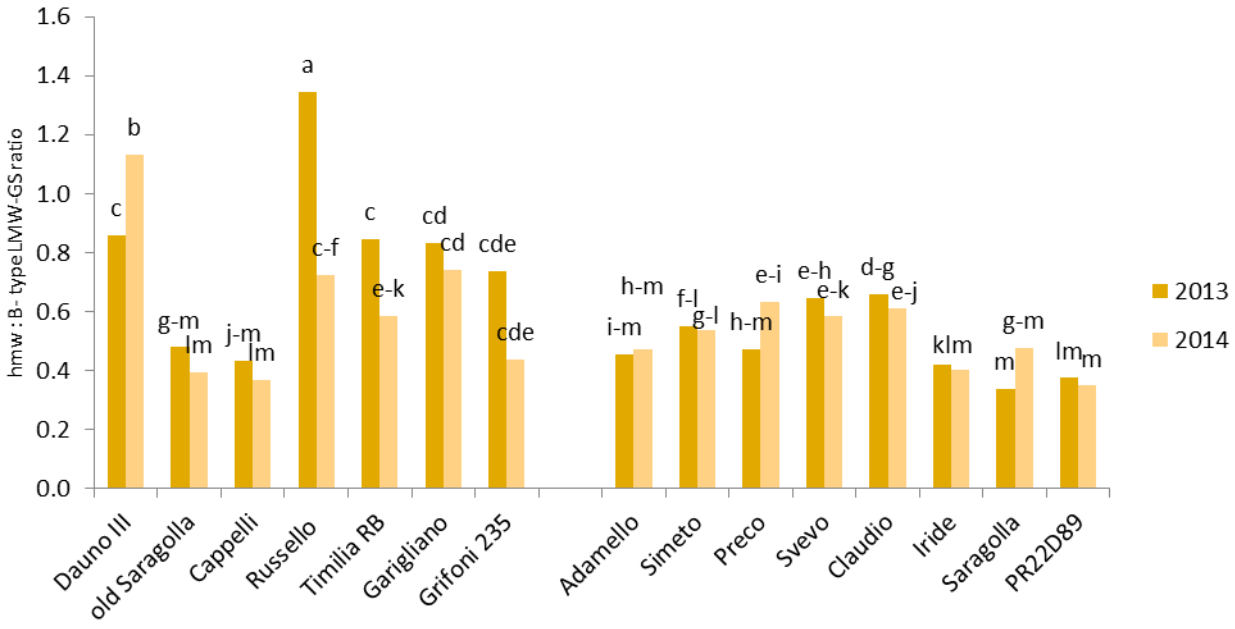


Figure 26 Effect of the interaction G x Y on the HMW-GS : B- type LMW-GS ratio. Different letters are significantly different at $P < 0.05$ according to Tukey's test

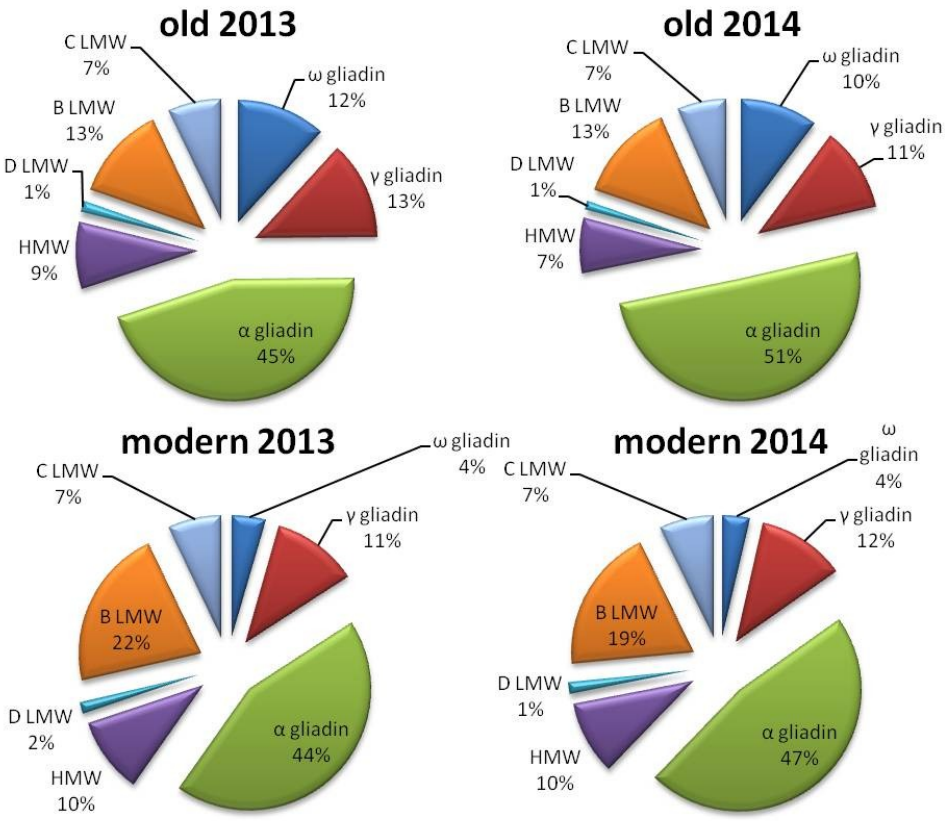


Figure 27 Storage protein composition in old (1900-1949) and modern (1985-2005) durum wheat groups normalised per protein unit

3.2.4. Principal Component Analysis

The correlations among the storage proteins expression, the year of release and the gluten protein composition are shown in Table 10. The year of release was highly positively correlated with gluten index and B- type LMW-GS expression and positively correlated HMW-GS expression. Instead, negative correlations were found between year of release and protein content, *glia : glut* ratio and ω– gliadin.

High significant positive correlation was found between semolina protein content (SPC) and ω- gliadin, and high significant negative correlation was found between protein content and gluten index (G.I.).

Gluten index showed high significant positive correlations (0.7) with the B- type LMW-GS and with HMW-GS and α- gliadin; high significant negative correlations with gliadin : glutenin ratio, ω- gliadin and HMW-GS : B- type LMW-GS ratio were observed.

ω- gliadin showed a significant negative correlation with both the high and low molecular weight glutenin subunits, especially with B- type LMW-GS (-0.77). α- gliadin correlated negatively and significantly with all glutenin subunits, in particular with HMW-GS (0.69).

Table 10 Correlation matrix of the kernel quality parameters with gliadin and glutenin relative composition

	SPC	**G. I.**	**glia : glut**	**ω- gliadin**	**α - gliadin**	**HMW-GS**	**B- type LMW-GS**	**C- type LMW-GS**	**HMW : B- type LMW**
Year of Release	-0.48^{*}	0.84^{***}	-0.48^{***}	-0.81^{***}	ns	0.38^{**}	0.63^{***}	ns	-0.45^{**}
Protein		-0.53^{**}	ns	0.44^{**}	ns	ns	-0.33^{**}	ns	0.29^{*}
G. I.			-0.6^{***}	-0.75^{***}	0.41^{**}	0.44^{**}	0.70^{***}	ns	-0.44^{**}
Glia : glut				0.69^{***}	0.66^{***}	-0.72^{***}	-0.77^{***}	-0.52^{**}	0.30^{**}
ω- gliadin					ns	-0.48^{**}	-0.75^{***}	-0.22^{*}	0.49^{**}
α- gliadin						-0.69^{***}	-0.54^{**}	-0.46^{**}	ns
HMW-GS							0.55^{**}	0.45^{**}	ns
B- type LMW-GS								ns	-0.68^{***}

n.s. = not significant. *P ≤ 0.05. **P ≤ 0.001. ***P ≤ 1x10-6

Principal component analysis (PCA) was applied to the correlation matrix. The main two components individuated by the PCA explained 55% and 23%, respectively of the total variability observed. The first factor was highly and positively associated with the year of release, G.I., B-type LMW-GS and negatively with ω– gliadin, grain protein content and HMW-GS : B- type LMW-GS ratio (Table 11). The second factor showed a positive

correlation with *glia : glut* ratio, α- gliadin and γ- gliadin and a negative correlation with HMW-GS and C- type LMW-GS, as shown in Figure 28. Thus, the first and the second factors may be considered to be influenced by breeding and by gluten composition respectively. The distribution of the genotypes (in 2013 and 2014) along the two principal components is shown in Figure 29. A sharp discrimination between the old and modern genotypes was observed along the factor 1, with Saragolla showing the best performances. A slight discrimination between the two crop seasons was observed along the factor 2 for the 1900-1949 durum wheat genotypes, with higher values in the wetter crop season (2014). As result of the PCA it is possible to associate the modern genotypes with high gluten index values and B- type LMW-GS expression, and with a low *glia : glut* ratio, ω–5 gliadin and γ–gliadin expression. The cultivar Saragolla showed the best quality performance associated to a high LMW-GS B- type expression and a low ω–5 gliadin expression, but with a low protein content. The old group of genotypes showed a higher environmental variability in relation to gluten protein composition, with the exception of two cultivars showing a higher stability (Cappelli and Russello). The cultivar Grifoni 235 showed an intermediate level of gluten protein expression, according to an intermediate year of registration within the investigated genotypes.

Table 11 Varimax rotated factor matrix

	Factor 1	**Factor 2**
Year of release	0.836	-0.308
Protein semolina	-0.693	-0.108
G.I.	0.778	-0.441
Glia : glut	-0.382	0.833
ω- gliadin	-0.813	0.400
α- gliadin	0.034	0.877
HMW-GS	0.072	-0.914
B- type LMW-GS	0.686	-0.600
HMW : B- typeLMW-GS	-0.753	-0.058
Eigenvalue	4.972	1.785
Cumulate %	55%	75%
%	55%	20%

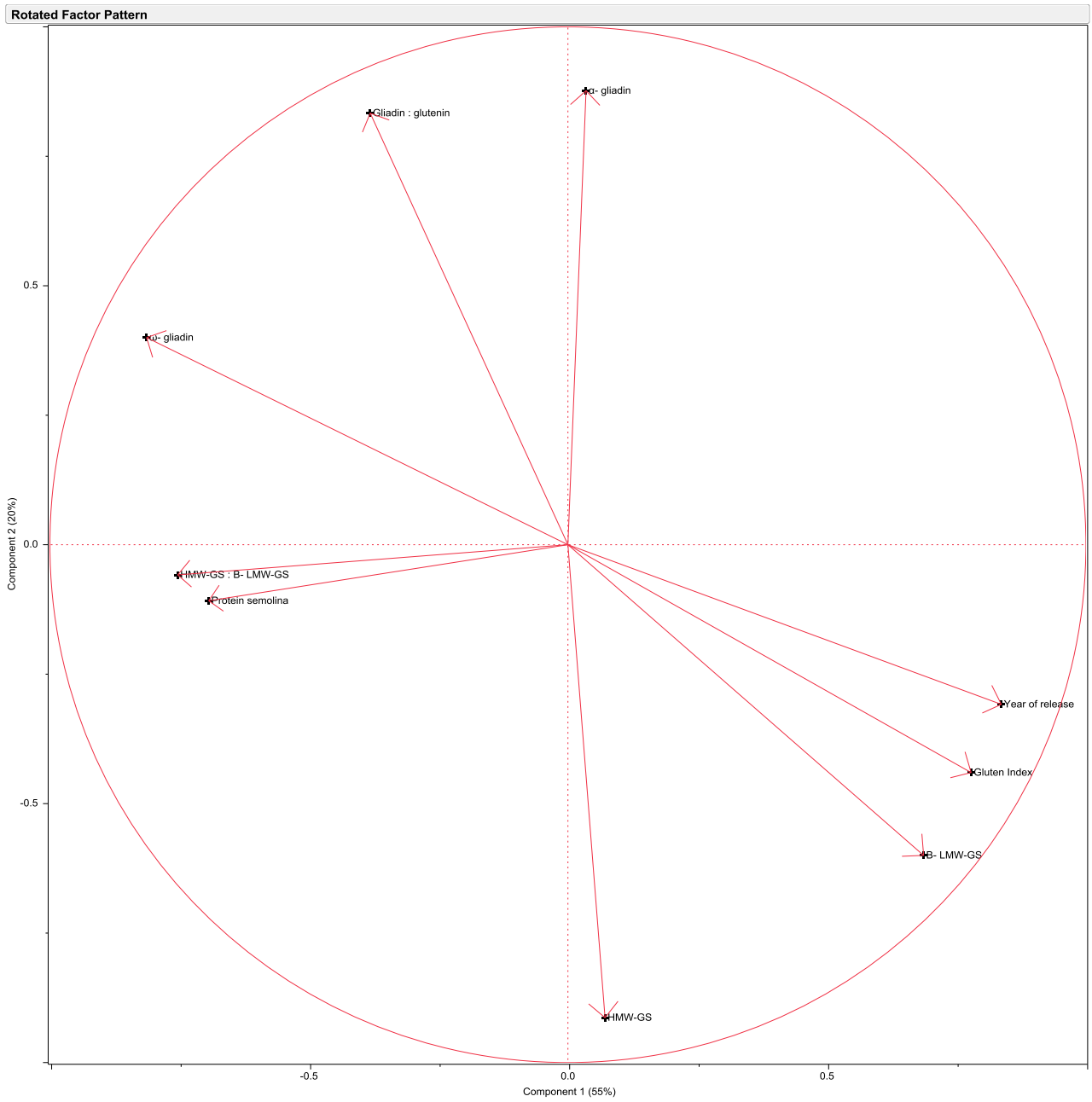


Figure 28 Representation of PCAbased on the storage protein compositional properties

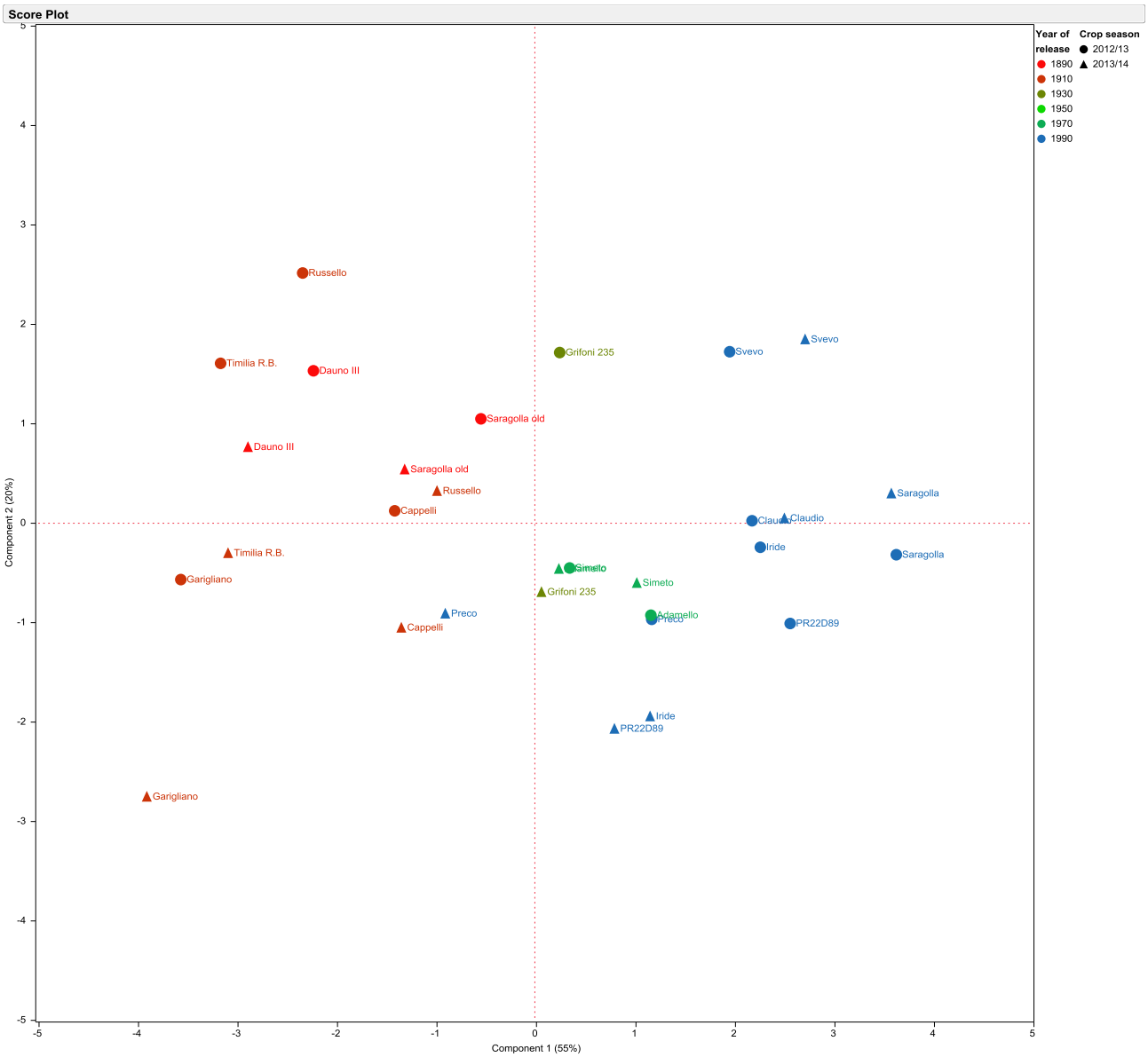


Figure 29 Score plot showing the distribution of old and modern genotypes grown in two crop seasons

3.2.5. Characterization of ω-5 gliadin by western blot

On the basis of the allelic homology, the analysis was carried out on old genotypes Dauno III, old Saragolla, Cappelli, Russello, Timilia RB and on the modern ones Claudio, Iride, Saragolla and PR22D89 (Figure 30). Protein bands with specific reactivity to monoclonal ω-5 gliadin antibody were observed in all investigated samples. Western blot analysis on the gliadins from old and modern genotypes confirmed the presence of ω-5 gliadin in all the investigated old and modern genotypes.

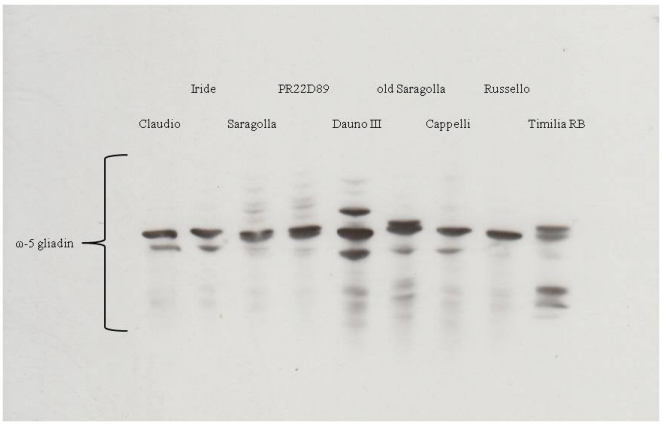


Figure 30 Western blot by monoclonal antibody specific to omega- 5 gliadin (Tri a 19) in old and modern genotypes

The number of bands detected by western blot corresponded to the allelic ω- gliadin observed in SDS-PAGE. Differences in intensity were not quantified because relative expression was previously measured by SDS-PAGE and 2DE SDS-PAGE. Based on the ω- gliadin expression level measured by SDS PAGE, gliadins from modern cultivar Saragolla (low ω-5 gliadin expression) and old landrace Dauno III (high ω-5 gliadin expression) were also analysed by 2DE SDS-PAGE. Proteins were separated based on the isoelectric point (IP) and the molecular weight (MW). Samples from 2013 (Figure 31), when a higher expression was found in both genotypes (Table 9), were analysed.

2DE analysis showed differences in spot number and position, previously found by SDS-PAGE. In particular, in cultivar Saragolla only three spots were expressed in the 60 kDa/6.97-7.02 pH gel region and no other spots were found. Those spots correspond to a previous identification (NCBI accession n. 73912496 ω-5 gliadin, QQYPQQQPSGSDVISISGL) performed by our group and published in Giuliani et al. (2015), on modern cultivars Svevo and Ciccio. No spot in the gel region of 50 kDa was found in Saragolla, as confirmed by SDS-PAGE and western blot. (Figure 31).

In Dauno III three large bands were found either in SDS-PAGE or in western blot. The analysis by 2DE found a higher number of spots (5) in gel region of 55-58 kDa, distributed in

a wider range of pH than in Saragolla (5.4- 7.2 pH). Also, 6 spots were found in 48-50kDa gel region in a large range of pH (5.0-7.8 pH). Finally three spots in a 40-47kDa gel region showed a slight reaction to monoclonal antibody.

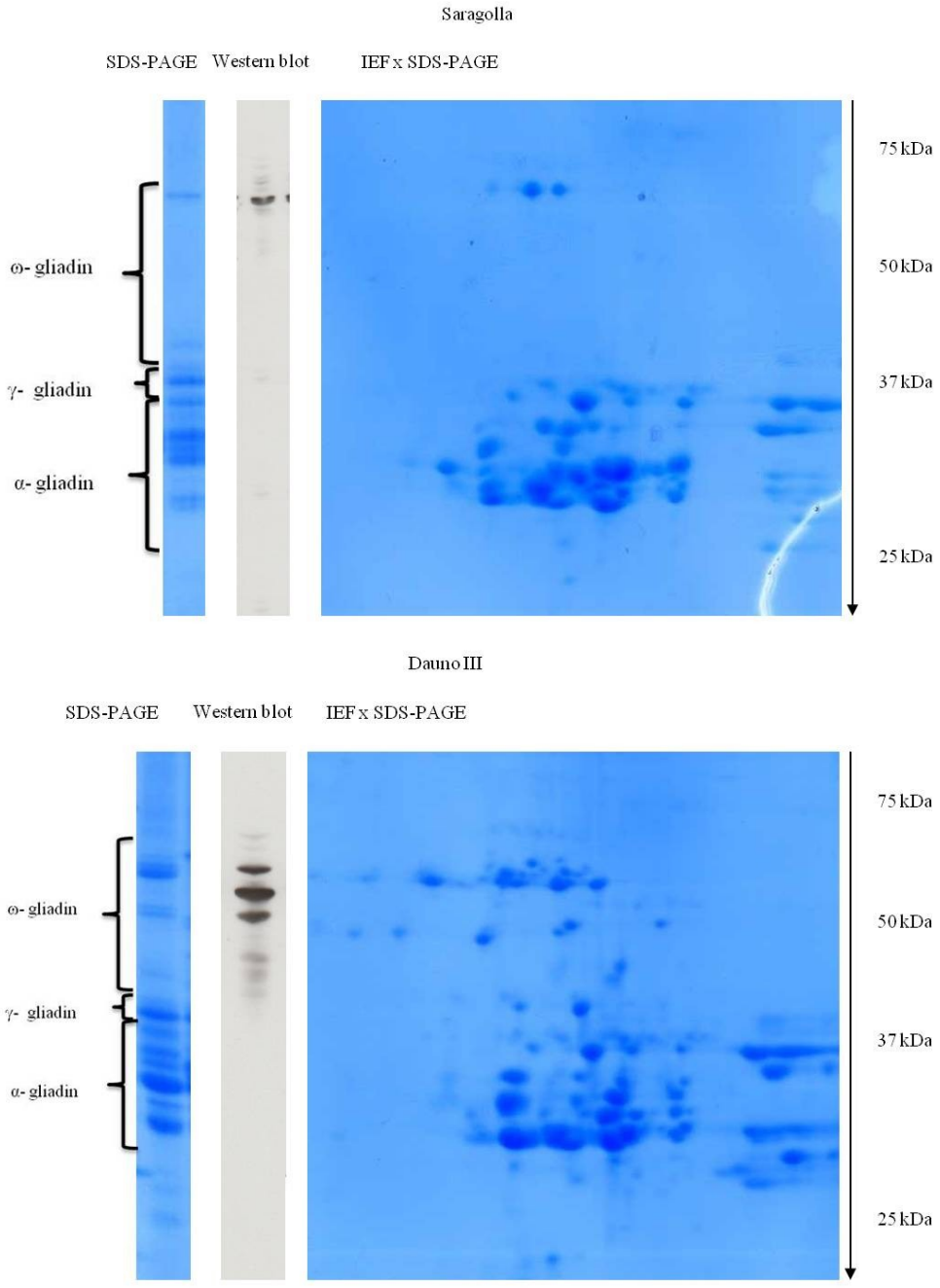


Figure 31 Analysis of gliadin composition by SDS-PAGE, western blot (ω-5 gliadin) and 2DE SDS-PAGE in Saragolla (top) and Dauno III (bottom)

3.2.6. Effect of the crop season analysed by 2DE SDS-PAGE

One old (Cappelli) and one modern (Simeto) genotype were analysed by 2DE SDS-PAGE to individuate differences in protein spot expression due to the effect of the crop seasons. The analysis was carried out on gliadins and glutenins separately.

3.2.6.1. 2DE of Gliadins

Gels of monomeric gliadins from Cappelli and Simeto are shown in Figure 32. Differential subunit expression was previously characterised in SDS-PAGE for relative ω-, γ- and α-gliadin expression.

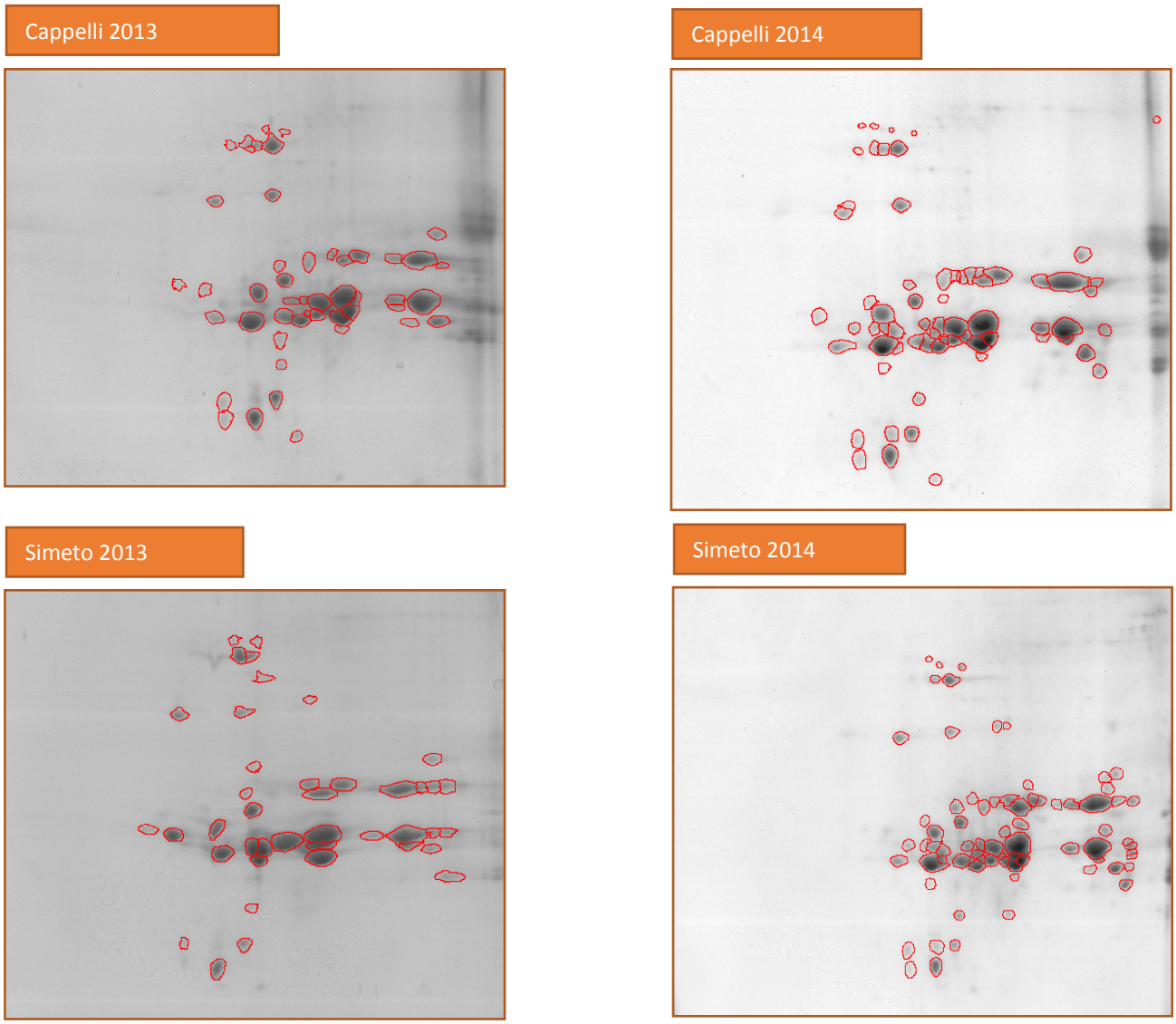


Figure 32 2DE gels of gliadin in two Cappelli (top) and Simeto (bottom) durum wheat genotypes in 2013 (left) and 2014 (right)

Three technical replicates were performed with two combined biological replicates. Gels were well resolved with a good reproducibility. Protein spots were individuated and relative percentage content was determined by gel image analysis (ImageMaster 2D Platinum 6.0). Gels of 2013 were matched with gels of 2014. Specific spots relative to crop seasons and differentially expressed spots that matched (overlapped) were individuated. Significant differences were analysed by Student's t test. Following the list of spots that showed statistically significant differences between crop seasons in Cappelli (Table 12) and Simeto (Table 13).

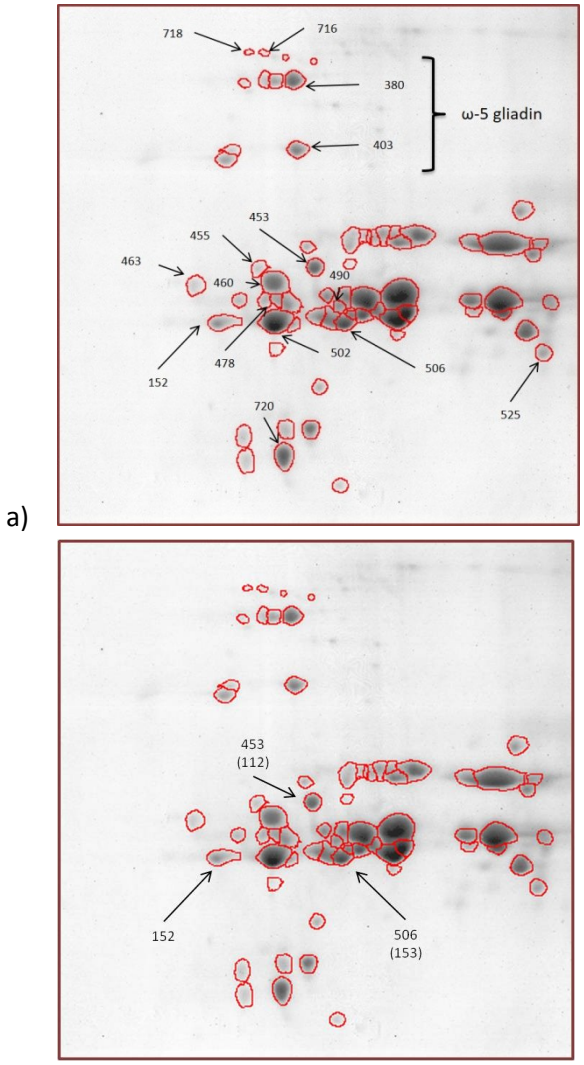


Figure 33 Image Analysis on master gel of Cappelli gliadin (a), and detail of identified spots (b)

Table 12 Effect of the crop season on gliadin composition in the old genotype Cappelli (2013 vs 2014).

Spot ID	Protein	MW (Da)	IP (pH)	2013 (%)	2014 (%)	2013 : 2014 ratio	T test
380	ω-5 gliadin	65501	7.30	4.610	1.937	2.38	*
403	ω- 5 gliadin	50280	7.32	2.024	1.294	1.56	*
716	ω- 5 gliadin	72706	7.04	-	0.090	specific 2014	-
718	ω- 5 gliadin	72822	6.91	-	0.074	specific 2014	-
453	α- gliadin	36392	7.47	2.549	1.599	1.59	*
455	α- gliadin	36356	6.99	-	0.503	specific 2014	-
460	α- gliadin	35136	7.13	4.803	2.989	1.61	*
463	α- gliadin	34999	6.46	0.262	0.687	0.38	*
478	α- gliadin	33858	7.05	-	0.596	specific 2014	-
479	α- gliadin	33949	7.09	-	0.596	specific 2014	-
486	α- gliadin	33132	7.12	-	0.596	specific 2014	-
490	α- gliadin	33366	7.68	-	0.344	specific 2014	-
492	α- gliadin	33010	7.99	-	0.621	specific 2014	-
498	α- gliadin	32642	7.88	2.041	1.247	1.64	*
499	α- gliadin	32706	7.78	-	1.566	specific 2014	*
502	α- gliadin	31871	7.13	10.918	5.961	1.83	**
506	α- gliadin	31965	7.72	4.638	2.544	1.82	*
525	α- gliadin	29987	9.40	-	0.652	specific 2014	-
720	α- gliadin	25110	7.23	-	0.833	specific 2014	-

Table 13 Effect of the crop season on gliadin composition in the modern genotype Simeto (2013 vs 2014)

Spot ID	Protein	MW (Da)	IP (pH)	2013 (%)	2014 (%)	2013 : 2014 ratio	T test
153	ω- 5 gliadin	63782	6.87	-	0.949	specific 2014	-
182	ω- 5 gliadin	48918	7.05	1.906	1.062	1.80	> *
233	γ- gliadin	35602	6.74	-	0.454	specific 2014	-
234	γ- gliadin	35409	7.18	2.818	1.479	1.91	> *
237	α- gliadin	35200	7.92	-	0.836	specific 2014	-
242	α- gliadin	34156	6.86	3.537	2.380	1.49	> *
255	α- gliadin	32671	7.59	9.860	2.738	3.60	> *
256	α- gliadin	32995	7.43	-	1.232	specific 2014	-
258	α- gliadin	32849	6.74	-	0.719	specific 2014	-
261	α- gliadin	32849	6.74	-	0.719	specific 2014	-
264	α- gliadin	32849	6.74	-	0.719	specific 2014	-
259	α- gliadin	32833	7.27	5.066	1.200	4.22	**
268	α- gliadin	32048	7.39	-	0.667	specific 2014	-
271	α- gliadin	31780	7.85	4.448	0.903	4.93	*
275	α- gliadin	31328	7.56	-	1.942	specific 2014	-
279	α- gliadin	31297	7.19	-	3.420	specific 2014	-
281	α- gliadin	31437	7.45	-	0.723	specific 2014	-
283	α- gliadin	31328	6.41	4.270	1.673	2.55	*
285	α- gliadin	31236	7.73	-	1.268	specific 2014	-
286	α- gliadin	30792	7.86	8.069	3.801	2.12	*
287	α- gliadin	30655	7.40	7.855	2.767	2.84	*

In both Cappelli (Table 12) and Simeto (Table 13) different specific and differentially expressed gliadin protein spots were found between 2013 and 2014. ω- gliadins were in general over-expressed in 2013; in addition, specific isoforms in 2014 were found in Cappelli (spot 716, 718) and in Simeto (spot 153).

In particular, spot 380 correspond to a previous identification (NCBI accession n. 73912496 ω-5 gliadin, QQYPQQQPSGSDVISISGL) performed by our group and published in Giuliani *et al.* (2015), on modern cultivars Svevo and Ciccio. This spot showed a significant higher expression in 2013 in Cappelli (fold variation 2.38). In Simeto no significant differences were found relative to this spot, but a specific isoform was expressed in 2014 (153).

Interesting crop season differences were found in both genotypes within α- gliadin gel region, in particular in 32.8 - 33.8 kDa and pI 6.81 - 7.05. A group of spots (α- gliadin) were found specific in 2014 both in Cappelli (478, 479 and 486 and shown in Figure 33a) and in Simeto (258, 261, 264).

Three gliadins overlapped with three drought responsive α- gliadin (Figure 33b) identified in a previous comparison performed by our research group on two durum wheat genotypes in water stress conditions (Giuliani *et al.*, 2015), and reported in Table 14. In particular spot 152 (Cappelli) was identified as a drought responsive α- gliadin, whose peptide sequence matched with an allergic protein involved in baker's asthma (Sander *et al.*, 2011). Spot 453 (112) corresponds to an α- gliadin precursor whose sequence results also involved in wheat allergy (Maruyama *et al.*, 1998). Finally spot 506 was previous identified as an α- gliadin whose sequence resulted triggering to CD (van Herpen *et al.*, 2006).

Table 14 List of common identified drought-responsive gliadin proteins from Giuliani *et al.* (2015)

spot	Accession (NCBI nr general index)	Proteins	species	Mascot Score	sequence coverage (%)	theor Mw (kDa); pI	no. of peptide matches	peptide sequence
152	1304264	α –gliadin	Triticum aestivum	84	14	30.3; 6.18	3	VRVPVPQLQPQ; NPSQQQPQEQVPL; RPQQPYPQPQPQY
112 (453)	283476402	α-gliadin precursor	Triticum aestivum	66	6	32.9; 6.62	1	QQPNIAHASSQVSQQSY
506 (153)	66393328	α-gliadin	Triticum monococcum	116	14	31.09; 7.14	3	LQLQPFPQPQLPY; RPQQPYPQPQPQY; QQPQQQYPLGQGSFRPSQQNPQA

3.2.6.2. 2DE of Glutenins

Polymeric glutenins were separated by 2DE, as shown in Figures 34 and 35. Comparison between 2013 and 2014 crop seasons was carried out on old Cappelli (Table 15) and modern Simeto (Table 16).

Figure 34 2DE gels of glutenin in two old (top) and two modern (bottom) durum wheat genotypes grown in 2013 (left) and 2014 (right)

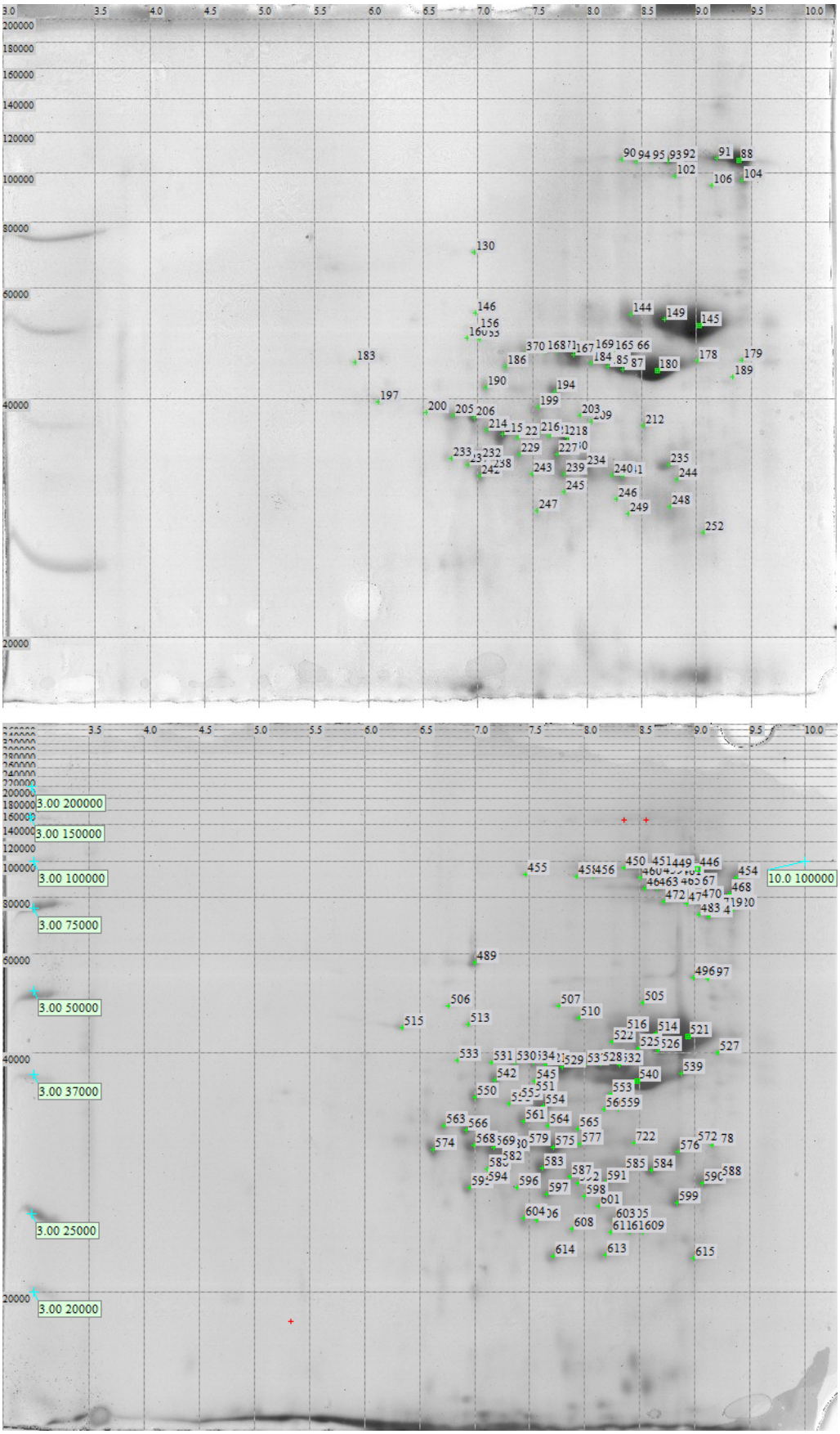


Figure 35 Image analysis on master gel of glutenin (2013) in Cappelli (a) and Simeto (a). Each spot is individuated by a code number

Table 15 Effect of the crop season on glutenin composition in the old genotype Cappelli (2013 vs 2014)

Spot ID	Group	MW (Da)	IP (pH)	2013 (%)	2014 (%)	2013 : 2014 ratio	T test
88	HMW-GS Bx20	105886	9.39	8.873	5.702	1.56	*
165	B- type LMW-GS	46650	8.24	1.762	2.966	0.59	**
171	B- type LMW-GS	46509	7.71	-	0.803	specific 2014	-
187	B- type LMW-GS	43924	8.32	-	2.582	specific 2014	-
199	C- type LMW-GS	38940	7.55	1.325	0.869	1.52	*
205	C- type LMW-GS	37981	6.78	0.492	1.009	0.49	*
212	C- type LMW-GS	36758	8.51	-	0.433	specific 2014	-
234	C- type LMW-GS	32681	7.99	0.978	2.919	0.33	*
252	C- type LMW-GS	26959	9.06	-	0.606	specific 2014	-

Table 16 Effect of the crop season on glutenin composition in the modern genotype Simeto (2013 vs 2014)

Spot ID	Group	MW (Da)	IP (pH)	2013 (%)	2014 (%)	2013 : 2014 ratio	T test
446	HMW-GS Bx7	95355	9.03	6.147	2.184	2.81	*
454	HMW-GS By8	90400	9.38	0.999	-	specific 2013	-
468	HMW-GS By8	81722	9.32	1.437	0.800	1.80	*
489	D- type LMW-GS	57568	7.00	0.996	0.224	4.45	*
510	D- type LMW-GS	45287	7.94	0.555	-	specific 2013	-
513	D- type LMW-GS	44218	6.94	0.331	-	specific 2013	-
514	B- type LMW-GS	42880	8.64	5.997	1.395	4.30	*
521	B- type LMW-GS	42356	8.93	16.577	9.937	1.67	*
550	C- type LMW-GS	34599	7.00	1.037	0.438	2.37	*
554	C- type LMW-GS	33786	7.62	1.809	1.053	1.72	*
574	C- type LMW-GS	29904	6.62	1.260	-	specific 2013	-
583	C- type LMW-GS	28440	7.61	1.774	0.549	3.23	*
590	C- type LMW-GS	27205	9.06	1.273	1.716	0.74	*
591	C- type LMW-GS	27306	8.19	0.446	1.485	0.30	*
599	C- type LMW-GS	25751	8.84	1.618	3.040	0.53	*
603	C- type LMW-GS	24484	8.26	0.949	1.619	0.59	*

In genotype Cappelli, a Bx20 HMW-GS spot (88) was over-expressed in 2013 with a fold variation of 1.56. A significant over-expression in 2013 was found also in Simeto for a Bx7 HMW-GS (spot 446) with a fold variation of 2.81. A specific spot (468) was also found in Simeto in 2013.

The most expressed spot in both genotypes resulted a B- type LMW-GS, individuated as spot 145 (Cappelli) and 521 (Simeto). This protein spot showed a significant over expression in 2013 only in Simeto (fold variation 1.67). This spot corresponds to a glutenin identified by our group in a previous investigation (Giuliani *et al.*, 2015), and identified as LMW glutenin subunit type 2 (*Triticum turgidum*, subsp. *durum*, 42.6 kDa; 8.736 pH – 5 peptides: SHIPGLERPSQQQPLPPQQTL; QQQIPFVHPSILQQLNPCKVF; EAIRAIVY; GQQPQQQQLAHGTF; YRTTTRVPF). This higher expression in Simeto was found also in the isoform protein 514 (fold variation 4.30).

As regards the C- type LMW glutenin subunits, a general variability was observed in response to the crop season. One spot was significantly over-expressed in 2013 in Cappelli (199) and three in Simeto (550, 554, 583); two spots were significantly down-expressed in 2013 in

Cappelli (205, 234) and four in Simeto (590, 591, 599, 603). Two spots resulted specific of 2014 crop season in Cappelli (212, 252) and one specific spot of 2013 in Simeto (574).

Finally, two spots (176, 199) in 2013 and five spots (718, 719, 437, 505, 564) in 2014 resulted specific of old Cappelli. Two of these were ω- 5 gliadins. Varietal differences were more marked in 2014, in particular for α- gliadin with five proteins over-expressed in Cappelli and Simeto.

Table 17 Spot differences in gliadin between Cappelli and Simeto (2013)

Spot ID	Group	MW (Da)	IP (pH)	Simeto (%)	Cappelli (%)	ratio	T test
	2013						
176	ω- gliadin	50000	7.75	-	8.884	specific	Cappelli
199	ω- gliadin	41999	9.10	-	4.638	specific	Cappelli
214	γ- gliadin	38223	7.77	10.097	6.764	1.493	> *
223	γ- gliadin	37522	8.56	5.424	10.918	0.497	< *
	2014						
718	ω- gliadin	72822	6.91		0.074	specific	Cappelli
719	ω- gliadin	64930	6.88	-	0.261	specific	Cappelli
437	γ- gliadin	39366	7.97	-	0.389	specific	Cappelli
441	γ- gliadin	38885	8.79	3.798	1.424	0.375	> **
471	α- gliadin	33792	7.90	2.738	6.222	0.440	< **
490	α- gliadin	33366	7.68	0.667	0.344	1.941	> *
502	α- gliadin	31871	7.13	6.992	5.961	1.171	> *
503	α- gliadin	32642	7.49	3.420	0.968	0.283	> **
505	α- gliadin	32309	7.58	-	1.865	specific	Cappelli
525	α- gliadin	29987	9.40	1.367	0.652	2.096	> **
545	α- gliadin	25134	7.43	0.669	1.381	0.485	< *
564	α- gliadin	21485	7.68	-	0.422	specific	Cappelli

3.3.Dietary fibre

3.3.1. Determination of pentosan content

Replicated spectrophotometric measures of AX content (pentosans) were performed on all investigated genotypes. Total (Tot-AX) and water extractable (WE-AX) arabinoxylans in wholemeal and semolina flour were measured in all old and modern genotypes in two crop seasons. %WE-AX was calculated as a ratio of WE-AX to Tot-AX multiplied by 100. Means of Tot-AX and WE-AX content (expressed as mg of AX/100mg of dm.) in semolina and wholemeal flour of old and modern genotypes grown in 2013 and 2014 crop seasons are shown below.

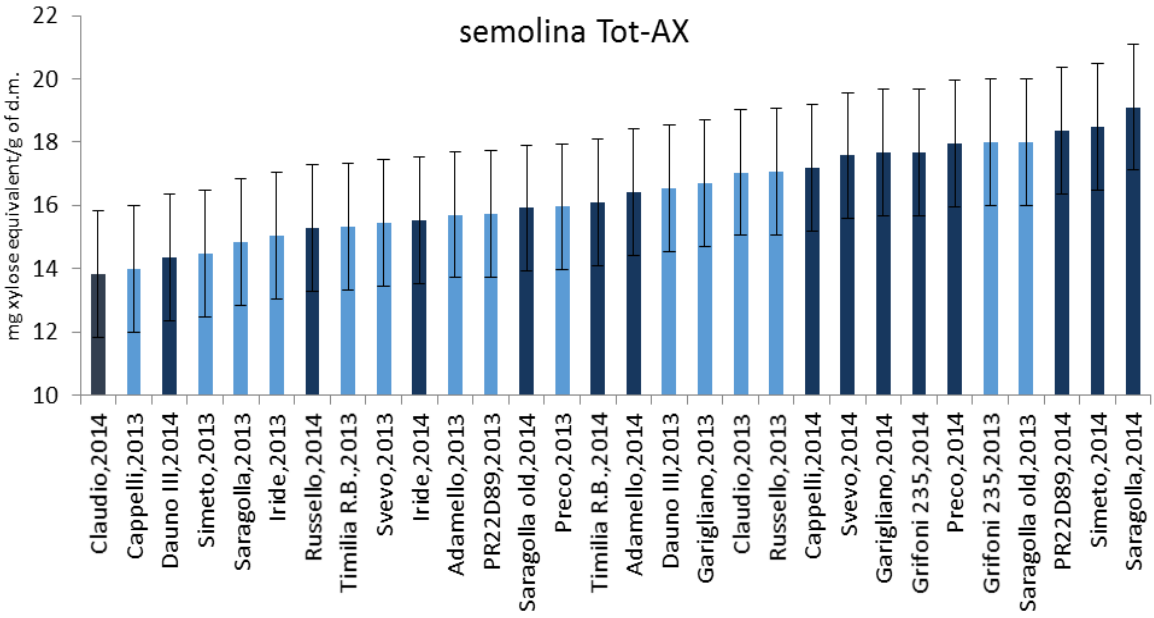


Figure 36 Tot-AX content in 100mg (d.m.) of semolina

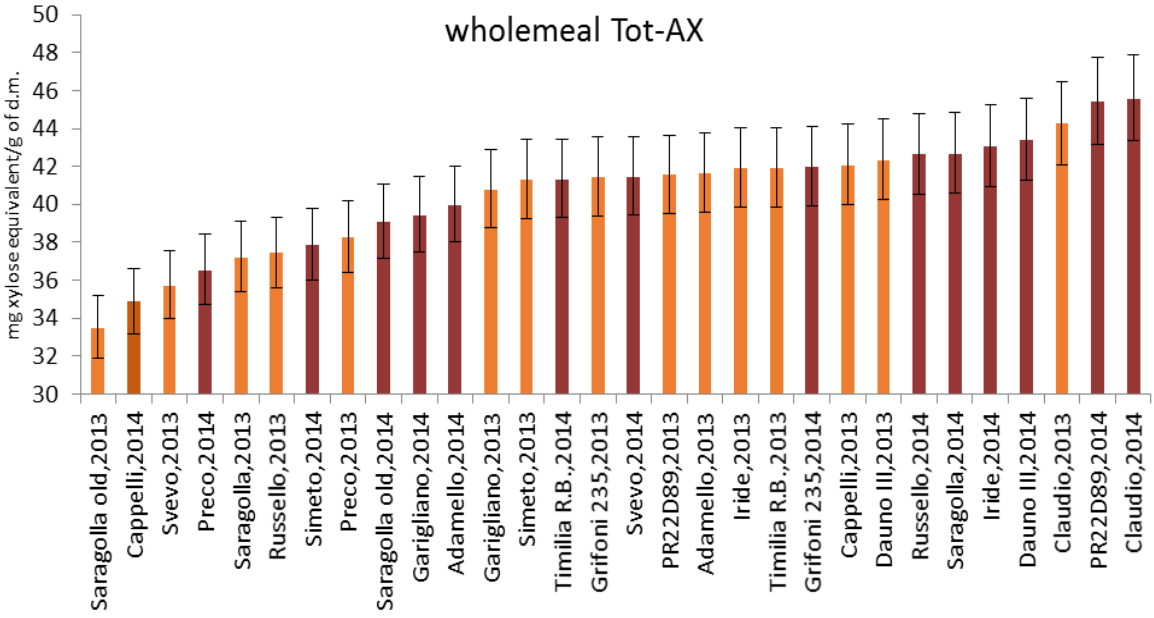


Figure 37 Tot-AX content in 100mg (d.m.) of wholemeal flour (b)

In semolina, (Figure 36) Tot-AX ranged from about 14 to 19 mg/g (dm.), without showing significant differences among investigated genotypes. The overall effect of the crop season resulted in a small but significant increase in 2014 (16.8 [a] vs 16,0 [b], P value 0.0302), however this trend was not significant in each genotype. In wholemeal flour (Figure 37) Tot-AX were higher than in semolina, as shown in Figure 2. Values ranged from 33.5 (old Saragolla, 2013) to 45.6 mg/1g (Claudio, 2014). The analysis of the variance (Anova) showed highly statistically significant differences due to the genotype, crop season (Y) and G x Y interaction (F significance < 0.0001). Significantly higher values were measured in 2014 (41.0 vs 40.1, P = 0.0087), as a general trend, and these differences were statistically significant in cultivars old Saragolla, Russello, Saragolla, Svevo. The exception was old cultivar Cappelli where the content of Tot-AX was greater in 2013 than in 2014.

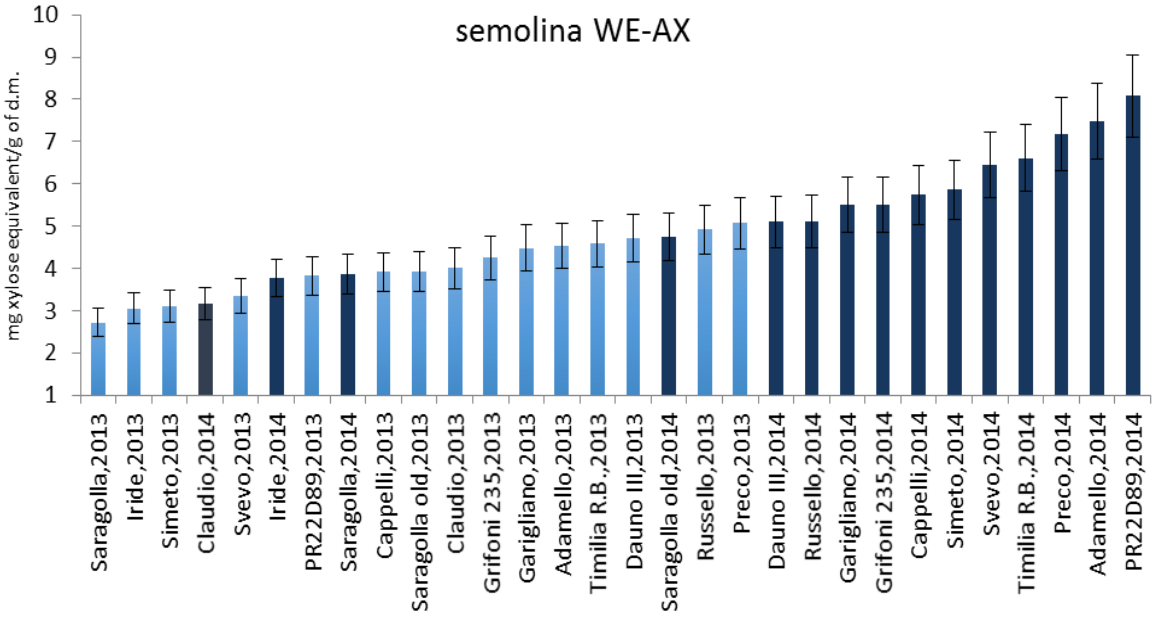


Figure 38 WE-AX content in 100mg (d.m.) of semolina

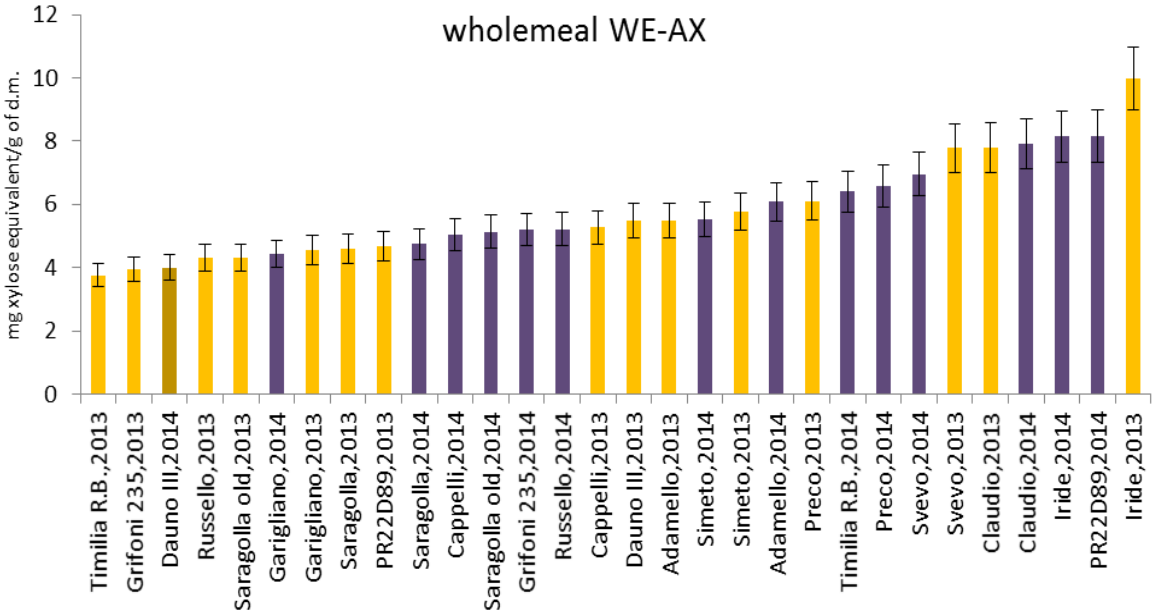


Figure 39 Tot-AX content in 100mg (d.m.) of semolina

WE-AX ranged from 2.7 to 8.1 in semolina (Figure 38), and from 3.8 to 9.9 in wholemeal (Figure 39). Most of the total grain WE-AX was present in the endosperm (about 70-98%, data not shown), and differences with WE-AX in wholemeal were minimal, i.e. little WE-AX is contained in the outer layers as the values for WE-AX semolina are similar to that for wholemeal. Only in cultivars Iride and Claudio about 40% of the total grain WE-AX were present in the endosperm (semolina). In both fractions, genetic and environmental differences were observed. Higher WE-AX were observed in semolina in cultivars Preco (both crop seasons), Adamello and PR22D89 (both in 2014), while the lowest content was measured in modern Saragolla. In general, a significantly higher content of WE AX was observed in 2014 both in semolina (5.6 vs 4.0, $P < 0.0001$), and in wholemeal flour (6.0 vs 5.5, $P < 0.0001$).

Results on the relative WE-AX compared to total pentose (%WE-AX) are reported below. %WE-AX in semolina was mostly influenced by WE-AX, rather than Tot-AX (total pentosan), as previously described.

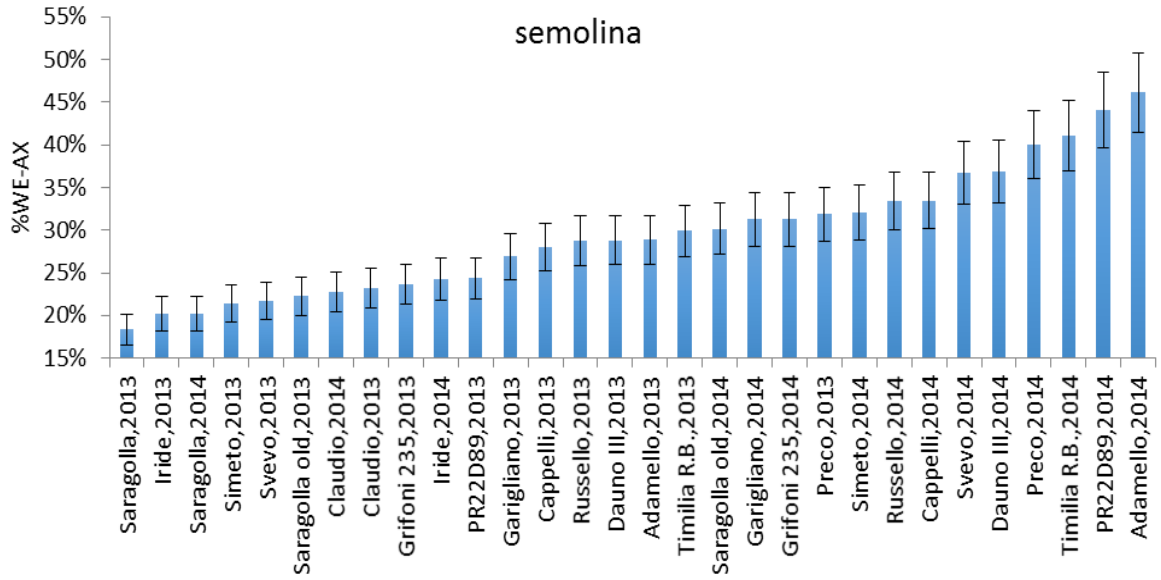


Figure 40 %WE content in semolina

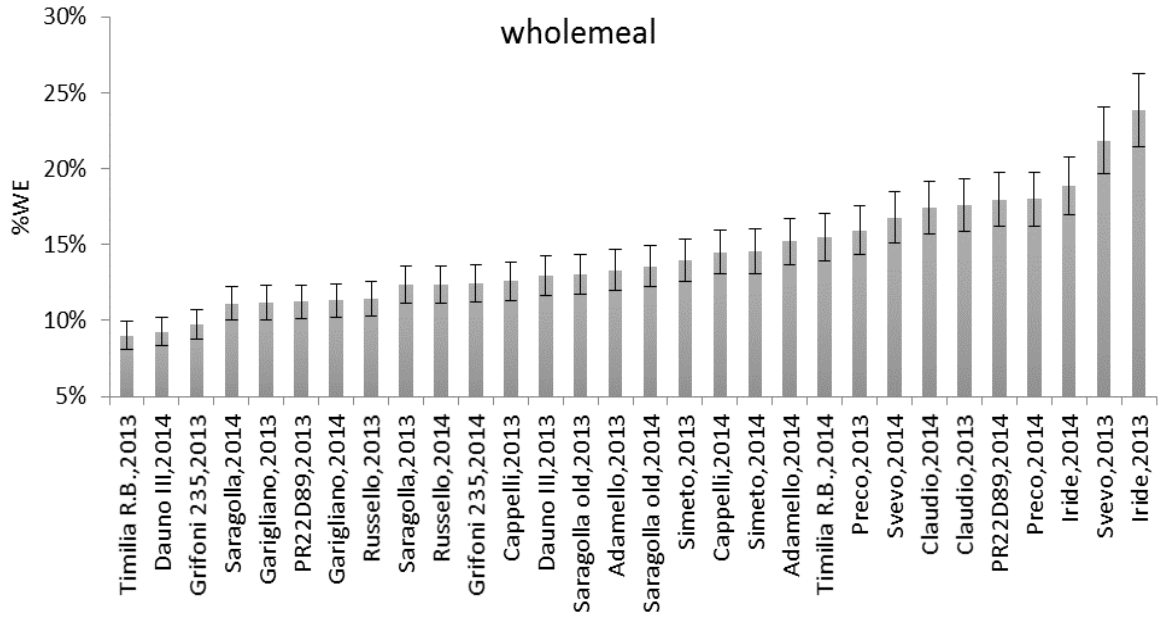


Figure 41 %WE content in wholemeal

Relative WE-AX ranged from 18 to 46% in semolina (Figure 40) and from 9 to 24% in wholemeal flour (Figure 41). A significant increase in AX content was observed in the 2014 crop season (in 2014, no significant differences in temperature occurred after anthesis, but there was higher rainfall) when water was freely available during grain filling in particular in modern varieties, which implies that the modern varieties might be more environmentally affected, in terms of AX content than the older varieties..

The effect of the modern intensive breeding was assessed by comparing genotypes in two groups, according to the year of release: a group of old (1900-1949) and a group of modern (1985-2005) durum wheat genotypes. In semolina no significant differences were found between the old and the modern group in response to crop season (Figure 42a); however, there was a marked difference in %WE-AX observed in wholemeal flour between the old and the modern groups, irrespective of crop season. Modern varieties showed, in both crop seasons, a higher %WE-AX content (Figure 42b).

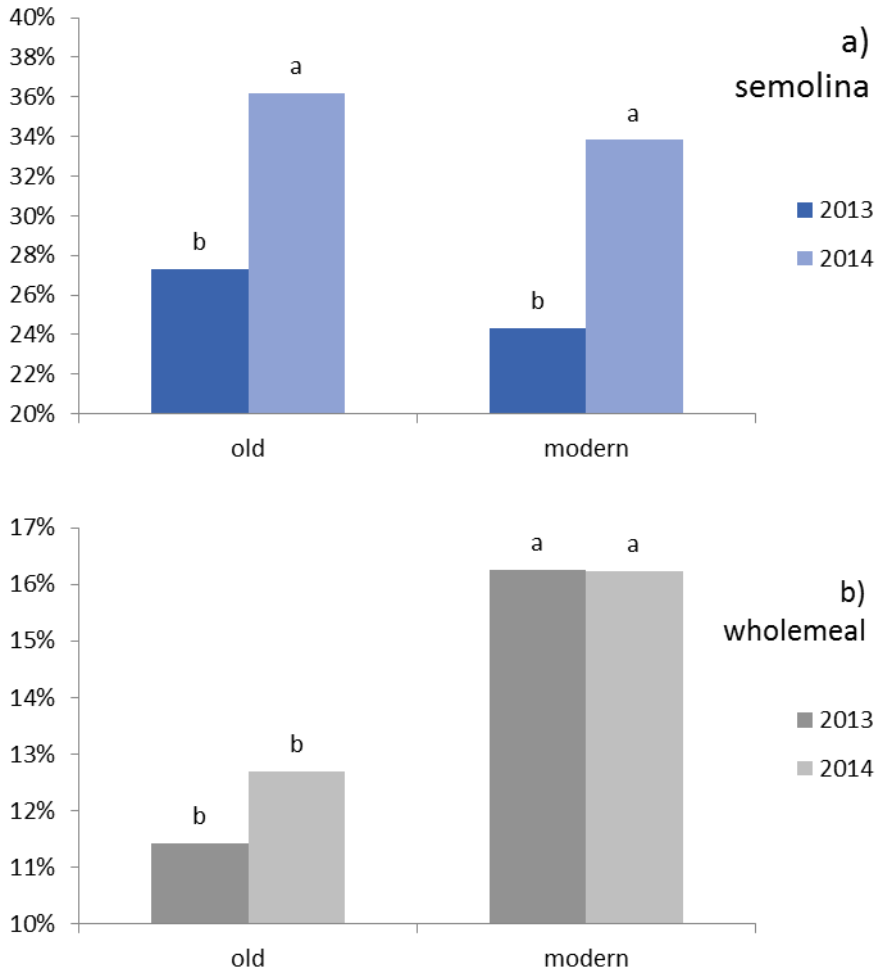


Figure 42 %WE-AX in semolina (a) and wholemeal flour (b), in old and modern groups in 2013 and 2014. Different letters are significantly different at $P < 0,05$ according to Tukey's test

3.3.2. *Relative viscosity*

Measurement of the relative viscosity on semolina aqueous extracts of DF were performed, as shown in Figure 43. The viscosity is expressed as relative viscosity (using distilled water as a standard).

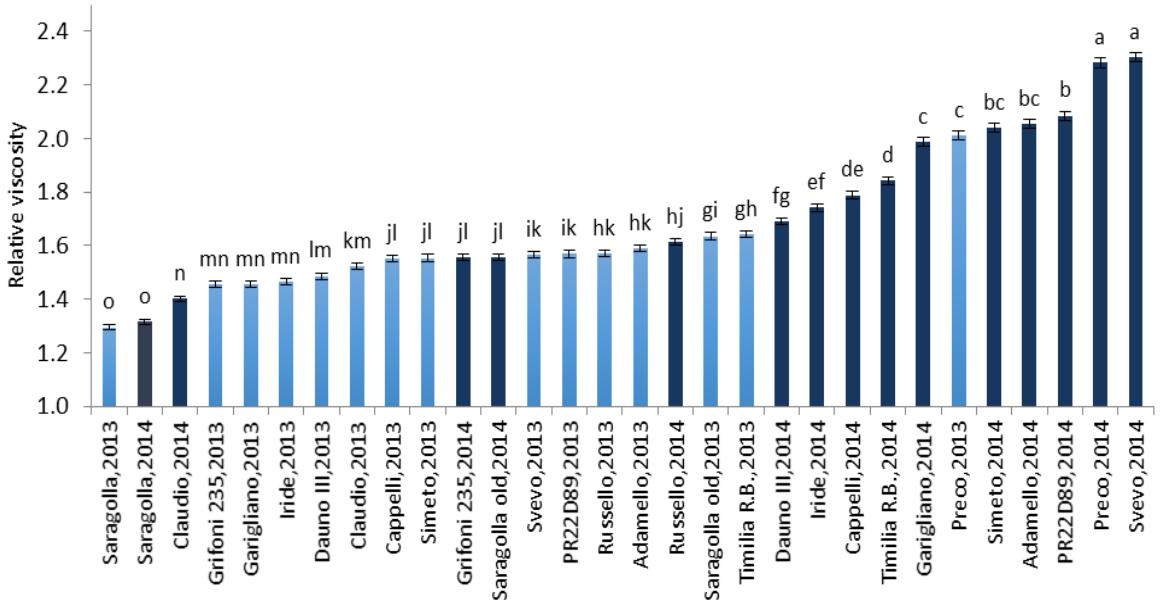


Figure 43 Relative viscosity of aqueous extract from old and modern durum wheat semolina. Different letters are significantly different at $P < 0.05$ according to Tukey's test

A large variability between the samples was found. Measures of relative viscosity ranged from 1.3 (Saragolla) to 2.3 (Preco and Svevo in 2014), as shown in Figure 43. Relative viscosity of aqueous extract, after removing protein fraction, is largely influenced by the content of soluble carbohydrates (arabinoxylan, β- glucan). The analysis of variance relative to the effect of the genotype and crop seasons showed high significant differences for genotype (G), crop season (year Y) and G x Y interaction (F significance < 0.001). Modern genotypes Preco, Svevo, PR22D89 and Adamello always showed the higher values, while in Saragolla, Claudio and Grifoni 235 a lower relative viscosity was measured. A high significant correlation was found between relative viscosity and WE-AX content in semolina (0.777, $P < 0.0001$). However, the content relative viscosity of a water extract will also be influenced by β- glucan content.

Comparison between crop seasons showed, in general, higher values in 2014 (1.82 vs 1.55, $p < 0.001$) and in particular for Garigliano, Adamello, Simeto, Svevo and PR22D89. A slight significant decrease in 2014 was observed for old Saragolla and Claudio. No differences between crop seasons were observed for Russello, Saragolla.

Again, in order to evaluate any effect of modern breeding upon relative viscosity of flour fractions, two groups of genotypes were considered: old (from 1900 to 1949) and modern (from 1985 to 2005). Analysis of variance (Tukey's test as post hoc) showed a higher statistically significant difference in relative viscosity in 2014 only in the modern group. Relative viscosity was higher in the 1985-2005 durum wheat group (1.73 vs 1.63, p 0.0304*), due to a higher response of the modern durum wheats in 2014, as shown in Figure 44. The higher relative viscosity in 2014 is in accordance with the higher %WE-AX observed in the same crop season.

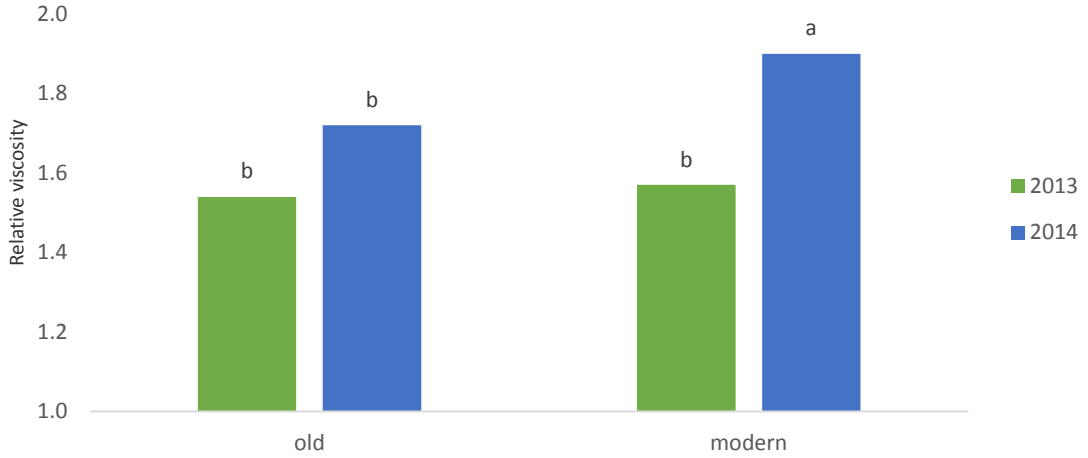


Figure 44 Effect of the interaction of the crop season and the age of the durum wheat groups for relative viscosity of semolina aqueous extract. Different letters are significantly different at $P < 0.05$ according to Tukey's test

3.3.3. Enzymatic fingerprinting

3.3.3.1.Arabinoxylan

Semolina and wholemeal flour were digested by enzymatic treatment (endo 1,4 β-xylanase (E.C.3.2.1.8), a xylanase of the GH11 group) for AX, endo 1,3(4) glucanase ('lichenase')(E.C.3.2.1.73) for MLG) and fragments were analysed by HP-AEC-PAD. Oligosaccharides (AXOS) released by enzyme digestion were grouped accordingly:4 unsubstituted (US: x, xx, xxx, xxxxx), 3 mono-substituted (MS: xa3xx, xa3a3xx, xa3xa3xx) and 3 di-substituted (DS: xa2+3xx, xa3a2+3xx, xa3xa2+3xx) (These peaks had previously been identified in the lab of Luc Saulnier (Ordaz-Ortiz *et al.*, 2005) and the method for enzymatic fingerprinting replicated at Rothamsted.)

Taking the sum of all the AXOS peak areas the total enzyme-extractable AX amount was analysed in old and modern durum wheat genotypes, grown in 2013 and 2014 crop seasons, either in semolina (Figure 45) or in wholemeal flour (Figure 46).

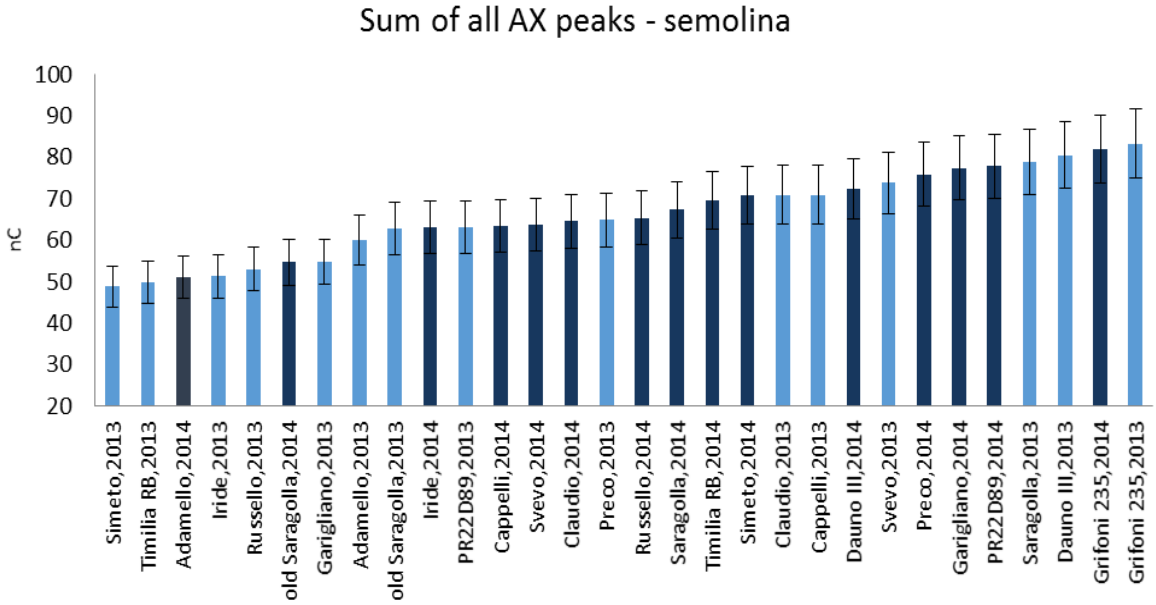


Figure 45 Sum of AX peak areas in semolina

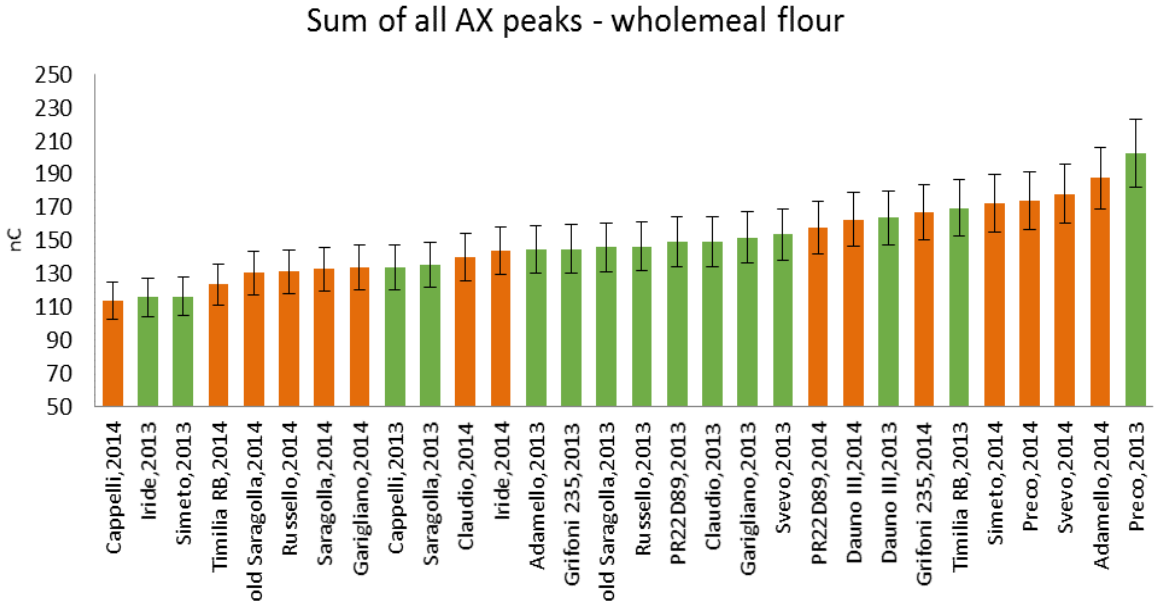


Figure 46 Sum of AX peak areas in wholemeal flour

As shown above, a variability within investigated genotypes, grown in two crop seasons, were found in both fractions. In semolina Adamello and old Saragolla showed the lower values in the two growing seasons, while Grifoni 235 and Dauno III the higher; in wholemeal lower

and higher values were found, respectively in Cappelli and Preco. No significant correlation with pentosan measures were found.

All peak areas were analysed for each sample. Table 18 illustrates the range of each AXOS are shown from semolina and wholemeal flour, and the means of the two crop seasons (2013 and 2014) in the old and modern durum wheat group are reported.

Table 18 Range of AXOS peak area and relative comparison of the old and modern group in two crop seasons. Different letters indicate significant differences at 0.05 p levels according to the Tukey's test

Semolina	min	max	old 2013	old 2014	modern 2013	modern 2014
x	16.1%	22.3%	19.2% a	19.2% a	18.3% a	19.3% a
xx	11.2%	19.8%	15.9% a	15.2% ab	14.0% b	14.7% ab
xxx	4.3%	8.5%	6.4% a	6.3% a	6.5% a	7.3% a
xxxxx	4.0%	18.0%	11.0% bc	9.0% c	13.9% a	12.6% ab
xa3xx	11.6%	17.1%	14.1% b	15.4% a	14.0% b	14.4% b
xa3a3xx	4.1%	9.1%	6.9% a	6.8% a	6.1% ab	5.4% b
xa3xa3xx	1.7%	3.1%	2.4% b	2.7% a	2.3% bc	2.2% c
xa2+3xx	14.0%	22.0%	16.9% ab	17.6% a	17.2% ab	16.1% b
xa3a2+3xx	3.7%	5.9%	4.3% b	4.8% a	4.5% ab	4.7% a
xa3xa2+3xx	2.5%	3.7%	2.8% c	3.0% b	3.2% a	3.3% a
Wholemeal	min	max	old 2013	old 2014	modern 2013	modern 2014
x	20.2%	28.5%	25.8% a	22.8% b	26.6% a	21.5% b
xx	18.2%	39.6%	20.7% c	34.9% b	21.9% c	37.3% a
xxx	6.5%	10.8%	9.0% a	7.8% b	8.8% a	8.0% b
xxxxx	2.0%	7.3%	5.2% a	4.0% b	5.2% a	4.3% b
xa3xx	10.5%	18.1%	16.4% a	12.6% c	15.5% b	11.6% d
xa3a3xx	1.5%	4.1%	3.7% a	3.0% b	3.0% b	2.3% c
xa3xa3xx	0.7%	1.7%	1.1% a	0.9% b	1.0% ab	1.0% ab
xa2+3xx	7.4%	13.2%	11.7% a	9.3% b	11.2% a	9.0% b
xa3a2+3xx	1.9%	3.2%	3.0% a	2.2% b	3.0% a	2.4%b
xa3xa2+3xx	2.3%	7.3%	3.4% a	2.5% b	3.9% a	2.7% b

Either in semolina or in wholemeal flour the most abundant AXOS were x, xx, xxxxx, xa3xx and xa2+3xx. For most of the AXOS peak areas, higher values were obtained in the wholemeal fraction as AXOS were released from the outer layers (bran); this trend was not confirmed for xxxxx (x_5), xa3a3xx and xa3xa3xx, which showed a comparable content both in semolina and in wholemeal, suggesting a large deposition in the endosperm fraction.

Strikingly high %x, %xx were observed in the wholemeal flour compared to semolina, which indicates these oligosaccharides concentrated in the brans layers.

As for the un-substituted AX, %x (the most abundant AXOS) no significant differences were found between old and modern group in the two crop seasons in semolina, while a significant higher content was found in 2013 in wholemeal. The %xx was not significantly affected by the effect of the crop season, a significant higher content was found in the old group in semolina only in 2013; in wholemeal the effect of the crop season was highly significant, with higher content in 2014. Relative to %xxx, no significant differences were found in semolina, while significant higher values were found in 2013 in wholemeal. The %xxxxx (x5) peak was much more abundant in semolina than wholemeal; in semolina either in 2013 or in 2014 group significantly higher values were observed in the modern group; in wholemeal the effect of the crop season was significant, with higher values in 2013.

Differences in the substituted AXOS content were also found within samples. Mono-substituted xa3xx was found, in semolina, significantly higher in 2014 only in the old durum wheat group; in wholemeal %xa3xx content was found significantly lower in the modern group, and significantly lower in 2014, in both genetic groups. Significant differences in %xa3a3xx were found only in 2014, in semolina, with a lower content in the modern group; in wholemeal significant higher relative content was found in the old group (in both crop seasons) and in 2013 rather than 2014. The xa3xa3xx digested AX fragment showed the lowest content both in semolina and in wholemeal. In semolina, a significant higher relative content was found in 2014 only in the old group; in the same crop seasons relative content was significantly lower in the modern group. In wholemeal flour a very low abundance was observed (0.7-1.7%), and a significant higher content was found only in the old group in 2013.

Three peaks presented a di-substitution in the xylose backbone: xa2+3xx, xa3a2+3xx, and xa3xa2+3xx. %xa2+3xx resulted the higher of them in both mill fractions, in particular in semolina. No statistically significant effect of the crop season was observed in semolina, and a significant higher content was found in the old group only in 2013. In wholemeal flour the effect of the crop season was significant with a general higher content in 2013. No significant differences were found, in semolina, for the %xa3a2+3xx, except for the modern group with a significant higher values in 2014; in wholemeal flour significant relative higher values were observed in 2013 in old and modern groups. Finally, the xa3xa2+3xx was found, in semolina, significantly higher in the modern group (both crop seasons) and significantly lower content was found only in 2013 crop season only in the old group. In wholemeal no significant

differences were found between old and modern group, while a statistically significant higher content was found in 2013 in both groups.

To better describe phenotypic differences observed in old and modern durum wheat genotypes in terms of AX composition, % US was analyzed in semolina (Figure 47) and wholemeal (Figure 48).

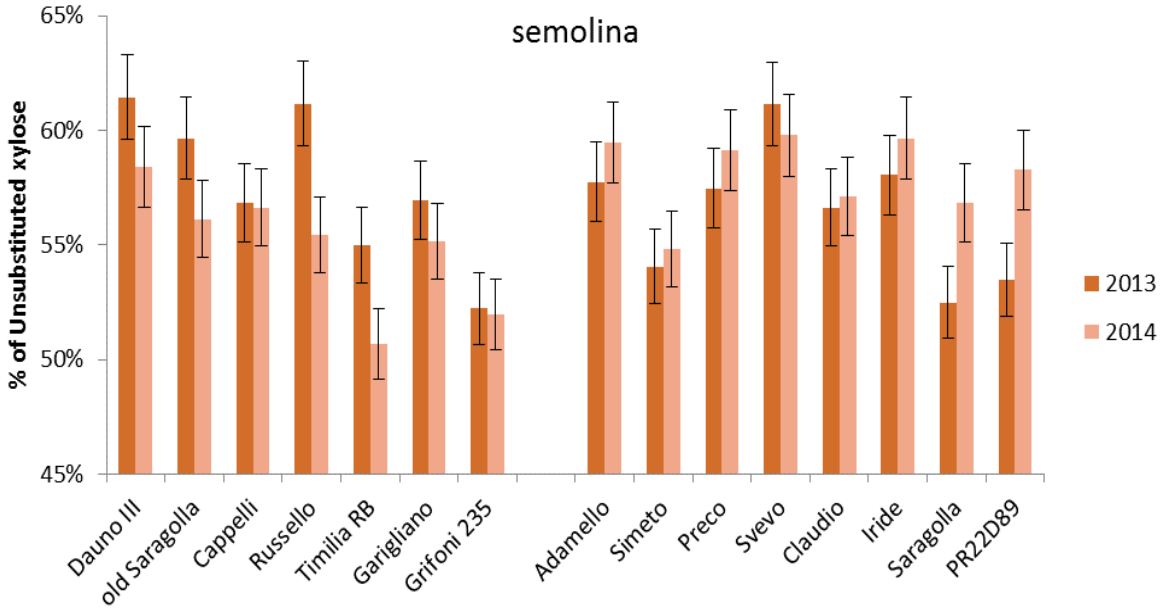


Figure 47 Relative content of Unsubstituted AXOS in semolina

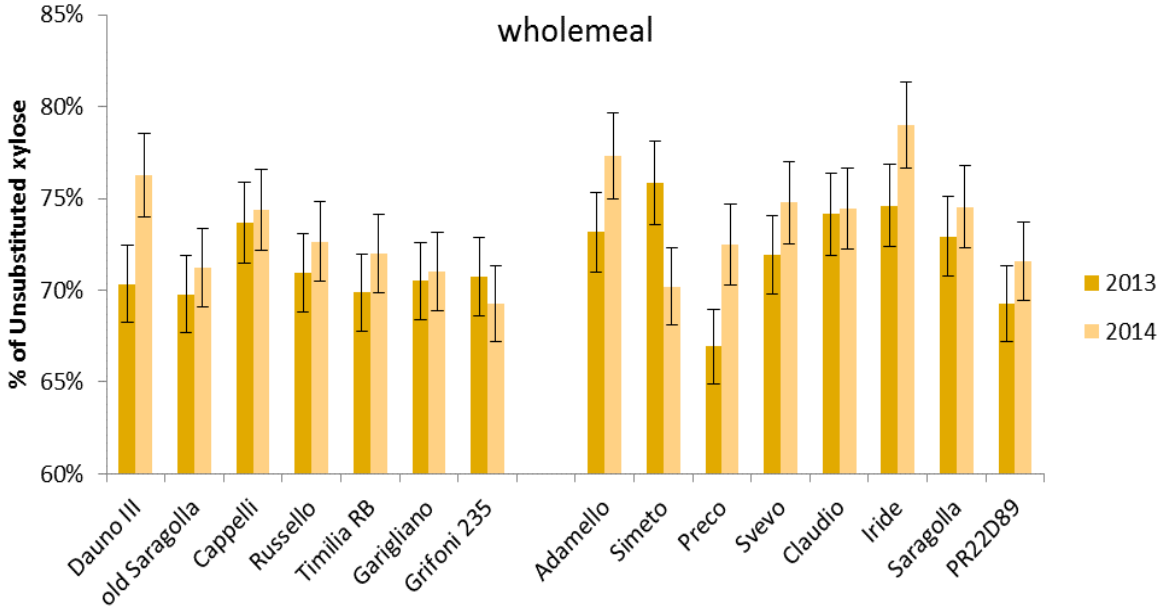


Figure 48 Relative content of Unsubstituted AXOS in wholemeal flour

Degree of arabinose substitution was measured in semolina and wholemeal flour. Two way analysis of variance (ANOVA) showed significant differences in G x Y interaction in both

mill fractions (P value < 0.05). In semolina, as showed in Figure 47, %US within the observed durum wheat genotypes ranged from about 51 % (Timilia RB, 2014) to 62% (Dauno III, 2013). In terms of means fortwo crop seasons, cultivar Grifoni 235 showed the lowest degree of substitution (52%) while modern cultivar Svevo showed the highest (61%). The effect of the crop season was statistically significant for old landraces, old Saragolla, Russello and Timilia RB with a higher %US in 2013 (associated to a lower rainfall during early grain development). In contrast, the modern cultivars Saragolla and PR22D89 showed a significant increase in % un-substituted xylose in the 2014 season. In wholemeal flour the %US was generally higher than in semolina (Figure 48). Values ranged from about 67% (Preco, 2013) to 78% (Iride, 2014). Genetic differences and differential environmental responses were observed. Cultivar Preco showed the lowest %US (70%) and Iride had the highest level of unsubstituted xylose (77%). T test between two crop seasons showed a significant general lower expression in 2013 (71.6% vs 73.4%, P < 0.05). This trend resulted in a statistically significant difference only for landrace Dauno III and for modern cultivars Preco.

3.3.3.2.Mixed-linkage β- glucan

Two main peaks obtained from enzymatic digestion of MLG by lichenase were obtained G3 and G4 which is a measure of oligosaccharides of 3 glucose residues linked together (β-1,4) or 4 glucose residues linked together (β-1,4). As for the total MLG (sum of G3 and G4 peak areas) genetic and environmental differences were found in semolina (Figure 49) and wholemeal flour (Figure 50).

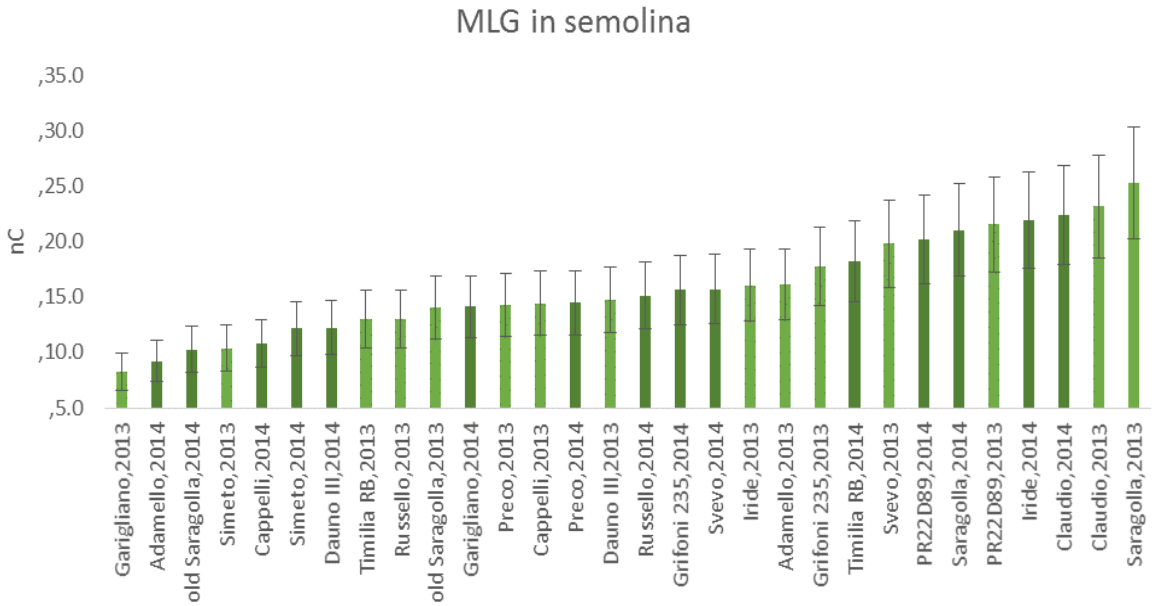


Figure 49 Sum of MLG peak area in semolina

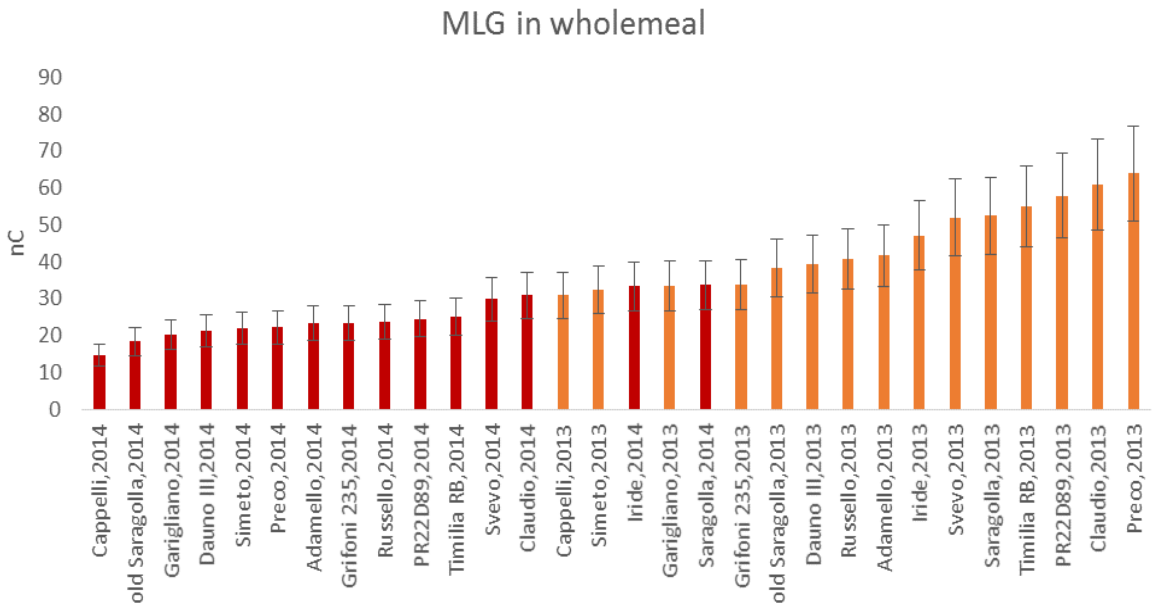


Figure 50 Sum of MLG peak area in wholemeal flour

A large variability of β- glucan content was observed within old and modern genotypes. Garigliano and Simeto, with very low levels of MLG, and Saragolla and Claudio the highest MLG content in semolina; in particular, Claudio and Saragolla showed a MLG content about 2 times higher than Garigliano and Simeto. Analysis of the variance (ANOVA) showed that the effect of the crop season was significant only for cultivar Adamello, with a higher content in 2013. In wholemeal flour the overall MLG content was about 2 times greater than semolina. Genetic diversity was found; in particular Cappelli, Simeto and Garigliano showed the lowest content, while Sarogolla, Preco, Claudio and PR22D89 the highest (about two times higher). Furthermore, the overall MLG content in wholemeal was highly statistically significant different (higher) in 2013 (45.3 vs 24.4 nC, $P < 0.001$) for all investigated genotypes than in the 2014 crop season.

G3 : G4 peak area ratio.

In semolina (Figure 51) the analysis of the variance showed a significant effect of the interaction of genotype x crop seasons (G x Y), of the genotype and of the crop season. G3 : G4 ratio ranged from 1.8 (Grifoni 235, 2013) to 3.8 (Adamello, 2014). Cappelli and Grifoni 235 showed the lowest ratio, while Adamello had the highest ratio, in both crop seasons. The effect of the crop season resulted in a generally significant higher ratio in 2014 (2.84^{a} vs 2.17^{b}, $P < 0.001$), in almost all genotypes, except Cappelli, Russello, Garigliano. In wholemeal flour (Figure 52) generally the ratio of G3 : G4 was higher than for semolina, and ranged from 2.3 (Grifoni 235, 2013) to 5.7 (Adamello, 2014). Overall, the effect of the crop season (environment) was significant; with an increase in 2014 (3.6^{a} vs 2.5^{b}, $P < 0.05$), in particular

for modern cultivars Adamello, Simeto, Preco and PR22D89 compared to 2013. However, for the cultivars Russello, Iride and Saragolla this was not the case.

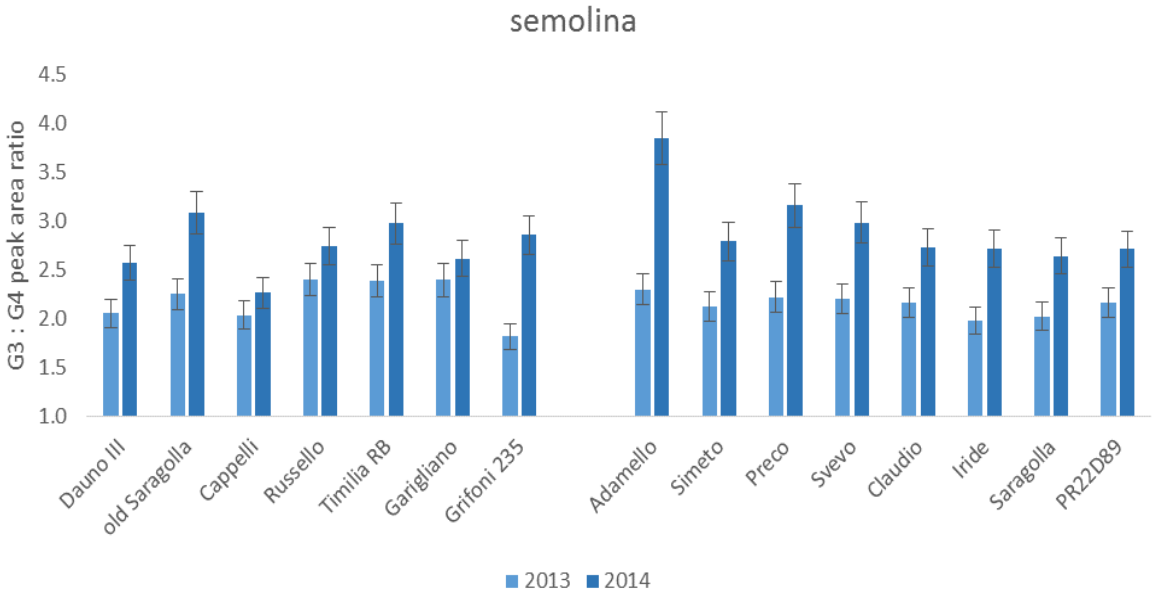


Figure 51 Peak area ratio of G3 and G4 glucans in semolina

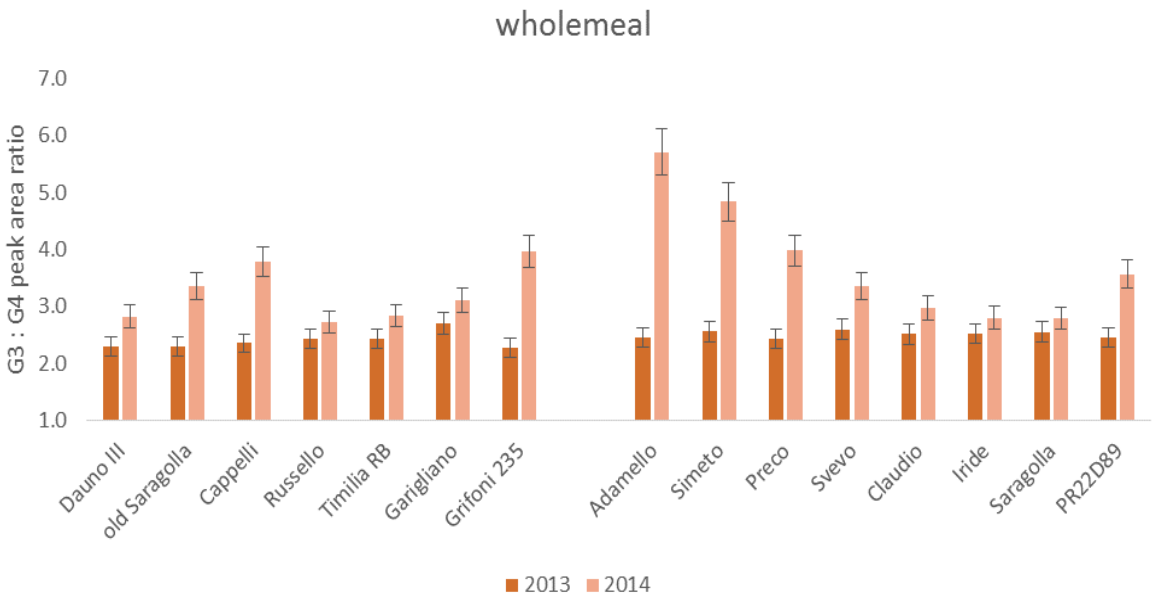


Figure 52 Peak area ratio of G3 and G4 glucans in wholemeal flour

As for AX, the effect of the breeding was evaluated in terms of MLG content and composition, by comparing old and modern groups in the two crop seasons, as shown in Table 19.

Briefly, β- glucans content exclusively from the endosperm (semolina flour) show a different response than those from wholemeal flour. In semolina total MLGs (G3 and G4) were (statistically significant) more abundant in the modern group of genotypes. Crop seasons did not affect the total amount, but the composition, with a higher %G3 and a reduction of the G4 fraction. In wholemeal flour the effect of the crop seasons was predominant, with a significant increase in 2013, when a slight water deficit occurred (during anthesis and grain development). However the G3 : G4 ratio drastically changed between the two crop seasons, with a marked increase in 2014. Modern group of varieties also showed a significantly higher β- glucans content in 2013, while in better rainfall conditions no significant differences were found. The G3 : G4 ratio was significantly lower only in 2014. Bran (grain outer layers) MLG content and composition were estimated by subtracting the peak areas of semolina from wholemeal flour. In bran a significant reduction of the MLG content occurred in 2014, with a significant increase of the G3 : G4 ratio. This might result from the reduction of the G3 fractions in the 2014 season.

Table 19 Mixed linkage beta glucan content and composition in old and modern durum wheat genotypes, in two crop seasons. Different letters indicate significant differences at 0.05 p levels according to the Tukey's test

		semolina		wholemeal		bran	
		MLG	G3:G4	MLG	G3:G4	MLG	G3:G4
Old	2013	13.6 b	2.19 b	38.8 b	2.40 c	25.2 a	2.54 b
Old	2014	13.8 b	2.8 a	20.9 c	3.23 b	7.1 b	4.26 a
Modern	2013	18.3 a	2.15 b	51.0 a	2.51 c	32.7 a	2.75 b
Modern	2014	18.8 a	2.95 a	27.4 c	3.74 a	8.6 b	4.97 a

3.3.4. PACE

Digested AX and MLG fragments were separated by Polysaccharide analysis using carbohydrate gel electrophoresis (PACE). Replicates from wholemeal and semolina samples were performed. Differences in bands expression were observed between genotypes, as in Figure 53. Bands correspond to AXOS, G3 and G4 peaks, as analysed in HPAEC and previously described.

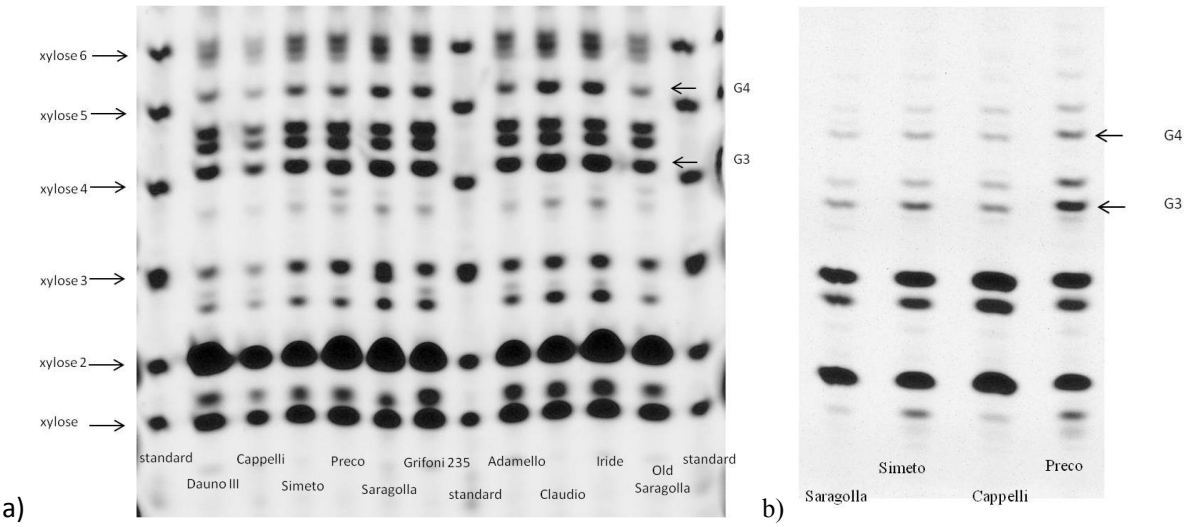


Figure 53 PACE gel from digested semolina AX and MLG (a), and focus on PACE with samples digested only by lichenase

The most obvious differences observed were in G3 and G4 β- glucans, as shown in Figure 53a. The proportions of MLG fractions are in good agreement with peak area data measured by HPAEC (G3 > G4). The detail in Figure 53b showed the highest expression of G3 and G4 bands in cultivar Preco (2013).

3.3.5. Analysis of monosaccharides

Aliquots of WE-AX, obtained by the protocol for pentosan quantification, were collected, hydrolysed and analysed by HPLC to determine total monosaccharide content, glucose, arabinose, xylose, mannose and galactose. Glucose was the most abundant sugar in semolina water extract, as shown in Figure 54 and 56. Glucose concentration ranged from 13 (Timilia RB, 2013) to 27 µg/mg (Svevo, 2014). Genetic and environmental differences were found. Cultivar Claudio and Grifoni 235 showed relative low levels in both crop seasons, while in Preco glucose concentration was always higher. A significant overall higher concentration was found in the 2014, especially in Simeto, Preco, Svevo and Iride. Glucose levels were 10 times higher than other saccharides, mostly presumably from starch in water extract.

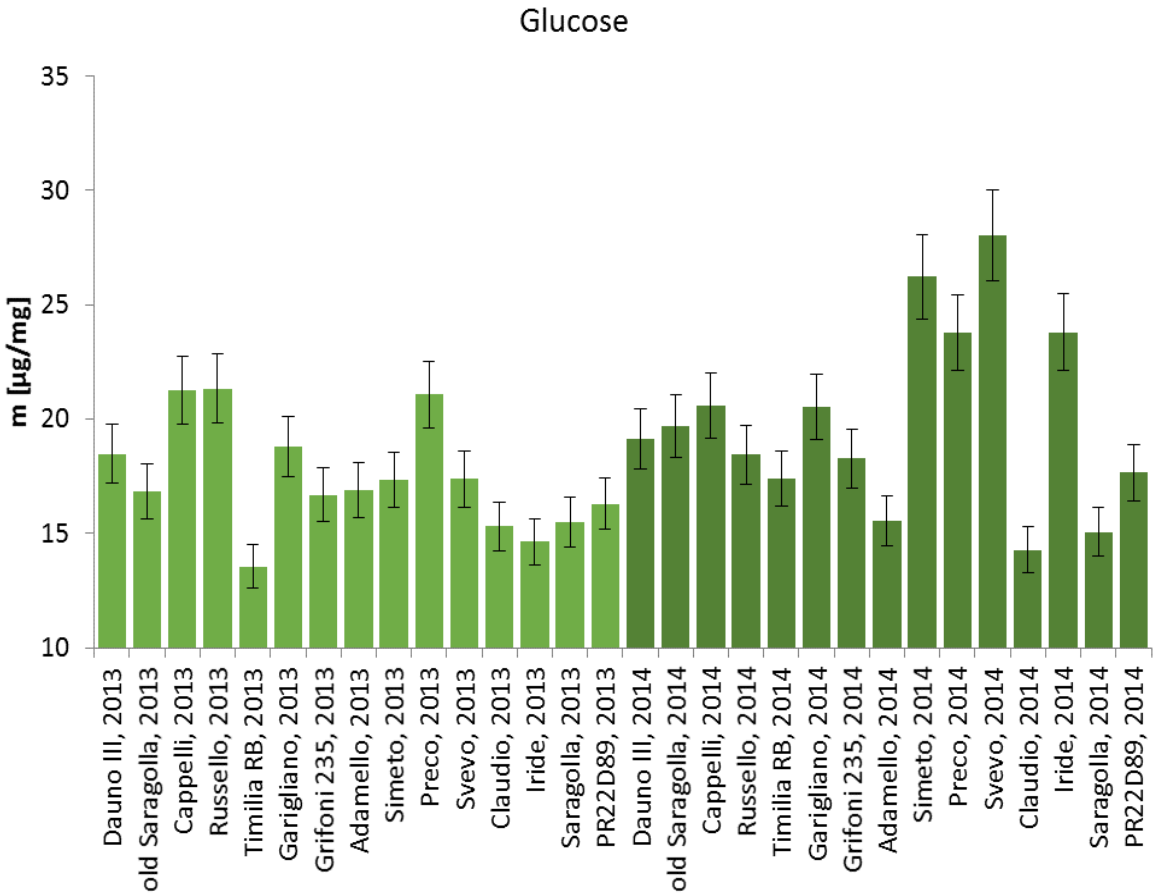


Figure 54 Glucose concentration in semolina water extract of old and modern genotypes in 2013 (former 15 samples) and 2014 (latter 15 samples)

Not significant differences were found between old and modern group (18.65 vs 18.68, T test, ns), but a different response to the crop season, as shown in Figure 55. In particular old group showed a higher stability in glucose concentration, while a significantly higher increase in 2014 occurred in the modern group.

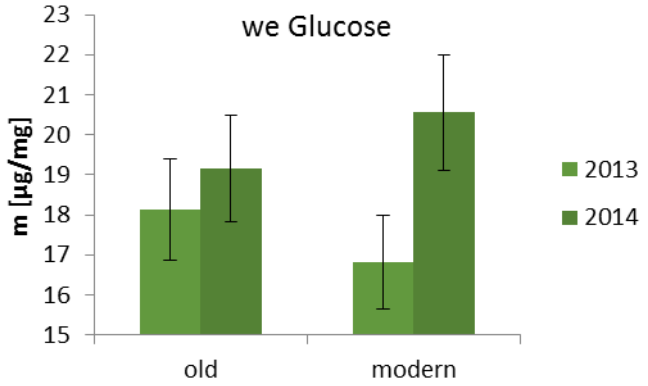


Figure 55 Glucose concentration in semolina water extract. Comparison between old and modern group in two crop seasons

Concentration of two hexose, Galactose and Mannose, was also determined. Those sugars averaged, respectively, 10% and 1% of the concentration of glucose in WE. No significant differences between crop seasons were found.

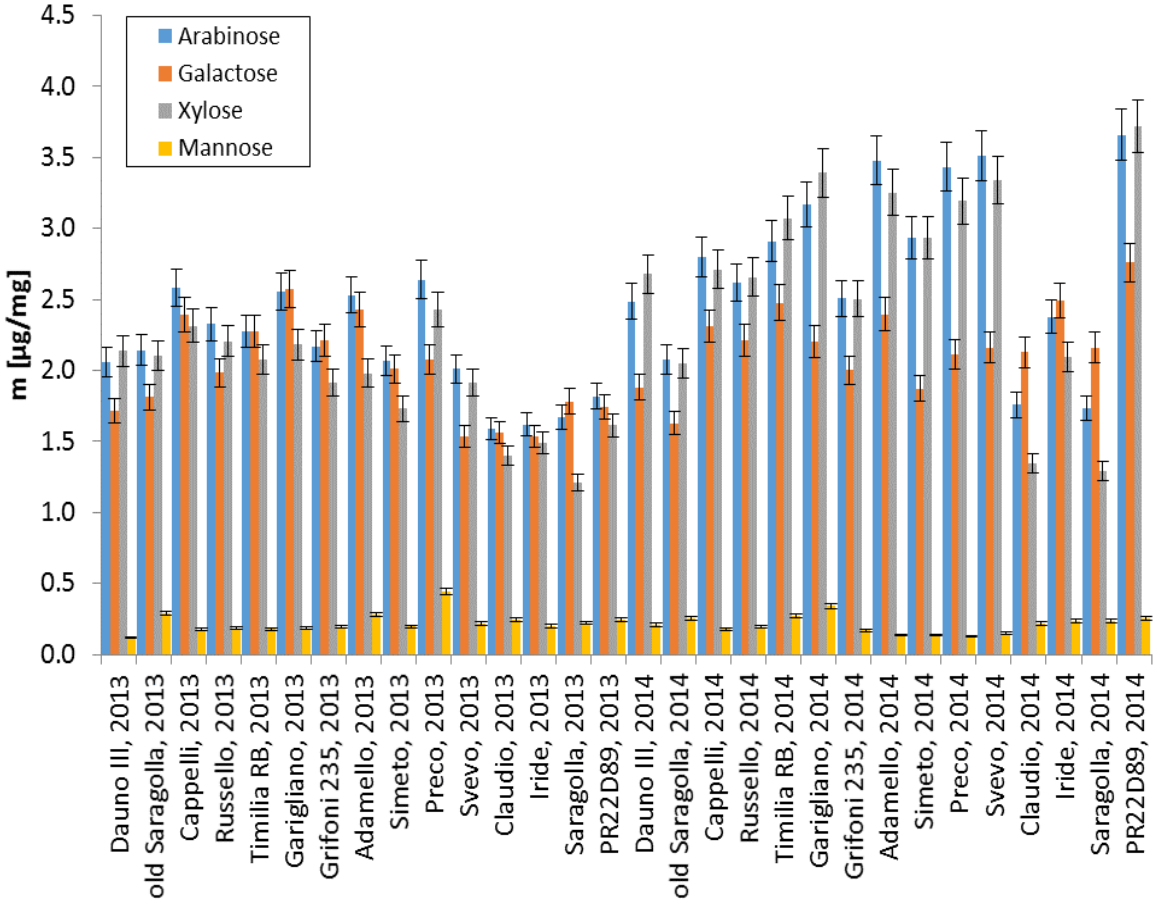


Figure 56 Arabinose, Galactose, Xylose and Mannose concentration in semolina water extract in 2013 (former 15 samples) and 2014 (latter 15 samples)

Pentose sugars, Xylose and Arabinose concentration ranged from 1.2 to 3.6 µg/mg (Figure 56). As for glucose, a significant increase in the amount arabinose and xylose was found in 2014. Differences in the proportion between pentose, expressed as arabinose / xylose ratio (A : X) were observed, as reported in Figure 57. A lower A : X ratio trend was observed in 2013 (1.04 vs 1.12, T test : $P < 0.05$). Genetic differences were found. Dauno showed the lowest ratio (0.94) and in general, old landraces, while the highest ratio was achieved by modern cultivar Saragolla (1.36), in both crop seasons.

Comparison between old and modern is shown in Figure 58. In particular A:X ratio was significantly higher in the modern group (1985-2005). Also, the reduction observed in 2013 was statistically significantly different only in the old group (1900-1949).

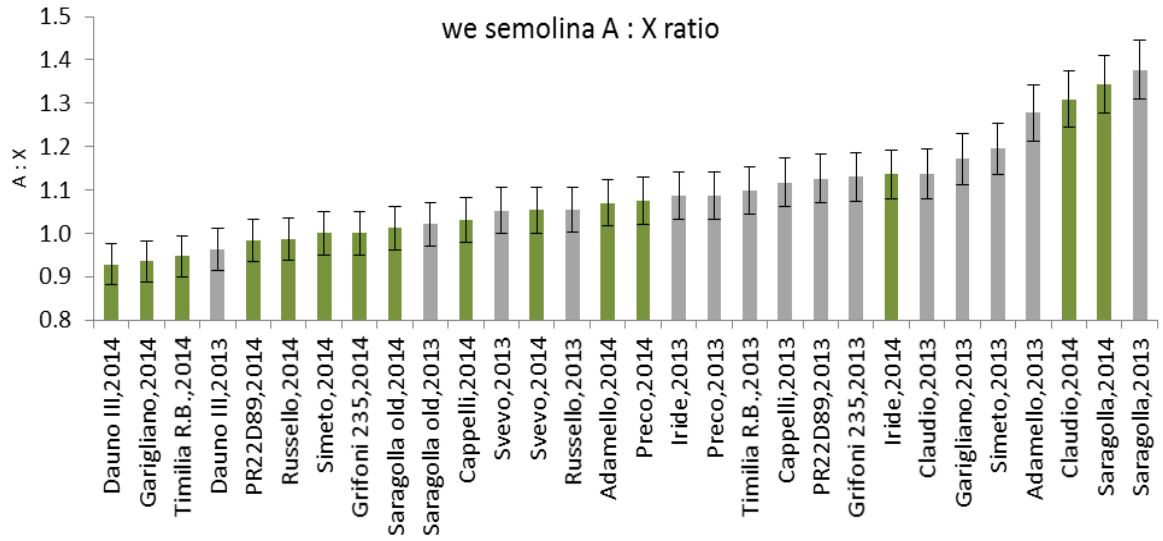


Figure 57 A:X ratio from semolina water extract

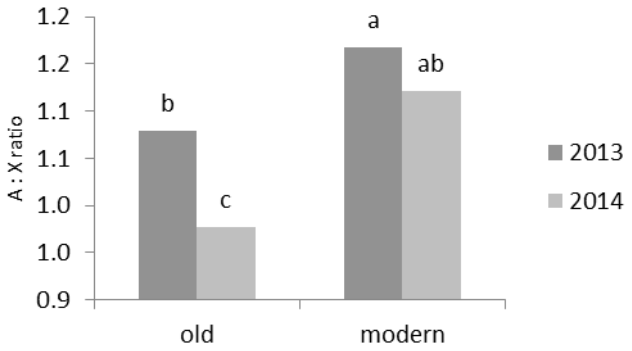


Figure 58 Effect of the interaction of old and modern group of genotypes with crop seasons on arabinose / xylose ratio in semolina water extract

Also, the ratio between pentose (arabinose + xylose) on the glucose in semolina water extract was considered to estimate the contribution of the AX in relation to sugars in solution. As shown in Figure 59, no significant differences were found between old and modern, while the effect of the crop season resulted in a higher ratio in 2014, significant only in for the modern group.

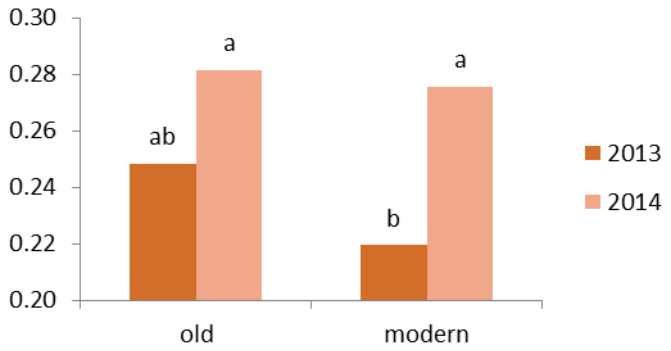


Figure 59 Effect of the interaction of old and modern group of genotypes with crop seasons on AX : glucose ratio in semolina water extract

3.3.6. *Evaluation of specific viscosity of semolina water extract by Size Exclusion Chromatography*

Semolina water extracts, previously analysed on the basis of the relative viscosity, were collected and further analysed by HPSEC-MALS high performance size exclusion chromatography with multi-angle laser detection. On the basis of the observed relative viscosity data four samples were selected for analysis, the two with the highest and the two with the lowest relative viscosity values; (Figure 8), the top 2 (Svevo and Preco, 2014) and bottom 2 samples (Claudio and Saragolla, 2014) was measured by HPSEC-MALS, as shown in Figure 60. Intrinsic viscosity of semolina WE ranged from 0.0015 (Saragolla, 2014) to 0.13 (Svevo, 2014), confirming the results of the relative viscosity (Svevo > Preco >> Claudio > Saragolla).

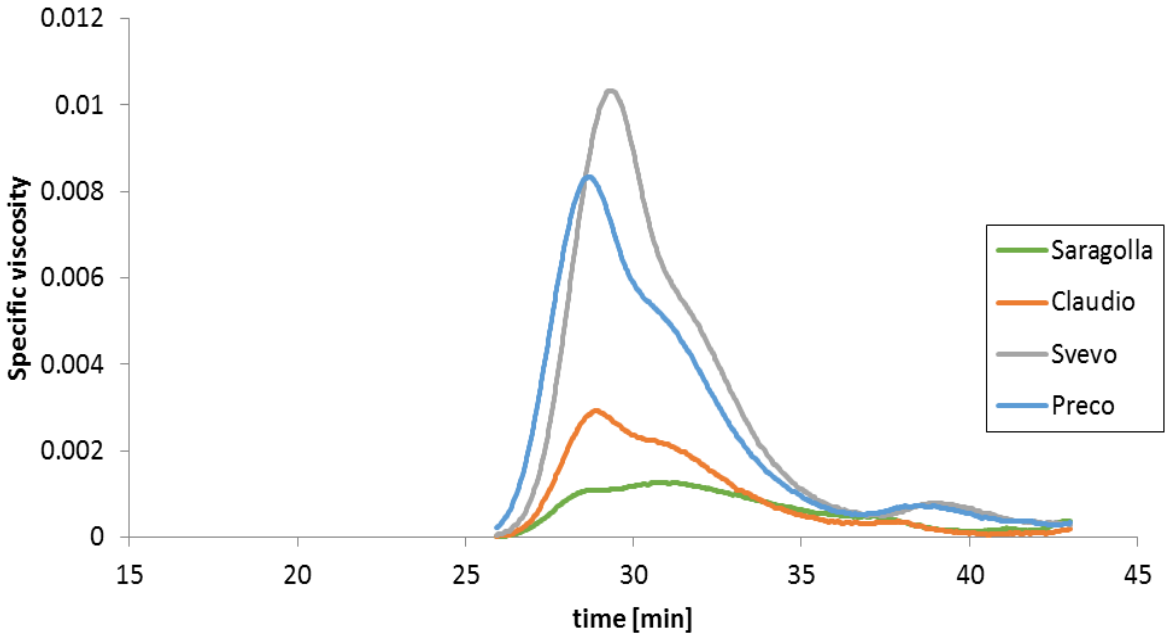


Figure 60 Specific viscosity and indirect AX and MLG backbone length of two modern and two old genotypes by SEC

To better evaluate the contribution of AX and MLG backbone, comparative enzymatic digestion trials were carried out on the sample with the lowest (Saragolla) and the highest (Svevo) specific viscosity.

The experiment consisted on:

digestion by endo 1,4 β-xylanase (E.C.3.2.1.8) xylanase - GH11 group for AX;

digestion by endo 1,3(4) glucanase ('lichenase')(E.C.3.2.1.73) for MLG;

digestion by both enzymes (xylanase GH11 and lichenase);

undigested blank.

The effect of the digestion is shown in Figure 61 for Svevo (high specific viscosity) and in Figure 62 for Saragolla (low specific viscosity).

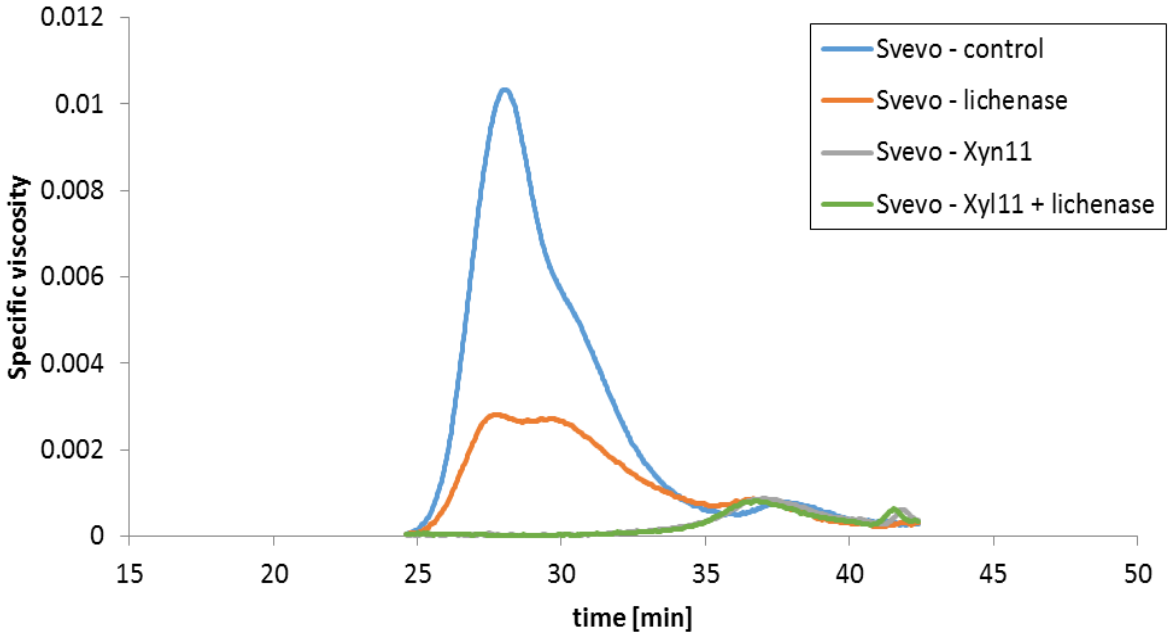


Figure 61 Specific viscosity of the genotype with the highest relative viscosity with different combination of enzymatic digestion and analysed by HPSEC-MALS

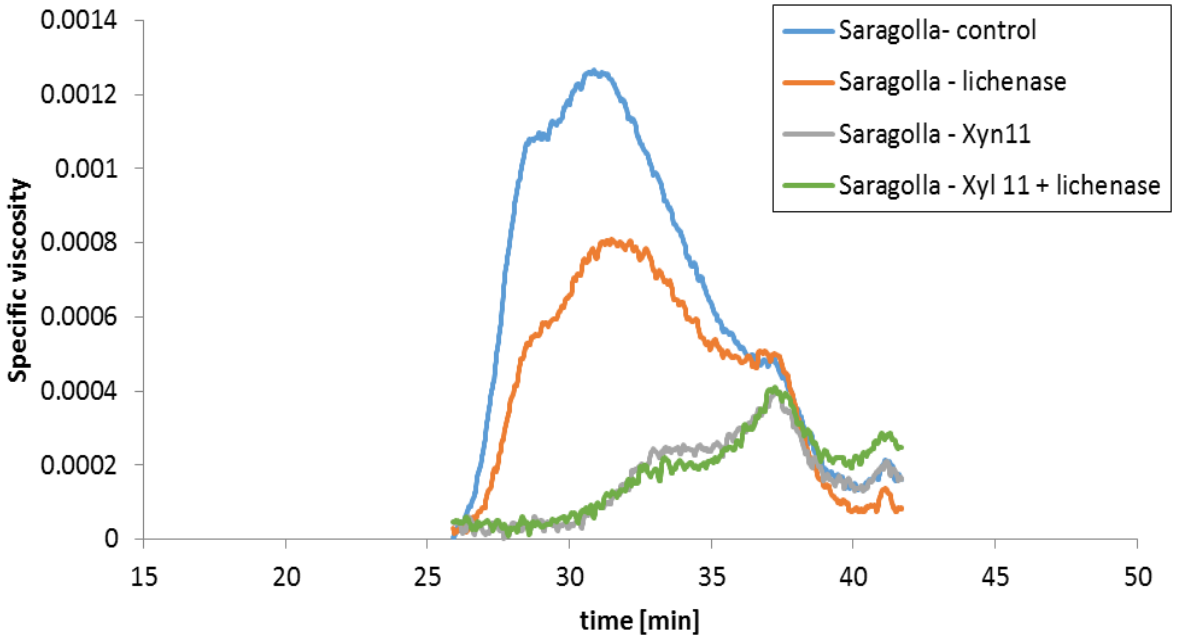


Figure 62 Specific viscosity of the genotype with the lowest relative viscosity with different combination of enzymatic digestion and analysed by HPSEC-MALS

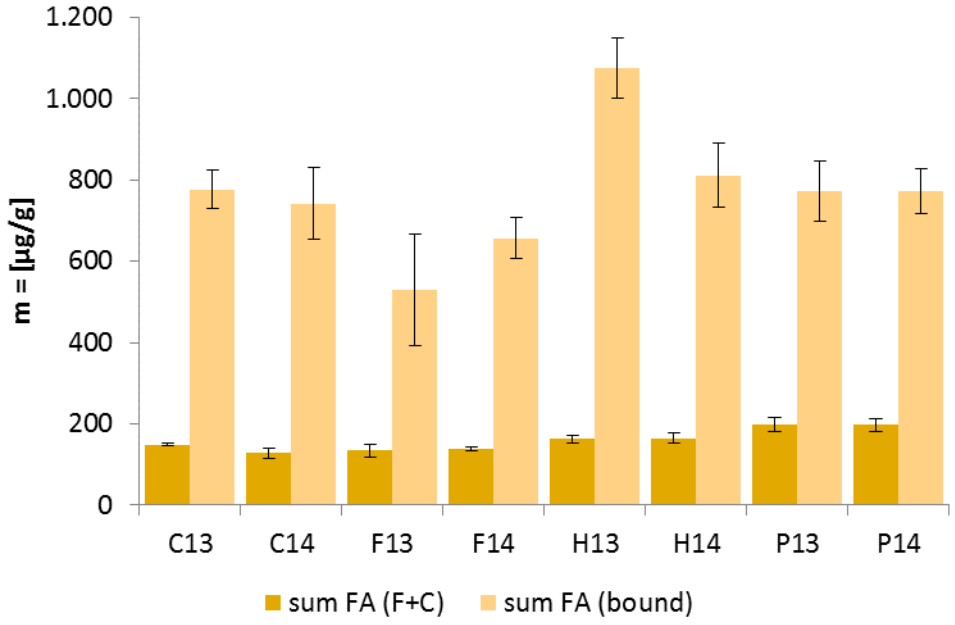
In both cultivars, a drastic reduction of the specific viscosity was observed after xylanase digestion.

Xylanase digestion had a more dramatic effect in cultivar Svevo, compared to Saragolla, The reduction in viscosity after lichenase digestion was less than after xylanase digestion. Which reflects the greater quantity of AX compared to MLG in flour and hence in water extracts of flour contributing to relative viscosity. In addition, specific viscosity after lichenase digestion was higher in cultivar Svevo, even if decrease in Saragolla (-38%) was proportionally lower than Svevo (-73%).

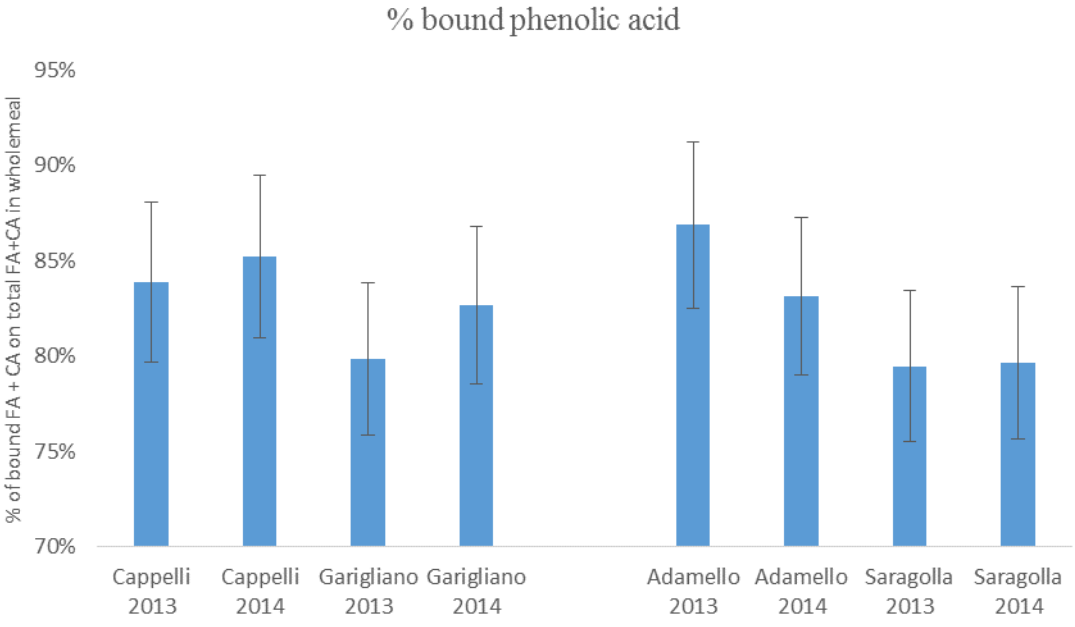
3.3.7. Phenolic compound

Ferulic and coumaric acid content.

Phenolic acids content was measured by HPLC in wholemeal and semolina of two old ("C" as Cappelli, "F" as Garigliano) and two modern genotypes ("H" as Adamello, "P" as Saragolla), grown in two crop seasons (2013, 2014). Genotypes were selected on the basis of the water unextractable (WU) AX content. The two most abundant classes (in wheat) of phenolic acid compounds were investigated: ferulic acid (FA) and coumaric acid (CA). Both phenolic compounds were present in a free + conjugated and bound form.



a)



b)

Figure 63 Ferulic acid content in two modern genotypes in free and bound form in wholemeal flour (a) and relative bound (%) phenolic acid content in wholemeal flour (b)

In Figure 63a, free and bound FA content was measured in wholemeal flour. Bound form (B) was higher than free and conjugated (F+C) in all samples. The relative bound FA (Figure 63b) content ranged from 79.7% (Garigliano, 2014) to 86.7% (Adamello, 2013). No significant differences were found between 2013 and 2014, except for bound FA in Adamello that resulted significantly higher in 2013.

CA content resulted in about 1% of the total phenolic acids observed (FA+CA), as shown in Figure 64. The relative CA bound content ranged from 61.2% (Saragolla, 2014) to 91.1% (Adamello, 2013 and 2014). As for FA, the significant highest bound CA content was measured in Adamello in 2013.

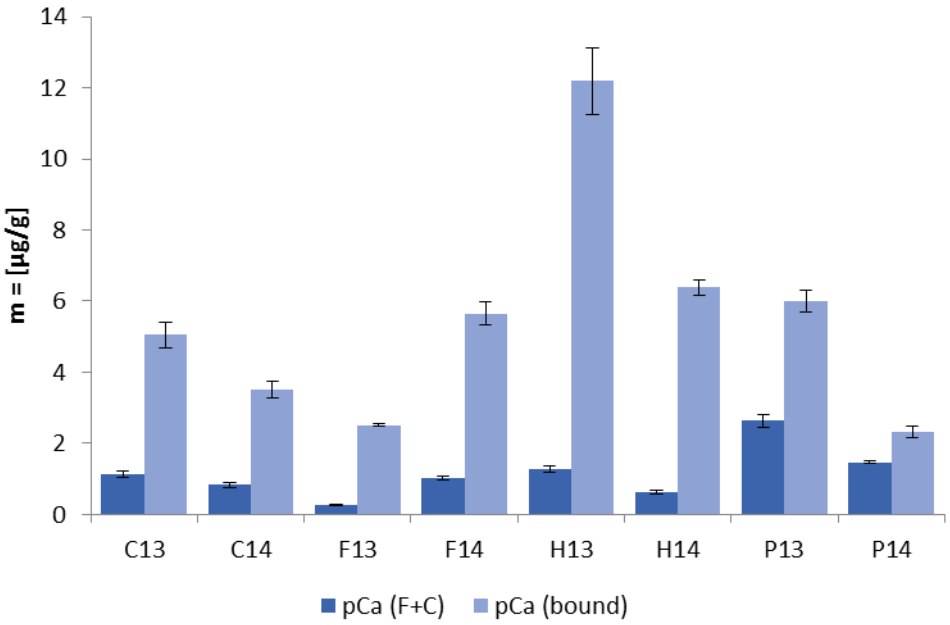


Figure 64 Coumaric acid content in wholemeal flour in two crop seasons

3.3.8. Analysis of the correlation of DF parameters

Phenotypic data on DF content and composition were correlated with the year of release of the genotypes, the kernel test weight and the relative viscosity from semolina aqueous extract (Table 20). Statistical analysis of the correlation (Pearson) was focused on semolina flour. Briefly, the year of release showed a positive significant correlation with the %xxxxx, %xa3a2+3xx, %xa3xa2+3xx, MLG : AX and we A : X, and a significant negative correlation with the %xx, %xa3a3xx, %xa3xa3xx, and M : D ratio.

Test weight, an important parameter of kernel quality and generally environmental affected, showed a positive significant correlation with %xxxxx, %xa3a3xx, M : D semolina, MLG : AX and we A : X ratio (monosaccharide), and a significant negative correlation with WE-AX,

%WE-AX, relative viscosity, %xa3a2+3xx, %xa3xa2+3xx, G3 : G4 (***), we A + X and we (A+X) : Glucose ratio (monosaccharide).

Relative viscosity showed a positive significant correlation with WE-AX, %WE-AX, %x, %xxx, G3 : G4 ratio, we A + X and we (A+X) : Glucose (monosaccharide) and a significant negative correlation with test weight, %xa3a3xx, %xa3xa3xx, MLG : AX and we A : X ratio (monosaccharide).

Table 20 Matrix of the correlation between year of release, test weight and relative viscosity of DF parameters

	Year of release	**P level**	**Test weight**	**P level**	**Relative viscosity**	**P level**
Test weight	-0.018	ns	1.000	-	-0.726	***
Tot-AX	0.046	ns	-0.336	*	0.271	*
WE-AX	-0.086	ns	-0.759	***	0.777	***
%WE-AX	-0.167	ns	-0.679	**	0.716	***
Relative viscosity	0.145	ns	-0.726	***	1.000	-
%x	-0.204	ns	-0.360	*	0.226	*
%xx	-0.356	*	-0.142	ns	0.113	ns
%xxx	0.030	ns	-0.162	ns	0.469	**
%xxxxx	0.603	**	0.255	*	-0.171	ns
%xa3a3xx	-0.413	*	0.455	*	-0.336	*
%xa3xa3xx	-0.510	**	0.153	ns	-0.302	*
%xa3a2+3xx	0.240	*	-0.336	*	0.062	ns
%xa3xa2+3xx	0.642	**	-0.215	*	0.103	ns
% U	0.041	ns	-0.168	ns	0.205	ns
G3 : G4	0.045	ns	-0.745	***	0.565	**
MLG : AX	0.580	**	0.565	**	-0.322	*
we A + X	-0.129	ns	-0.765	***	0.878	***
we A : X	0.537	**	0.390	*	-0.504	**
we (A+X) : Glucose	-0.153	ns	-0.533	**	0.541	**

The analysis of the correlations helped to explain the observed variability in DF. The year of the release is a key parameter to estimate genetic diversity by comparing old and modern genotypes. According to the results of the correlation a genetic diversity seems to be confirmed in terms of AX composition and with a higher proportion of total β- glucans on total AX. An interesting result is in the negative correlation of the test weight and AX content (in particular water extractable fraction) and its influence on the relative viscosity and the proportion of G3 : G4 β- glucans fractions. A high negative correlation of the relative viscosity and the %WE-AX confirm the important role of AX to determine viscosity

properties and gastrointestinal implications. The role of AX, in durum wheat, seems higher than MLG and a low arabinose : xylose ratio of the semolina water extract resulted to be statistically significant influent on viscosity.

3.3.9. PCA of DF composition

The analysis on the principal components (PCA), on semolina flour, was performed according to the best results obtained by analysis of the correlations, as shown in Figure 65. The first two factors individuated by PCA explained respectively 42% and 28% (Table 21) of the observed variability for AX and β- glucan composition in semolina in old and modern durum wheat genotypes. Component 1 showed a positive correlation with the relative viscosity, the G3 : G4 glucans ratio and the %WE-AX, and a high negative correlation with the test weight (0.88) and negative with arabinose : xylose ratio in water extract (we A : X), the mixed-linkage β- glucan on arabino-xylan ratio (MLG : AX) and %xa3a3xx, and may be considered the "environmental response". Component 2 showed a high positive correlation with the year of release (0.95) and with %xa3xa2+3, "*we A : X*" and semolina MLG : AX, and negative with M : D arabino-xylan ratio, and may be considered "effect of the breeding".

Table 21 Varimax rotated factor matrix

	Factor 1	**Factor 2**
Year of release	-0.022	0.923
Test weight	-0.928	-0.074
Sem WE-AX%	0.822	-0.221
Relative viscosity	0.852	0.060
G3 : G4	0.780	0.143
MLG : AX	-0.569	0.532
we A : X	-0.562	0.633
%xa3xa2+3xx	0.154	0.826
%xa3a3xx	-0.481	-0.510
Eigenvalue	3.820	2.504
Cumulate %	42%	70%
%	42%	28%

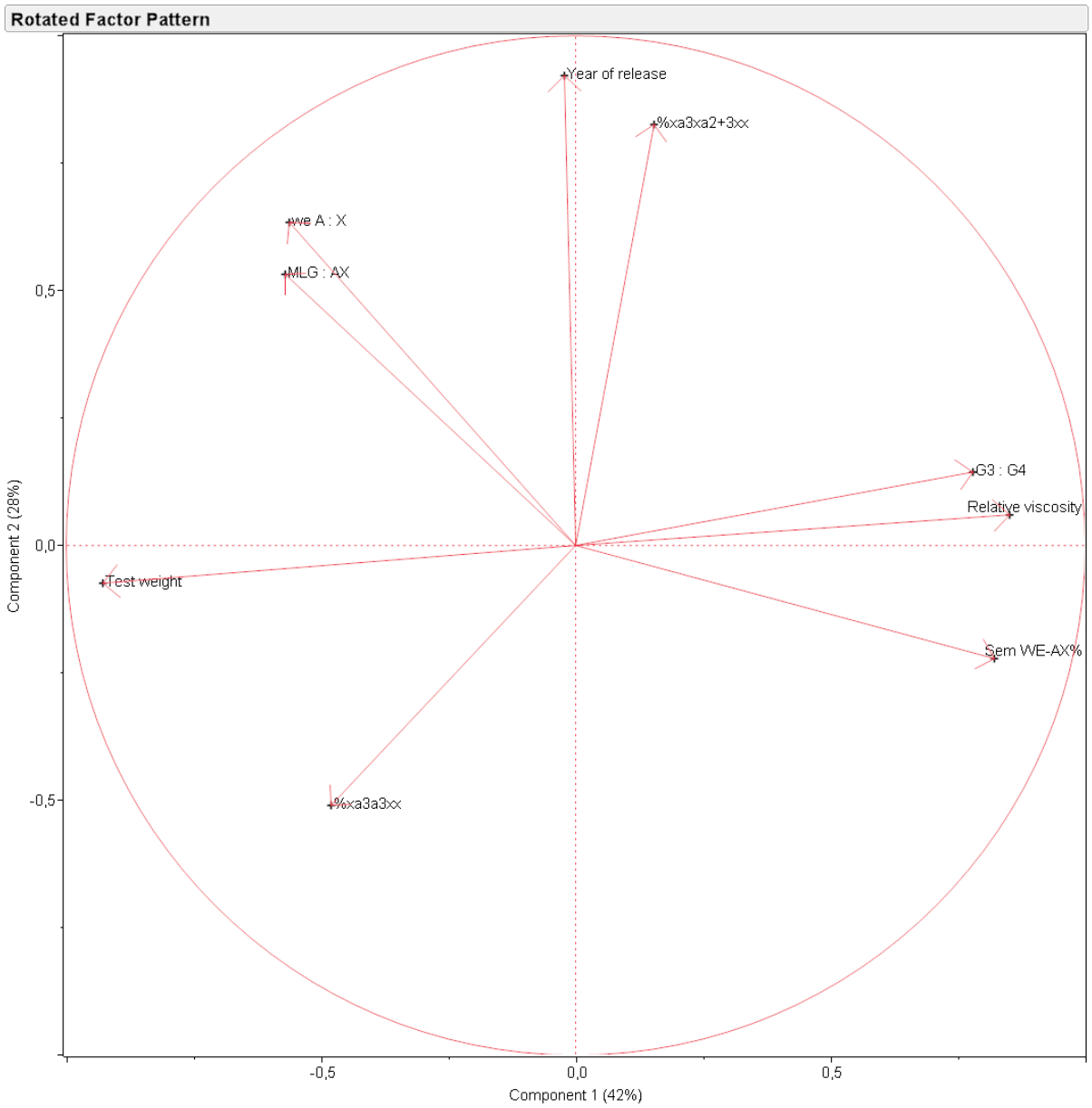


Figure 65 Representation of PCA based on the DF compositional properties in semolina fom old and modern genotypes

The distribution of the samples along the two factors is shown in Figure 66. A discrimination between old and modern genotypes was observed along factor 2 being the old genotypes distributed on the bottom part. Instead, samples from the two crop seasons were discriminated along factor 1 being the more favourable season 2014 on the left. The higher stability in terms of DF content and composition was found in old landrace Dauno III (left side) and in modern cultivars Claudio and Saragolla.

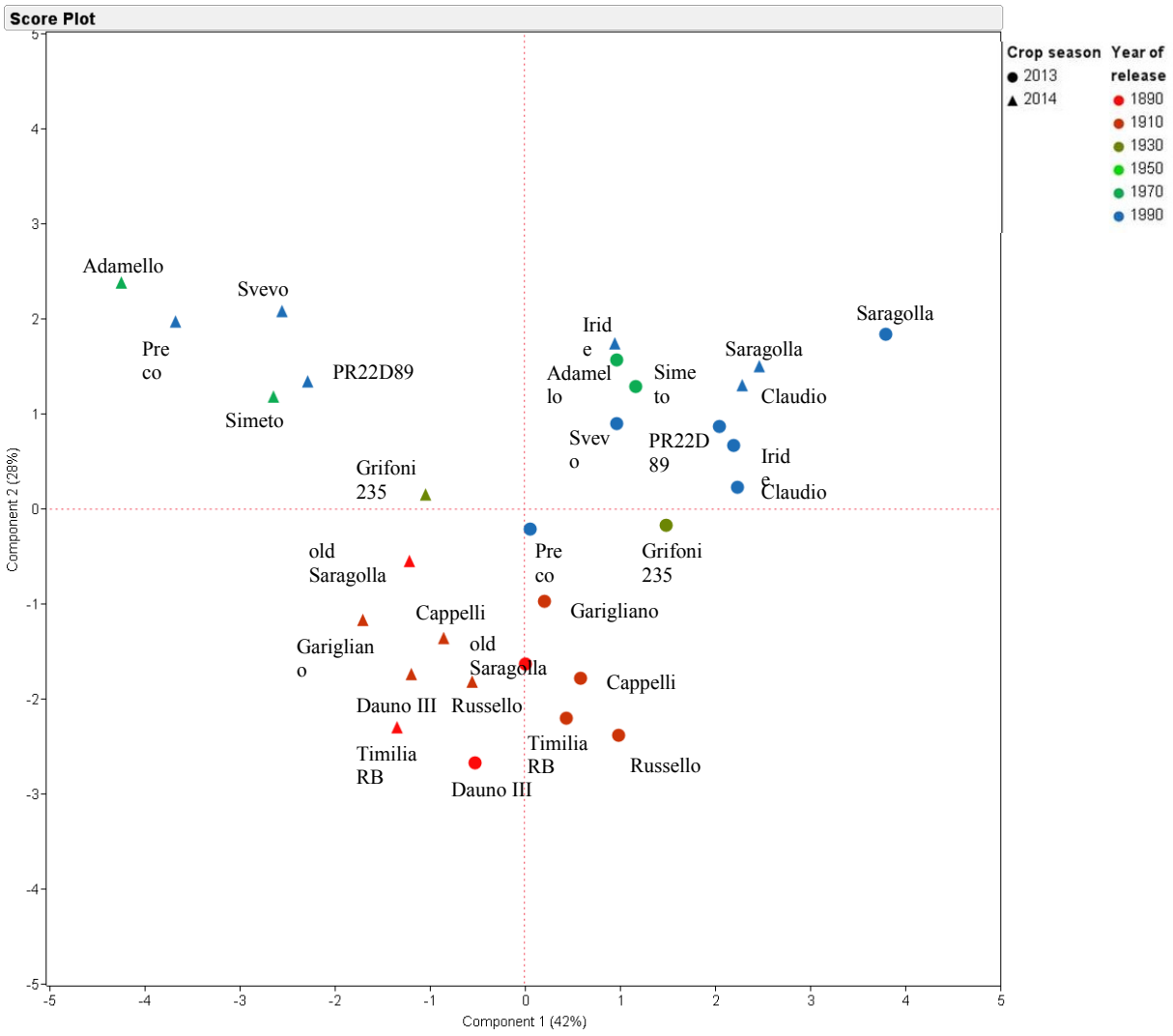


Figure 66 Score plot showing the distribution of old and modern genotypes grown in two crop seasons

4. DISCUSSIONS

4.1. Yield and quality parameters

The two crop seasons were characterized by thermal conditions typical of the Mediterranean area, with lower temperature during fall/winter period and an increasing trend from spring (Royo *et al.*, 2014). Rainfall distribution was not markedly different between the two crop seasons; however, in 2013 a higher rainfall occurred in vegetative stage, while in 2014 a slight water deficit occurred in the same period with higher rainfall in reproductive stage. Significant differences in yield and its components were observed between investigated old and modern groups of durum wheat genotypes. The primary target of the breeding was the increase of yield. The lower plant size and the higher earliness of the modern durum wheat genotypes fully explain the higher values of yield and its components observed (De Vita *et al.*, 2007). TKW and number of kernel per unit area were affected by the year of release and by the crop season. The modern genotypes showed a significant higher number of kernel per unit area and at the same time a significant decrease of the TKW when a higher yield occurred (except for Saragolla) in 2014 crop season. On the contrary, the old group of durum wheat genotypes was not significantly affected by the crop season, showing only Timilia RB and Grifoni 235 a significant increase of both parameters when yield increase was observed. The differences between the old and the modern group in relation to yield and its components indicate that in the old genotypes the principal determinant of yield is grain weight (TKW) while in the modern varieties the number of grains per unit area, explained by a higher number of spikes per unit area and of grains per spikes. Those results are in agreement with Subira *et al.* (2015) that compared Italian and Spanish durum wheat genotypes characterized by different release dates; the authors observed higher number of spikes per unit area and of grains per spikes in the modern genotypes. Also De Vita *et al.* (2007) showed a significant correlation of the number of kernel per area unit with the year of release, in particular under low N input.

Higher grain protein content (GPC) was found in the old genotypes, in agreement with De Vita *et al.* (2007) and due to the negative correlation existing between yield and protein content. Among modern genotypes, cultivar Svevo showed a high GPC in both crop seasons; this genotype has been recently characterised by the Fd-GOGAT gene in chromosome A, as major QTL for GPC in durum wheat (Nigro *et al.*, 2014). In contrast, two old (Timilia RB, Grifoni 235) and three modern (Adamello, Preco and PR22D89) genotypes, showed a higher protein content when higher yield and TKW occurred. In this case, better environmental conditions may have promoted at the same time both a higher yield and a better nitrogen assimilation.

Semolina yellow index is an important quality parameter (Flagella, 2006) and represents a target of the breeding required by industry. This parameter is correlated to β-carotene and lutein content (Fratianni *et al.*, 2013) and a genetic variability in durum wheat exists (Adom *et al.*, 2003). Yellow index measured on semolina flour was significantly higher in the modern group of genotypes; the same trend was observed by Subira *et al.* (2014) that found an increase of the yellow index in the modern cultivars (in particular Italian ones) in a comparison of 12 Italian and Spanish durum wheat varieties released in different periods. The effect of the crop season was not significant in the two groups for this parameter.

A general higher yield and a lower test weight and GPC were observed in the 2014, when higher rainfall during grain development occurred. It is known that yield reduction that generally occurs under limiting water conditions, is generally associated with an increase in protein content (Rharrabti *et al.*, 2003; Garrido-Lestache *et al.*, 2005; Dupont *et al.*, 2006; Flagella *et al.*, 2010; Giuliani *et al.*, 2011). This trend resulted more marked in the modern genotypes group, while a generally higher yield stability was observed in the old genotypes group. In some old durum wheat genotypes, a lower yield was found in the wetter year 2014. Differential response to crop seasons might be due to heading date implications (De Vita *et al.*, 2007) and to phytopatological conditions, since old genotypes are in general characterized by a lower resistance to plant disease.

The Gluten Index is a widely accepted indicator of gluten strength (Sissons *et al.*, 2008). The analysis of G.I. in old and modern durum wheat genotypes showed the presence of two separated groups in relation to the release date. G.I. was significantly influenced by the genotype, rather than by environment (crop season), according to Sissons *et al.* (2008) and Flagella *et al.* (2010); indeed except for cultivar Preco, Adamello and PR22D89, the effect of the crop season was not significant. Several studies demonstrated that the effect of dough strength is highly influenced by gene combinations of storage proteins alleles (mainly glutenins). It has been demonstrated that the improvement of pasta making quality during 20th century selection was firstly due to the LMW-2 / γ- 45 gliadin allele presence, that was more abundant in the intermediate and modern Mediterranean durum wheat cultivars (De Vita *et al.*, 2007; Nazco *et al.*; 2013, Subira *et al.*; 2014). In addition, HMW-GS allelic combination has a role in determining gluten quality. The genotypes investigated in this thesis were representative of the most frequent HMW allelic forms of Mediterranean durum wheat genotypes (Subira *et al.*, 2014) (Glu B1 20, 7+8 and 6+8). According to the literature (De Vita *et al.*, 2007; Nazco *et al.*; 2013, Subira *et al.*; 2014) differences observed in relation to the G.I.

were also explained by the presence of poor (Glu B1 20, 13+16, Gli B1 LMW 1) and good (Glu B1 7+8, 6+8, Gli B1 LMW 2) pasta making quality HMW-GS alleles.

4.2.Storage proteins

4.2.1. Effect of the breeding on gluten protein composition

Most of the studies focused on genetic allele configuration (De Vita *et al.*, 2007; Nazco *et al.*; 2013, Subira *et al.*; 2014) do not explain thoroughly the differences in gluten quality (G.I.) among genotypes characterized by a different year of constitution. In particular, differences in gluten strength between genotypes with the same genetic configuration might be explained by differences in storage protein subunits expression.

Glutenin content was significantly and positively correlated with the year of release (R^2 0.32, $P < 0.05$), while a more significant negative correlation (R^2 -0.44, $P < 0.01$) was observed for gliadin content. As a consequence the gliadin (monomeric) / glutenin (polymeric) proteins ratio (glia : glut) showed a significant negative correlation (R^2 -0.48, $P < 0.01$) with the year of release. It is known that a positive correlation between the *glia : glut* ratio and the GPC exists (Gupta *et al.*, 1992). However no significant correlation was found in this study between protein content and *glia : glut* ratio. This might be due to the fact that two very different (genetic distance) groups of genotypes, old and modern (pre and after Rht gene introduction), were investigated. Indeed, among the modern genotypes Svevo showed both a high GPC (> 15%) and a low *glia : glut* ratio (1.4) due to a high overall glutenin content.

The *glia : glut* ratio showed also a significant negative correlation (R^2-0.53, $P < 0.01$) with G.I. This result is in accordance with Sissons *et al.* (2005) who found that increasing glutenin/gliadin ratio increased the mixograph dough strength, even testing with glutenin-enriched flours.

A high positive significant correlation with the year of release was found for the B- type LMW-GS (R^2 0.63, $P < 0.001$). Except for Dauno III, the investigated genotypes were characterized by the allelic form LMW II. The impact of the LMW II has been widely studied. Several studies on allelic LMW composition and dough strength (Pogna *et al.*, 1990; Sissons *et al.*, 2005) demonstrated the role of LMW II. Edwards *et al.* (2007) found an association between gluten strength and LMW II. Masci *et al.* (2000) found a strong correlation of 42 kDa LMW band with gluten strength. Within that protein band, the major protein was identified as LMW-GS m (D'Ovidio and Masci, 2004; Muccilli *et al.*, 2010; Mamone *et al.*, 2011, Giuliani *et al.*, 2015). Gluten quality, measured as gluten index, resulted mostly affected by B- type LMW-GS relative expression (R^2 0.7, $P < 0.001$). This effect was

more evident in the modern cultivars, characterized by 7+8/6+8 HMW Glu B1 allele configurations, where the B- type LMW-GS expression mostly influenced the gluten index. Among investigated genotypes, Saragolla showed at the same time the highest gluten index (> 75) and the highest B- type LMW-GS expression (> 26% of total storage proteins). Probably, the indirect selection of durum wheat for higher pasta making quality (gluten index, alveograph mixograph properties, De Vita *et al.*, 2007) resulted in the selection of genotypes with a superior relative expression of B- type LMW-GS, in particular of the 42 kDa band. This protein has a key role on dough resistance and elasticity, and it is characterised by the presence of two cysteines available for inter-chain linking (D'Ovidio and Masci, 2004). Masci *et al.* (2000) observed a weak dough strength durum wheat cultivar, Demetra, characterised by LMW 2 allele configuration probably due to low expression this subunit observed. The C-type LMW-GS, suggested as chain terminator (D'Ovidio and Masci, 2004) in the gluten dough, showed a not significant correlation with the year of release.

The HMW-GS showed a slight increased relative expression in the modern genotypes (R^2 0.38, $P < 0.01$). HMW-GS expression was in general about 9-11% of total storage proteins according to Shewry (2009). The role of the HMW-GS (Shewry, 2009) and of their allelic configuration (De Vita *et al.*, 2007; Sissons, 2008; Subira *et al.*, 2015) on gluten technological properties is known.

The ratio between HMW-GS and B- type LMW-GS significantly decreased with the year of release; this correlation resulted mainly explained by the increased expression of B- type LMW-GS in the modern varieties. No significant evidence on the effect of the breeding was found for α- and γ- gliadins expression level.

A marked higher ω- gliadin expression was observed in the old genotypes group (correlation with year of release: R^2 -0.8, $P < 0.001$). At our best knowledge, no specific studies on the evidence of higher ω- gliadin proportion on total storage proteins in old genotypes are reported in the literature. Since ω- gliadin expression showed a significant correlation with semolina protein content (SPC), the relative proportion of this gliadin sub-fraction was evaluated after normalization per protein unit (SPC). Even after this normalization, the average ω- gliadin expression level was about three times higher in the old genotypes group. Among the modern genotypes Saragolla showed the best gluten quality composition presenting at the same time, the lowest ω- gliadin and the highest B- type LMW-GS. The S-poor ω- gliadins does not have a direct important role in pasta quality (Sissons *et al.*, 2008). Results obtained by SDS-PAGE were integrated by bi-dimensional gel electrophoresis approach (2DE) and by western blot. In particular, the ω- 5 gliadin over-expression observed

in the old landrace Dauno III and, in contrast, the down-expression in modern Saragolla, was explained not only by a different number of MW bands (higher in Dauno III), but also by a different relative expression of each band, which showed a different number of isoforms individuated by 2DE in each MW gel region.

4.2.2. Effect of the crop season on gluten protein composition

The observed changes in gliadin : glutenin ratio resulted significantly influenced by the crop season in only three old (Dauno III, Timilia RB and Garigliano) and three modern (Preco, Iride and PR22D89) genotypes. Gliadins and glutenins proportion is suggested to be influenced by the grain protein content (Triboi 2000; Triboi *et al.*, 2003); however no significant correlation was found in this study between protein content and *glia : glut* ratio. It may be possible that the observed differences were more affected by genetic differences in gluten protein composition and expression level between the old and the modern group rather than by differences in protein content due to the effect of the crop season.

It is known that ω- gliadin expression is high environment dependent (Wan *et al.*, 2013, Hurkman *et al.*, 2013, Altenbach *et al.*, 2015). The ω- 5 gliadin showed higher expression in 2013, when a lower rainfall occurred during grain filling. This result was confirmed also by 2DE analysis between one old (Cappelli) and one modern (Simeto) genotype and is in accordance with Giuliani *et al.* (2015) who observed a higher ω- 5 gliadin expression when water deficit conditions occurred during grain filling. 2DE approach showed also a number of spots over expressed in 2013, in particular in the α- gliadin gel region. The highest fold variations (between 2013 and 2014) were observed in modern cultivar Simeto, while the old genotype Cappelli showed a higher stability in gliadin composition. Within the B- type LMW, a specific spot, identified by Giuliani *et al.* (2015) as LMW 2 (m) showed a higher expression in 2013. This result is in accordance with the results obtained by Giuliani *et al.* (2015) on two modern durum wheat genotypes (Svevo and Ciccio) under drought conditions.

4.2.3. Differences in gluten protein composition in relation to WA and CD

ω- 5 gliadins are of particular interest because of their implications in wheat allergy (Matsuo *et al.*, 2015). In particular, ω-5 gliadins are individuated as major components responsible for triggering wheat-dependent exercise-induced anaphylaxis (WDEIA). The differences in ω-gliadin expression observed between the old and the modern group of durum wheat genotypes are of great interest in relation to the potential allergenicity of pasta wheat. In a recent study with transgenic bread wheat in which ω- 5 gliadin genes were silenced by RNA interference

(Altenbach *et al.*, 2015) a reduced allergy response was observed by testing allergic patient sera. However, results obtained in this PhD investigation, suggest interesting perspective in the exploration of wheat germplasm to individuate genotypes with reduced ω- 5 gliadin amount.

Finally, the results obtained by 2DE approach showed an environmental variability of two specific α- gliadins involved in wheat allergy (spot n. 152 and 453) and one specific α- gliadin involved in celiac disease (506), both in one old (Cappelli) and in one modern (Simeto) genotype. Spot 152 overlaps with a protein in which the identified sequences (in Giuliani *et al.*, 2015) match to an allergic protein involved in baker's asthma (Sanders *et al.*, 2011). Spot 453 corresponds to a specific α- gliadins identified in Giuliani *et al.* (2015) and involved in wheat allergy (Tri a 21) (Maruyama *et al.*, 1998). Spot 506 corresponds to a specific α-gliadin identified by Giuliani *et al.* (2015) and its amino acids identified sequence match with a triggering sequence for celiac disease, reported in van Herpen *et al.* (2006). All the three spots showed a higher expression in 2013, when a lower rainfall occurred during grain development. This result is in accordance with Giuliani *et al.* (2015) that found an over-expression of these α- gliadins under drought conditions.

4.3.Dietary fibre

The analysis of pentosan content in old and modern durum wheat genotypes showed Tot-AX content ranging from 1.4 to 1.8% in semolina flour. In the HEALTHGRAIN diversity screen project, 10 durum wheat cultivars, grown in different environments, showed a Tot-AX content in semolina flour ranging from 1.7 to 2.35% (Gebruers *et al.*, 2008). Tot-AX measured in wholemeal flours in this study were higher than in semolina, ranging from 3.3 to 4.5%. Wholemeal AX content is in agreement with Ciccoritti *et al.*, (2008) who found a mean total AX content in whole grain flour in 19 durum wheat genotypes of 4.6%. Similarly, Marcotuli *et al.* (2015) in a wide investigation of 104 tetraploid genotypes, including 61 durum wheats characterized by a different year of release, observed a mean total AX content of 4.0%, with a range of 1.8 to 5.5%. Regarding water extractable arabinoxylan (WE-AX) or soluble AX, the current study of old and modern durum wheat genotypes found a range from 0.3 to 0.8% in semolina and from 0.4 to 1.0% in wholemeal flour. In the HEALTHGRAIN project, (Gebruers *et al.*, 2008, Shewry and Hey, 2015) a range of 0.3-0.55% in endosperm flour, bran and wholemeal flour, was observed. In the current study, the %WE-AX of the total AX, ranged from 18 to 46% in semolina, a similar range was reported by Shewry and Hey (2015), of 25-55%. A lower %WE-AX was observed in *wholemeal* flour (9-24%) is in agreement

with observations by Finnie *et al.* (2006) in bread wheat varieties. A lower concentration of the water extractable AX (soluble AX) in wholemeal with respect to white flour is to be expected as AX in the outer layers is more crosslinked and less soluble.

The relative viscosity of the semolina flour (1.3-2.3) is within the range reported flour of bread wheat varieties (1.3-3.4) by Ordaz-Ortis *et al.* (2005).

Enzymatic fingerprinting provided information about the structure and composition of AX and MLG cell wall polymers in durum wheats of different year of release. Fragments coresponding to xylose (x), xylosebiose (xx) and mono-substituted xa3xx oligosaccharide digest fragments were most abundant, with x and xx being predominant in the outer grain layers (wholemeal flour). Similar results were obtained by Ordaz-Ortiz *et al.* (2005) in five milling fractions of two French bread wheat varieties (Scipion and Baroudeur). Differences in the proportions of G3 and G4 components and total amounts of β– glucans were also observed.

Relative viscosity is affected by the degree of aribinose substitution of the xylan backbone and by size of the AX and MLG polimers wheareas intrinsic viscosity is directly related to AX chain lenght (Dervilly-Pinel *et al*., 2001). The intrinsic viscosity of water exctract, determined SEC-MALS, confirmed the differences observed in terms of relative viscosity.

The greater decrease in intrinsic viscosity in cultivar Svevo after xylanase 11 digestion, suggests the presence of higher AX chain length than in cultivar Saragolla, which in agreement with the effect of enzymatic digestion observed by Dervilly-Pinel *et al*. (2004).

The arabinose / xylose ratio, determined by analysis of monosaccharides in semolina water extracts, were higher than those observed by Ordaz-Ortiz *et al*. (2005) in bread wheat flour (0.47-0.66); however, Dervilly-Pinel *et al*. (2004) reported higher AX ratios in water-soluble AX.

The phenolic acid contents determined in the old and modern genotypes showed more ferulic acid (FA) than *p*-coumaric acid (CA), in particular in the bound form in wholemeal flour. This confirms the results of Li *et al.* (2008) who found about that 85% of total bound phenols was represented by FA in 10 durum wheat cultivars within the HEALTHGRAIN project. The range of the % bound FA observed in wholemeal (79-87%) is consistent with previous investigations. In particular, Nicoletti *et al.* (2013) observed that the percentage of bound FA ranged from 61 to 83.3% in durum wheat semolina and wholemeal flour while Laus *et al.* (2012) also observed higher values in durum wheat (90%).

4.3.1. Effect of the breeding on DF composition

The durum wheat genotypes were characterised by a range of year of release from the beginning of the 20th century (old Italian landraces) to 2005 (modern varieties). Correlation analysis showed that five parameters in semolina were highly significantly correlated (linear Pearson correlation $R^2 \pm 0.50$, $P < 0.01$) with release date. A positive correlation was observed with the %xxxxx (x5) and %xa3xa2+3xx fragments, the proportions of β- glucan and AX (i.e. the ratio between MLG (G3+G4) and AX (sum of the AXOS peaks)) and the arabinose : xylose ratio in the water extractable AX of semolina; a negative correlation was found with the %xa3xa3xx AX fragment. These results suggest a decrease of the proportion of mono-substituted xa3a3xx and an increase in di-substituted xa3xa2+3xx in the modern varieties. The %WE-AX in wholemeal flour was significant higher in the modern group of durum wheat genotypes. To our best knowledge, no information in literature on enzymatic fingerprinting of AX and β- glucans in relation to breeding effects are available on durum wheat genotypes. Marcotuli *et al.* (2015), investigating the AX content in whole grain, did not find evidence of a significant trend in three groups of durum wheat genotypes with different year of release. In the same study, the authors found a lower amount in wild species (*Triticum turgidum* subsp. *dicoccum*). A further comparison including other with other Triticum species and, in particular ancient related tetraploids such as emmer, was carried out within the EU HEALTHGRAIN project in relation to DF content and composition. In that study, reported in Shewry and Hey (2015), the ranges of total and water extractable AX were differed among wheat (bread and durum), einkorn, emmer and spelt. In addition, the authors reported a lower %WE-AX in the ancient species, in particular in emmer. The same trend was also found in relation to total dietary fibre (TDF) and β– glucans, which tended to be lower in ancient species, in particular einkorn and emmer. Consistent with these results, the analyses reported here also showed lower %WE-AX in wholemeal flour and mixed-linkage β– glucan content in semolina of the old genotypes. Thus, there appear to be subtle differences in AX structure, which correlate with release date. However, since dietary fibre content was not a breeding target when these genotypes were selected, we are not able to draw conclusions.

Relative to bound phenolic acids, no significant differences were observed between the old and the modern genotypes investigated. This result is in accordance with Dinelli *et al.* (2009) who did not found significant differences in relation to relative bound phenolic acid content; instead, the same authors found variability in this class of phenolic compounds.

4.3.2. Effect of the crop season on DF composition

Effect of the crop season resulted in a significant reduction of test weight in 2014, in particular in the modern varieties. Significant negative correlations (R^2 < -0.66) with test weight were found with WE-AX and %WE-AX and the proportions of β- glucan G3 : G4 in semolina, with the relative viscosity of aqueous extracts of semolina (-0.73). In the HEALTHGRAIN project, Shewry *et al.* (2010) demonstrated that DF composition is mainly affected by the genotype; in particular, flour tot-AX and WE-AX are about 65% heritable; however, the environment can also influence AX composition. The increased %WE-AX and relative viscosity in 2014, in particular in some modern durum wheat genotypes, might be due to higher rainfall under grain formation and development (from heading to ripening). These results are in accordance to Shewry *et al.* (2010) that found a positive correlation between rainfall from heading to harvest with the flour WE-AX (0.692) and the bran WE-AX (0.737) content, in modern bread wheat cultivars. The reduction of the G3:G4 ratio with the increase in test weight also affected the relative viscosity, with a significant positive correlation between these two parameters (0.565). In addition, the determination of intrinsic viscosity by HPSEC-MALS indicated an increase in the length of AX polymer in 2014, under higher rainfall during grain development.

5. CONCLUSIONS

In this PhD thesis differences due to breeding between old and a modern groups of Italian durum wheat genotypes, were evaluated in relation to dietary fibre and gluten composition.

The phenotyping was performed taking in account ordinary conditions occurring in the Mediterranean area, where water availability is the key factor responsible for agronomical and qualitative aspects.

Several interesting and novel results on the effects of durum wheat breeding in Italy in the 20th century were obtained.

As is well known, the durum wheat breeding was responsible for both an increase of grain yield and improvement of technological quality (gluten strength). In this study it was shown that the superior quality observed in the modern varieties is not only the effect of the improving glutenin allelic composition due to the introduction of high quality alleles of *Glu B1* and *Gli B1* genes, but is also due to differential expression of specific storage proteins. In particular the higher gluten index observed in modern genotypes was correlated with an increased expression of glutenins, especially B- type LMW-GS and in particular 42 kDa band (LMW-GS m). B- type LMW-GS expression was, on average, two times higher in the modern group of durum wheat genotypes, accounting for 30% of total storage proteins in cultivar Saragolla. No significant differences were found between old and modern durum wheat genotypes in relation to α- gliadins, one of the most important fractions determining the toxicity of wheat in celiac disease. Furthermore, a drastic reduction in the expression of ω-5 gliadin, also known as Tri a 19 a major allergen in food wheat allergy (WDEIA), was observed in the modern genotypes, in particular in the top performing genotype, Saragolla. 2DE analysis showed that differences in ω-5 gliadin expression were also due to a different number of specific isoforms.

Differences in rainfall during grain development in the two crop seasons resulted in higher expression of the B- type LMW-GS, of the ω- gliadin, and of specific α- gliadins involved in wheat allergy and celiac disease, as previously characterised by our research group by mass spectrometry under water limiting conditions.

The effect of breeding on cell wall dietary fibre was studied using enzymatic fingerprinting to determine arabinoxylan (AX) and β- glucan (MLG) composition in wholemeal and semolina flour.

Although no significant changes were observed in the total amount of AX, a higher proportion of water soluble AX was observed in wholemeal flour of the modern varieties.

Variation in the AX polymer composition may have occurred as a consequence of breeding with an increase of one unsubstitued (xxxxx), one di-substituted (xa3xa2+3xx) and a

simultaneous decrease of one mono-substituted (xa3xa3xx) AX chain fragment in semolina of modern varieties. In addition, the water soluble AX extracted from semolina flour showed a lower arabinose : xylose ratio in the old genotypes. A secondary effect of breeding resulted in the higher MLG content in semolina in modern varieties.

Our results did not show an effect of breeding on the viscosity of soluble DF extracts but considerable variability was observed between the durum wheat genotypes.

Several effects were observed when higher rainfall occurred during the reproductive period. In particular, a higher content of water soluble AX and, as a consequence, a higher relative viscosity of the soluble DF extracts was observed in the wetter season. These changes in viscosity were associated with variation in AX and MLG composition, with a lower arabino : xylose ratio (A : X) and with a higher proportion of the G3 (three glucose) compared with G4 (four glucose) β- (1→4) glucans fragments, determined after enzymatic fingerprinting. These effects of environment were more marked in the modern varieties, indicating a higher stability in DF in the old genotypes.

In conclusion, 20th century breeding seems to have improved both the technological and health quality of Italian durum wheat genotypes. Higher contents of glutenin and B- type LMW-GS were responsible for better gluten quality while a lower content of ω- 5 gliadin may indicate a lower allergic potential of gluten from modern genotypes. An increase in the proportion of water soluble AX in wholemeal flour, changes in the AX polymer composition and a higher MLG content in semolina have also occurred as a consequence of breeding in modern Italian durum wheat varieties.

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