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Nutraceutical value of durum wheat: influence of environment and genotype in a large-scale experimental trial

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ABSTRACT

Grain quality is well known as one of the most interesting breeding objectives in Mediterranean countries. It still has great importance in wheat markets because of the increased interest of the consumers for high-quality staple food such as pasta, couscous and various types of bread. The performance of many quality characteristics depends greatly on environmental conditions and, in this context, organic agriculture could guarantee a durum wheat material with high nutraceutical value for healthy food production and special dietary uses. Among organic wheat production, KAMUT® khorasan wheat (*Triticum turgidum* ssp. *turanicum* (Jakubz.)) has attracted great attention due to its specific nutritional and functional properties (antioxidant, anti-inflammatory, and prebiotic activities). The aim of this thesis was to evaluate the environmental and climatic effects on the nutritional and nutraceutical quality of organic durum and durum-type wheat varieties. The work was subdivided into three main sections aimed at understanding the dynamic affecting the accumulation of nutritional and functional compounds in wheat caryopsis of durum and durum type wheat varieties. The first two section provide a complete characterization of KAMUT® khorasan grain. This is a distinctive study: first a collection of the same organically grown genotype collected during two decades of cultivation was characterized for nutritional and functional properties; then the investigation has shifted to the same crop harvested in a vast region (180000 km^2), including several different environments. In the third section 24 old and modern durum and durum-type wheat varieties, cropped in the same location and growing season, were analyzed in order to determine and compare the phenolic composition. Results obtained gives a fundamental understanding of durum wheat grains composition in terms of nutrient and bioactive compounds.

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INTRODUCTION

The recent attention to environmental, economical and social challenges has increased the need for an alternative to conventional agriculture. Conventional or chemical agriculture involves a massive use of fertilizers and pesticides that can contaminate the environment and damage the soil structure, leading to soil erosion, organic matter loss and reduced soil fertility (Herencia et al., 2008). Organic agriculture is a viable alternative to conventional farming and one of the key values is to maintain high yields and improve the quality utilizing farming practices that have acceptable environmental impacts and take into account the health of the consumer (Murphy et al., 2007).

In the present work several organic durum wheat varieties have been analyzed. Durum wheat (*Triticum turgidum* ssp. *durum*) has been chosen for the analysis because it is one of the most important cereal crops used in the Mediterranean-type temperate zones for the production of staple food, such as pasta, couscous, and various types of bread. Durum wheat is also one of the most common crops in Italy with a cultivated area of 1.31 million hectares in 2015 and a production of 4.38 million tons (Database - Eurostat). Furthermore organic wheat represents an important nutraceutical raw material for healthy food production and special dietary uses. Several epidemiological studies have evidenced that regular consumption of whole wheat derived staple food has positive effects on health, as it contributes to proper regulation of blood glucose levels and the management of obesity. It was also demonstrated that whole wheat consumption helps to decrease cardiovascular mortality and the incidence of colorectal cancer (Gil et al., 2011; Sahyoun et al., 2006; Truswell, 2002). Other studies recently evidenced in humans that a replacement diet with KAMUT® khorasan (an ancient organic grain) products has positive effects in circulatory blood parameters in both a healthy population and in chronic disease populations. In a similar study ancient grain significantly reduced symptoms of irritable bowel syndrome (IBS) (Sofi et al., 2014, 2013; Whittaker et al., 2016, 2015). Nevertheless nowadays, most of the wheat products we consume derive from modern wheat varieties bred after the so-called “Green Revolution” (in the 1960s). The introduction of dwarf genes led to new durum wheat cultivars straw lines with high yield and improved technological quality (De Vita et al., 2007a;

2007b). In fact, the breeding programs have been focused on improving primary metabolism and technological properties (i.e. gluten quality) of durum wheat and, consequently, they have almost not taken into account the nutraceutical and health- promoting properties of wheat consumption in the human diet (Motzo et al., 2004; Pecetti and Annicchiarico, 1998). Conversely, KAMUT® khorasan is the trademark of an ancient wheat cultivar, the khorasan wheat (*Triticum turgidum* ssp. *turanicum* (Jakubz.)) derived from a natural hybrid between *T. durum* and *Triticum polonicum*, which occurred in the Fertile Crescent (Khlestkina et al., 2006). Cultivation of KAMUT® khorasan wheat is exclusively managed by a license agreement which requires organic certification of the crop and several quality specifications related both to nutritional characteristics and growing conditions (Quinn, 1999). In particular, the grain must be the ancient khorasan variety of wheat, never hybridised or cross, grown only as a certified organic grains, untouched by modern varieties of wheat to 99%, have a protein content between 12 and 18%, be free of signs of disease at 98% and contain between 400 and 1000 ppb of selenium. So far these specific standards are consistently satisfied only in the growing region of North America and in particular in the Upper Great Plains of Alberta, Saskatchewan, Montana and North Dakota (Grausgruber et al., 2004). Climate change is nowadays evident, particularly in terms of increasing temperature, increasing CO_2 concentration, widespread melting of snow and ice and rising global average sea level (Ceccarelli et al., 2010). In fact, over the past sixty years (1950–2010) there has been a significant alteration of the climatic conditions. For example, in Canada the temperature has increased and the warming is slightly more evident in the minimum temperature than in the maximum (Vincent et al., 2012). It is known that wheat is a species adapted to different agricultural environments and end-uses, and is exposed to variable environmental conditions, both within and over several crop years (Graybosch et al., 1995). The manifestation of those environmental conditions in the developing kernel affects the quality of the crop by influencing yield, grain characteristics and flour quality (Altenbach et al., 2003). In particular, fibre components have been commonly considered as inherent characteristics, although climatic conditions may have a certain effect on the grain fibre quantity and quality, by influencing grain size and the

proportional amounts of each kernel tissue (endosperm, aleurone, germ) (Gebruers et al., 2008; Shewry et al., 2010). Additionally, the growing conditions are known to strongly influence the expression levels of plant secondary metabolites such as polyphenols and, therefore, may affect the accumulation of phenolic compounds during kernel development (Mpofu et al., 2006; Shewry et al., 2010). These phytochemical compounds with antioxidant activity are plant secondary metabolites belonging to the phenylpropanoid pathway and contain one or more aromatic rings and one or several hydroxyl groups, including phenolic acids, such as ferulic, p-coumaric, p-hydroxybenzoic, vanillic, and syringic acids. (Adom et al., 2005, 2003; Liu, 2004; Yu et al., 2002). As per definition, antioxidant compounds, can delay or avoid the oxidative damage of substrates as DNA, enzymes and cell wall molecules, neutralizing free radicals (e.g., ROS) that are the cause of many chronic diseases (i.e. cardiovascular disease and cancer). *In vitro* investigations of whole wheat grains established that phenolic compounds possess high antiradical power and contribute to most of the total antioxidant activity (Adom et al., 2005, 2003; Okarter et al., 2010; Yu et al., 2002). Phenolic compounds in wheat are mainly concentrated in the outer layers of the kernel, such as aleurone, pericarp, and embryo cell walls (Adom et al., 2005). They are mainly present as soluble free acids, soluble conjugates esterified to sugars and other low molecular weight molecules, as well as insoluble forms bound to cell wall components, such as polysaccharides and lignin. The latter phenolic components contribute to 70 - 80% of the total phenolic content (Adom et al., 2005; Dinelli et al., 2011; Leoncini et al., 2012). Due to the fact that phenolic acids occur in the outer layers of the grain, they may be lost during the refinement processes, thus significantly affecting the nutritional and nutraceutical values of the final grain products (Di Silvestro et al., 2014; Slavin, 2004). On the other hand, antioxidant compounds as well as dietary fiber, vitamins and minerals are preserved in whole meal products (Hirawan et al., 2010). Currently, few data about nutritional and functional composition of organically grown KAMUT® khorasan grains are available in the literature, especially regarding the changes as a function of cropping year and growing location.

The purpose of the research reported in this thesis was to evaluate the environmental and climatic effects on the nutritional and nutraceutical quality of organic durum and durum-type wheat varieties. The work was subdivided into three main sections aimed at understanding the dynamic affecting the accumulation of nutritional and functional compounds in wheat caryopsis of durum and durum type wheat varieties. The first two section of the present work gives a complete characterization of a durum-type wheat variety: KAMUT® khorasan grain. This is a unique study: first a collection of the same organically grown genotype collected during twenty-one years of cultivation was analyzed (SECTION 1); then the researched has shifted to the same crop harvested in a region of such broad extension (180000 km^2), that consequently includes several different environments (SECTION 2). In the third section of this thesis different old and modern durum and durum-type wheat varieties, cropped in the same location and growing season, were analyzed in order to determine and compare the phenolic composition (SECTION 3). This last section aimed to contribute to the understanding of wheat whole grain nutraceutical properties, fostering the development of wheat varieties with high level of health-promoting phytochemicals.

The specific of this thesis were:

- a complete description of the quality of KAMUT® khorasan grains harvested in the last two decades (1989-2012) on a specific farm in Montana and, therefore, an evaluation of the environmental effects on phytochemical accumulation. The project included the analysis of dietary fibre components (both insoluble and soluble fractions), starch compositions (total and resistant starch, amylose and amylopectin fractions) and the characterization of antioxidant properties through the determination of polyphenol and flavonoid content (both free and bound phenolic compounds) (SECTION 1).
- the determination of the nutrient, fibre and antioxidant composition of KAMUT® grain as a function of the growing location in order to understand the environmental dynamics affecting the phytochemical profile. The study involved an area covering approximately 180000 km^2 (Canada and USA) which includes several different farms and

environments. The grain collection consisted of grain samples harvested at 109 different locations in two cropping years, 2010 and 2011. Each grain sample was analyzed for the antioxidant compounds (polyphenols, flavonoids) and dietary fibre components (soluble and insoluble dietary fibres, resistant starch) and results were elaborated using Geographic Information System (GIS) to develop *quality maps*. Moreover, the elaboration of data concerning grain yield, test weight and protein content allowed a comparison between the agronomic performance and the nutritional/nutraceutical profile of KAMUT® grain (SECTION 2).

- the determination and comparison of the phenolic composition of different durum and durum-type wheat varieties, including old and modern genotypes, cropped in the same location and growing season. In particular, the characterization and identification of individual polyphenols was obtained using *ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS)* (SECTION 3).

SECTION 1

Paper 1

Under submission

Nutritional and nutraceutical aspects of KAMUT® khorasan wheat grown during the last two decades

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SUMMARY

Recently, organic farming system has attracted the attention of consumers because of its low environmental impact. Organic agriculture is a valid alternative to conventional farming, and ancient wheat, such as KAMUT® khorasan wheat, has emerged as a leader in this category for its nutritional and functional properties (anti-inflammatory, antioxidant and prebiotic). It is known in literature that the environmental condition may affect bioactive compound composition of wheat kernels.

The aim of the present study is to evaluate the environmental effect on the quality of KAMUT® khorasan grains harvested in the last two decades (1989-2012) on one farm in Montana (USA), through the evaluation of phytochemical accumulation. The characterization of a collection of KAMUT® khorasan wheat collected during twenty-one years of cultivation is a unique study and, as far as we know, there are few similar articles available in literature. Results evidenced a great variability in the amounts of macronutrient and nutraceuticals. In particular from 1989 to 2012, it was observed a decrease of the trend line in the content of total starch (ranging from 50.54 ± 1.31 to 70.87 ± 1.61 g/100g) and amylose (from 41.48 ± 1.01 to 31.46 ± 0.15 % of total starch) and a slight increase of insoluble dietary fiber (IDF) amount (from 12.14 ± 0.41 to 17.75 ± 0.44 g/100g), while the soluble dietary fiber (SDF) content varied among the years of cultivation even if the general trend remained constant (4.57 ± 0.12 - 2.82 ± 0.02 g/100g). High variability of the total polyphenols content was observed among the investigated wheat samples. The free soluble fraction varied between 52.07 ± 2.36 mg/100g and 119.98 ± 4.72 mg/100g, while the range observed for bound polyphenols is comprised between 73.22 ± 3.15 and 136.20 ± 11.07 mg/100g. Moreover an inverse correlation between free and bound

fractions of polyphenols was observed ($r = -0.528$; $p = 0.0139$). The results obtained in the present study evidenced that, under organic farming systems, the influence of environmental conditions plays a fundamental role on the accumulation of primary and secondary metabolites in wheat kernels and strongly modulates the nutritional and nutraceutical value of flour.

INTRODUCTION

The recent attention to environmental, economical and social problems has increased the need for an alternative to conventional agriculture. Conventional or chemical agriculture involves a massive use of fertilizers and pesticides that can contaminate the environment and damage the soil structure, leading to soil erosion, organic matter loss and reduced soil fertility (Herencia *et al.*, 2008). Organic agriculture is a viable alternative to conventional farming and one of the key values is to maintain high yields and improve the quality utilizing farming practices that have acceptable environmental impacts and take into account the health of the consumer (Murphy *et al.*, 2007). It raises a great deal of interest that organic wheat represents an important nutraceutical raw material for healthy food production and special dietary uses. Above all, the ancient KAMUT® khorasan wheat (*Triticum turgidum* ssp. *turanicum* (Jakubz.)) has attracted great attention due to its specific nutritional and functional properties (antioxidant, anti-inflammatory, and prebiotic activities) (Benedetti *et al*., 2012; Carnevali *et al*., 2014; Gianotti *et al*., 2011; Marotti *et al*., 2012; Valerii *et al*., 2015; Valli *et al.*, 2015). In particular, KAMUT® khorasan wheat contains a higher protein content than common wheat, in addition to greater variability in the qualitative composition of amino acids and a greater quantity of lipids and fatty acids (Grausgruber *et al*., 2005; Angioloni & Collar 2011). Furthermore, other studies recently evidenced in humans that a replacement diet with KAMUT® khorasan products has positive effects in circulatory blood parameters in both a healthy population and in chronic disease populations. In a similar study ancient grain significantly reduced symptoms of irritable bowel syndrome (IBS) (Sofi *et al*., 2013; Sofi *et al*., 2014; Whittaker *et al*., 2015; Whittaker *et al*., 2016).

KAMUT® is a registered trademark used in marketing foodstuffs of the ancient cultivated khorasan wheat variety called “QK-77”, which was registered by the USDA in 1990. Cultivation of KAMUT® khorasan wheat is exclusively managed by a license agreement which requires organic certification of the crop and several quality specifications related both to nutritional characteristics and growing conditions (Quinn, R.M., 1999). For example the grain must descend

unmodified from the original seed stock first used under the KAMUT® brand. Additionally, the grain must have a protein content of between12 and 18% and must contain between 400 and 1000 ppb selenium. So far these specific standards are consistently satisfied only in the growing region of North America and in particular in the Upper Great Plains of Alberta, Saskatchewan, Montana and North Dakota (Grausgruber *et al*., 2004). Climate change is nowadays evident, particularly in terms of increasing temperature, increasing CO_2 concentration, widespread melting of snow and ice and rising global average sea level (Ceccarelli *et al*., 2010). In fact, over the past sixty years (1950–2010) there has been a significant alteration of the climatic conditions. In particular, in Canada the temperature has increased and the warming is slightly more evident in the minimum temperature than in the maximum (Vincent *et al.,* 2012). It is known that wheat is a species adapted to different agricultural environments and end-uses, and is exposed to variable environmental conditions, both within and over several crop years (Graybosh *et al*., 1995). The manifestation of those environmental conditions in the developing kernel affects the quality of the crop by influencing yield, grain characteristics and flour quality (Altenbach *et al.*, 2003). In particular, fibre components have been commonly considered as inherent characteristics, although climatic conditions may have a certain effect on the grain fibre quantity and quality, by influencing grain size and the proportional amounts of each kernel tissue (endosperm, aleurone, germ) (Gebruers *et al*., 2008; Shewry *et al*., 2010). Additionally, the growing conditions are known to strongly influence the expression levels of plant secondary metabolites such as polyphenols and, therefore, may affect the accumulation of phenolic compounds during kernel development (Mpofu et al., 2006; Shewry et al., 2010). Currently, few data about nutritional and functional composition of organically grown KAMUT® khorasan grains are available in the literature, especially regarding the changes as a function of cropping year and growing location.

The study aims at providing a description of the quality of KAMUT® khorasan grains harvested in the last two decades (1989-2012) on a specific farm in Montana and, therefore, evaluating the environmental effects on phytochemical accumulation. The project includes the analysis of dietary fibre components

(both insoluble and soluble fractions), starch compositions (total and resistant starch, amylose and amylopectin fractions) and the characterization of antioxidant properties through the determination of polyphenol and flavonoid content (both free and bound phenolic compounds).

MATERIAL AND METHODS

Grain samples

The study was carried out using a collection of KAMUT® khorasan wheat (*Triticum turgidum* spp. *turanicum*) harvested in North America for 21 growing season (from 1989 to 2012). The wheat samples were cropped on the "Quinn Organic Farm" located near Big Sandy, Montana (USA) in accordance with the strictly quality specifications required by Kamut International, Ltd. Mean temperatures and rainfall amounts recorded at the experimental field are reported in Figure 1. Whole-grain samples were ground to semolina using a domestic stone mil (100% flour extraction) (Billy 200, Hawos Mulini, Bad Homburg, Germany). All determinations were replicated three times and results expressed on a dry weight (DW) basis.

Chemicals

Folin-Ciocalteu, gallic acid and catechin reagent were purchased from Sigma-Aldrich (St Louis, MO, USA). All other chemicals and solvents were of analytical grade.

Macronutrients Analyses

The determination of the protein content was carried out according to the Kjeldahl procedure (N x 5.7) (AACC, 1983). The determination of total starch content was carried out using the Megazyme assay kit (Megazyme Int. Ireland Ltd., Wicklow, Ireland) based on the use of glucose oxidase-peroxidase reagent (GOPOD) after hydrolytic digestion with α-amylase and amyloglucosidase. The amount of amylose was obtained using the Amylose/Amylopectin Megazyme assay kit (Megazyme Int. Ireland Ltd., Wicklow, Ireland). The method was based on the use of lectin concanavalin A (Con A) for the precipitation and

removal of the amylopectin-Con A complex. Afterwards, the amylose molecules were hydrolyzed to D-glucose with α-amylase/amiloglucosidase and spectrophotometrically quantified using GOPOD reagent. According to McCleary & Monaghan (2002), resistant starch content was determined using a Megazyme assay kit. After an overnight digestion with α-amylase and amyloglucosidase, soluble starch was removed with 95% and 50% ethanol consecutive washes. Then, the pellet was extracted with 2 mol L^{-1} KOH to dissolve resistant starch, hydrolysed with amyloglucosidase and quantified using a GOPOD reagent (Di Silvestro *et al.*, 2012).

Dietary Fibre components

Insoluble (IDF), soluble (SDF) and total (TDF) dietary fibre contents were determined following the enzymatic/gravimetric procedure described by Prosky *et al*. (1998) using the Megazyme assay kit (Megazyme Int. Ireland Ltd, Bray, Ireland). The procedure was based on the sequential enzymatic digested using heat stable α-amylase, protease and amyloglucosidase, allowing the determination of IDF, SDF and TDF amounts as previously described (Di Silvestro *et al*., 2012).

The arabinoxylans (AX) content was determined according to the procedure of the enzymatic assay kit D-Xylose (Megazyme International Ireland Ltd, Wicklow, Ireland) with some modifications. Wholegrain samples were subjected to acid hydrolyzation in boiling water bath, followed by re-equilibration of pH with NaOH 1.3M. After the hydrolysis, samples were clarified by treatment with solutions of hexacyanoferrate (II) and zinc sulphate. This assay kit allowed the indirect measurement of D-xylose amount, through the spectrophotometric determination of NADH produced during the reaction of β-D-xylose with NAD^+ in the presence of β-xylose dehydrogenase. The AX concentration was calculated by considering the estimated D-xylose content in wheat flour arabinoxylans (62%).

The determination of betaglucans (BG) content was carried out on the basis of the method described previously by our research group (Marotti *et al*., 2012) using the Megazyme assay kit (Megazyme International Ireland Ltd, Wicklow, Ireland).

Determination of phenolic compounds

Free and bound phenolic compounds were extracted as previously described by Dinelli *et al*. (2011). Whole-grain flours were extracted with cold 80% ethanol to dissolve the free soluble compounds, followed by acid and alkaline hydrolyses to release the bound forms. Extracts were analyzed for the polyphenol quantification following the colorimetric procedure based on the Folin–Ciocalteu reagent, as described by Singleton *et al.* (1999). Furthermore, extracts containing free and bound phenolic compounds were analyzed for flavonoid content following the spectrophotometric method previously described (Dinelli *et al.*, 2011).

Statistical analysis

One-way analysis of variance (ANOVA, Tukey's honestly significant difference multiple comparison) was performed for comparing the flour samples.
Linear discriminate analysis (LDA) was performed on the standardised matrix of the nutrient and phytochemical content of grain samples, including a total of 16 variables (protein, starch, amylose, amylopectin, RS, IDF, SDF, TDF, AX, BG, FP, BP, TP, FF, BF, TF). All statistical analyses were conducted using Statistica 6.0 software (2001, StatSoft, Tulsa, OK, USA).

RESULTS

Macronutrient composition

Macronutrient composition of the wheat grains analyzed is presented in Table 1 for protein starch, amylose, and amylopectin content. Regarding the protein content, significantly differences were observed among wheat sample analyzed in the present study. In particular, protein values ranged between 12.3 g/100g to 23.7 g/100g, respectively for samples collected in 1993 and 2003 with a mean value of 16.8 g/100g (Table 1).Total starch content varied significantly among the sample analyzed. KAMUT® khorasan grain harvested in 1993 has evidenced the highest amount of starch, while samples collected in 1999, 2003 and 2004 have showed the lowest starch content (Table 1). Besides total starch, statistically significant differences were observed in the amylose and

amylopectin content. In particular, a reduction of the amylose percentage fraction was observed from 1989 to 2012 in KAMUT® khorasan grains. The opposite trend was observed in the amylopectin content. Total starch content is in agreement with data reported in literature by Merendino *et al.*, (2006) regarding durum wheat harvested in Italy, while percentage fraction of amylose is lower than observed in two durum wheat cultivars cropped in Spain by Guzman *et al.*, (2011).

Dietary fibre and resistant starch content

The bran layer of the kernel is particularly rich in dietary fibre, including insoluble (IDF), soluble (SDF) and resistant starch. Dietary fibre composition of the investigated wheat samples is presented in Table 2. In our study, dietary fibre content varied greatly among the 21 KAMUT® khorasan samples. The highest IDF amount was observed in the sample harvested in 1997, while the lowest was in samples collected in 1989, 1993, 1994 and 1998 (Table 2). In general, a positive trend in the IDF amounts was observed in the last two decades. Regarding SDF, significant differences were observed among the different years of cultivation. KAMUT® khorasan wheat harvested in 1989, 2000, 2003, 2005,2006 and 2009 had the highest level, while samples collected in 1990, 1991, 1993, 1995, 2004, 2011 and 2012 showed the lowest amounts However, the general trend in the content of soluble dietary fibre has remained stable during the last twenty-one years, although with some exceptions. The arabinoxylan (AX) content of whole grains observed in the present study was reported in Table 2. KAMUT® khorasan wheat harvested in 1990, 1991 1992 and 1995 showed the highest content of AX, while the grain collected in 1989 had the lowest content. Along with AX, β-glucans are one of the most important dietary fibre components. Significant differences in β-glucan amount were observed among samples of different cropping year. From 1989 to 2012 the tendency in the content of β-glucan remained constant (Table 2). Resistant starch represents the fraction of the starch escaping digestion and not absorbed in the small intestine of healthy humans. In this study were observed significant differences among the KAMUT® khorasan wheat samples analyzed (Table 2). The trend of resistant starch content did not change during the twenty-one years

of the study. The amounts of dietary fibre component, except for SDF, β-glucan and resistant starch, observed in the present study are significantly higher than those previously reported for KAMUT® khorasan and closely related durum wheat, while for SDF, β-glucan and resistant starch the average amounts were lower than reported in literature on durum wheat cultivars and KAMUT® khorasan harvested in Italy (Merendino *et al.*, 2006 and Marotti *et.al*., 2012) and with respect to durum wheat varieties cropped in Hungary in 2004 (Gebruers *et al*., 2008)

Antioxidant components

Polyphenols are the most representative class of antioxidant in wheat, mainly concentrated in the bran layer of the kernel. They are for the most part made up of phenolic acids and flavonoids (Dinelli *et al*.,2009) In wheat, phenolic compound exist in the soluble (free) and insoluble (bound) forms. The phenolic composition of the investigated grains is reported in Table 3. High variability of the total polyphenols content was observed among the investigated wheat samples, with the sample harvested in 1992 showing the highest amount. Regading the free soluble fraction, higher amounts were obtained for samples collected in 2008, 2009 and 2012. The lowest free phenolic accumulation was observed for grain harvested in 1990, 1994, 1996, 1997 and 1998 (Table 3). An inverse correlation between free and bound fractions of polyphenols was observed ($r = -0.528$; $p = 0.0139$). Moreover, in the last twenty-one years the trend in the amount of the free fraction of polyphenols increased, while an opposite tendency was evidenced for the bound fraction of polyphenols. Regarding the accumulation of total flavonoids in KAMUT® khorasan kernel, samples collected in 1992, 1997, 2008 and 2012 have shown the highest amount, while samples harvested in 1990, 1993, 1995 and 2006 were characterized by the lowest content of total flavonoids (Table 3). The highest free soluble flavonoid amount was recorded by sample collected in 1992,whereas the highest bound flavonoid content was obtained by grains grown in 1997 and 1998 (Table 3).

Linear Discriminant Analyses (LDA)

The multivariate technique of data analyses has been used to explain the observed variability in nutrient and phytochemical content of wheat samples harvested in Montana (USA) from 1989 to 2012. LDA allowed to obtain more information as regards the variables that mainly influenced the sample similarities and differences (Rodriguez- Delgado *et al.*,2002; Di Silvestro *et al.*, 2012). Figure 2 shows the scatterplot of the grain samples on the space defined by the first two canonical functions.

KAMUT® khorasan grains harvested in 2006, 2008, 2009, 2011 and 2012 are clustered together in a separate group. A second cluster is identified by sample collected in 1991, 1992, 1995, 1996, 1997, 1999, 2001, 2004, and 2005. A third but less homogeneous cluster is identified by KAMUT® khorasan wheat harvested in 1989, 1990, 1993, and 1994. As revealed from the values of canonical functions standardised within variances for each variable on root 1, the described clusterisation of the cases seen were strongly influenced by protein, betaglucans, insoluble and soluble dietary fibre and amylose content (values of first canonical discriminant function (Root 1) equal to 1.38, 0.57, -0.52, -0.72 and -0.69, respectively). The clusterisation of the cases along second canonical function (Root 2) seen were strongly influenced by amylose, free polyphenols, bound flavonoids, resistant and total starch and betaglucans amount (values of canonical discriminant function 2 equal to 1.62 1.23, 1.25, 0.72, -1.29 and -0.68, respectively). Indeed, cluster A included grain samples with the highest amount of amylose, free polyphenols and bound flavonoids, whereas cluster C had the highest total starch content. Wheat samples grouped together in cluster B are characterized by an highest average content of soluble dietary fibre and by the lowest protein content.

According to the results obtained, the year of cropping induced strong effects on kernel composition and induced huge variation in the content of primary and secondary metabolites.

DISCUSSION

Recently, reports in the literature evidenced the effects of genotype and environment, as well as their interaction, on bioactive compound composition of wheat kernels (Mpofu *et al*., 2006; Shewry *et al*., 2010). However, the available literature is quite scarce on this topic and, as far as we know, the changes of fibre components and antioxidants of the ancient KAMUT® khorasan grains as a function of cropping year has never been investigated. The ancient grains may constitute a rich genetic resource for the low-input and organic sector, as they could guarantee a superior nitrogen (N) extraction in low-N environments, good competitiveness on weed control (due to higher plant height) and a general higher resistance to biotic and abiotic stresses (Lammerts van Bueren *et al*., 2011). Several minor cereals and ancient crops constitute valuable nutritional grain ingredients for healthy food production, thanks to their better health-related composition. In organic wheat production, one of the most important point is the grain quality, particularly as regards the protein content. Data obtained in this study were higher than those recorded in literature by Dinelli *et al*., (2014), but Merendino *et al.*, (2006) reported higher values in KAMUT® khorasan grains. Protein content resulted positively correlated with temperature recorded during the grain-filling stage ($r = 0.536$; $p = 0.012$). The effect of environmental growing conditions on macronutrient accumulation was observed comparing the amount of starch in wheat kernel. It was observed a decrease of the trend line in the content of total starch from 1989 to 2012. As previously described by Hucl and Chibbar (1996), a negative correlation exists between protein and starch content in the wheat grain. This statement was confirmed by the presence of an inverse relationship between protein and starch levels was observed ($r = -0.582$; $p = 0.005$). In addition, it was also observed a negative correlation ($r = -0.450$; $p=0.0407$) between mean temperature during the grain-filling stage and the lower ratio of starch in the endosperm. This is in accordance with Labuschagne *et al*., (2009) and can be attributed to the heat inactivation of starch synthase, the key enzyme in the starch biosynthesis reaction pathway. Differently from what reported in literature by Labuschagne et al. (2009), the ratio amylose:amylopectin decreased with increasing temperature during the last two decades. Literature data report that durum wheat starch granules contain 25-

30% amylose (BeMiller & Whistler, 1996; Hansen *et al*., 2010), while in the present study a higher amylose percentage was observed. This divergence could be related to the influence of agronomic growing conditions on starch granule composition (Singh *et al*., 2010) or the limitation in comparing different amylose/amylopectin analytical techniques, as previously reported by Zhu *et al*., (2008).

Resistant starch and soluble dietary fibre (as beta-glucans, arabinoxylans) are characterized by high prebiotic activity, indeed they can resist digestion and reach the colon where functioning as substrate for intestinal microbial flora (Marotti *et al*., 2012). Usually, a positive correlation between dietary fibre and mean temperature between heading and harvest is expected. (Shewry *et al*., 2010). In the present study, the content of dietary fibre was found to slightly increase from 1989 to 2012 evidencing the strong effect of environmental condition on its accumulation in wheat kernel, as previously described (Gebruers *et al*., 2008; Marotti *et al*., 2012).

The bran layer of wheat grains is relatively rich in antioxidants compounds. Polyphenols are a large group of phytochemicals including flavonoids (the most representative in wheat kernel), phenolic acids, coumarins, tannins, lignans and stilbens. In wheat kernel polyphenols exist in three forms: soluble, soluble conjugated (linked to low molecular weight sugars) and bound to cell wall components. All polyphenols present high antioxidant activity, arresting the damaging oxidative chain reaction caused by free radicals (peroxide, O_2), thus preventing many degenerative human diseases (i.e. heart disease and cancer). Especially bound polyphenols possess strong anti-cancer properties as they survive the digestion process and reach the colon, where the endothelial cells may absorb phenolics and gain antioxidant protection (Kroon *et al*., 1997). The free phenolic contents observed in the present study were higher than those reported in literature for soluble compounds in durum wheat varieties harvested in Italy during one year of cultivation (Bellato *et al*., 2013; Ciccoritti *et al*., 2013), while were in line with those recorded by Fares *et al* (2010), taking into account the insoluble fractions. As reported in Table 3, a great variability was observed in the amount of antioxidant compounds during the twenty-one cropping years of the present study. The only correlation observed was a

positive correlation with total precipitation was observed for free and total polyphenols and flavonoids ($r = 0.576$, $p = 0.0062$; $r=0.947$, $p=0.0001$; $r=0.775$, $p=0.0001$; $r=0.729$, $p= 0.0002$). The results were expected as phenolic biosynthesis can be induced by various biotic stress and therefore the observed variability among years of cultivation may also be related to plant pathologies present at different levels of severity (Dixon and Paiva, 1995). Moreover it wasn't observed any significant correlations between phenolic compounds and temperature during the cropping years taking into account in this study.

The characterization of nutritional and nutraceutical properties of wheat grains harvested in Montana (USA) from 1989 to 2012 allowed the investigation of the nutrient and phytochemical accumulation as a function of changing environmental conditions in a long term experiment. The analyses showed great variability among samples collected in different years of cultivation. In particular, the greater environmental effects were observed for polysaccharides (starch and some dietary fibre components) and for phenolic compounds. Due to the major exposure of the crop to climatic changes, lower nutrients availability and pest diseases, the effects of the environmental conditions in agricultural low-input systems may exert higher influence as compared to the conventional ones. (Bilsborrow *et al*., 2013). More research is needed to investigate the cause of location effects, the correlations with soil samples and previous crops planted in rotation, extend the study to several genotype, and determine the heritability of the antioxidant properties. In conclusion, the present study has a unique value, because in literature are present very few work in which the same genotype is cultivated during twenty-one cropping years with the same agricultural practice.

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REFERENCES

AACC, (1983). Approved method 46-12. American Association of Cereal Chemists, St. Paul, MN, USA.

ALTENBACH, S.B., DUPONT, F.M., KOTHARI, K.M. CHAN, R., JOHNSON, E.L. & LIEU D. (2003). Temperature, water and fertilizer influence the timing of key events during spring grain development in a US spring wheat. *Journal of Cereal Science* **37**, 9-20.

ANGIOLONI, A. & COLLAR, C. (2011) Nutritional and functional added value of oat, Kamut®, spelt, rye, and buckwheat versus commonwheat in breadmaking. *Journal of Science and Food Agriculture* **91**, 1283-1292.

BELLATO, S., CICCORITTI, R., DEL FRATE, V., SGRULLETTA, D.& CARBONE, K. (2013). Influence of genotype and environment on the content of 5-n alkylresorcinols, total phenols and on the antiradical activity of whole durum wheat grains. *Journal of Cereal Science* **57**, 162-169.

BeMILLER, J.N. & WHISTLER, R.L. (1996). Carbohydrates. In *Food Chemistry*, third ed. (Eds. Fennema O.R), pp. 157-204. New York: Marcel Dekker Inc.

BENEDETTI, S., PRIMITERRA, M., TAGLIAMONTE, M. C., CARNEVALI, A., GIANOTTI, A., BORDONI, A. & CANESTRARI, F. (2012). Counteraction of oxidative damage in the rat liver by an ancient grain (Kamut brand khorasan wheat). *Nutrition* **28**, 436-441.

BILSBORROW, P., COOPER, J., TETARD-JONES, C., SREDNICKA-TOBER, D., BARANSKI, M., EYRE, M., SCHMIDT, C., SHOTTON, P., VOLAKAKIS, N., CAKMAK, I., OZTURK, L., LEIFERT, C. & WILCOCKSON, S. (2013). The effect of organic and conventional management on the yield and quality of wheat grown in a long-term field trial. *European Journal of Agronomy* **51**, 71-80.

CARNEVALI, A., GIANOTTI, A., BENEDETTI, S., TAGLIAMONTE, M.C., PRIMITERRA, M., LAGHI, L., DANESI, F., VALLI, V., NDAGHIJIMANA, M., CAPOZZI, F., CANESTRARI, F. & BORDONI, A. (2014). Role of Kamut® brand khorasan wheat in the counteraction of non-celiac wheat sensitivity and oxidative damage. *Food Research International* **63**, 218-226.

CECCARELLI, S., GRANDO, S., MAATOUGUI, M., MICHAEL, M., SLASH, M., HAGHPARAST, R., RAHMANIAN, M., TAHERI, A., AL-YASSIN, A., BENBELKACEM, A., LABDI, M., MIMOUN, H. & NACHIT, M. (2010). Plant breeding and climate changes. *Journal of Agricultural Science* **148**, 627-637.

CICCORITTI, R., CARBONE, K., BELLATO, S., POGNA, N. & SGRULLETTA, D. (2013). Content and relative composition of some phytochemicals in diploid, tetraploid and hexaploid Triticum species with potential nutraceutical properties. *Journal of Cereal Science* **57**, 200-206.

DI SILVESTRO, R., MAROTTI, I., BOSI, S., BREGOLA, V., SEGURA-CARRETERO, A., SEDEJ, I., MANDIC, A., SAKAC, M., BENEDETTELLI, S. & DINELLI, G. (2012). Health-promoting phytochemicals of Italian common wheat varieties grown under low-input agricultural management. *Journal of the Science of Food and Agriculture* **92**, 2800-2810.

DINELLI, G., SEGURA-CARRETERO, A., DI SILVESTRO, R., MAROTTI, I., FU, S., BENEDETTELLI, S. & GHISELLI, L. (2009). Determination of phenolic compounds in modern and old varieties of durum wheat using liquid chromatography coupled with time-of-flight mass spectrometry. *Journal.of Chromatography A* **1216**, 7229-7240.

DINELLI, G., SEGURA-CARRETERO, A., DI SILVESTRO, R., MAROTTI, I., FU, S., BENEDETTELLI, S., GHISELLI, L. & FERNANDEZ-GUTIERREZ, A. (2011). Profiles of phenolic compounds in modern and old common wheat varieties determined by liquid chromatography coupled with time-of-flight mass spectrometry. *Journal of Chromatography A* **1218**, 7670-7681.

DINELLI, G., DI SILVESTRO, R., MAROTTI, I., BOSI, S., BREGOLA, V., DI LORETO, A., NIPOTI, P., PRODI, A. & CATIZONE, P. (2014). Agronomic traits and deoxynivalenol contamination of two tetraploid wheat species (*Triticum turgidum* spp. *durum*, *Triticum turgidum*, spp. *turanicum*) grown strictly under low input condition. *Italian Journal of Agronomy* 9, 127-135.

DIXON, R.A. & PAIVA, N.L. (1995). Stress–induced phenylpropanoid metabolism. *The Plant Cell* **7**, 1085–1097.

FARES, C., PLATANI, C., BAIANO, A. & MENGA, V. (2010). Effect of processing and cooking on phenolic acid profile and antioxidant capacity of durum wheat pasta enriched with debranning fractions of wheat. *Food Chemistry* **119**, 1023-1029.

GEBRUERS, K., DORNEZ, E., BOROS, D., FRAS, A., DYNKOWSKA, W., BEDO, Z., RAKSZEGI, M., DELCOUR, J.A. & COURTIN, C.M. (2008). Variation in the content of dietary fiber and components thereof in wheats in the HEALTHGRAIN diversity screen. *Journal of Agricultural and Food Chemistry* **56**, 9740-9749.

GIANOTTI, A., DANESI, F., VERARDO, V., SENZATETTI, D.I., VALLI, V., RUSSO, A., RICIPUTI, Y., TOSSANI, N., CARBONI, M.F., GUERZONI, M.E. & BORDONI, A. (2011). Role of cereal type and processing in whole grain in vivo protection from oxidative stress. *Frontiers of Bioscience* **16**, 1609-1618.

GRAUSGRUBER, H., OBERFORSTER, M., GHAMBASHIDZE, G. & RUCKENBAUER, P. (2005) Yield and agronomic traits of Khorasan wheat (*Triticum turanicum* Jakubz.). *Field Crop Research* **91**, 319-327.

GRAYBOSH, R.A., PETERSON, C.J., BAENZIGER, P.S. & SHELTON, D.R. (1995). Environmental modification of hard red winter wheat flour protein composition. *Journal of Cereal Science* **22**, 45-51.

GUZMAN, C., CABALLERO, L., ALVARE, J.B. & YAMAMORI, M. (2011). Amylose content and starch properties in emmer and durum wheat lines with different waxy protein composition. *Journal of the Science of Food and Agriculture* **91**, 1625-1629.

HANSEN, L.E., JACKSON, D.S., WEHLING, R.L., WILSON, J.D. & GRAYBOSH, R.A. (2010). Functionality of native tetraploid wheat starches: Effects of waxy loci alleles and amylase concentration in blends. *Journal of Cereal Science* **52**, 39-45.

HERENCIA, J.F., RUIZ, J.C., MELERO, S., GARCIA GALAVIS, P.A. & MAQUEDA C. (2008). A short-term comparison of organic v. conventional agriculture in a silty loam soil using two organic amendents. *Journal of Agricultural Science* **146**, 677-687.

KROON, P.A., FAULDS, C.B., RYDEN, P., ROBERTSON, J.A. & WILLIAM, G (1997). Release of Covalently Bound Ferulic Acid from Fiber in the Human Colon. *Journal of Agricoltural and Food Chemistry* **45**, 661–667.

LABUSCHAGNE, M.T., ELAGO, O. & KOEN, E. (2009). The influence of temperature extremes on some quality and starch characteristics in bread, biscuit and durum wheat. *Journal of Cereal Science* **49**, 184-189.

LAMMERTS VAN BUEREN, E.T., JONES, S.S., TAMM, L., MURPHY, K.M., MYERS, J.R., LEIFERT, C. & MESSMER, M.M. (2011) The need to breed crop varieties suitable for organic farming, using wheat, tomato and broccoli as examples: a review. *NJAS – Wageningen Journal of Life Sciences*, **58**, 193–205.

MAROTTI, I., BREGOLA, V., ALOISIO, I., DI GIOIA, D., BOSI, S., DI SILVESTRO, R., QUINN, R. & DINELLI, G. (2012). Prebiotic effect of soluble fibres from modern and old durum-type wheat varieties on *Lactobacillus* and *Bifidobacterium* strains. *Journal of the Science of Food and Agriculture* **92**, 2133-2140.

McCLEARY, B.V. & MONAGHAN, D.A. (2002). Measurement of resistant starch. *Journal of the Association of Official Analytical Chemists*, **85**, 665–675.

MERENDINO, N., D'AQUINO, M., MOLINARI, R., DE GARA, L., D'EGIDIO M.G., PARADISO, A., CECCHINI, C., CORRADINI, C. & TOMASSI, G. (2006). Chemical characterization and biological effects of immature durum wheat in rats. *Journal of Cereal Science* **43**, 129-136.

MPOFU, A., SAPIRSTEIN, H.D. & BETA, T. (2006). Genotype and environmental variation in phenolic content, phenolic acid composition and antioxidant activity of hard spring wheat. *Journal of Agricultural and Food Chemistry* **54**, 1265-1270.

MURPHY, K.M., CAMPBELL K.G., LYON, S.R. & JONES, S.S. (2007). Evidence of varietal adaptation to organic farming systems. *Field Crop Research* **102**, 172-177.

PROSKY, L., ASP, N.G., SCHWEIZER, T.F., DEVRIES, J.W. & FURDA, I. (1988). Determination of insoluble, soluble, and total dietary fibre in foods and food products. Interlaboratory study. *Journal of the Association of Official Analytical Chemists* **71**, 1017-1023.

QUINN, R.M. (1999). Kamut®: ancient grain, new cereal. In *Perspectives on new crops and new uses*. (Eds. Janick J.), pp. 182-183. ASHS Press, Alexandria, VA.

RODRIGUEZ-DELGADO, M.A., GONZALEZ-HERNANDEZ, G., CONDE-GONZALEZ, J.E. & PEREZ-TRUJILLO, J.P. (2002). Principal component analysis of the polyphenols content in young red wines. *Food Chemistry* **78**, 523-532.

SHEWRY, P.R., PIIRONEN, V., LAMPI, A.M., EDELMANN, M., KARILUOTO, S., NURMI, T., FERNANDEZ-OROZCO, R., RAVEL, C., CHARMET, G., ANDERSSON, A.A.M., AMAN, P., BOROS, D., GEBRUERS, K., DORNEZ, E., COURTIN, C.M., DELCOUR, J.A., RAKSZEGI, M., BEDO, Z. & WARD, J.L. (2010) The HEALTHGRAIN Wheat Diversity Screen: Effect of Genotype and Enviroment on Phytochemicals and Dietary Fiber Components. *Journal of Agricultural and Food Chemistry* **58**, 9291-9298.

SINGH, S., GUPTA, A.K., GUPTA, S.K. & KAUR, N. (2010) Effect of sowing time on protein quality and starch pasting characteristics in wheat (*Triticum aestivum* L.) genotypes grown under irrigated and rain-fed conditions. *Food Chemistry* **122**, 559-565.

SINGLETON, V.L., ORTHOFER, R. & LAMUELA-RAVENTOS, R.M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. *Methods in Enzymology* **299**, 152–178.

SOFI, F., WHITTAKER, A., CESARI, F., GORI, A.M., FIORILLO, C., BECATTI, M., MAROTTI, I., DINELLI, G., CASINI, A., ABBATE, R., GENSINI, D.F., & BENEDETTELLI, S. (2013). Characterization of Khorasan wheat (Kamut) and impact of a replacement diet on cardiovascular risk factors: cross-over dietary intervention study. *European Journal of Clinical Nutrition* **67**, 190-195.

SOFI, F., WHITTAKER, A., GORI, A.M., CESARI, F., SURRENTI, E., ABBATE, R., GENSINI, D.F., BENEDETTELLI, S. & CASINI, A. (2014). Effect of *Triticum turgidum* subsp. *turanicum* wheat on irritable bowel syndrome: a double-blinded randomised dietary intervention trial. *British Journal of Nutrition* **111**, 1992-1999.

VALERII, M.C., RICCI, C., SPISNI, E., DI SILVESTRO, R., DE FAZIO, L., CAVAZZA, E., LANZINI, A., CAMPIERI, M., DALPIAZ, A., PAVAN, B., VOLTA, U. & DINELLI, G. (2015). Responses of peripheral blood mononucleated cells from non-celiac gluten sensitive patients to various cereal sources. *Food Chemistry* **176**, 167-174.

VALLI, V., DANESI, F., GIANOTTI, A., DI NUNZIO, M., TANEYO SAA, D.L & BORDONI, A. (2015). Antioxidative and anti-inflammatory effect of *in vitro* digested cookies baked using different types of flours and fermentation methods. *Food Research International* http://dx.doi.org/10.1016/j.foodres.2015.12.010.

VINCENT, L.A., WANG, X.L., MILEWSKA, E. J., WAN, H., YANG, F. & SWAIL, V. (2012). A second generation of homogenized Canadian monthly surface air temperature for climate trend analysis. Journal of Geophysical Research **117**, D18110.

WHITTAKER, A., SOFI, F., LUISI, M.L.E., RAFANELLI, E., FIORILLO, C., BECATTI, M., ABBATE, R., CASINI, A., GENSINI, G.F. & BENEDETTELLI, S. (2015). An Organic Khorasan Wheat-Based Replacement Diet Improves Risk Profile of Patients with Acute Coronary Syndrome: A Randomized Crossover Trial. *Nutrients* **7**, 3401-3415.

WHITTAKER, A., DINU, M., CESARI, F., GORI, A.M., FIORILLO, C., BECATTI, M., CASINI, A., MARCUCCI, R., BENEDETTELLI, S. & SOFI, F. (2016). A khorasan wheat-based replacement diet improbe risk profile of patients with type 2 diabetes mellitus (T2DM): a randomized crossover trial. European Journal of Nutrition. DOI 10.1007/s00394-016-1168-2

ZHU, T., JACKSON, D.S., WEHLING, R.L. & GEERA, B. (2008). Comparison of amylose determination methods and the development of a dual wavelength iodine binding technique. *Cereal Chemistry* **81**, 51-58.

Tables

Table 1: Protein, starch, amylose and amylopectin composition of the investigated wheat grains. Values are mean of three different replications ± standard deviation. Means followed by the same letter are not significantly different at $p<0.05$ (Tukey's HSD)

YEAR	Protein (g/100g)	Starch (g/100g)	Amylose (% Starch)	Amylopectin (% Starch)
1989	20.9 ± 0.14 (b)	60.15 ± 1.22 (d-f)	41.48 ± 1.01 (a)	58.52 ± 1.01 (j)
1990	17.7 ± 0.16 (ef)	67.23 ±1.44 (b)	37.69 ± 2.85 (cd)	62.31 ± 2.85 (gh)
1991	12.8 ± 0.14 (l)	65.88 ± 2.38 (b)	40.40 ± 1.23 (ab)	59.60 ± 1.23 (ij)
1992	16.1 ± 0.15 (hi)	61.85 ± 2.20 (c-e)	35.20 ± 1.96 (e-h)	64.80 ± 1.96 (c-f)
1993	12.3 ± 0.14 (l)	70.87 ± 1.61 (a)	35.48 ± 1.24 (d-g)	64.52 ± 1.24 (d-g)
1994	15.7 ± 0.18 (ij)	67.11 ± 0.68 (b)	36.97 ± 1.16 (c-f)	63.03 ± 1.16 (e-h)
1995	17.3 ± 0.12 (fg)	64.50 ± 2.30 (bc)	34.61 ± 0.47 (f-i)	65.39 ± 0.47 (b-e)
1996	14.0 ± 0.13 (k)	62.59 ± 2.97 (cd)	35.13 ± 1.66 (f-h)	64.87 ± 1.66 (c-e)
1997	16.3 ± 0.14 (h)	60.56 ± 0.50 (d-f)	37.67 ± 0.02 (c-e)	62.33 ± 0.02 (f-h)
1998	15.4 ± 0.11 (j)	60.95 ± 0.56 (c-f)	37.71 ± 0.81 (cd)	62.29 ± 0.81 (gh)
1999	17.1 ± 0.12 (g)	52.82 ± 0.61 (jk)	38.97 ± 0.52 (bc)	61.03 ± 0.52 (hi)
2000	20.3 ± 0.13 (c)	54.67 ± 0.23 (ij)	32.61 ± 0.53 (h-j)	67.39 ± 0.53 (a-c)
2001	16.0 ±0.14 (hi)	55.61 ± 1.78 (g-j)	32.05 ± 0.52 (ij)	67.95 ± 0.52 (ab)
2003	23.7 ± 0.11 (a)	50.54 ± 1.31 (k)	32.72 ± 0.20 (h-j)	67.28 ± 0.20 (a-c)
2004	15.6 ± 0.19 (ij)	53.31 ± 1.55 (jk)	31.46 ± 0.15 (j)	68.54 ± 0.15 (a)
2005	17.9 ± 0.19 (e)	54.90 ± 0.51 (ij)	34.53 ± 0.31 (f-i)	65.47 ± 0.31 (b-e)
2006	18.5 ± 0.14 (d)	54.98 ± 1.26 (h-j)	35.34 ± 0.59 (d-h)	64.66 ± 0.59 (c-g)
2008	15.3 ±0.21 (j)	58.80 ± 2.23 (e-h)	33.13 ± 1.30 (g-j)	66.87 ± 1.30 (a-d)
2009	16.5 ± 0.15 (h)	56.65 ± 0.92 (g-j)	31.51 ± 0.70 (j)	68.49 ± 0.70 (a)
2011	15.7 ± 0.17 (ij)	58.30 ± 2.70 (f-i)	32.78 ± 0.09 (h-j)	67.22 ± 0.09 (a-c)
2012	18.6 ± 0.14 (d)	59.40 ± 2.32 (e-g)	31.96 ± 1.50 (ij)	68.04 ± 1.50 (ab)

Table 2: Dietary fibre, arabinoxylan, β-glucans and resistant starch composition of the investigated wheat grains. Values are mean of three different replications ± standard deviation. Means followed by the same letter are not significantly different at $p<0.05$ (Tukey's HSD)

YEAR	IDF (g/100g)	SDF (g/100g)	AX (g/100g)	BG (g/100g)	RS (g/100g)
1989	12.14 ± 0.41 (k)	4.16 ± 0.14 (ab)	2.24 ± 0.09(j)	0.24 ± 0.02(c-e)	0.48 (b-d)
1990	13.42 ± 0.94 (h-j)	2.85 ± 0.27 (g)	3.35 ± 0.08(a-c)	0.25 ± 0.02(a-d)	0.51 (a-c)
1991	13.73 ± 0.52 (g-j)	3.32 ± 0.48 (d-g)	3.54 ± 0.16(a)	0.27 ± 0.01(ab)	0.44 (b-d)
1992	13.76 ± 0.71 (g-j)	3.87 ±0.31 (b-d)	3.39 ± 0.17(ab)	0.26 ± 0.01(a-d)	0.39 (d)
1993	12.13 ± 0.98 (k)	3.24 ± 0.34 (d-g)	3.13 ± 0.26(b-e)	0.27 ± 0.03(ab)	0.47 (b-d)
1994	12.65 ± 0.31 (jk)	3.59 ±0.07 (b-f)	2.67 ± 0.05(hi)	0.21 ± 0.01(e)	0.55 (ab)
1995	14.90 ± 0.72 (d-g)	3.49 ± 0.56 (c-g)	3.51 ± 0.25(a)	0.22 ± 0.01(de)	0.46 (b-d)
1996	13.39 ± 0.65 (ij)	3.85 ± 0.26 (b-d)	3.18 ± 0.31(b-d)	0.23 ± 0.01(c-e)	0.54 (ab)
1997	19.65 ± 0.49 (a)	3.62 ± 0.20 (b-e)	2.61 ± 0.01(i)	0.27 ± 0.02(a-c)	0.63 (a)
1998	13.14 ± 0.61 (i-k)	3.78 ± 0.44 (b-d)	2.88 ± 0.01(e-i)	0.25 ± 0.02(a-d)	0.55 (ab)
1999	14.63 ± 0.54 (e-g)	3.69 ± 0.42 (b-d)	2.82 ± 0.01(f-i)	0.26 ± 0.01(a-d)	0.42 (b-d)
2000	16.15 ± 0.39 (cd)	3.91 ± 0.66 (a-d)	2.94 ± 0.06(d-i)	0.22 ± 0.01(de)	0.62 (a)
2001	14.90 ± 0.51 (d-g)	3.67 ± 0.62 (b-e)	2.98 ± 0.03(d-h)	0.24 ± 0.01(b-e)	0.44 (b-d)
2003	15.97 ± 0.74 (c-e)	4.53 ± 0.11 (a)	2.97 ± 0.07(d-h)	0.24 ± 0.01(a-e)	0.40 (cd)
2004	14.51 ± 0.06 (f-i)	3.22 ± 0.13 (d-g)	2.94 ± 0.05(d-i)	0.28 ± 0.01(a)	0.44 (b-d)
2005	14.60 ± 1.53 (e-h)	4.57 ± 0.12 (a)	3.00 ± 0.03(d-h)	0.24 ± 0.01(a-e)	0.44 (b-d)
2006	16.87 ± 0.52 (bc)	4.04 ± 0.13 (a-c)	3.05 ± 0.12(c-g)	0.21 ± 0.01(e)	0.54 (ab)
2008	16.50 ± 0.62 (bc)	2.88 ± 0.60 (fg)	3.01 ± 0.06(d-g)	0.24 ± 0.04(b-e)	0.52 (a-c)
2009	15.87 ± 0.06 (c-f)	3.91 ± 0.73 (a-d)	2.72 ± 0.10(g-i)	0.23 ± 0.02(c-e)	0.49 (a-d)
2011	16.42 ± 0.18 (bc)	2.95 ± 0.36 (e-g)	3.13 ± 0.06(b-f)	0.25 ± 0.01(a-d)	0.53 (a-c)
2012	17.75 ± 0.44 (b)	2.82 ± 0.02 (g)	3.09 ± 0.06(c-f)	0.24 ± 0.01(c-e)	0.51 (a-d)

Abbreviations: IDF, insoluble dietary fibre; SDF, soluble dietary fibre; AX, arabinoxylan; BG, β-glucans; RS, resistant starch

Table 3: Free, bound and total polyphenol and flavonoid contents of the investigated wheat grains. Values are mean of three different replications ± standard deviation. Means followed by the same letter are not significantly different at $p<0.05$ (Tukey's HSD)

YEAR	Free Polyphenols (mg/100g)	Bound Polyphenols (mg/100g)	Total Polyphenols (mg/100g)	Free Flavonoids (mg/100g)	Bound Flavonoids (mg/100g)	Total Flavonoids (mg/100g)
1989	68.59 ± 7.16 (gh)	97.55 ± 7.39 (e-g)	166.14 ± 9.92 (f-h)	29.90 ± 4.11 (cd)	10.61 ± 1.95 (de)	40.51 ± 4.20 (d-f)
1990	59.91 ± 4.57 (ij)	100.88 ± 4.69 (d-f)	160.78 ± 4.83 (h)	19.04 ± 2.57 (fg)	7.48 ± 1.00 (e)	26.51 ± 3.49 (g)
1991	77.28 ± 4.37 (ef)	116.15 ± 4.18 (bc)	193.43 ± 6.02 (bc)	26.28 ± 1.39 (de)	12.22 ± 1.62 (de)	38.50 ± 1.03 (d-f)
1992	104.64 ± 8.36 (b)	136.20 ± 11.07 (a)	240.84 ± 13.12 (a)	46.62 ± 4.66 (a)	13.14 ± 1.57 (c-e)	59.77 ± 5.79 (a)
1993	67.15 ± 5.64 (g-i)	125.28 ± 3.83 (ab)	192.43 ± 8.70 (b-d)	13.83 ± 1.46 (h)	10.80 ± 0.96 (de)	24.64 ± 2.31 (g)
1994	62.10 ± 5.68 (h-j)	102.93 ± 12.31 (c-f)	165.02 ± 6.63 (f-h)	27.27 ± 4.19 (c-e)	11.79 ± 0.37 (de)	39.06 ± 3.82 (d-f)
1995	84.66 ± 2.05 (c-e)	91.55 ± 7.57 (e-h)	176.21 ± 5.53 (c-h)	24.11 ± 1.40 (ef)	9.95 ± 0.56 (de)	34.05 ± 0.84 (e-g)
1996	57.96 ± 5.59 (j)	112.07 ± 17.78 (cd)	170.04 ± 20.52 (f-h)	23.01 ± 5.92 (ef)	18.02 ± 9.36 (bc)	41.03 ± 14.09 (d-f)
1997	58.75 ± 0.79 (ij)	130.83 ± 10.63 (ab)	189.58 ± 11.41 (b-e)	27.54 ± 1.34 (c-e)	27.54 ± 1.34 (a)	55.08 ± 2.67 (ab)
1998	52.07 ± 2.36 (j)	115.53 ± 0.79 (b-d)	167.59 ± 1.57 (f-h)	23.18 ± 0.31 (ef)	23.18 ± 0.31 (ab)	46.36 ± 0.62 (b-d)
1999	69.88 ± 3.94 (f-h)	91.03 ± 3.15 (e-i)	160.91 ± 0.79 (gh)	25.65 ± 0.10 (de)	19.55 ± 0.72 (bc)	45.20 ± 0.62 (b-e)
2000	74.61 ± 1.97 (e-g)	89.09 ± 0.39 (e-i)	163.70 ± 2.36 (gh)	26.16 ± 0.01 (de)	16.64 ± 0.92 (b-d)	42.80 ± 0.92 (c-f)
2001	81.85 ± 2.76 (de)	88.81 ± 3.15 (f-i)	170.66 ± 0.39 (e-h)	24.34 ± 0.31 (ef)	14.90 ± 0.10 (cd)	39.24 ± 0.41 (d-f)
2003	89.09 ± 0.39 (cd)	83.80 ± 1.57 (g-i)	172.88 ± 1.18 (d-h)	22.74 ± 0.10 (ef)	14.53 ± 0.82 (cd)	37.28 ± 0.92 (d-f)
2004	92.70 ± 3.94 (c)	106.90 ± 1.97 (c-e)	199.60 ± 5.90 (b)	24.56 ± 0.41 (d-f)	12.93 ± 0.41 (c-e)	37.49 ± 0.01 (d-f)
2005	76.28 ± 3.54 (e-g)	90.76 ± 5.12 (e-i)	167.04 ± 8.66 (f-h)	23.69 ± 0.41 (ef)	15.91 ± 1.13 (b-d)	39.60 ± 0.72 (d-f)
2006	104.39 ± 5.51 (b)	81.85 ± 4.33 (hi)	186.24 ± 9.84 (b-f)	15.77 ± 1.14 (gh)	16.49 ± 1.54 (b-d)	32.27 ± 2.68 (d-f)
2008	119.98 ± 4.72 (a)	73.22 ± 3.15 (i)	193.20 ± 1.57 (b-d)	38.00 ± 0.72 (b)	14.17 ± 1.13 (cd)	52.17 ± 0.41 (a-c)
2009	112.19 ± 7.87 (ab)	82.96 ± 5.90 (g-i)	195.15 ± 13.78 (bc)	33.35 ± 0.31 (bc)	13.59 ± 0.92 (c-e)	46.94 ± 1.23 (b-d)
2011	108.01 ± 6.69 (b)	73.22 ± 3.15 (i)	181.23 ± 9.84 (b-g)	27.18 ± 0.21 (c-e)	13.88 ± 0.31 (c-e)	41.05 ± 0.10 (c-f)
2012	111.63 ± 1.57 (ab)	87.14 ± 4.72 (f-i)	198.77 ± 6.30 (b)	37.64 ± 0.41 (b)	14.24 ± 0.82 (cd)	51.88 ± 1.23 (a-c)

Figures

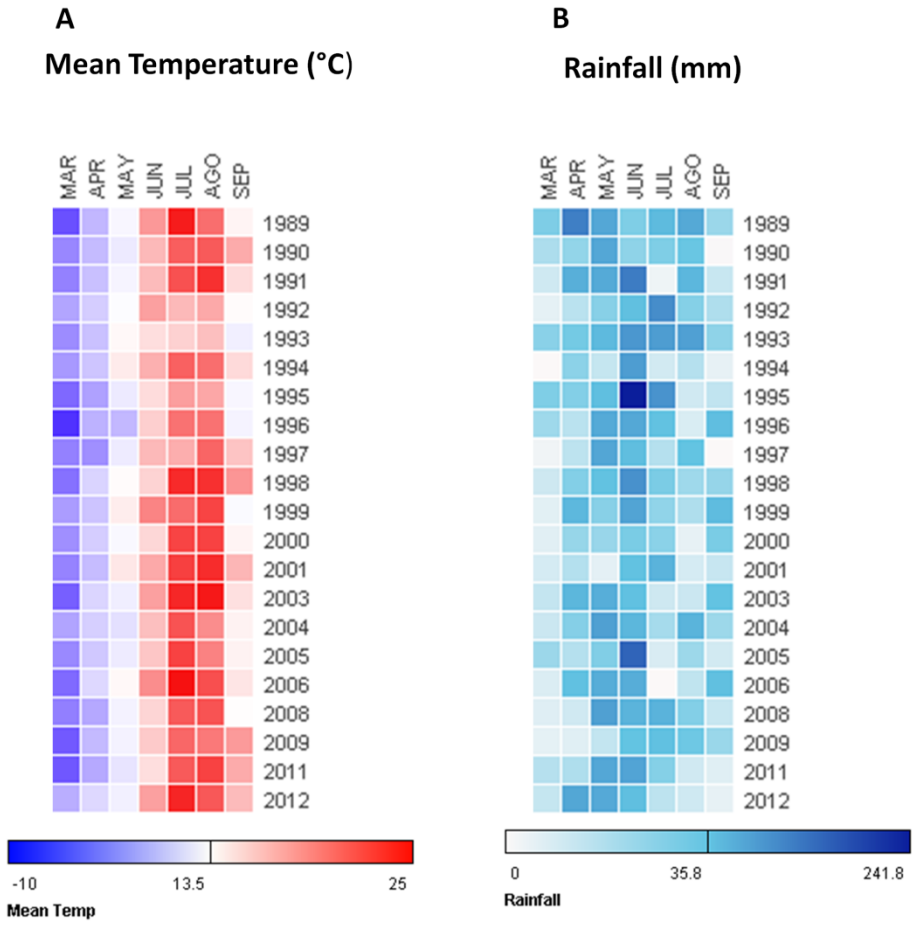

Figure 1: Heat maps showing the temperature (A) and precipitation (B) recorded for the trial site during the years of the study. Values are the means (A) and totals (B) for a month periods.

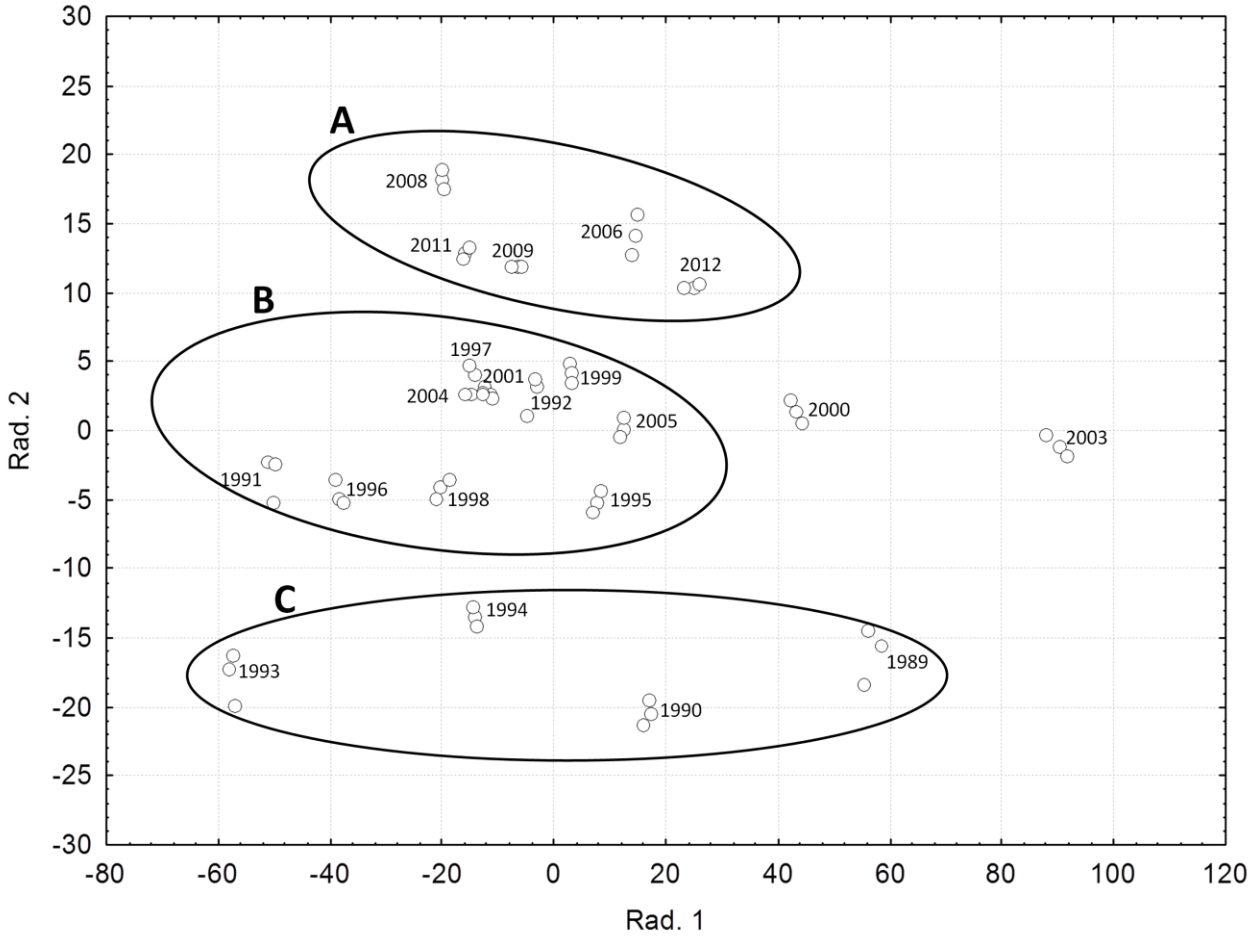

Figure 2: Scatterplot of the KAMUT khorasan grains collected during the twenty-one years of cultivation according to the nutrient and phytochemical content defined by the first two canonical functions (Root 1and Root 2)

SECTION 2

SECTION 2

Study of phytochemical profile of khorasan wheat (*T. turgidum* ssp *turanicum*): spatial analyses of KAMUT® production

1. Introduction

The recent attention to environmental, economical and social problems has increased the interest in organic food products. Several epidemiological studies have evidenced that regular consumption of whole wheat derived staple food has positive effects on health, as it contributes to proper regulation of blood glucose levels and the management of obesity. It was also demonstrated that whole wheat consumption helps to decrease cardiovascular mortality and the incidence of colorectal cancer (Gil et al., 2011; Sahyoun et al., 2006; Truswell, 2002). KAMUT® khorasan is the trademark of an ancient wheat cultivar, the khorasan wheat (*Triticum turgidum* ssp. *turanicum* (Jakubz.)) derived from a natural hybrid between *T. durum* and *Triticum polonicum*, which occurred in the Fertile Crescent (Khlestkina et al., 2006). Cultivation of KAMUT® khorasan wheat is exclusively managed by a license agreement which requires organic certification of the crop and several quality specifications related both to nutritional characteristics and growing conditions (Quinn, 1999). In particular, the grain must be the ancient khorasan variety of wheat, never hybridised or cross, grown only as a certified organic grains, untouched by modern varieties of wheat to 99%, have a protein content between 12 and 18%, be free of signs of disease at 98% and contain between 400 and 1000 ppb of selenium. So far these specific standards are consistently satisfied only in the growing region of North America and in particular in the Upper Great Plains of Alberta, Saskatchewan, Montana and North Dakota (Grausgruber et al., 2004). Durum wheat is produced in virtually every region of the US, where in fact the main cereal grown, occupying an area of about 240000 km² and showing characteristics that vary from region to region. 60% of the total US production of durum wheat is produced in North Dakota, while the remaining 40% is divided among Montana, California, South Dakota and Idaho, with a total production of about 58 million tons per year, making the United States the third largest producer of durum wheat (https://www.ag.ndsu.edu/plantsciences/research/durum/production, 2012;

"United States wheat Production", 2002). In Canada, an area of about 100,000 square kilometers, produces about 27 million tons of wheat a year, making the country the seventh largest producer in the world, mainly due to agricultural activity carried out in Alberta and Saskatchewan (http://www.albertawheat.com/about-wheat/canada/, 2013). It is known that wheat is a species adapted to different agricultural environments and end-uses, and is exposed to variable environmental conditions, both within and over several crop years (Graybosch et al., 1995). The manifestation of those environmental conditions in the developing caryopsis may affects the quality of the crop by influencing yield, grain characteristics and flour quality (Altenbach et al., 2003). In particular, fibre components have been commonly considered as inherent characteristics, although climatic conditions may have a certain effect on the grain fibre quantity and quality, by influencing grain size and the proportional amounts of each kernel tissue (endosperm, aleurone, germ) (Gebruers et al., 2008; Shewry et al., 2010). In addition, the growing environmental conditions are known to strongly influence the expression levels of plant secondary metabolites and, therefore, may affect the accumulation of phenolic compounds during kernel development (Mpofu et al., 2006; Shewry et al., 2010). Currently, few data about nutritional and functional composition of organically grown KAMUT® khorasan grains are available in the literature, especially regarding the changes as a function of cropping year and growing location. Moreover, this is the first study in which an organically grown crop is investigated in a region of such broad extension, including several different environments.

The research aimed at determining the nutrient, fibre and antioxidant composition of KAMUT® grain as a function of the growing location and understanding the environmental dynamics affecting the phytochemical profile. The study involved an area covering approximately 180000 km^2 (Canada and USA) which included several different farms and environments. The grain collection consisted of grain samples harvested at 109 different locations in two cropping years, 2010 and 2011. Each grain sample was analyzed for the antioxidant compounds (polyphenols, flavonoids) and dietary fibre components (soluble and insoluble dietary fibres, resistant starch) and results were elaborated

using Geographic Information System (GIS) to develop *quality maps*. Moreover, the elaboration of data concerning grain yield, test weight and protein content allowed a comparison between the agronomic performance and the nutritional/nutraceutical profile of KAMUT® grain. The study, however, is in progress and results obtained here will be integrated with the third year of KAMUT® khorasan cultivation.

2. Material and Methods

Grain samples and chemicals

The study was carried out using a collection of KAMUT® khorasan wheat (*Triticum turgidum* spp. *turanicum*) harvested in North America in 2010 and 2011. The collection consisted of 109 samples for the first year and 109 for the second. Each sample identified a KAMUT® khorasan producer and was cropped in accordance with the strictly specifications required by Kamut International, Ltd. Whole-grain samples were ground to semolina using a domestic stone mil (100% flour extraction) (Billy 200, Hawos Mulini, Bad Homburg, Germany). All determinations were replicated three times and results expressed on a dry weight (DW) basis. Folin-Ciocalteu, gallic acid and catechin reagent were purchased from Sigma-Aldrich (St Louis, MO, USA). All other chemicals and solvents were of analytical grade.

Starch analysis

Starch was measured using a Megazyme assay kit (Megazyme International Ireland Ltd, Wicklow, Ireland), as described by Di Silvestro et al., (2012). Resistant starch (RS) was determined with the Megazyme assay kit (Megazyme International Ireland Ltd), as detailed previously by Marotti et al., (2012). After an a-amylase and amyloglucosidase overnight digestion, soluble starch was removed with consecutive washes of 95% and 50% ethanol. The pellet was dissolved in KOH solution, hydrolysed with amyloglucosidase and spectrophotometrically quantified using GOPOD reagent.

Dietary Fibre components

Total (TDF), insoluble (IDF) and soluble (SDF) die- tary fibre contents were determined following the enzy- matic/gravimetric method described by Prosky et al. (1988) using a Megazyme assay kit (Megazyme Inter- national Ireland Ltd) The procedure was based on the sequential enzymatic digested using heat stable α-amylase, protease and amyloglucosidase, allowing the determination of IDF, SDF and TDF amounts as previously described (Di Silvestro et al., 2012).

Phenolic compounds

Free and bound phenolic compounds were extracted as previously described by Dinelli et al., (2011). Whole grain flours were extracted with cold 80% ethanol (4°C) in order to dissolve the free soluble compounds, followed by acid and alkaline hydrolyses to release the bound forms. Extracts were analysed for the polyphenol quantification following the colorimetric procedure based on the Folin–Ciocalteu reagent, as described by Singleton et al., (1999). Furthermore, extracts containing free and bound phenolic compounds were analysed for flavonoid content following the spectrophotometric method previously described (Dinelli et al., 2011). The absorbance values were converted using gallic acid and catechin as standard for polyphenols and flavonoid, respectively.

Statistical analysis

Whole data set of 2010 and 2011 KAMUT® khorasan production were elaborated using open source QGIS 2.6.1 - Brighton software. Two different type of maps were elaborated using two different geostatistical methods, the circles around points and interpolation using the algorithms inverse distance to a power. One-way analysis of variance (ANOVA) in conjunction with Tukey's honest significant difference was performed for comparing the flour samples. Significance between means was determined by least significant difference values for $P < 0.05$. Correlation analyses was performed on the standardised matrix of the nutrient and phytochemical content of 2010 and 2011 data set using Statistica 6.0 software (2001, StatSoft, Tulsa, OK, USA). One-way analysis of variance (ANOVA) in conjunction with Tukey's honest significant difference was performed for comparing the flour samples. Significance

between means was determined by least significant difference values for $P < 0.05$

3. Results and discussion

Yield and commercial quality

The main agronomic traits and commercial quality parameters recorded in the present work are yield and protein content.(Table 1). Grain yield significantly varied among samples harvested in 2010 and ranged from 0.3 to 2.3 t/ha, with a mean values of 1.3 t/ha (Table 1). Data distribution is not in agreement with normal distribution. Most of the farms (47%) had grain yield comprised within 1.0-1.5 t/ha. In 30 farms out of 108 (28%) the grain yield was higher than the mean value observed for the whole data set. KAMUT® khorasan grains cropped in 2011 showed a mean values of 1.1 t/ha, slightly lower than 2010. The range of production was comprised between 0.1 and 2.2 t/ha (Table 1). Data distribution is in agreement with normal distribution. Most of the farms (70%) had grain yield comprised within 0.5-1.5 t/ha. In 56 farms out of 109 (51%) the grain yield was higher than the mean value observed for the whole data set. *Quality maps* showed a sort of gradient of yield grains moving from east to west of the KAMUT® khorasan region of production (Figure 1). Grain yield recorded in samples cropped in 2010 was positive correlated with total starch ($r<0.3$). In addition a negative correlation was observed between yield and free and total polyphenols ($-0.3<r<0$). Grain yield of 2011 showed a positive correlation with altitude, protein content and test weight. As evidenced in literature, the environmental conditions significantly affected the grain yield. In facts, the air temperature during grain filling may affect translocation rates of carbon and nitrogen compounds, determining the yield and the final protein concentration of wheat kernel (Vaccari et al., 2007).

Protein content of KAMUT® khorasan production in 2010 ranged from 11% to 16% with an average value of 13% (Table 1). Great variability was observed among samples analysed. In 42 farms out of 108 (38%) the protein content was higher than the mean value observed for the whole data set. In 2011 the mean protein content was 12% and ranged from 9% to 16% (Table 1). Data

distribution was in agreement with normal distribution, and in the same way observed in 2010 samples, there was a huge range for protein. In 52 farms out of 109 (48%) the protein content was higher than the mean value observed for the whole data set. The protein accumulation was shown to be highly dependent on genotype, soil fertility (available nitrogen) and environmental conditions (temperature, water) (Dupont et al., 2006; Giuliani et al., 2011). Generally a negative correlation would be expected between grain yield and protein content (Blanco et al., 2012; Kibite and Evans, 1984). In the present study this statement was confirmed only by 2011 production which evidenced a negative correlation between protein content and grain yield. In facts, farms located in the East area providing the lowest yield but the highest protein levels. Instead, the farms located on the West margin provided the KAMUT® grains richest in proteins while maintaining high productivity (Figure 3, 4). Further studies are necessary to fully understand the dynamics affecting the grain yield level and protein content, also taking into account temperature, precipitations and soil structure.

Total and resistant starch

Starch is the most important polysaccharide in human diet and is the major component of wheat caryopsis, representing more than 70% of its dry weight. Resistant starch (RS) is a starch fraction that cannot be digested in the small intestine, and it ferments in the large intestine resulting in the production of hydrogen, carbon dioxide, methane and short chain fatty acids (Mann et al., 2007). In the present study RS content observed for the 2010 was comprised between 0.22 and 1.78 g/100g and data distribution was not in agreement with normal distribution (Table 1). In 57 farms out of 109 (52%) the resistant starch content was higher than the mean value (0.65 ± 0.21 g/100g) observed for the whole data set. Samples harvested in 2011 showed a lower average content of RS (0.55 g/100g) in comparison with 2010 samples. Data distribution is not in agreement with normal distribution and ranged from 0.18 g/100g to 2.15 g/100g (Table 1). A huge range for RS was observed. In facts, 50 farms out of 109 (47%) showed a RS content higher than the mean value observed for the whole data set. Comparing with other studies in literature, the obtained RS content was lower (Di Silvestro et al., 2014; Marotti et al., 2012). The total starch (TS) amount obtained in 2010 grains varied within a huge range (52.22 - 69.19

g/100g) but in most of the farms (56%) the starch content aligned with the mean value observed for the whole data set (63.6 ± 2.9 g/100g) and only 36 locations showed higher TS values (Table 1). This is reflected in the *quality map* reported in Figure 7, as the majority of the production area provided KAMUT® grain with starch levels comprised between 61.0 and 67.0 g/100g (green/blue colours). The comparison between samples cropped in 2010 and 2011 showed significant differences in the TS content. In particular, KAMUT® khorasan production of 2011 showed an average content higher TS content if compared with the 2010 one (Table 1). As previously evidenced in literature a negative correlation exists between protein and starch content in the wheat grain and this statement was confirmed by the correlation analyses in the 2011 KAMUT® khorasan production (Hucl and Chibbar, 1996). Moreover, also GIS map confirmed this statement, with the area of the region that was characterized by the lowest protein content provided the highest starch amounts (Figures 6, 8).

Dietary fibre

Dietary fibre is the predominant bioactive component of whole wheat grain. Due to its several health benefits, which include prevention of colon cancer, prebiotic activity and modulation of blood glucose and insulin levels, dietary fibre makes a big contribution to the nutraceutical value of wheat-based products (Charalampopoulos et al., 2002). As regards dietary fibre content, there was a significant difference ($p<0.001$) between the wheat varieties cropped in 2010 and in 2011 in terms of insoluble fibre components (Table 1). Grain samples harvested in 2010 ranged from 11.16 g/100g to 19.15 g/100g with a mean value of 14.29 g/100g. KAMUT® khorasan cropped in 2011 showed an average insoluble dietary fibre (IDF) content of 16.13 g/100g, ranging between 10.76 g/100g to 23.05 g/100g (Table 1). Great variability was observed among data distribution in the year for what concern IDF amounts (Figure 9). In 60 farms out of 108 (55%) the IDF content was higher than the mean value observed for the whole data set. Data distribution for the year 2011, instead is in agreement with normal distribution. In 58 farms out of 109 (53%) the IDF content was higher than the mean value observed for the whole data set. As regards the soluble dietary (SDF) components, data distribution for samples cropped in 2010 was in agreement with normal distribution and the mean values was 3.27

g/100g (Table 1). The SDF values were comprised within a huge range of variation. In 42 farms out of 109 (38%) the SDF content was higher than the observed mean value and they are located throughout the production area, from East to West (Figure 11). Significant differences ($p<0.001$) were observed comparing the two years of cultivation, with samples harvested in 2011 showing a higher content of soluble dietary fibre. This content ranged between 1.99 g/100g and 6.43 g/100g, with a mean values of 3.64 g/100g (Table 1). The total dietary fibre (TDF) content of KAMUT® khorasan harvested in 2010 varied within a huge range of values (13.58-22.62 g/100g) and data distribution was not in agreement with normal distribution (Table 1). In 44 farms out of 109 (40%) the TDF amount was higher than the mean value observed for the whole data set (17.58 g/100g). Once again significant differences ($p<0.001$) were observed comparing the two years of cultivation. Grain samples from 2011 showed a mean value higher (19.76 g/100g) than that of 2010 (Table 1). The quality map obtained for TDF was highly influenced by the IDF contribution and again evidenced the highest accumulation of fibre components in the central area of the region (Figures 13, 14). Data obtained in the present study however were in line with previous studies for what concerns the year 2010, while data from 2011 were higher (Gebruers et al., 2008; Marotti et al., 2012). These results again underline the important effect of climate and growing factors and the need for in-depth investigation of the environment effects on the accumulation of fibres in the caryopsis.

Antioxidant compounds

Polyphenols are the most representative class of antioxidant in wheat, mainly concentrated in the outer layer of the kernel. They are for the most part made up of phenolic acids and flavonoids and exert high antioxidant activity acting as radical scavengers (Dinelli et al., 2009). Moreover, their role in the prevention of cancer and several chronic diseases were widely demonstrated in literature (Carter et al., 2006; Fardet, 2010). In wheat, phenolic compounds exist in the soluble (free) and insoluble (bound) forms. Both phenolic fractions have been shown to possess valuable health–promoting properties, acting as radical scavengers and preventing several chronic disease. In addition, the bound

fraction, which is crosslinked with cell wall components , may resist upper digestive process and reach the colon where they are liberated by the intestinal microflora. The digested phenolics directly exert their health benefits reducing the incidence of colon cancer; indeed, colonic endothelial cells may absorb the liberated phenolics and gain powerful antioxidant protection (Kroon et al., 1997). In the present work significant differences ($p<0.001$) were observed between 2010 and 2011 samples in the free polyphenols (FP) content, with mean values of 81.62 mg/100g and 68.57 mg/100g, respectively (Table 2). The FP content of 2010 varied within a huge range of values (34.50 - 130.72 mg/100g) and data distribution was in agreement with the Gaussian normal distribution. In 58 farms out of 109 (53%) the free polyphenols content was higher than the mean value observed for the whole set of data. The data distribution of FP content in 2011, instead, was not in agreement with normal distribution with 72 farms out of 109 (64%) containing a FP content lower than the mean value. Regarding the bound polyphenols content in 2010 a huge range of variation was observed and it was comprised between 56.59 and 115.33 mg/100g (Table 2). 65% of the farms (75 out of 109) showed bound phenolic amounts higher or aligned with the mean values observed for the whole data set (84.84 mg/100g). In 2011, data distribution was in agreement with the Gaussian distribution. In 50 farms out of 109 (46%) the BP content was higher than the mean value observed for the whole data set (88.32 mg/100g). The total polyphenol (TP) content in samples harvested in 2010 ranged from 117.24 to 223.38 mg/100g and data distribution was not in agreement with normal distribution (Table 2). Since the total polyphenol amount was given by the sum of the free and bound fractions for which a normal distribution was observed, it is plausible that some locations favoured the accumulation of both free and bound polyphenols. TP amount in KAMUT® khorasan cropped in 2011 was significantly lower than 2010 production, with an average values of 156.90 mg/100g and 166.46 mg/100g, respectively (Table 2). In 2011 KAMUT® khorasan production, data distribution was in agreement with Gaussian normal distribution. In 54 farms out of 109 (50%) the TP content was higher than the mean value observed for the whole data set. Data obtained in the present work were slightly lower than reported in literature by Di Silvestro et al., (2014), but higher than reported by Fares et al.,

(2010) and Dinelli et al., (2009). Considering the *quality map* obtained for FP and BP (Figures 15, 16, 17, 18), a sort of compensation between the two phenolic fractions was observed. In particular, locations with high FP content exhibited a general low BP amount and vice-versa. Concerning flavonoids, the range of variation observed for the free fraction of 2010 samples was narrow with respect to free polyphenols and ranged between 14.04 and 72.67 mg/100g (Table 2). Most of the farms (51 out of 109) produced KAMUT® grain samples with free flavonoid amount comprised within 20-40 mg/100g, while in 35 locations (32% of the total farms) the FF content was higher than the mean value observed for the whole data set (27.59 mg/100g). The *quality map* clearly shows that most of the investigated areas provide grain with medium free flavonoids levels (Figure 21). In the map, some locations emerged for the highest accumulation of soluble flavonoids and are mainly located in the central area of the map. Significant differences ($p<0.01$) were observed comparing the two years of cultivation, with a higher accumulation of FF in 2010 samples with respect to 2011 (Figures 21, 22). Data distribution is not in agreement with normal distribution and ranged from 4.68 mg/100 to 51.67 mg/100, with a mean value of 23.50 mg/100g (Table 2). In 41% of farms (45 out of 109) the FF content was higher than the mean value observed. The bound flavonoid fraction in 2010 samples ranged from 6.63 and 20.54 mg/100g (Table 2). Data distribution was in agreement with normal distribution, with a mean value of 11.22 mg/100g. In 39 farms out of 109, accounting for 36% of the total locations, the bound flavonoid content was higher than the observed mean value. KAMUT khorasan production in 2011 showed an average BF amount lower than 2010 (14.85 mg/100g), ranging from 0.47 to 39.10 mg/100g (Table 2).. The total flavonoid (TF) determination highlighted that in 2010 KAMUT® accumulates on average 38.81 mg/100g in the kernel. 47 farms out of 109 (43%) showed total flavonoids values higher than the observed mean value. Most of them provided grain samples with TF amounts comprised within 36-49 mg/100g, that is the yellow, red/pale area of the map (Figures 25, 26). As previously observed for polyphenols, there is a sort of compensation between the soluble and insoluble flavonoid fractions that results in a quite homogenous distribution of total flavonoids values, with the exception of few farms

corresponding to the locations with the highest free flavonoids levels. In 2011 KAMUT® khorasan showed a total flavonoids amount similar than 2010, with a mean value of 38.35 mg/100g. The range of the TF amounts was comprised between 18.51 and 84.72 mg/100g (Table 2). In addition 48 farms out of 109 showed a content higher than the mean value observed for the whole data set. Data obtained in the present work for TF were in line with those reported in literature (Di Silvestro et al., 2014) and higher than reported by Dinelli et al., (2009).

4. Conclusion

It raises a great deal of recent interest that organic wheat constitutes valuable nutraceutical raw material for healthy food production and special dietary uses. In particular, khorasan wheat (*T. turgidum* ssp. *turanicum)* has attracted great attention because of its specific nutritional and functional properties (antioxidant, antitumoral and prebiotic activities). Currently, few data are available in literature about fibre and antioxidant composition and the changes of their amounts as a function of cropping year and growing location of organically grown KAMUT® khorasan grains. To the best of our knowledge, this is the first study in which an organically grown crop is investigated in a region of such broad extension (180000 km^2), including several different environments (Canada, USA). The research aimed at determining the productivity, nutrient and nutraceutic composition of KAMUT® grain as a function of the growing location and understanding the environmental dynamics affecting the phytochemical profile. The maps showed the variability of the phytochemical amounts among North American farms and allowed the identification of areas in which bioactive compounds had accumulated at a higher level in the wheat grains. The research underlined the strong influence of the environmental factors on crop productivity and quality, in terms of nutritional and nutraceutical value. According to the present results, the choice of a growing location in which the accumulation of health-promoting compounds (i.e. polyphenols, fibres) is stimulated by specific climatic conditions emerges as a key factor for the production of organic wheat foodstuff in the functional food scenario. However this is a 2 year study and further study

are necessary to full understand the environmental factors which affected the accumulation of nutrients and antioxidant compounds in wheat kernel.

5. Bibliography

Altenbach, S.B., DuPont, F.M., Kothari, K.M., Chan, R., Johnson, E.L., Lieu, D., 2003. Temperature, Water and Fertilizer Influence the Timing of Key Events During Grain Development in a US Spring Wheat. J. Cereal Sci. 37, 9–20. doi:10.1006/jcrs.2002.0483

Blanco, A., Mangini, G., Giancaspro, A., Giove, S., Colasuonno, P., Simeone, R., Signorile, A., De Vita, P., Mastrangelo, A.M., Cattivelli, L., Gadaleta, A., 2012. Relationships between grain protein content and grain yield components through quantitative trait locus analyses in a recombinant inbred line population derived from two elite durum wheat cultivars. Mol. Breed. 30, 79–92. doi:10.1007/s11032-011-9600-z

Carter, J.W., Madl, R., Padula, F., 2006. Wheat antioxidants suppress intestinal tumor activity in Min mice. Nutr. Res. 26, 33–38. doi:10.1016/j.nutres.2005.10.005

Charalampopoulos, D., Wang, R., Pandiella, S.S., Webb, C., 2002. Application of cereals and cereal components in functional foods: A review. Int. J. Food Microbiol. 79, 131–141. doi:10.1016/S0168-1605(02)00187-3

Di Silvestro, R., Di Loreto, A., Marotti, I., Bosi, S., Bregola, V., Gianotti, A., Quinn, R., Dinelli, G., 2014. Effects of flour storage and heat generated during milling on starch, dietary fibre and polyphenols in stoneground flours from two durum-type wheats. Int. J. Food Sci. Technol. 49, 2230–2236. doi:10.1111/ijfs.12536

Di Silvestro, R., Marotti, I., Bosi, S., Bregola, V., Carretero, A.S., Sedej, I., Mandic, A., Sakac, M., Benedettelli, S., Dinelli, G., 2012. Health-promoting phytochemicals of Italian common wheat varieties grown under low-input agricultural management. J. Sci. Food Agric. 92, 2800–10. doi:10.1002/jsfa.5590

Dinelli, G., Carretero, A.S., Di Silvestro, R., Marotti, I., Fu, S., Benedettelli, S., Ghiselli, L., Gutiérrez, A.F., 2009. Determination of phenolic compounds in modern and old varieties of durum wheat using liquid chromatography coupled with time-of-flight mass spectrometry. J. Chromatogr. A 1216, 7229–40. doi:10.1016/j.chroma.2009.08.041

Dinelli, G., Segura-Carretero, A., Di Silvestro, R., Marotti, I., Arráez-Román,

D., Benedettelli, S., Ghiselli, L., Fernadez-Gutierrez, A., 2011. Profiles of phenolic compounds in modern and old common wheat varieties determined by liquid chromatography coupled with time-of-flight mass spectrometry. J. Chromatogr. A 1218, 7670–81. doi:10.1016/j.chroma.2011.05.065

Dupont, F.M., Hurkman, W.J., Vensel, W.H., Tanaka, C., Kothari, K.M., Chung, O.K., Altenbach, S.B., 2006. Protein accumulation and composition in wheat grains: Effects of mineral nutrients and high temperature. Eur. J. Agron. 25, 96–107. doi:10.1016/j.eja.2006.04.003

Fardet, A., 2010. New hypotheses for the health-protective mechanisms of whole-grain cereals: what is beyond fibre? Nutr. Res. Rev. 23, 65–134. doi:10.1017/S0954422410000041

Fares, C., Platani, C., Baiano, A., Menga, V., 2010. Effect of processing and cooking on phenolic acid profile and antioxidant capacity of durum wheat pasta enriched with debranning fractions of wheat. Food Chem. 119, 1023–1029. doi:10.1016/j.foodchem.2009.08.006

Gebruers, K., Dornez, E., Boros, D., Fraś, A., Dynkowska, W., Bedo, Z., Rakszegi, M., Delcour, J. a., Courtin, C.M., 2008. Variation in the content of dietary fiber and components thereof in wheats in the healthgrain diversity screen. J. Agric. Food Chem. 56, 9740–9749. doi:10.1021/jf800975w

Gil, A., Ortega, R.M., Maldonado, J., 2011. Wholegrain cereals and bread: a duet of the Mediterranean diet for the prevention of chronic diseases. Public Health Nutr. 14, 2316–2322. doi:10.1017/S1368980011002576

Giuliani, M.M., Giuzio, L., de Caro, A., Flagella, Z., 2011. Relationships between nitrogen utilization and grain technological quality in durum wheat: I. Nitrogen translocation and nitrogen use efficiency for protein. Agron. J. 103, 1487–1494. doi:10.2134/agronj2011.0153

Grausgruber, H., Sailer, C., Ruckenbauer, P., 2004. Khorasan wheat, Kamut® and “Pharaonenkorn”: Origin, characteristics and potential 75–80.

Graybosch, R. a., Peterson, C.J., Baenziger, P.S., Shelton, D.R., 1995. Environmental modification of hard red winter wheat flour protein composition. J. Cereal Sci. 22, 45–51. doi:10.1016/S0733-5210(05)80006-

2

Hucl, P., Chibbar, R.N., 1996. Variation for starch concentration in spring wheat and its repeatability relative to protein concentration. Cereal Chem. 73, 756–758.

Khlestkina, E.K., Roder, M.S., Grausgruber, H., Borner, A., 2006. A DNA fingerprinting-based taxonomic allocation of Kamut wheat. doi:10.1079/PGR2006120

Kibite, S., Evans, L.E., 1984. Causes of negative correlations between grain yield and grain protein concentration in common wheat. Euphytica 33, 801–810. doi:10.1007/BF00021906

Kroon, P.A., Faulds, C.B., Ryden, P., Robertson, J.A., Williamson, G., 1997. Release of Covalently Bound Ferulic Acid from Fiber in the Human Colon. J. Agric. Food Chem. 45, 661–667. doi:10.1021/jf9604403

Mann, J., Cummings, J.H., Englyst, H.N., Key, T., Liu, S., Riccardi, G., Summerbell, C., Uauy, R., van Dam, R.M., Venn, B., Vorster, H.H., Wiseman, M., 2007. FAO/WHO scientific update on carbohydrates in human nutrition: conclusions. Eur. J. Clin. Nutr. 61 Suppl 1, S132–S137. doi:10.1038/sj.ejcn.1602943

Marotti, I., Bregola, V., Aloisio, I., Di Gioia, D., Bosi, S., Di Silvestro, R., Quinn, R., Dinelli, G., 2012. Prebiotic effect of soluble fibres from modern and old durum-type wheat varieties on Lactobacillus and Bifidobacterium strains. J. Sci. Food Agric. 92, 2133–40. doi:10.1002/jsfa.5597

Mpofu, A., Sapirstein, H.D., Beta, T., 2006. Genotype and environmental variation in phenolic content, phenolic acid composition, and antioxidant activity of hard spring wheat. J. Agric. Food Chem. 54, 1265–1270. doi:10.1021/jf052683d

Quinn, R.M., 1999. Kamut: ancient grain, new cereal. Perspect. new Crop. new uses 182–183.

Sahyoun, N.R., Jacques, P.F., Zhang, X.L.L., Juan, W.Y., McKeown, N.M., 2006. Whole-grain intake is inversely associated with the metabolic syndrome and mortality in older adults. Am. J. Clin. Nutr. 83, 124–131.

Shewry, P.R., Piironen, V., Lampi, A.M., Edelmann, M., Kariluoto, S., Nurmi, T., Fernandez-Orozco, R., Ravel, C., Charmet, G., Andersson, A. a M.,

Åman, P., Boros, D., Gebruers, K., Dornez, E., Courtin, C.M., Delcour, J. a., Rakszegi, M., Bedo, Z., Ward, J.L., 2010. The HEALTHGRAIN wheat diversity screen: Effects of genotype and environment on phytochemicals and dietary fiber components. J. Agric. Food Chem. 58, 9291–9298. doi:10.1021/jf100039b

Singleton, V.L., Orthofer, R., Lamuela-Raventós, R.M., 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods Enzymol., Methods in Enzymology 299, 152–178. doi:10.1016/S0076-6879(99)99017-1

Truswell, A.S., 2002. Cereal grains and coronary heart disease. Eur. J. Clin. Nutr. 56, 1–14. doi:10.1038/sj.ejcn.1601283

Vaccari, F.P., Ranieri, R., Matese, A., Miglietta, F., 2007. Enhanced Temperature During Grain Filling Reduces Protein Concentration of Durum Wheat 393–400.

Sitography

https://www.ag.ndsu.edu/plantsciences/research/durum/production, 2012; "United States wheat Production", 2002

http://www.albertawheat.com/about-wheat/canada/, 2013

Tables

Table 1: Range of concentration and mean values of agronomical traits, starch and dietary fibre components. Different letters in a column indicate statistically different values.

Years of production	Yield (t/ha)	Protein (%)	Total Starch (g/100g)	Resistant Starch (g/100g)	Insoluble Dietary Fibre (g/100g)	Soluble Dietary Fibre (g/100g)	Total Dietary Fibre (g/100g)
2010	0.28-2.81	11-16	52.22-69.19	0.22-1.78	11.16-19.15	1.26-4.89	13.58-22.62
	(1.30) a	**(13) a**	**(63.56) b**	**(0.65) a**	**(14.29) b**	**(3.27) b**	**(17.57) b**
2011	0.15-2.17	9-16	55.94-71.98	0.18-2.15	10.76-23.05	1.99-6.43	13.86-26.09
	(1.05) b	**(12) b**	**(65.29) a**	**(0.55) b**	**(16.13) a**	**(3.64) a**	**(19.76) a**

Table 2: Range of concentration and mean values of antioxidant compounds. Different letters in a column indicate statistically different values.

Years of production	Free Polyphenols (mg/100g)	Bound Polyphenols (mg/100g)	Total Polyphenols (mg/100g)	Free Flavonoids (mg/100g)	Bound Flavonoids (mg/100g)	Total Flavonoids (mg/100g)
2010	34.50-130.72	56.59-115.33	117.24-223.38	14.04-72.67	6.63-20.54	22.45-83.60
	(81.62) a	**(84.84) a**	**(166.46) a**	**(27.59) a**	**(11.22) b**	**(38.81) a**
2011	41.21-131.11	44.00-143.64	98.57-217.69	4.68-51.67	0.47-39.10	18.51-84.72
	(68.57) b	**(88.32) a**	**(156.90) b**	**(23.50) b**	**(14.85) a**	**(38.35) a**

Figures

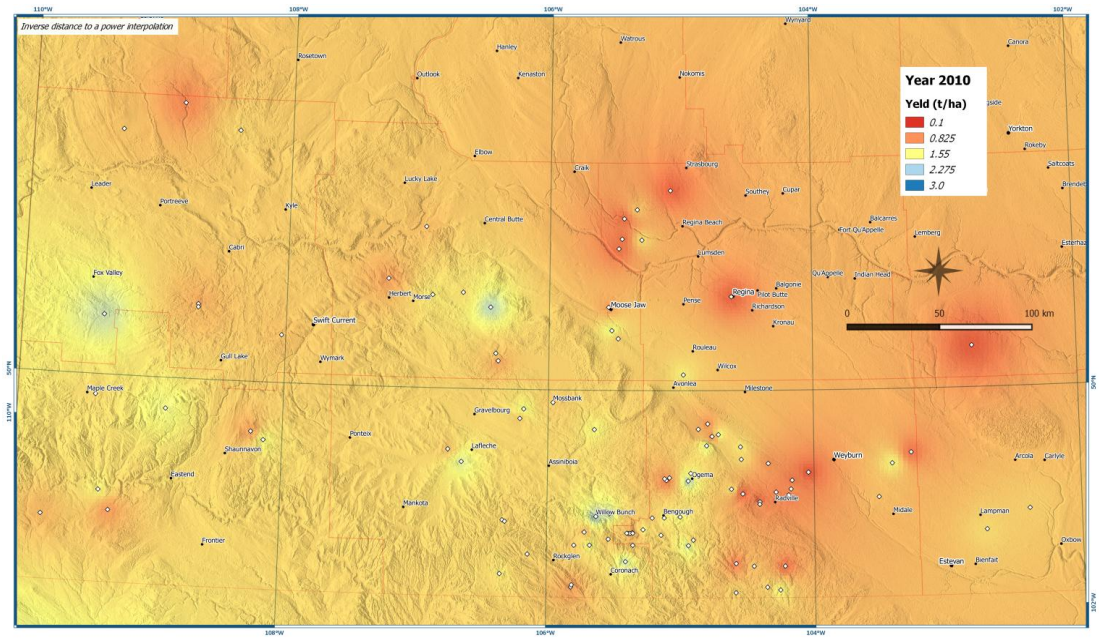

Figure 1: GIS map obtained for grain yield of KAMUT® collection harvested in North America in 2010 (red colour = low; blue colour = high).

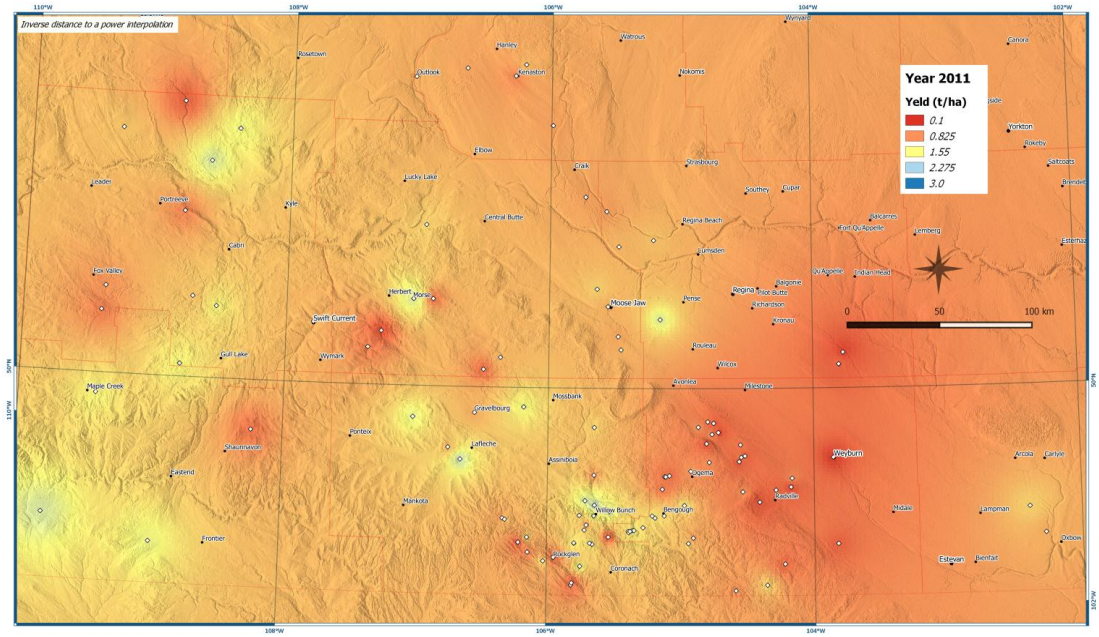

Figure 2: GIS map obtained for grain yield of KAMUT® collection harvested in North America in 2011 (red colour = low; blue colour = high).

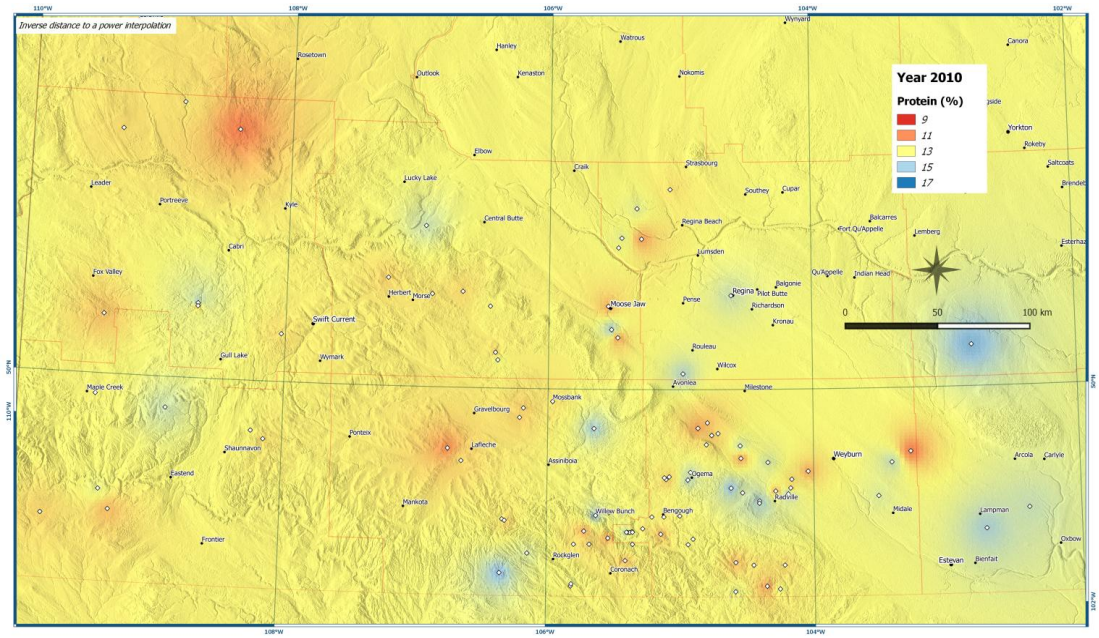

Figure 3: GIS map obtained for protein content of KAMUT® collection harvested in North America in 2010 (red colour = low; blue colour = high).

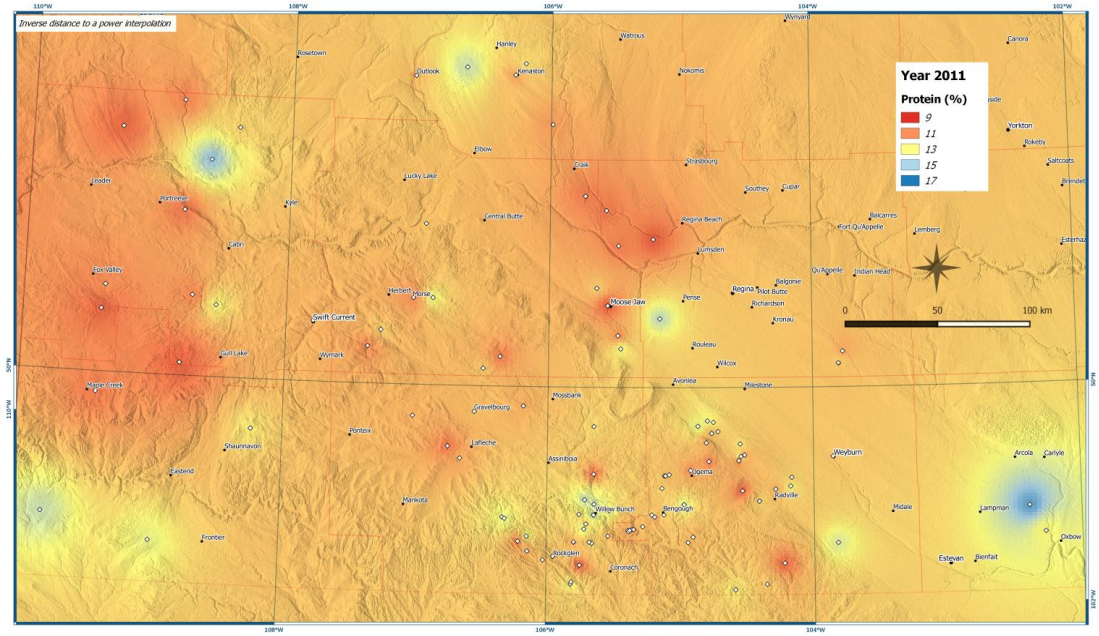

Figure 4: GIS map obtained for protein content of KAMUT® collection harvested in North America in 2010 (red colour = low; blue colour = high).

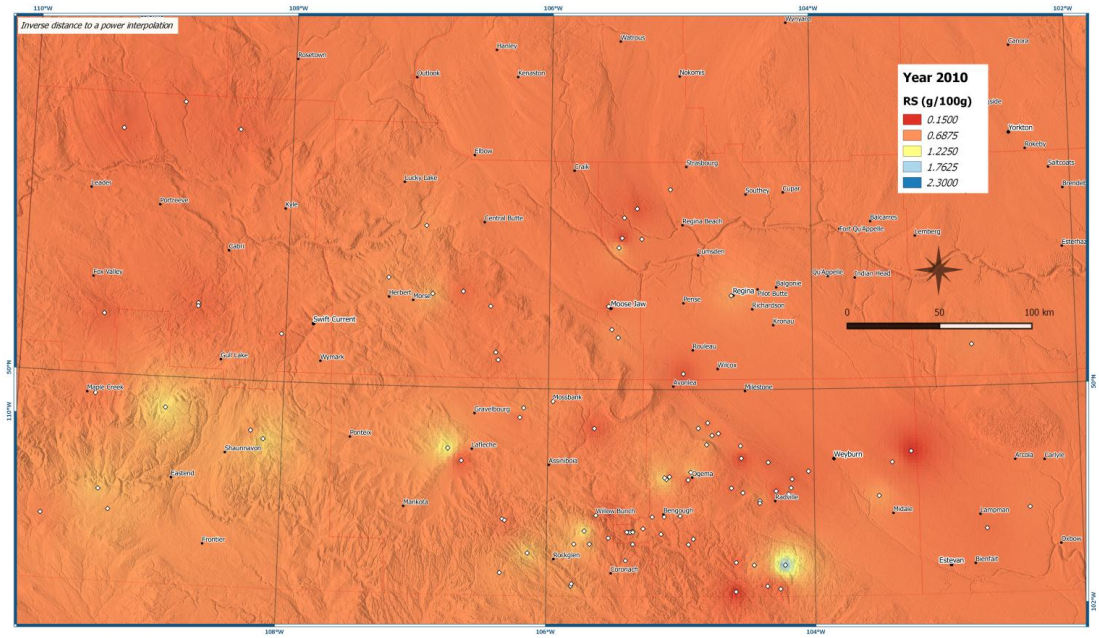

Figure 5: GIS map obtained for resistant starch content of KAMUT® collection harvested in North America in 2010 (red colour = low; blue colour = high).

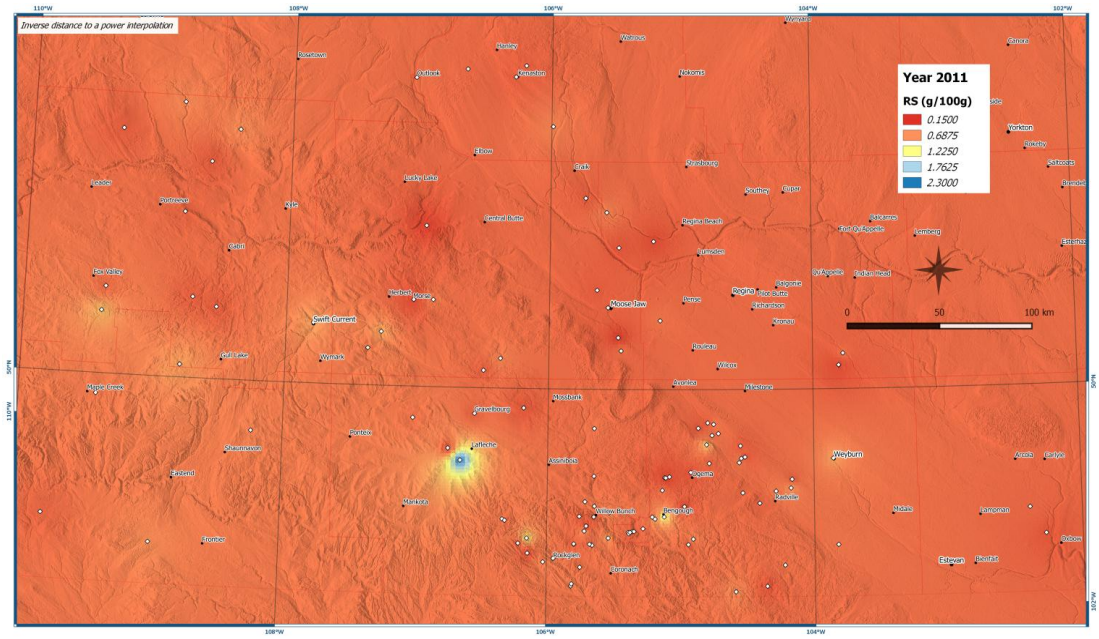

Figure 6: GIS map obtained for resistant starch content of KAMUT® collection harvested in North America in 2011 (red colour = low; blue colour = high).

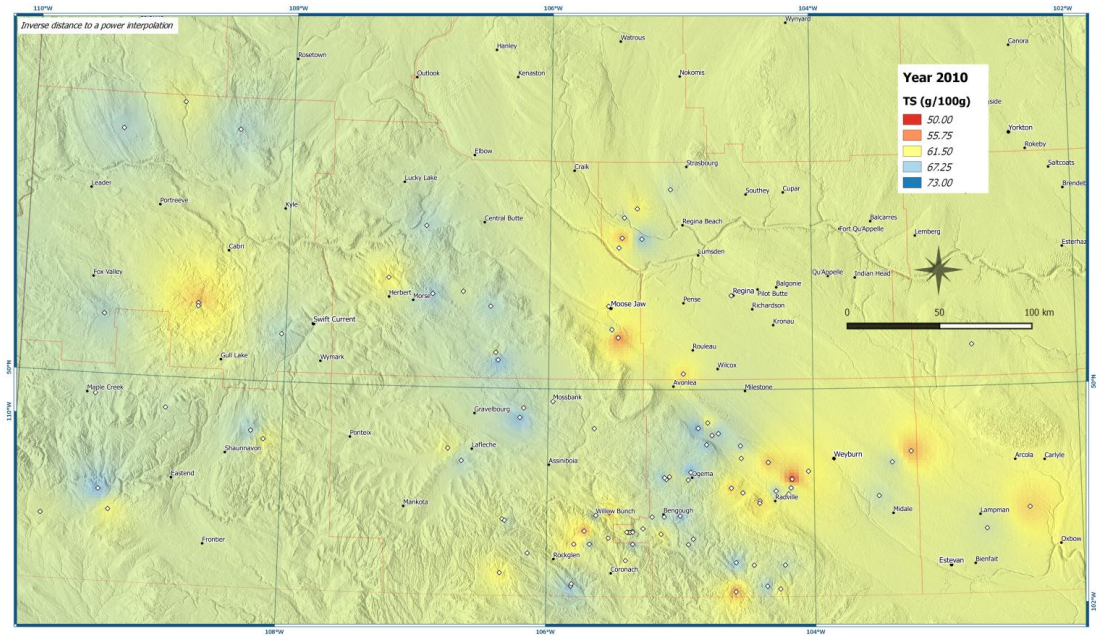

Figure 7: GIS map obtained for total starch content of KAMUT® collection harvested in North America in 2010 (red colour = low; blue colour = high).

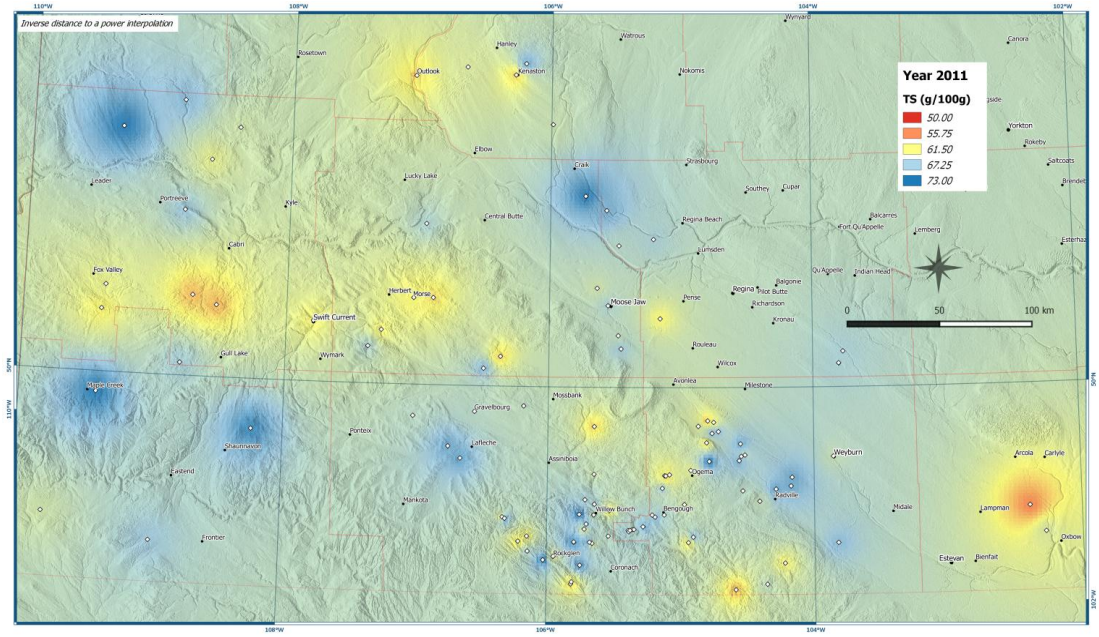

Figure 8: GIS map obtained for total starch content of KAMUT® collection harvested in North America in 2011 (red colour = low; blue colour = high).

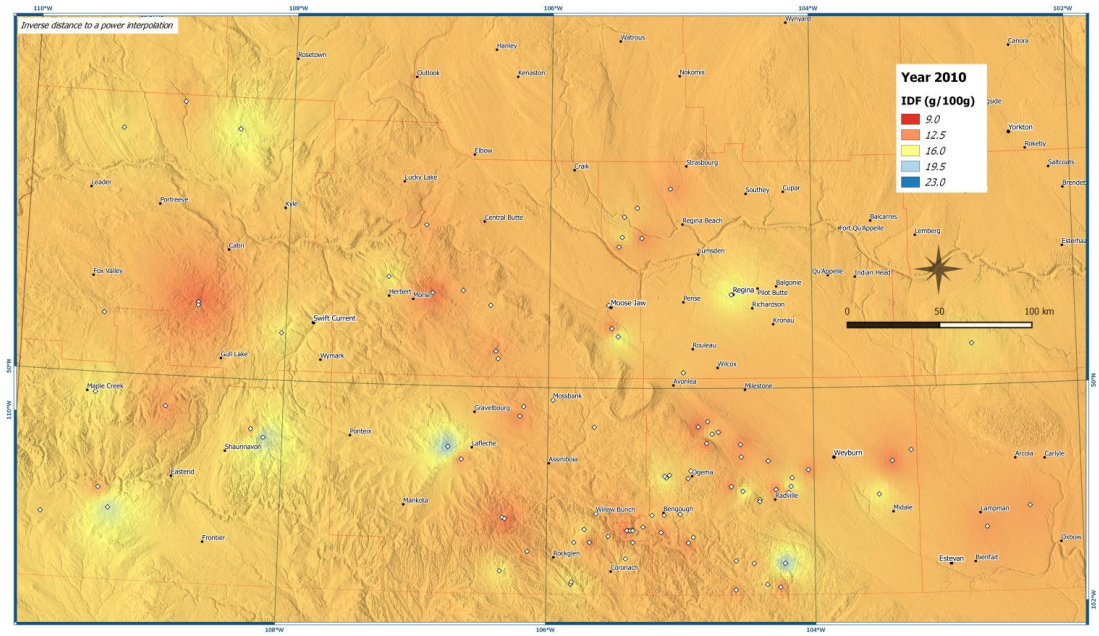

Figure 9: GIS map obtained for Insoluble Dietary Fibre content of KAMUT® collection harvested in North America in 2010 (red colour = low; blue colour = high).

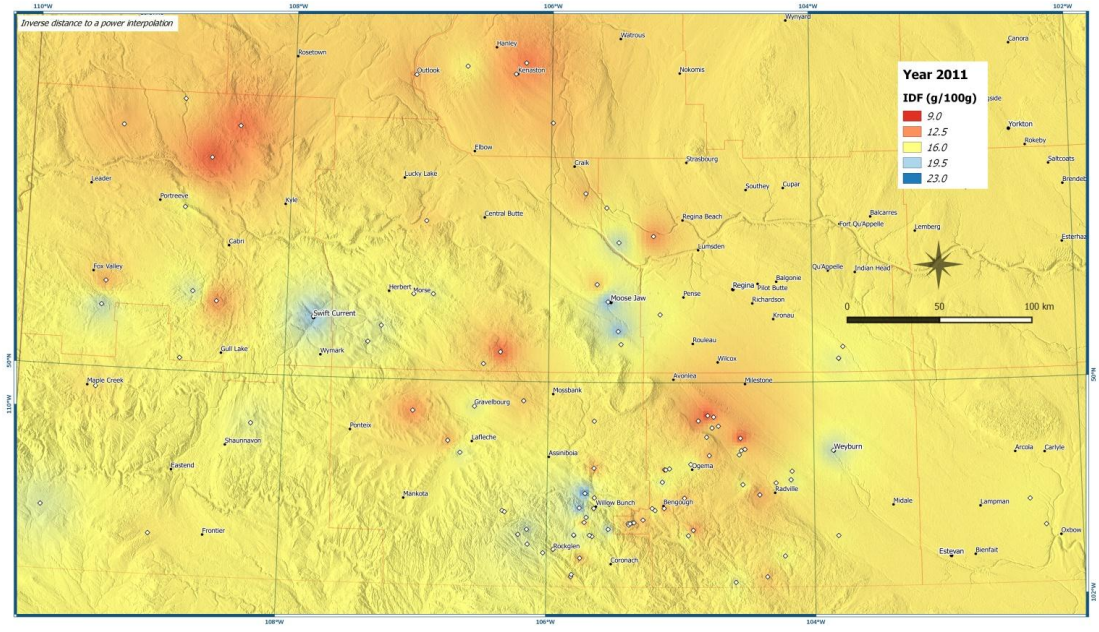

Figure 10: GIS map obtained for Insoluble Dietary Fibre content of KAMUT® collection harvested in North America in 2011 (red colour = low; blue colour = high).

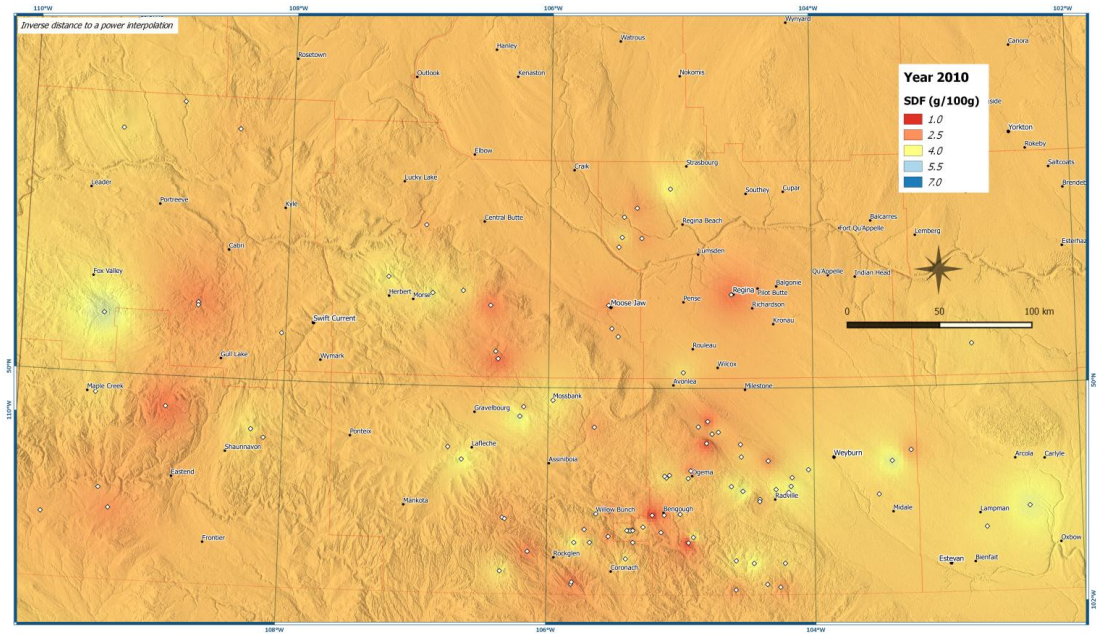

Figure 11: GIS map obtained for Soluble Dietary Fibre content of KAMUT® collection harvested in North America in 2010 (red colour = low; blue colour = high).

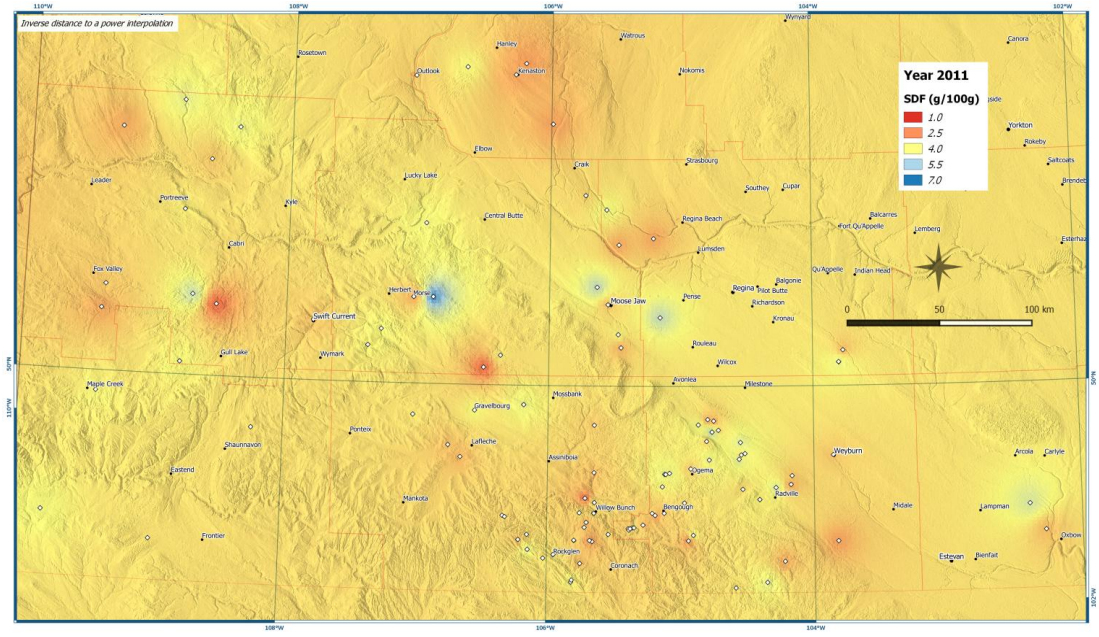

Figure 12: GIS map obtained for Soluble Dietary Fibre content of KAMUT® collection harvested in North America in 2011 (red colour = low; blue colour = high).

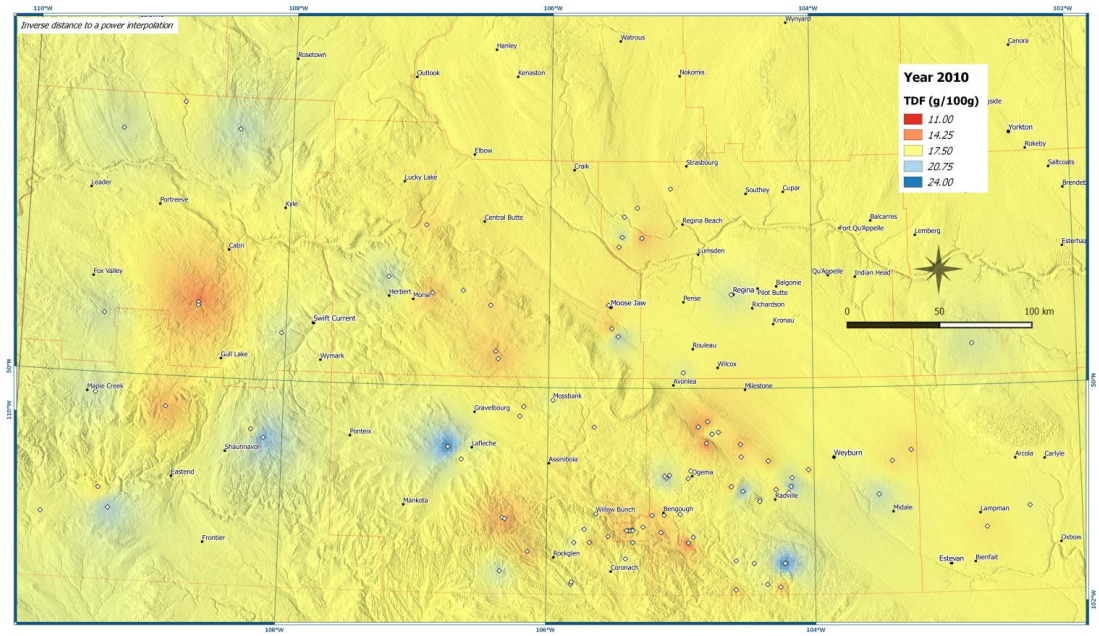

Figure 13: GIS map obtained for Total Dietary Fibre content of KAMUT® collection harvested in North America in 2010 (red colour = low; blue colour = high).

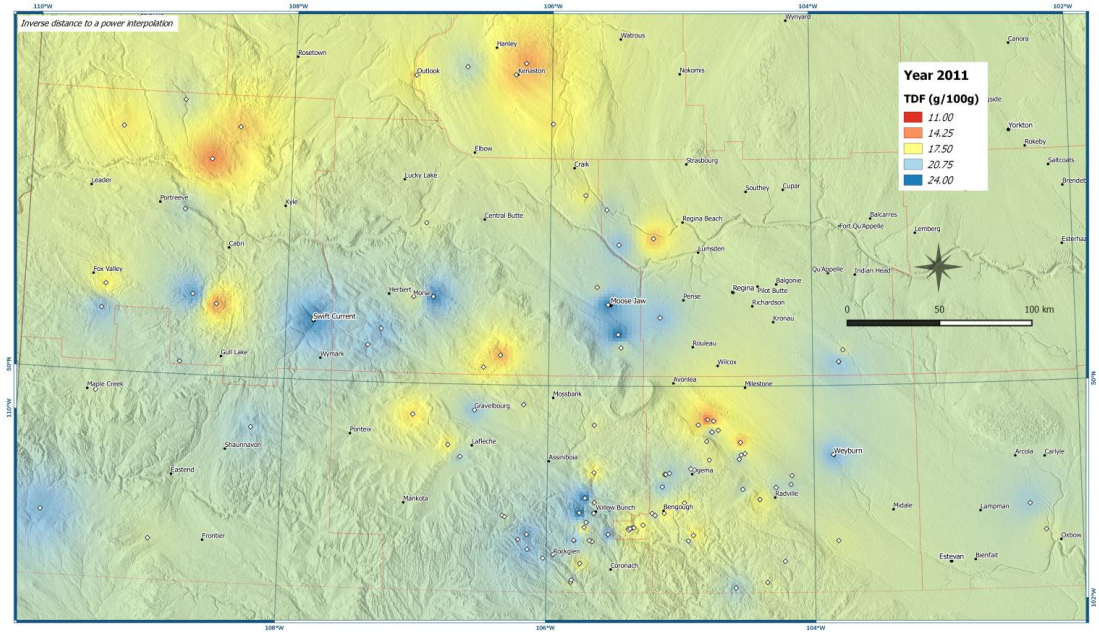

Figure 14: GIS map obtained for Total Dietary Fibre content of KAMUT® collection harvested in North America in 2011 (red colour = low; blue colour = high).

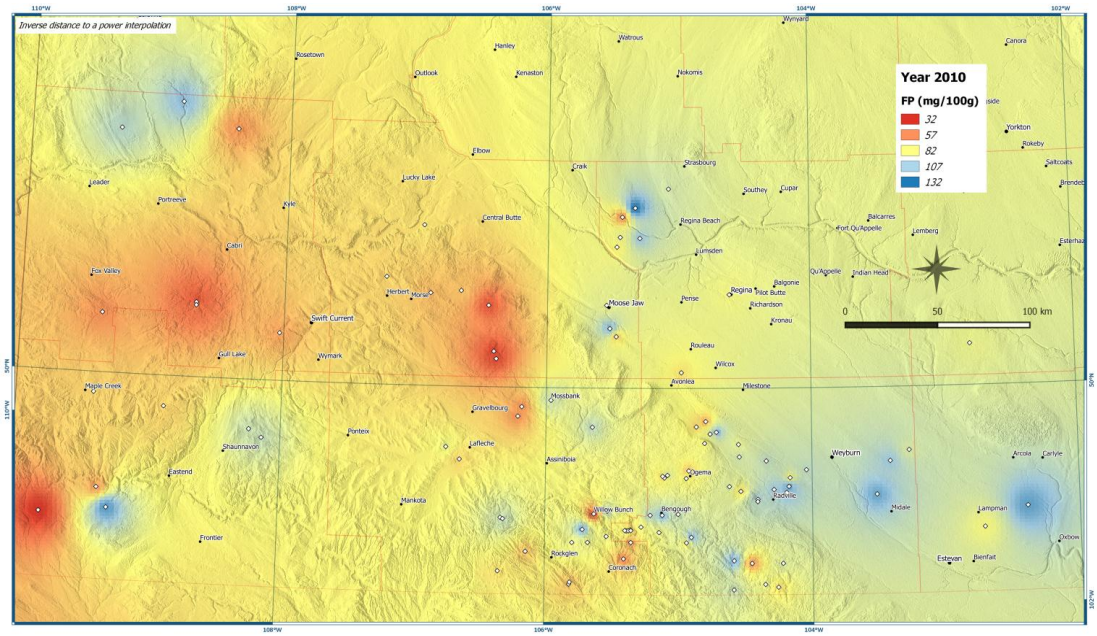

Figure 15: GIS map obtained for free polyphenols content of KAMUT® collection harvested in North America in 2010 (red colour = low; blue colour = high).

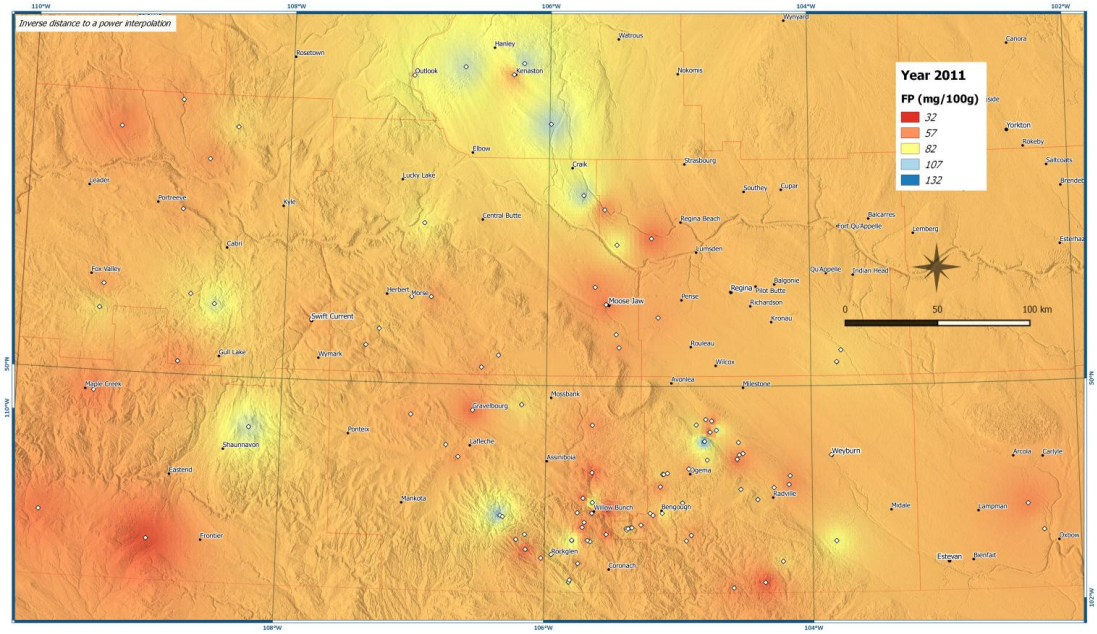

Figure 16: GIS map obtained for free polyphenols content of KAMUT® collection harvested in North America in 2011 (red colour = low; blue colour = high).

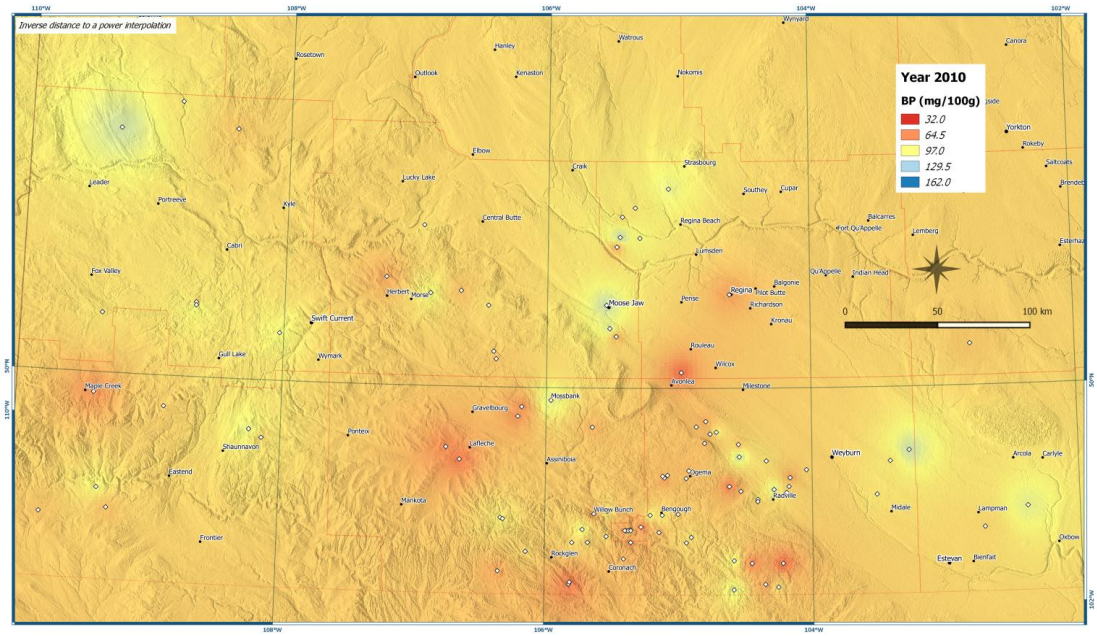

Figure 17: GIS map obtained for bound polyphenols content of KAMUT® collection harvested in North America in 2010 (red colour = low; blue colour = high).

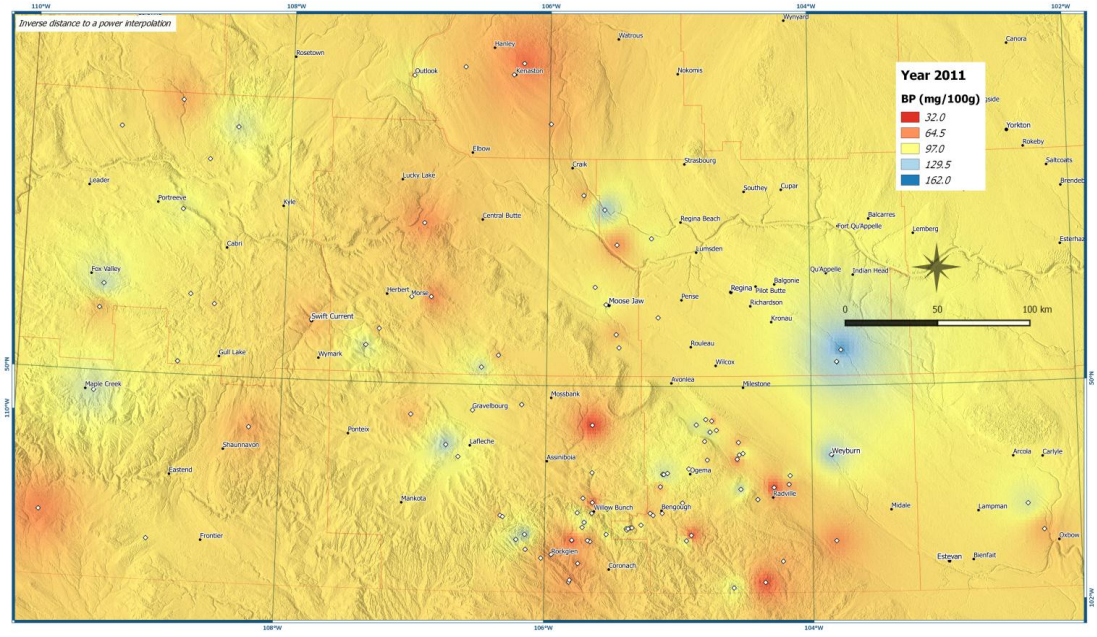

Figure 18: GIS map obtained for bound polyphenols content of KAMUT® collection harvested in North America in 2011 (red colour = low; blue colour = high).

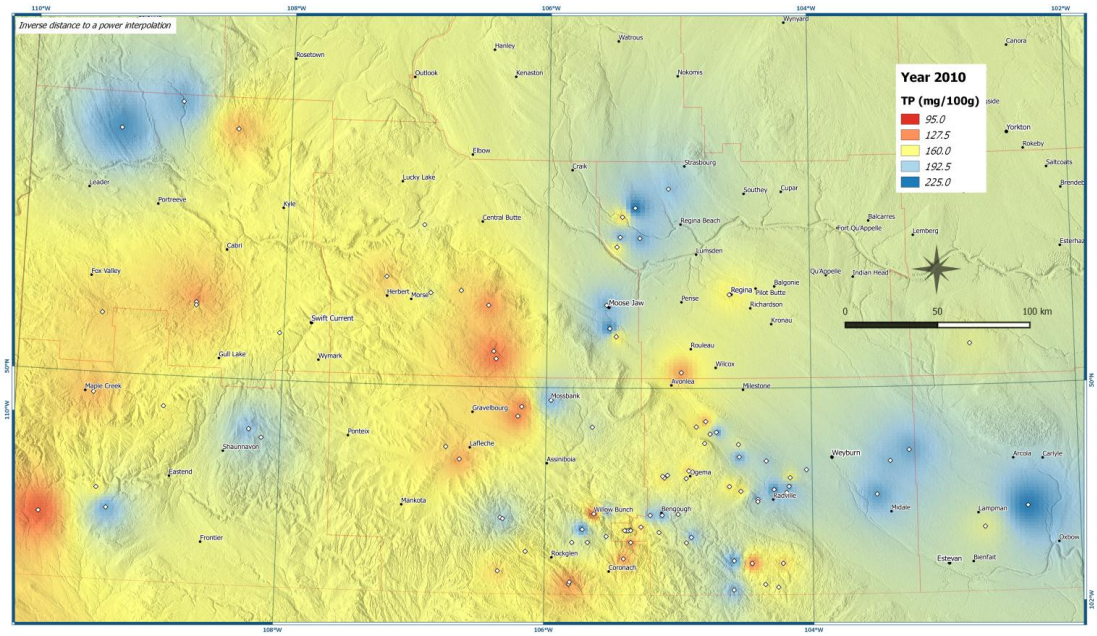

Figure 19: GIS map obtained for total polyphenols content of KAMUT® collection harvested in North America in 2010 (red colour = low; blue colour = high).

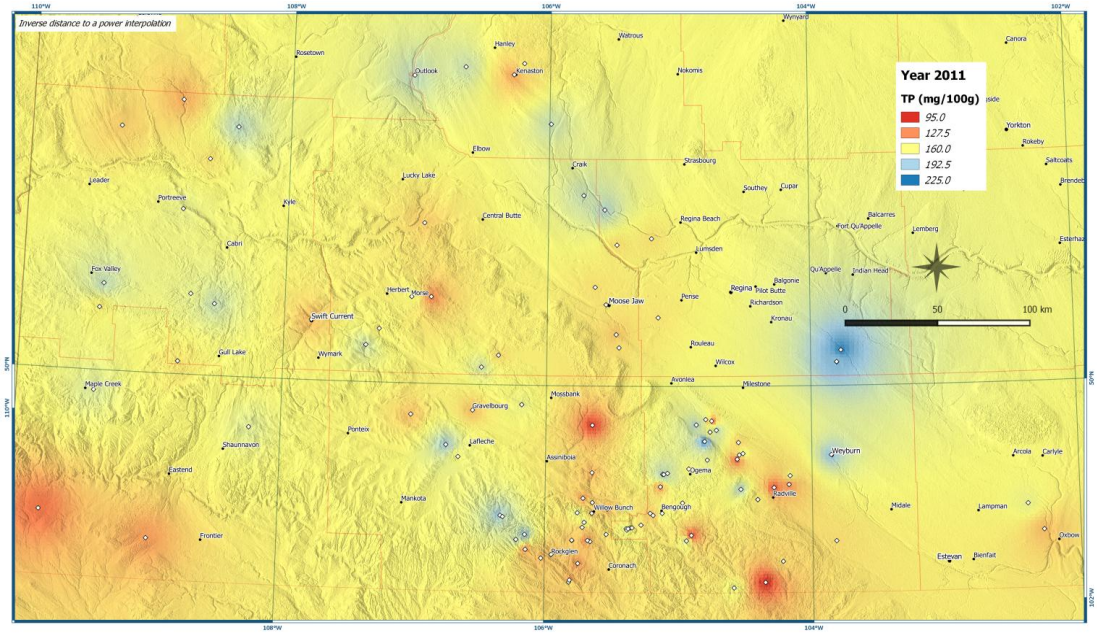

Figure 20: GIS map obtained for total polyphenols content of KAMUT® collection harvested in North America in 2011 (red colour = low; blue colour = high).

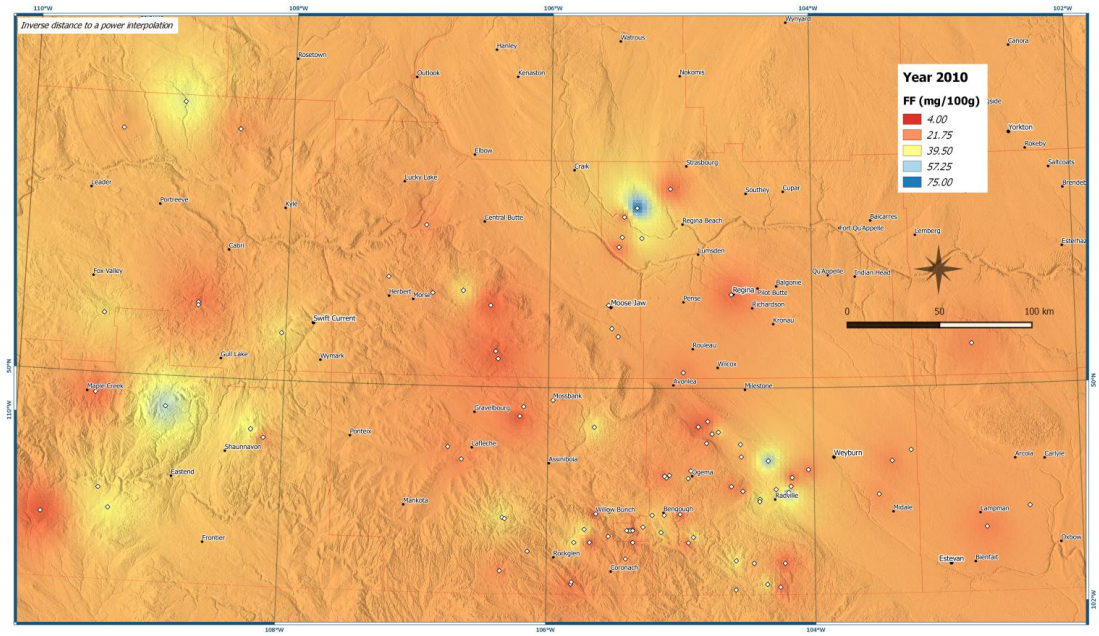

Figure 21: GIS map obtained for free flavonoids content of KAMUT® collection harvested in North America in 2010 (red colour = low; blue colour = high).

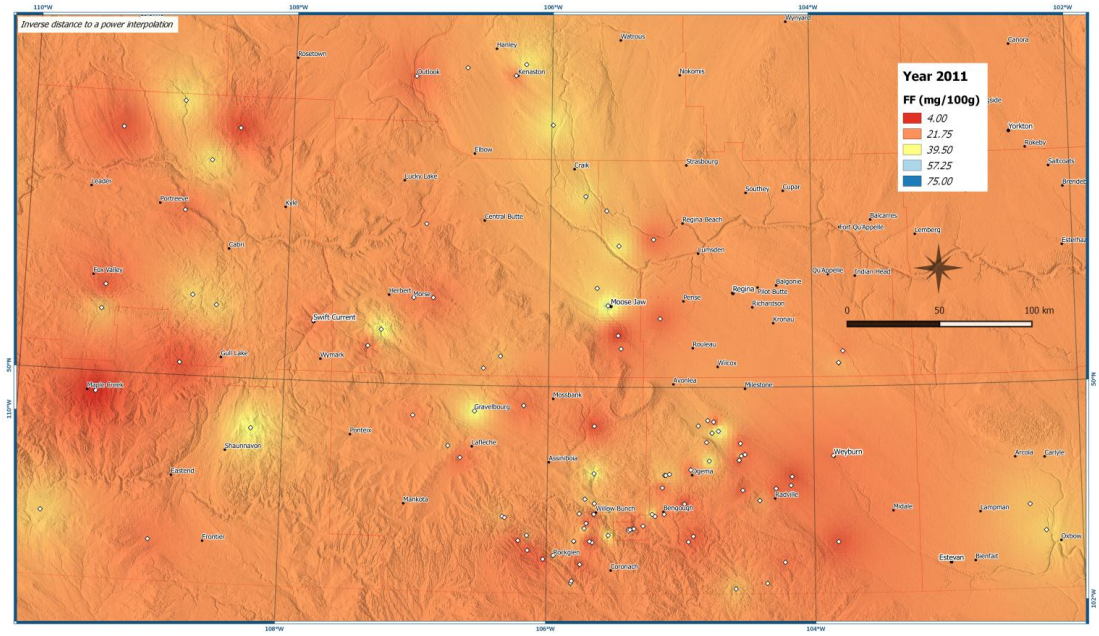

Figure 22: GIS map obtained for free flavonoids content of KAMUT® collection harvested in North America in 2011 (red colour = low; blue colour = high).

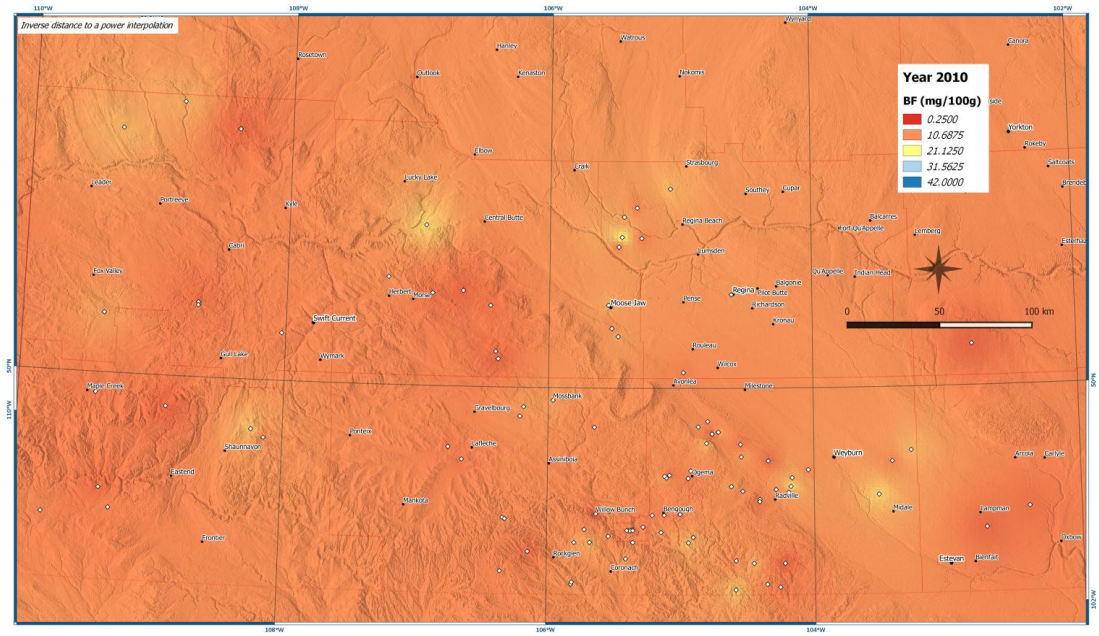

Figure 23: GIS map obtained for bound flavonoids content of KAMUT® collection harvested in North America in 2010 (red colour = low; blue colour = high).

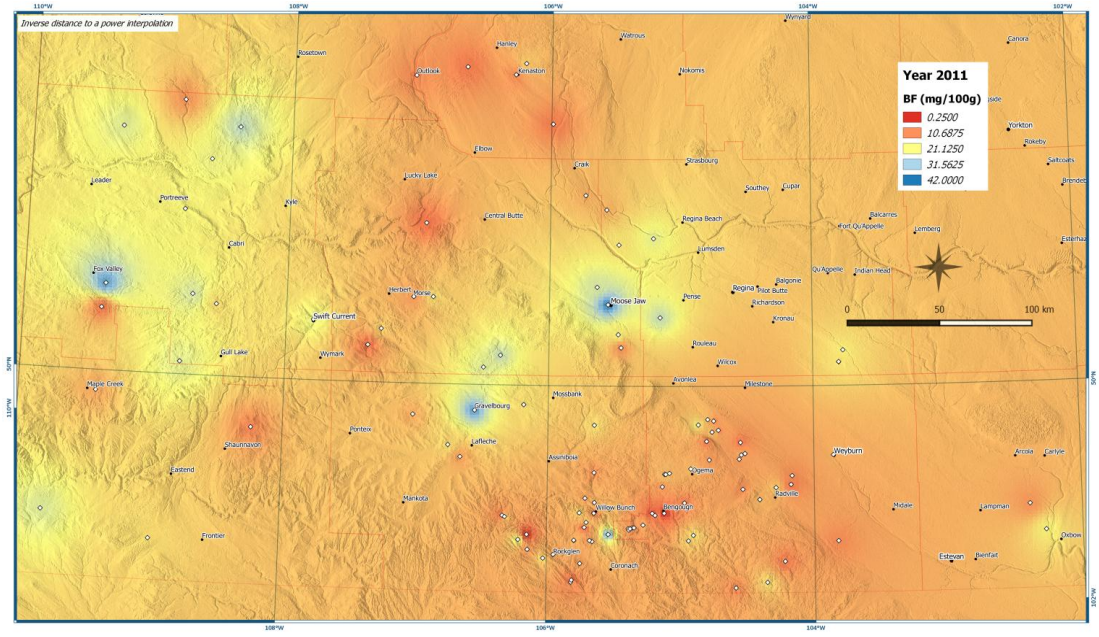

Figure 24: GIS map obtained for bound flavonoids content of KAMUT® collection harvested in North America in 2011 (red colour = low; blue colour = high).

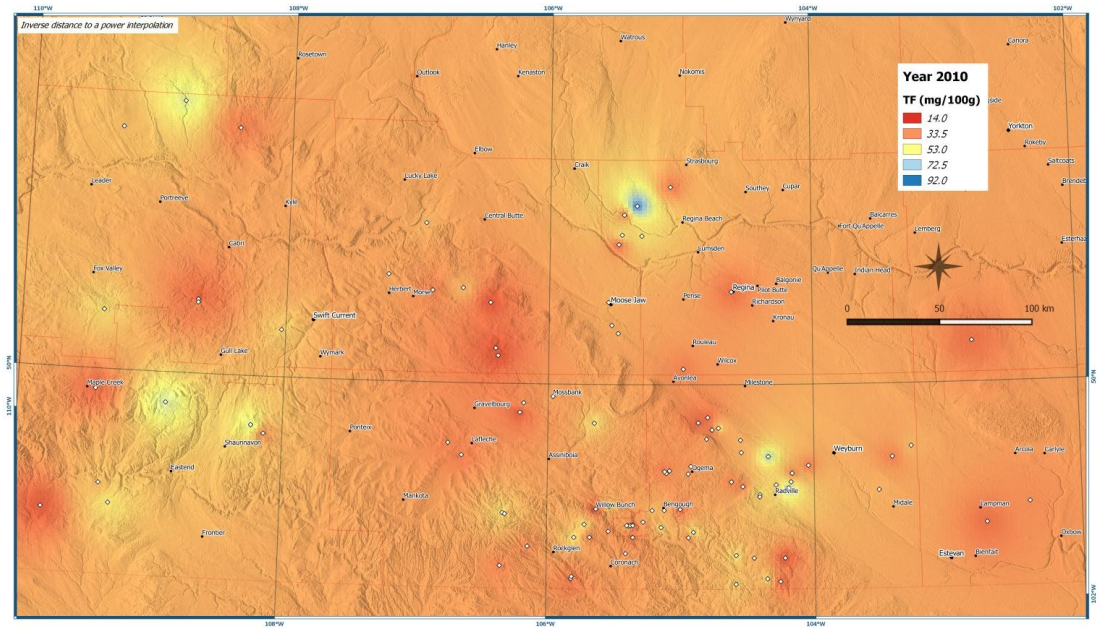

Figure 25: GIS map obtained for total flavonoids content of KAMUT® collection harvested in North America in 2010 (red colour = low; blue colour = high).

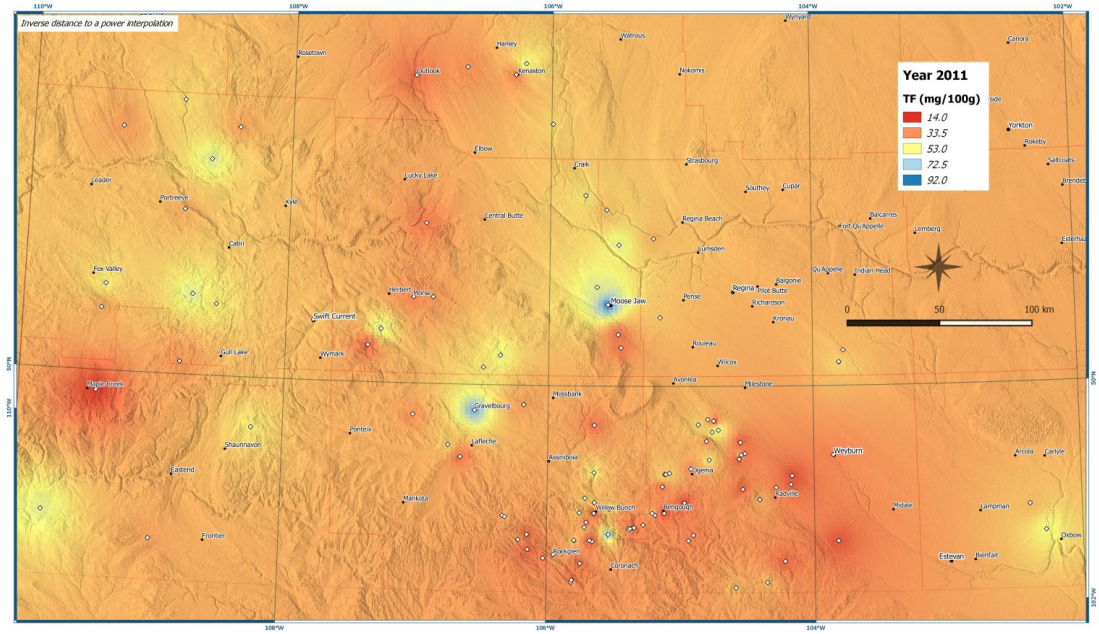

Figure 26: GIS map obtained for total flavonoids content of KAMUT® collection harvested in North America in 2011 (red colour = low; blue colour = high).

SECTION 3

Paper 2

Under submission

Quantification of free and bound phenolic compounds in old and modern durum wheat varieties using ultra high performance liquid chromatography coupled to tandem mass spectrometry

A. Di Loreto, L. Montero, R. Di Silvestro, V. Bregola, I. Marotti, R.E. Sferrazza, V. Stenico, G. Dinelli, M. Herrero, S. Bosi , A. Cifuentes.

Abstract

In this work, the evaluation of grain functional components of durum wheat cultivars is carried out. Due to its significant amount of antioxidants, durum wheat (*Triticum turgidum* ssp. *durum*) could potentially contribute to the dietary antiradical protection against a number of chronic diseases, such as diabetes, cardiovascular disease and cancer. The increasing interest towards healthy food among both consumers and scientists, led to focus on the phytochemical content of whole wheat grains, with particular interest in the peculiar antioxidant properties of old wheat varieties. The aim of this study was to identify the phytochemical composition of 24 varieties belonging to old and modern durum wheat genotypes, including quantitative phenolic and flavonoid content (free and bound forms) and antioxidant capacity (DPPH and FRAP tests). Phenolic extracts were also chemically characterized by *ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS).* Results showed that among the investigated phenolic compounds, trans-ferulic acid was the most abundant, ranging from 5.5 µg/g to 259.8 µg/g. All the other identified compounds, even if present at low concentration compared to ferulic acid, showed great variability among the 24 old and modern durum wheat varieties. Differences were also observed in the content of polyphenols and flavonoids, considering the free and bound fractions. Moreover, DPPH seemed to be correlated with the content of total polyphenols, being the old variety Inglesa the one showing the highest amount of both parameters (7.44 µmol/g and 2.88 mg GAE/g, respectively). Comparison between modern and old durum wheat varieties evidenced significant differences in the content of free and total flavonoids and in the antioxidant activity measured by DPPH test. However,

further studies are necessary to fully comprehend the dynamic affecting the concentration of antioxidant compounds in durum wheat.

Keywords: UHPLC-MS/MS; durum wheat; phenolics; antioxidants; DPPH and FRAP

1. Introduction

Durum wheat (*Triticum turgidum* ssp. *durum*) is one of the most important cereal crops used in the Mediterranean-type temperate zones for the production of staple food, such as pasta, couscous, and various types of bread. Durum wheat is also one of the most common crops in Italy with a cultivated area of 1.31 million hectares in 2015 and a production of 4.38 million tons [1].

Nowadays, most of the wheat products we consume derive from modern wheat varieties bred after the so-called "Green Revolution" (in the 1960s). The introduction of dwarf genes led to new durum wheat cultivars straw lines with high yield and improved technological quality [2,3]. In fact, the breeding programs have been focused on improving primary metabolism and technological properties (i.e. gluten quality) of durum wheat and, consequently, they have almost not taken into account the nutraceutical and health- promoting properties of wheat consumption in the human diet [4,5].

Several epidemiological researches have shown that, due to their phytochemical contents, whole grain flour helps to lower the incidence of chronic diseases such as diabetes [6], cardiovascular disease [7,8], and cancer [7,9–11].

These phytochemical compounds with antioxidant activity are plant secondary metabolites belonging to the phenylpropanoid pathway and contain one or more aromatic rings and one or several hydroxyl groups, including phenolic acids, such as ferulic, p-coumaric, p-hydroxybenzoic, vanillic, and syringic acids. [12–15]. As per definition, antioxidant compounds, can delay or avoid the oxidative damage of substrates as DNA, enzymes and cell wall molecules, neutralizing free radicals (e.g., ROS) that are the cause of many chronic diseases (i.e. cardiovascular disease and cancer). *In vitro* investigations of whole wheat grains established that phenolic compounds possess high antiradical power and contribute to most of the total antioxidant activity [12–14,16].

Phenolic compounds in wheat are mainly concentrated in the outer layers of the kernel, such as aleurone, pericarp, and embryo cell walls [14]. They are mainly present as soluble free acids, soluble conjugates esterified to sugars and other low molecular weight molecules, as well as insoluble forms bound to cell wall components, such as polysaccharides and lignin. The latter phenolic components

contribute to 70 - 80% of the total phenolic content [14,17,18]. Due to the fact that phenolic acids occur in the outer layers of the grain, they may be lost during the refinement processes, thus significantly affecting the nutritional and nutraceutical values of the final grain products [19,20]. On the other hand, antioxidant compounds as well as dietary fiber, vitamins and minerals are preserved in whole meal products [21].

In addition, literature reports that the environmental conditions strongly influence the plant secondary metabolism affecting the accumulation of phenolic compounds, such as polyphenols and flavonoids, during kernel development [22,23].

The increasing interest in healthy food production and consumption has led to an increased focus on the phytochemical content of different grains and grain varieties. In this context, old wheat varieties have been recognized to give unique nutraceutical values for their particular contents in health-beneficial phytochemicals [24–26].

Previous studies investigated the phenolic content of whole wheat grains mainly using colorimetric methods. Recently, the wide diffusion of more sensitive tools for the characterization and identification of individual polyphenols, such as separation techniques coupled to mass spectrometry (HPLC–MS, UHPLC–MS, CE–MS) have replaced the traditional methods [27–34].

The present research is conducted to determine and compare the phenolic composition of different durum and durum-type wheat varieties, including old and modern genotypes, cropped in the same location and growing season. The research aims to contribute to the understanding of wheat whole grain nutraceutical properties, fostering the development of wheat varieties with high level of health-promoting phytochemicals.

2. Materials and methods

2.1 Samples

The study was carried on 23 durum wheat samples (*Triticum turgidum* spp. *durum*) and a sample of KAMUT® khorasan wheat (*Triticum turgidum* spp. *turanicum*). The grains were cultivated at Argelato (Italy, latitude 44°39’57’’N, longitude 11°19’43’’E, 25 m a.s.l.) during the cropping year 2014-2015,

according to the biodynamic agro-technique. Whole grain samples were ground to semolina using a domestic stone mil (100% flour extraction) (Billy 200, Hawos Mulini, Bad Homburg, Germany). All determinations were carried out on three replicates and results expressed on a dry weight (DW) basis. The names and type of old and modern wheat varieties studied are shown in Table 1.

2.2 Chemicals

Folin-Ciocalteu`s phenol reagent, gallic acid, catechin, 4-hydroxybenzaldehyde, vanillin, p-coumaric acid, syringaldehyde, trans-ferulic acid, formononetin and apigenin (Figure 1) were purchased from Sigma-Aldrich (St Louis, MO, USA). All other chemicals and solvents were of analytical grade.

2.3 Extraction and in-vitro estimation of phenolic compounds

Free and bound phenolic compounds were extracted as previously described by Dinelli et al. [17]. Whole grain flours were extracted with cold 80% ethanol (4°C) in order to dissolve the free soluble compounds, followed by acid and alkaline hydrolyses to release the bound forms. Extracts were analysed for their polyphenol content following the colorimetric procedure based on the Folin–Ciocalteu's reagent, as described by Singleton et al. [35] Furthermore, extracts containing free and bound phenolic compounds were analysed for flavonoid content following the spectrophotometric method previously described [17]. The absorbance values were converted using gallic acid and catechin as standards for polyphenols and flavonoid, respectively.

2.4 Antioxidant activity

The DPPH assay was carried out according to the procedure described by Floegel et al. [36]. Briefly, a solution of 1 mM DPPH in 80% (v/v) methanol was prepared. Absorbance of the solution was adjusted to 0.650 ± 0.020 AU at 517 nm using fresh 80% (v/v) methanol. Subsequently, 50 µL of sample were mixed with 2.95 mL of DPPH solution and incubated for 30 min in the dark at room temperature. The absorbance at 517 nm was monitored for each sample, along with control against a blank of pure methanol. A calibration curve of Trolox (0-500 mg/L) was performed as a function of the percentage of DPPH

radical scavenging activity and the final results expressed as micromoles of Trolox equivalents (TE) per gram of whole wheat flour (µmol TE/g). The FRAP test was carried out according to Benzie and Strain [37] with some modifications. The FRAP working solution (WS) was prepared freshly as a mixture of 300 mM acetate buffer pH 3.6 (containing 3.1 g of sodium acetate trihydrate and 16 ml glacial acetic acid), 10 mM TPTZ (in 40 mmol/l HCl) and 20 mM ferric chloride (10:1:1, v:v:v). 80 µl of diluted (1:1 v/v) free and bound phenolic extracts were mixed with 2.4 ml of WS and the absorbance was measured at 593 nm after 1 hour in the dark. Absorbance values were compared with those of ferrous sulphate ($FeSO_4$ 7H2O) (0-1000 µmol/L) and results expressed as mmol Fe^{2+} per 100 g of flour. Each measurement was performed in triplicate.

2.5 Analysis and quantification of phenolic compounds by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS).

Phenolic extracts were analysed using an UHPLC Accela liquid chromatograph (Thermo Scientific, San Jose, CA) equipped with a diode array detector (DAD). A Zorbax Eclipse Plus C-18 (50 mm x 2.1 mm, d.p. 1.8 µm) (Agilent) column thermostated at 40 °C was used. The mobile phases used were (A) 0.5% formic acid in MilliQ water and (B) acetonitrile, eluted according to the following gradient: 0 min, 95% B; 0.5 min, 10% B;1 min, 20% B; 7 min, 40% B; 9 min, 95% B; 10 min, 5%B. A 5 min re-equilibration time was used after each analysis. The flow rate was set at 500 µL/min throughout the gradient, whereas the injection volume used was 5 µL. The DAD recorded the spectra from 190 to 550 nm. Moreover, the UHPLC system was coupled to a TSQ Quantum triple quadrupole mass spectrometer (Thermo Scientific) via an electrospray interface, operated under negative ionization mode. Parameters for analysis were set using negative ion mode with MS spectra acquired over a mass range from m/z 50 to 800. The optimum values of the MS parameters were: scan time, 0.1 s; capillary voltage, 3500 V; capillary temperature, 350°C; sheath gas pressure, 35 arbitrary units; auxiliary gas pressure, 5 arbitrary units. Peak identification of each phenolic compound was based on comparison of relative retention time (RT),

spectral data and MS parameters with those of phenolic standards. Moreover, quantification of main phenolic compounds (chemical structures shown in Figure 1) was performed by selected reaction monitoring (SRM) using scan width and the scan time values at 0.100 (m/z) and 0.100 s, whereas collision gas (Ar) pressure was set at 1.5 mTorr. SRM parameters were optimized by direct infusion of standards. Two transition ions were monitored for identification but only the most intense one for each precursor ion was used for quantification. The values corresponding to the tube lens offset voltage and collision energy for each selected ion transitions are indicated in Table 2. All phenolic compounds were quantified via rationing to the internal standard (diosmetin) added to every sample and using calibration curves of phenolic standards. Diosmetin was appropriately diluted in DMSO to a concentration of 0.1 mg/ml and *ten microliters of internal standard was added to each sample extracts.* All samples were analyzed in triplicate, and concentrations of individual phenolic acids were expressed in micrograms per gram of dry matter.

2.6 Statistical analyses

One-way analysis of variance (ANOVA) in conjunction with Tukey's honest significant difference was performed for comparing the flour samples. Significance between means was determined by least significant difference values for $P < 0.05$. Linear discriminate analysis (LDA) was performed on the standardised matrix of the content of the 7 compounds used as standards in each sample analysed and on the results of all the parameters investigated. All statistical analyses were conducted using Statistica 6.0 software (2001, StatSoft, Tulsa, OK, USA).

3. Results and discussion

3.1 Phenolic acids and flavonoids contents

The most investigated class of phytochemical compounds extracted from plants are phenolic acids and flavonoids, which have been extensively studied for their role in the prevention of cancer and other chronic diseases due to their ability to act as antioxidants and radical scavengers [24,38,39]. In wheat, phenolic

compounds are mainly concentrated in the bran layer of the kernel and exist in two forms: the soluble (free) and the insoluble (bound) forms [14]. The biosynthesis and accumulation of phenolic compounds in the wheat caryopsis vary depending on the wheat genotype, the environmental and growing conditions. The phenolic acid contents of the investigated wheat genotypes are reported in Table 1, expressed as milligrams of gallic acid equivalent (GAE) per 100 grams. The determination of the phenolic content of whole wheat grains evidenced a high variability among the 15 old and 9 modern wheat genotypes investigated. The free polyphenols (FP) content ranged from 1.48 ± 0.11 mg GAE/g of grain in Inglesa to 0.47 ± 0.04 mg GAE/g of grain in Claudio and a mean value of 0.97 ± 0.06 mg GAE/g was obtained (Table 1). In addition, no significant differences were observed comparing the mean FP values obtained for old wheat varieties with respect to the modern ones (Figure 2). Regarding the bound fraction, phenolic content ranged from 1.76 ± 0.04 mg GAE/g in Senatore Cappelli to 0.90 ± 0.03 mg GAE/g in modern wheat Duilio. On average, the bound fraction contributed to the total phenolic content for 55%. This result is not fully in agreement with previous findings highlighting that in wheat kernel polyphenols primarily exist in the bound form, associated with cell wall components [13,16,40]. No significant differences were observed between mean values of old and modern genotypes for bound and total phenolic amount (Figure 2). As evidenced, significant differences were observed for the free and total flavonoids in comparison with bound fraction of polyphenols, thus, suggesting a strong genotypic effect on the soluble phenolic forms. These results are in accordance with those reported in literature. In fact, Shewry et al. [23] indicated the existence of substantial genetic variation in the amounts and compositions of bioactive components in wheat. This can be related to a different response of genotype to growing conditions, especially to biotic/abiotic stress. Moreover, this physiological reaction could be stronger if wheat varieties are cultivated under low-input agriculture management. In facts, Moore et al. [41] showed that wheat grain and fractions from individual wheat samples may differ in their contents of natural antioxidants, suggesting the potential to enhance wheat antioxidant capacity though improved agricultural practices such as growing a selected wheat variety under optimal conditions.

Flavonoids are the predominant class of phytochemicals described in wheat phenolic content investigations, accounting for approximately two-thirds of the dietary phenols [42]. Flavonoid content of the wheat samples analysed in the present study is presented in Table 1, expressed as milligrams of catechin equivalents (CE) per grams of grain. The observed flavonoid amounts were significantly different among the 24 tested wheat varieties for free, bound and total flavonoid fractions. Free soluble fraction ranged from 0.86 ± 0.05 mg CE/g in old wheat variety Chiattulidda to 0.30 ± 0.03 mg CE/g in Senatore Cappelli with a mean value of 0.54 ± 0.04 mg CE/g. The bound insoluble fraction of flavonoids in old and modern durum wheat varieties investigated ranged from 0.37 ± 0.07 mg CE/g in cultivar Inglesa to 0.13 ± 0.01 mg CE/g in Urria 12. The calculated mean value is 0.21 ± 0.01mg CE/g. Significant differences between old and modern wheat varieties were observed comparing the average amounts of free and total flavonoids. The obtained flavonoid amounts were higher than previously reported in literature for both free and bound forms [24,43,44]. This can be related to genotypes as well as environmental growing conditions. Flavonoids are the ecological response of plants, therefore their accumulation in the wheat caryopsis is known to be strictly dependent on genotype, biotic and abiotic stress [45–47].

3.2 Radical scavenging activity (DPPH and FRAP tests)

Antioxidant capacities determined by both assays were more strongly correlated with total phenolics than with total flavonoids content as previously reported [48,49]. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) is a stable free radical which has an unpaired valence electron at one atom of nitrogen bridge [50]. Scavenging of DPPH radical is the basis of the popular DPPH antioxidant assay [51,52]. The obtained results are summarized in Table 1, expressed as μmol of trolox equivalents (TE) per grams of whole flour. The antioxidant activity determined by DPPH test revealed great variability among the analysed wheat samples. The highest value was observed in the old wheat cultivar Inglesa (7.44 ± 0.53 μmol TE/g), while the lowest activity was found in the modern durum wheat varieties Claudio (4.06 ± 0.24 μmol TE/g). The other samples showed an antioxidant activity around the mean value (5.37 ± 0.22 μmol TE/g).

On the other hand, the FRAP test measures the chelating activity of the phenolic extract, namely the reduction and stabilisation of transition metals (i.e. Fe^{2+}) which are the cause of the free radical formation and consequently of the oxidative chain inception [12]. The values obtained for the 24 varieties object of the present study are also reported in Table 1. On average, wheat varieties showed an antioxidant activity equal to 1.43 ± 0.05 mmol/100g and ranged from 1.94± 0.02 mmol/100g grains in Anco Marzio to 1.05 ± 0.01 mmol/100g grains in Claudio. The comparison between the old and modern wheat genotypes evidenced a significant difference only for the DPPH test, whereas the FRAP assay showed, on average, the same values (Figure 2).

Previous studies in literature reported that antioxidant and chelating activity vary as a function of the wheat genotype and environmental conditions [12,53,54]. Results of the present work are referred to only one year of cultivation, but suggested the need for further studies to better understand the role of the genotype and, eventually, of the environment on the antioxidant properties of wheat.

3.3 UHPLC-DAD-MS/MS characterization and quantification of phenolic compounds

A new RP-UHPLC-DAD-MS/MS method was developed for the characterization of phenolic extracts obtained from the 24 wheat varieties. The optimization allowed the identification of the chromatographic conditions providing lower pressure, greater baseline stability, higher ionization efficiency and good separation of phenolic compounds in the extracts. As an example, Figure 3A shows the chromatogram (280 nm) and the multiple reaction monitoring (MRM) chromatograms corresponding to the mixture of the seven phenolic compounds quantified in the 24 old and modern wheat varieties, while Figure 3B shows the chromatogram (280 nm) and the MRM chromatograms of the quantified compounds obtained for the old wheat variety Senatore Cappelli. Seven of the most abundant phenolic standards were selected based on previous reports [24,55] in order to investigate the polyphenol composition of durum wheat samples. For quantitative analysis, calibration curves were constructed by injecting known concentrations of the different standard compounds,

appropriately diluted, using seven concentration points. Details about retention time (RT), linear range, regression values, limits of detection (LOD), limits of quantification (LOQ), mass, fragment and concentration range are presented in Table 2. Great variability was observed in the content of the seven investigated compounds among the 24 wheat varieties. Among the investigated compounds, apigenin was not detected either in old and modern durum wheat samples. The most abundant compound found was trans-ferulic acid ranging from 259.75 ± 26.30 µg/g of flour in Urria 12 to 5.53 ± 0.93 µg/g of flour in Inglesa. Ferulic acid is the most common phenolic acid in monocotyledonous cell walls [56,57] and was identified according to its molecular ion $[M-H]^-$ at *m/z* 193 and characteristic fragment ions at *m/z* 134 and 178 (Table 2). In wheat flour, the trans isomer is predominant and accounts for 90% of the total phenolic acids [58]. The amount of trans-ferulic acid was significantly different among the 24 varieties analysed. This is in accordance with Lempereur et al. [59], who suggested that there was a very high genetic influence on the amounts of ferulic acid. The other compounds were present at very low concentration if compared with trans-ferulic acid, with mean values of 0.97, 0.41, 0.15, 0.04 and 0.01 µg/g for p-coumaric acid, syringaldeyde, vanillin, 4-hydroxybenzaldehyde and formononetin, respectively (Table 3). 4-hydroxybenzaldehyde was characterized by a molecular ion $[M-H]^-$ at *m/z* 121 and only one fragment ion at *m/z* 92 (Table 2) and the concentration ranged from 0.005 to 0.147 µg/g among the analysed samples. p-Coumaric acid ranged between 0.108 µg/g grain in Anco Marzio and 4.104 µg/g grain in Urria 12 and was identified based on its molecular ion ($[M-H]^-$) at *m/z* 163 and its corresponding fragment ions (at *m/z* 93 and 119). Phenolic compound with a molecular ion $[M-H]^-$ at *m/z* 151 and a fragmentation ions at *m/z* 123 and 136 was identified as vanillin. The highest amount was observed in the old variety Senatore Cappelli (0.600 µg/g), while the lowest value was found in the modern variety Quadrato (0.028 µg/g) (Table 3). Another phenolic acid was identified as syringaldehyde and was characterized by a molecular ion $[M-H]^-$ at *m/z* 181 and fragmentation ions at *m/z* 151 and 166 (Table 2). The highest amount (1.270 µg/g) was reported in old wheat variety Urria 12, whereas the lowest (0.102 µg/g) was observed in the modern Anco Marzio. The last eluted peak was identified as an isoflavone,

namely formononetin (molecular ion $[M-H]^-$ at *m/z* 267 and fragmentation ions at *m/z* 135 and 252) (Table 2). The range of the content of syringaldehyde among the 24 analysed samples was comprised between 0.003 µg/g in Inglesa and 0.044 µg/g in Urria 12 (Table 3).

Comparing the mean content of the old and the modern wheat varieties, significant differences ($p< 0.05$) were observed only for the content of vanillin and p-coumaric acid. In particular, the mean values obtained in old wheat varieties are higher than the average of modern ones (Table 4).

Very few works have been published so far studying phenolic acids in durum wheat grains. Results obtained in the present work show lower values than those previously described for ferulic acid and p-coumaric acid [55,60,61]. To the best of our knowledge, this is the first work in which vanillin, 4-hydroxybenzaldehyde and formononetin were studied in durum wheat. Results obtained in the present work underlined great variability among different durum wheat genotypes. However, further investigations are necessary to evaluate also the effect of the environment on the amount of phenolic acids.

3.4 Linear Discriminant Analyses

Linear Discriminant Analyses (LDA) was used to analyse the complete set of data, in order to explain the variability observed among the 24 investigated wheat genotypes. The scatterplot of the grain samples on the space defined by the first two canonical functions is shown in Figure 4. LDA allowed to obtain more information as regards the variables that mainly influenced the sample similarities and differences [54,62]. As revealed from the values of canonical functions standardised within variances for each variable on Root 1, the described clusterisation of the cases resulted strongly influenced by the content of trans-ferulic acid, formononetin, free flavonoids and polyphenols (values of first canonical discriminant function (Root 1) equal to 1.06, -1.05, -0.79, 0.68, respectively). The clusterisation of the cases along second canonical function (Root 2) resulted strongly influenced by syringaldehyde, vanillin, DPPH, free and bound flavonoids amount (values of canonical discriminant function 2 equal to 1.56, -0.68, -0.89, 0.84 and -0.68, respectively). It is possible to identify a big cluster with most of the analysed varieties in the right part of the scatterplot and

a separated group of old wheat varieties Tripolino, Margherito, Scorsonera and Bidì (3, 4, 5, 6) in the central area (Figure 5). Senatore Cappelli (1), Urria 12 (2), Inglesa (9) and the modern variety Claudio (16) formed completely separated single-variety clusters, highlighting a peculiar composition in antioxidant compounds.

4. Conclusions

To the best of our knowledge, this is the first time that UHPLC coupled to tandem mass spectrometry is used to quantify phenolic compounds in durum wheat grains. Remarkable quantitative differences were detected among the 15 old and the 9 modern wheat genotypes investigated in this work. Among the single phenolic acids the most abundant in durum wheat was the trans isomer of ferulic acid. On average, significant differences were observed between old and modern varieties only in the amount of vanillin and p-coumaric acid. This is the first work in which the content of vanillin, 4-hydroxybenzaldehyde and formononetin were studied in durum wheat. Great variability was observed in the content of total polyphenols and flavonoids, considering the free and bound fractions. Moreover, DPPH values seemed to correlate with the content of total polyphenols, with the old variety Inglesa showing the highest values for both parameters. Significant differences were observed comparing old and modern wheat varieties in terms of free fraction of polyphenols and flavonoids and in the antioxidant activity measured with DPPH test. However, further studies are necessary to fully understand the dynamic affecting the concentration of antioxidant compounds in durum wheat caryopsis, including genotypic and environmental influence.

References

[1] Database - Eurostat. http://ec.europa.eu/eurostat/data/database.

[2] P. De Vita, E. Di Paolo, G. Fecondo, N. Di Fonzo, M. Pisante, No-tillage and conventional tillage effects on durum wheat yield, grain quality and soil moisture content in southern Italy, Soil Tillage Res. 92 (2007) 69–78. doi:10.1016/j.still.2006.01.012.

[3] P. De Vita, O.L.D. Nicosia, F. Nigro, C. Platani, C. Riefolo, N. Di Fonzo, et al., Breeding progress in morpho-physiological, agronomical and qualitative traits of durum wheat cultivars released in Italy during the 20th century, Eur. J. Agron. 26 (2007) 39–53. doi:10.1016/j.eja.2006.08.009.

[4] L. Pecetti, P. Annicchiarico, Agronomic value and plant type of Italian durum wheat cultivars from different eras of breeding, Euphytica. 99 (1998) 9–15.

[5] R. Motzo, S. Fois, F. Giunta, Relationship between grain yield and quality of durum wheats from different eras of breeding, Euphytica. 140 (2004) 147–154. doi:10.1007/s10681-004-2034-5.

[6] K. a. Meyer, L.H. Kushi, D.R. Jacobs, J. Slavin, T. a. Sellers, A.R. Folsom, Carbohydrates, dietary fiber, and incident type 2 diabetes in older women, Am. J. Clin. Nutr. 71 (2000) 921–930. doi:10.1093/jnci/dji276.

[7] L.U. Thompson, Antioxidants and hormone-mediated health benefits of whole grains., Crit. Rev. Food Sci. Nutr. 34 (1994) 473–97. doi:10.1080/10408399409527676.

[8] D.R. Jacobs, K.A. Meyer, L.H. Kushi, A.R. Folsom, Whole-grain intake may reduce the risk of ischemic heart disease death in postmenopausal women: the Iowa Women's Health Study., Am. J. Clin. Nutr. 68 (1998) 248–57. http://www.ncbi.nlm.nih.gov/pubmed/9701180 (accessed February 29, 2016).

[9] K. Smigel, Fewer colon polyps found in men with high-fiber, low-fat diets., J. Natl. Cancer Inst. 84 (1992) 80–1.

http://www.ncbi.nlm.nih.gov/pubmed/1310510 (accessed February 29, 2016).

[10] C.M. Kasum, D.R. Jacobs, K. Nicodemus, A.R. Folsom, Dietary risk factors for upper aerodigestive tract cancers, Int. J. Cancer. 99 (2002) 267–272. doi:10.1002/ijc.10341.

[11] K.K. Nicodemus, D.R. Jacobs, A.R. Folsom, Whole and refined grain intake and risk of incident postmenopausal breast cancer (United States)., Cancer Causes Control. 12 (2001) 917–25. http://www.ncbi.nlm.nih.gov/pubmed/11808711 (accessed February 29, 2016).

[12] L. Yu, S. Haley, J. Perret, Free radical scavenging properties of wheat extracts, J. Agric. Food Chem. 50 (2002) 1619–1624. http://pubs.acs.org/doi/abs/10.1021/jf010964p.

[13] K.K. Adom, M.E. Sorrells, R.H. Liu, Phytochemical Profiles and Antioxidant Activity of Wheat Varieties, J. Agric. Food Chem. 51 (2003) 7825–7834. doi:10.1021/jf030404l.

[14] K.K. Adom, M.E. Sorrells, H.L. Rui, Phytochemicals and antioxidant activity of milled fractions of different wheat varieties, J. Agric. Food Chem. 53 (2005) 2297–2306. doi:10.1021/jf048456d.

[15] R.H. Liu, Potential synergy of phytochemicals in cancer prevention: mechanism of action., J. Nutr. 134 (2004) 3479S–3485S. doi:134/12/3479S [pii].

[16] N. Okarter, C.S. Liu, M.E. Sorrells, R.H. Liu, Phytochemical content and antioxidant activity of six diverse varieties of whole wheat, Food Chem. 119 (2010) 249–257. doi:10.1016/j.foodchem.2009.06.021.

[17] G. Dinelli, A. Segura-Carretero, R. Di Silvestro, I. Marotti, D. Arráez-Román, S. Benedettelli, et al., Profiles of phenolic compounds in modern and old common wheat varieties determined by liquid chromatography coupled with time-of-flight mass spectrometry., J. Chromatogr. A. 1218 (2011) 7670–81. doi:10.1016/j.chroma.2011.05.065.

[18] E. Leoncini, C. Prata, M. Malaguti, I. Marotti, A. Segura-Carretero, P. Catizone, et al., Phytochemical Profile and Nutraceutical Value of Old and Modern Common Wheat Cultivars, PLoS One. 7(9): e45997 (2012).

doi:10.1371/journal.pone.0045997.

[19] J. Slavin, Whole grains and human health., Nutr. Res. Rev. 17 (2004) 99–110. doi:10.1079/NRR200374.

[20] R. Di Silvestro, A. Di Loreto, I. Marotti, S. Bosi, V. Bregola, A. Gianotti, et al., Effects of flour storage and heat generated during milling on starch, dietary fibre and polyphenols in stoneground flours from two durum-type wheats, Int. J. Food Sci. Technol. 49 (2014) 2230–2236. doi:10.1111/ijfs.12536.

[21] R. Hirawan, W.Y. Ser, S.D. Arntfield, T. Beta, Antioxidant properties of commercial, regular- and whole-wheat spaghetti, Food Chem. 119 (2010) 258–264. doi:10.1016/j.foodchem.2009.06.022.

[22] A. Mpofu, H.D. Sapirstein, T. Beta, Genotype and environmental variation in phenolic content, phenolic acid composition, and antioxidant activity of hard spring wheat, J. Agric. Food Chem. 54 (2006) 1265–1270. doi:10.1021/jf052683d.

[23] P.R. Shewry, V. Piironen, A.M. Lampi, M. Edelmann, S. Kariluoto, T. Nurmi, et al., The HEALTHGRAIN wheat diversity screen: Effects of genotype and environment on phytochemicals and dietary fiber components, J. Agric. Food Chem. 58 (2010) 9291–9298. doi:10.1021/jf100039b.

[24] G. Dinelli, A.S. Carretero, R. Di Silvestro, I. Marotti, S. Fu, S. Benedettelli, et al., Determination of phenolic compounds in modern and old varieties of durum wheat using liquid chromatography coupled with time-of-flight mass spectrometry., J. Chromatogr. A. 1216 (2009) 7229–40. doi:10.1016/j.chroma.2009.08.041.

[25] A. Gianotti, F. Danesi, V. Verardo, D.I. Serrazanetti, V. Valli, A. Russo, et al., Role of cereal type and processing in whole grain in vivo protection from oxidative stress Andrea, (2011) 1609–1618.

[26] A. Carnevali, A. Gianotti, S. Benedetti, M.C. Tagliamonte, M. Primiterra, L. Laghi, et al., Role of Kamut® brand khorasan wheat in the counteraction of non-celiac wheat sensitivity and oxidative damage, Food Res. Int. 63 (2014) 218–226. doi:10.1016/j.foodres.2014.01.065.

[27] H. Wu, T. Haig, J. Pratley, D. Lemerle, M. An, Simultaneous

determination of phenolic acids and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one in wheat (Triticum aestivum L.) by gas chromatography-tandem mass spectrometry, J. Chromatogr. A. 864 (1999) 315–321. doi:10.1016/S0021-9673(99)01034-1.

[28] C. Cavaliere, P. Foglia, E. Pastorini, R. Samperi, A. Lagan??, Identification and mass spectrometric characterization of glycosylated flavonoids in Triticum durum plants by high-performance liquid chromatography with tandem mass spectrometry, Rapid Commun. Mass Spectrom. 19 (2005) 3143–3158. doi:10.1002/rcm.2185.

[29] C. Gallardo, L. Jiménez, M.-T. García-Conesa, Hydroxycinnamic acid composition and in vitro antioxidant activity of selected grain fractions, Food Chem. 99 (2006) 455–463. doi:10.1016/j.foodchem.2005.07.053.

[30] R.E. Asenstorfer, Y. Wang, D.J. Mares, Chemical structure of flavonoid compounds in wheat (Triticum aestivum L.) flour that contribute to the yellow colour of Asian alkaline noodles, J. Cereal Sci. 43 (2006) 108–119. doi:10.1016/j.jcs.2005.09.001.

[31] G. Dinelli, I. Marotti, S. Bosi, S. Benedettelli, L. Ghiselli, S. Cortacero-Ram??rez, et al., Lignan profile in seeds of modern and old Italian soft wheat (Triticum aestivum L.) cultivars as revealed by CE-MS analyses, Electrophoresis. 28 (2007) 4212–4219. doi:10.1002/elps.200700301.

[32] C. Hu, Y.Z. Cai, W. Li, H. Corke, D.D. Kitts, Anthocyanin characterization and bioactivity assessment of a dark blue grained wheat (Triticum aestivum L. cv. Hedong Wumai) extract, Food Chem. 104 (2007) 955–961. doi:10.1016/j.foodchem.2006.12.064.

[33] F.S. Hosseinian, W. Li, T. Beta, Measurement of anthocyanins and other phytochemicals in purple wheat, Food Chem. 109 (2008) 916–924. doi:10.1016/j.foodchem.2007.12.083.

[34] M.A. Matus-Cádiz, T.E. Daskalchuk, B. Verma, D. Puttick, R.N. Chibbar, G.R. Gray, et al., Phenolic compounds contribute to dark bran pigmentation in hard white wheat, J. Agric. Food Chem. 56 (2008) 1644–1653. doi:10.1021/jf072970c.

[35] V.L. Singleton, R. Orthofer, R.M. Lamuela-Raventós, Analysis of total phenols and other oxidation substrates and antioxidants by means of

folin-ciocalteu reagent, Methods Enzymol. 299 (1999) 152–178. doi:10.1016/S0076-6879(99)99017-1.

[36] A. Floegel, D.O. Kim, S.J. Chung, S.I. Koo, O.K. Chun, Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods, J. Food Compos. Anal. 24 (2011) 1043–1048. doi:10.1016/j.jfca.2011.01.008.

[37] I.F. Benzie, J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay., Anal. Biochem. 239 (1996) 70–6. doi:10.1006/abio.1996.0292.

[38] J.W. Carter, R. Madl, F. Padula, Wheat antioxidants suppress intestinal tumor activity in Min mice, Nutr. Res. 26 (2006) 33–38. doi:10.1016/j.nutres.2005.10.005.

[39] A. Fardet, New hypotheses for the health-protective mechanisms of whole-grain cereals: what is beyond fibre?, Nutr. Res. Rev. 23 (2010) 65–134. doi:10.1017/S0954422410000041.

[40] P. Van Hung, D.W. Hatcher, W. Barker, Phenolic acid composition of sprouted wheats by ultra-performance liquid chromatography (UPLC) and their antioxidant activities, Food Chem. 126 (2011) 1896–1901. doi:10.1016/j.foodchem.2010.12.015.

[41] J. Moore, J.-G. Liu, K. Zhou, L.L. Yu, Effects of genotype and environment on the antioxidant properties of hard winter wheat bran., J. Agric. Food Chem. 54 (2006) 5313–5322. doi:10.1021/jf060381l.

[42] R.J. Robbins, Phenolic acids in foods: An overview of analytical methodology, J. Agric. Food Chem. 51 (2003) 2866–2887. doi:10.1021/jf026182t.

[43] Y. Feng, C.E. Mc Donald, Comparison of Flavonoids in Bran of Four Classes of Wheat, Cereal Chem. 66 (1989) 516–518.

[44] G. Dinelli, I. Marotti, R. Di Silvestro, S. Bosi, V. Bregola, M. Accorsi, et al., Agronomic, nutritional and nutraceutical aspects of durum wheat (Triticum durum Desf.) cultivars under low input agricultural management, Ital. J. Agron. 8 (2013) e12. doi:10.4081/ija.2013.e12.

[45] D. Treutter, Significance of flavonoids in plant resistance: A review, Environ. Chem. Lett. 4 (2006) 147–157. doi:10.1007/s10311-006-0068-8.

[46] N.J. Atkinson, T.P. Dew, C. Orfila, P.E. Urwin, Influence of Combined Biotic and Abiotic Stress on Nutritional Quality Parameters in Tomato (Solanum lycopersicum), J. Agric. Food Chem. 59 (2011) 9673–9682. doi:10.1021/jf202081t.

[47] N.J. Atkinson, P.E. Urwin, The interaction of plant biotic and abiotic stresses: From genes to the field, J. Exp. Bot. 63 (2012) 3523–3544. doi:10.1093/jxb/ers100.

[48] O.K. Chun, D.-O. Kim, H.Y. Moon, H.G. Kang, C.Y. Lee, Contribution of individul polyphenolics to total antioxidant capacity of plums., J. Agric. Food Chem. 51 (2003) 7240–7245.

[49] D.O. Kim, O.K. Chun, Y.J. Kim, H.Y. Moon, C.Y. Lee, Quantification of Polyphenolics and Their Antioxidant Capacity in Fresh Plums, J. Agric. Food Chem. 51 (2003) 6509–6515. doi:10.1021/jf0343074.

[50] P.C. Eklund, O.K. Långvik, J.P. Wärnå, T.O. Salmi, S.M. Willför, R.E. Sjöholm, Chemical studies on antioxidant mechanisms and free radical scavenging properties of lignans., Org. Biomol. Chem. 3 (2005) 3336–47. doi:10.1039/b506739a.

[51] M.H. Alma, A. Mavi, A. Yildirim, M. Digrak, T. Hirata, Screening chemical composition and in vitro antioxidant and antimicrobial activities of the essential oils from Origanum syriacum L. growing in Turkey., Biol. Pharm. Bull. 26 (2003) 1725–1729. doi:10.1248/bpb.26.1725.

[52] O.P. Sharma, T.K. Bhat, DPPH antioxidant assay revisited, Food Chem. 113 (2009) 1202–1205. doi:10.1016/j.foodchem.2008.08.008.

[53] L. Yu, S. Haley, J. Perret, M. Harris, Comparison of wheat flours grown at different locations for their antioxidant properties, Food Chem. 86 (2004) 11–16. doi:10.1016/j.foodchem.2003.08.037.

[54] R. Di Silvestro, I. Marotti, S. Bosi, V. Bregola, A.S. Carretero, I. Sedej, et al., Health-promoting phytochemicals of Italian common wheat varieties grown under low-input agricultural management., J. Sci. Food Agric. 92 (2012) 2800–10. doi:10.1002/jsfa.5590.

[55] I. Nicoletti, D. Martini, A. De Rossi, F. Taddei, M.G. D'Egidio, D. Corradini, Identification and quantification of soluble free, soluble conjugated, and insoluble bound phenolic acids in durum wheat (triticum

turgidum L. var. durum) and derived products by RP-HPLC on a semimicro separation scale, J. Agric. Food Chem. 61 (2013) 11800–11807. doi:10.1021/jf403568c.

[56] M.M. Smith, R.D. Hartley, Occurrence and nature of ferulic acid substitution of cell-wall polysaccharides in graminaceous plants, Carbohydr. Res. 118 (1983) 65–80. doi:10.1016/0008-6215(83)88036-7.

[57] W. Li, F. Shan, S. Sun, H. Corke, T. Beta, Free Radical Scavenging Properties and Phenolic Content of Chinese Black-Grained Wheat, (2005) 10–13.

[58] F. Sosulski, K. Krygier, L. Hogge, Free, Esterified, and insoluble-Bound Phenolic Acids. 3. Composition of Phenolic Acids in Cereal and Potato Flours, J. Agric. Food Chem. 30 (1982) 337–340. doi:10.1021/jf00110a030.

[59] I. Lempereur, X. Rouau, J. Abecassis, Genetic and Agronomic Variation in Arabinoxylan and Ferulic Acid Contents of Durum Wheat (Triticum durumL.) Grain and Its Milling Fractions, J. Cereal Sci. 25 (1997) 103–110. doi:10.1006/jcrs.1996.0090.

[60] L. Li, P.R. Shewry, J.L. Ward, Phenolic acids in wheat varieties in the healthgrain diversity screen, J. Agric. Food Chem. 56 (2008) 9732–9739. doi:10.1021/jf801069s.

[61] D. Martini, F. Taddei, I. Nicoletti, R. Ciccoritti, D. Corradini, M.G. D'Egidio, Effects of Genotype and Environment on Phenolic Acids Content and Total Antioxidant Capacity in Durum Wheat, Cereal Chem. J. 91 (2014) 310–317. doi:10.1094/CCHEM-09-13-0201-CESI.

[62] M.Á. Rodríguez-Delgado, G. González-Hernández, J.E. Conde-González, J.P. Pérez-Trujillo, Principal component analysis of the polyphenol content in young red wines, Food Chem. 78 (2002) 523–532. doi:10.1016/S0308-8146(02)00206-6.

Tables

Table 1: Mean values of the antioxidant composition obtained for each investigated wheat variety. Different letters in a column indicate statistically different values

Old Wheat	**FP (mg g^{-1})**	**BP (mg g^{-1})**	**TP (mg g^{-1})**	**FF (mg g^{-1})**	**BF (mg g^{-1})**	**TF (mg g^{-1})**	**DPPH (µmol g^{-1})**	**FRAP (mmol $100g^{-1}$)**
Senatore Cappelli	1.08 (**bcdefg**)	1.76 (**a**)	2.84 (**ab**)	0.30 (**m**)	0.23 (**cdef**)	0.53 (**g**)	5.22 (**bcdef**)	1.33 (**efgh**)
Urria 12	0.92 (**defgh**)	0.90 (**f**)	1.82 (**ef**)	0.51 (**efghij**)	0.13 (**g**)	0.64 (**defg**)	5.06 (**cdefg**)	1.49 (**bcdef**)
Tripolino	1.17 (**abcde**)	1.73 (**a**)	2.90 (**a**)	0.72 (**abcd**)	0.25 (**bcde**)	0.98 (**a**)	5.68 (**bcde**)	1.40 (**cdefg**)
Margherito	0.80 (**fghi**)	0.97 (**ef**)	1.77 (**f**)	0.67 (**bcde**)	0.19 (**efg**)	0.86 (**abcd**)	5.46 (**bcdef**)	1.54 (**bcde**)
Scorsonera	1.15 (**abcde**)	1.50 (**ab**)	2.65 (**abc**)	0.47 (**fghijklm**)	0.29 (**abcd**)	0.76 (**bcdef**)	5.21 (**bcdef**)	1.38 (**defg**)
Bidì	0.71 (**hij**)	1.03 (**def**)	1.74 (**f**)	0.49 (**fghijkl**)	0.16 (**fg**)	0.65 (**cdefg**)	5.32 (**bcdef**)	1.49 (**bcdef**)
Russello	0.75 (**gijh**)	1.12 (**bcdef**)	1.87 (**ef**)	0.65 (**bcdef**)	0.16 (**fg**)	0.81 (**abcde**)	5.90 (**bcd**)	1.68 (**b**)
Timilia	0.75 (**gijh**)	1.09 (**cdef**)	1.84 (**ef**)	0.75 (**abc**)	0.25 (**bcde**)	1.01 (**a**)	6.18 (**bc**)	1.69 (**b**)
Inglesa	1.48 (**a**)	1.40 (**abcd**)	2.88 (**a**)	0.53 (**efghi**)	0.37 (**a**)	0.90 (**ab**)	7.44 (**a**)	1.54 (**bcde**)
Tunisina	1.27 (**abc**)	1.46 (**abc**)	2.73 (**abc**)	0.35 (**jklm**)	0.31 (**abc**)	0.66 (**cdefg**)	4.98 (**defg**)	1.23 (**ghi**)
Regina	0.95 (**cdefgh**)	0.88 (**f**)	1.83 (**ef**)	0.51 (**efghijk**)	0.16 (**fg**)	0.67 (**cdefg**)	5.23 (**bcdef**)	1.51 (**bcde**)
Manto di Maria	1.22 (**abcde**)	1.07 (**def**)	2.28 (**bcdef**)	0.72 (**abcd**)	0.14 (**g**)	0.87 (**abc**)	4.98 (**defg**)	1.54 (**bcde**)
Chiattulidda	0.77 (**ghij**)	1.04 (**def**)	1.82 (**ef**)	0.86 (**a**)	0.15 (**fg**)	1.00 (**a**)	5.73 (**bcde**)	1.61 (**bcd**)
Ruscia	1.14 (**bcdef**)	1.04 (**def**)	2.18 (**cdef**)	0.82 (**ab**)	0.17 (**efg**)	0.99 (**a**)	5.67 (**bcde**)	1.66 (**b**)
Kamut	1.29 (**ab**)	0.92 (**f**)	2.21 (**cdef**)	0.68 (**abcde**)	0.31 (**abc**)	0.99 (**a**)	5.33 (**bcdef**)	1.09 (**hi**)
Modern Wheat								
Anco Marzio	1.26 (**abc**)	1.24 (**bcdef**)	2.51 (**abcd**)	0.62 (**cdefg**)	0.17 (**efg**)	0.79 (**abcde**)	6.24 (**b**)	1.94 (**a**)
Claudio	0.47 (**j**)	1.32 (**bcde**)	1.79 (**f**)	0.45 (**ghijklm**)	0.14 (**g**)	0.59 (**efg**)	4.06 (**g**)	1.05 (**i**)
Iride	0.99 (**bcdefgh**)	1.41 (**abcd**)	2.40 (**abcde**)	0.34 (**klm**)	0.21 (**defg**)	0.55 (**fg**)	4.99 (**defg**)	1.18 (**ghi**)
Simeto	0.91 (**efgh**)	0.95 (**ef**)	1.86 (**ef**)	0.36 (**ijklm**)	0.33 (**ab**)	0.69 (**bcdefg**)	4.34 (**fg**)	1.26 (**fghi**)
Quadrato	0.99 (**bcdefgh**)	0.99 (**ef**)	1.98 (**def**)	0.52 (**efghij**)	0.15 (**fg**)	0.67 (**cdefg**)	5.46 (**bcdef**)	1.64 (**bc**)
Ciccio	1.25 (**abcd**)	1.48 (**abc**)	2.72 (**abc**)	0.32 (**lm**)	0.30 (**abcd**)	0.62 (**efg**)	4.78 (**defg**)	1.19 (**ghi**)
Duilio	0.93 (**defgh**)	0.90 (**f**)	1.84 (**ef**)	0.57 (**defgh**)	0.16 (**fg**)	0.73 (**bcdefg**)	5.59 (**bcde**)	1.53 (**bcde**)
Alemanno	0.54 (**ij**)	1.48 (**abc**)	2.01 (**def**)	0.41 (**hijklm**)	0.16 (**fg**)	0.57 (**fg**)	5.32 (**bcdef**)	1.25 (**ghi**)
Creso	0.53 (**ij**)	1.20 (**bcdef**)	1.73 (**f**)	0.38 (**ijklm**)	0.16 (**fg**)	0.54 (**fg**)	4.67 (**efg**)	1.14 (**hi**)
Mean Value	0.97	1.20	2.18	0.54	0.21	0.75	5.37	1.43

FP = Free Polyphenols; BP = Bound Polyphenols; TP = Total Polyphenols; FF = Free Flavonoids; BF = Bound Flavonoids; TF = Total Flavonoids.

Table 2: Retention time (RT), linear range, regression values, limits of detection (LOD), limits of quantification (LOQ), mass, fragment and concentration range of the 7 standards identified.

Compound	Retention Time (min)	Linear Range (µg/ml)	R^2	LOD (ng/ml)	LOQ (ng/ml)	Precursor ion (*m/z*) $[M - H]^-$	Product ions (*m/z*) (Collision Energy, V)	Tube lens offset (V)
4-Hydroxybenzaldehyde	2.48 ± 0.04	0.2 – 9.9	0.9892	19.40	64.68	121.20	92.3 (26)	62
p-Coumaric acid	2.70 ± 0.02	0.2 – 9.9	0.9898	197.83	659.44	163.10	93.3 (40), 119.2 (19)	42, 76
Vanillin	2.80 ± 0.02	0.2 – 9.9	0.9966	43.31	144.38	151.20	123.2 (14), 136.1 (15)	68
Syringaldehyde	2.93 ± 0.06	0.2 – 9.9	0.9951	41.77	139.24	181.10	151.1 (22), 166.1 (16)	45, 42
trans-Ferulic acid	3.00 ± 0.01	0.2 – 9.9	0.9953	187.75	625.83	193.10	134.1 (19), 178.1 (16)	62, 31
Formononetin	7.00 ± 0.07	0.2 – 9.9	0.9892	535.23	1784.09	267.10	135.1 (34), 252.1 (23)	67
Apigenin	5.64 ± 0.07	0.2 – 9.9	0.9823	94.50	315.02	269.10	117.2 (39), 149.1 (25)	31, 70
Diosmetin (internal standard)	6.00 ± 0.01	-	-	-	-	299.10	151.1 (36), 283.9 (25)	58

Table3: Amount of phenolic compounds found in the studied wheat varieties expressed as average values ± standard deviation (µg/g DW) and mean values of phenolic compounds in old and modern wheat varieties. Different letters in a column indicate statistically different values. Ns, not significant; *, $P < 0.05$.

Old Wheat	**4-hydroxybenzaldehyde**	**p-Coumaric acid**	**Vanillin**	**Syringaldehyde**	**Ferulic acid**	**Formononetin**	**Apigenin**
Senatore Cappelli	0.130 ± 0.019	1.59 ± 0.097	0.600 ± 0.042	0.651 ± 0.057	137.58 ± 13.57	0.023 ± 0.001	NF
Urria 12	0.147 ± 0.007	4.104 ± 0.207	0.458 ± 0.050	1.270 ± 0.079	259.75 ± 26.30	0.044 ± 0.001	NF
Tripolino	0.086 ± 0.004	2.186 ± 0.288	0.240 ± 0.016	0.710 ± 0.035	127.44 ± 17.68	0.026 ± 0.001	NF
Margherito	0.057 ± 0.013	1.653 ± 0.212	0.345 ± 0.034	0.645 ± 0.075	133.15 ± 18.12	0.031 ± 0.002	NF
Scorsonera	0.032 ± 0.002	1.593 ± 0.066	0.249 ± 0.031	0.576 ± 0.045	115.76 ± 8.98	0.022 ± 0.001	NF
Bidì	0.026 ± 0.004	1.393 ± 0.131	0.182 ± 0.013	0.529 ± 0.025	121.00 ± 11.36	0.025 ± 0.001	NF
Russello	0.044 ± 0.004	0.955 ± 0.041	0.157 ± 0.019	0.343 ± 0.037	72.05 ± 6.28	0.005 ± 0.001	NF
Timilia	0.088 ± 0.009	0.774 ± 0.006	0.317 ± 0.005	0.528 ± 0.032	68.01 ± 2.28	0.005 ± 0.001	NF
Inglesa	0.040 ± 0.004	0.149 ± 0.021	0.178 ± 0.024	0.150 ± 0.019	5.53 ± 0.93	0.003 ± 0.001	NF
Tunisina	0.024 ± 0.003	0.439 ± 0.042	0.041 ± 0.006	0.208 ± 0.014	37.40 ± 5.37	0.008 ± 0.001	NF
Regina	0.008 ± 0.001	0.475 ± 0.021	0.058 ± 0.004	0.193 ± 0.016	35.21 ± 2.44	0.009 ± 0.001	NF
Manto di Maria	0.025 ± 0.001	1.035 ± 0.054	0.092 ± 0.002	0.320 ± 0.017	60.52 ± 6.06	0.010 ± 0.001	NF
Chiattulidda	0.020 ± 0.001	0.752 ± 0.018	0.075 ± 0.004	0.346 ± 0.012	74.15 ± 6.93	0.009 ± 0.001	NF
Ruscia	0.019 ± 0.001	1.140 ± 0.036	0.060 ±0.005	0.396 ± 0.022	80.78 ± 9.76	0.013 ± 0.001	NF
Kamut	0.021 ± 0.005	0.482 ± 0.056	0.028 ± 0.004	0.186 ± 0.022	60.53 ± 5.41	0.014 ± 0.001	NF
Modern Wheat							
Anco Marzio	0.005 ± 0.001	0.108 ± 0.005	0.033 ± 0.003	0.102 ± 0.003	10.62 ± 0.36	0.005 ± 0.001	NF
Claudio	0.092 ± 0.002	0.965 ± 0.021	0.248 ± 0.021	1.193 ± 0.050	127.17 ± 9.55	0.005 ± 0.001	NF
Iride	0.047 ± 0.002	0.908 ± 0.037	0.106 ± 0.007	0.640 ± 0.030	87.27 ± 8.00	0.013 ± 0.001	NF
Simeto	0.014 ± 0.002	0.598 ± 0.027	0.038 ± 0.003	0.248 ± 0.009	62.38 ± 2.18	0.009 ± 0.001	NF
Quadrato	0.016 ± 0.002	0.300 ± 0.017	0.028 ± 0.003	0.140 ± 0.014	19.29 ± 2.33	0.009 ± 0.001	NF
Ciccio	0.022 ± 0.004	0.253 ± 0.010	0.048 ± 0.004	0.106 ± 0.010	15.84 ± 2.53	0.007 ± 0.001	NF
Duilio	0.020 ± 0.005	0.764 ± 0.105	0.055 ± 0.006	0.184 ± 0.034	41.99 ± 6.64	0.011 ± 0.001	NF
Alemanno	0.013 ± 0.002	0.436 ± 0.027	0.053 ± 0.006	0.131 ± 0.007	35.13 ± 1.05	0.012 ± 0.001	NF
Creso	0.011 ± 0.002	0.466 ± 0.009	0.028 ± 0.002	0.130 ± 0.010	43.40 ± 1.79	0.013 ± 0.002	NF
Old	0.049 (a) ns	1.237 (a) *	0.197 (a) *	0.447 (a) ns	88.538 (a) ns	0.016 (a) ns	NF
Modern	0.027 (a) ns	0.533 (b) *	0.071 (b) *	0.319 (a) ns	49.232 (a) ns	0.009 (a) ns	NF

NF = not found

Figure captions

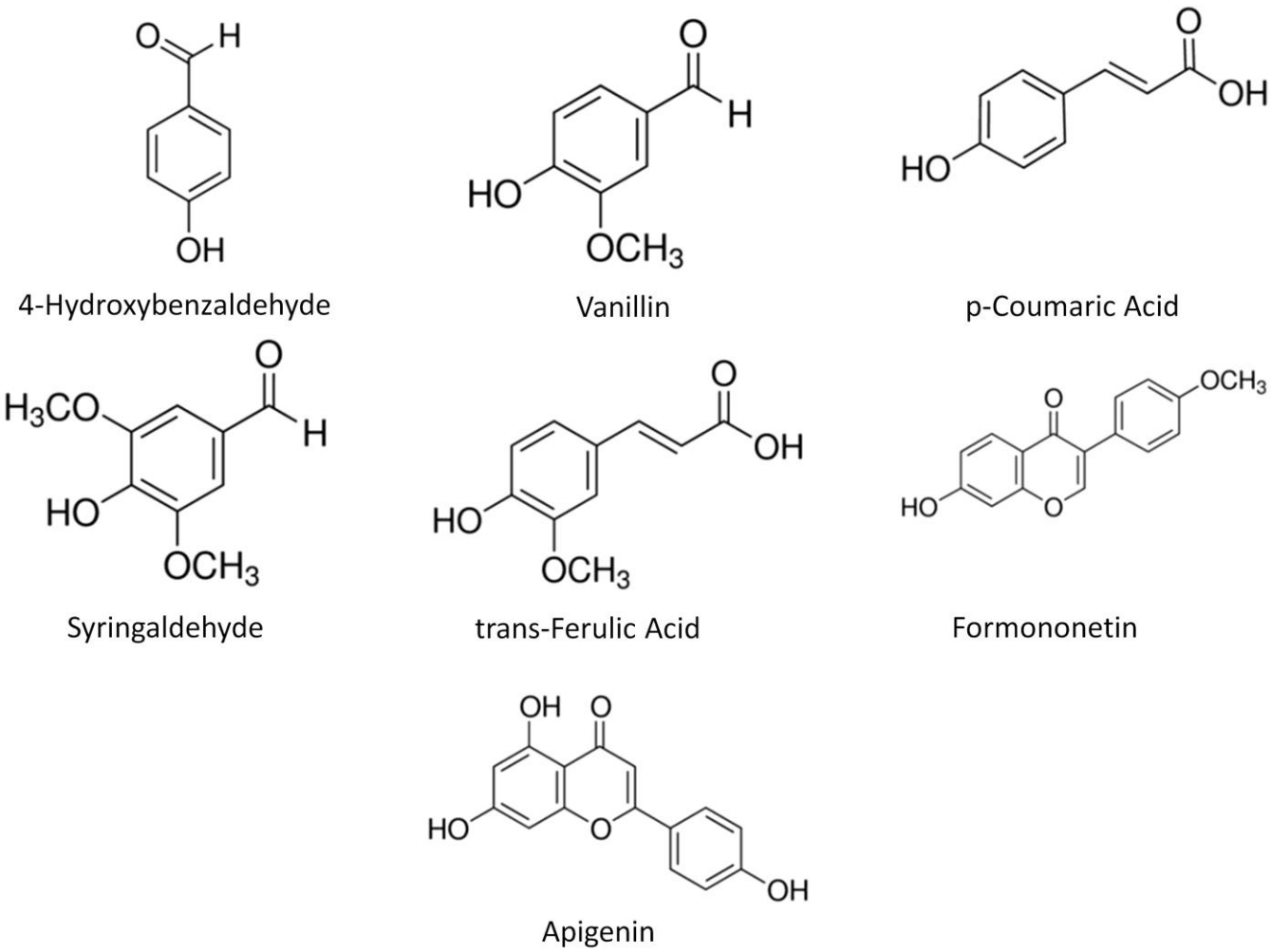

Figure 1: Chemical structures of the seven phenolic compounds identified in the wheat samples.

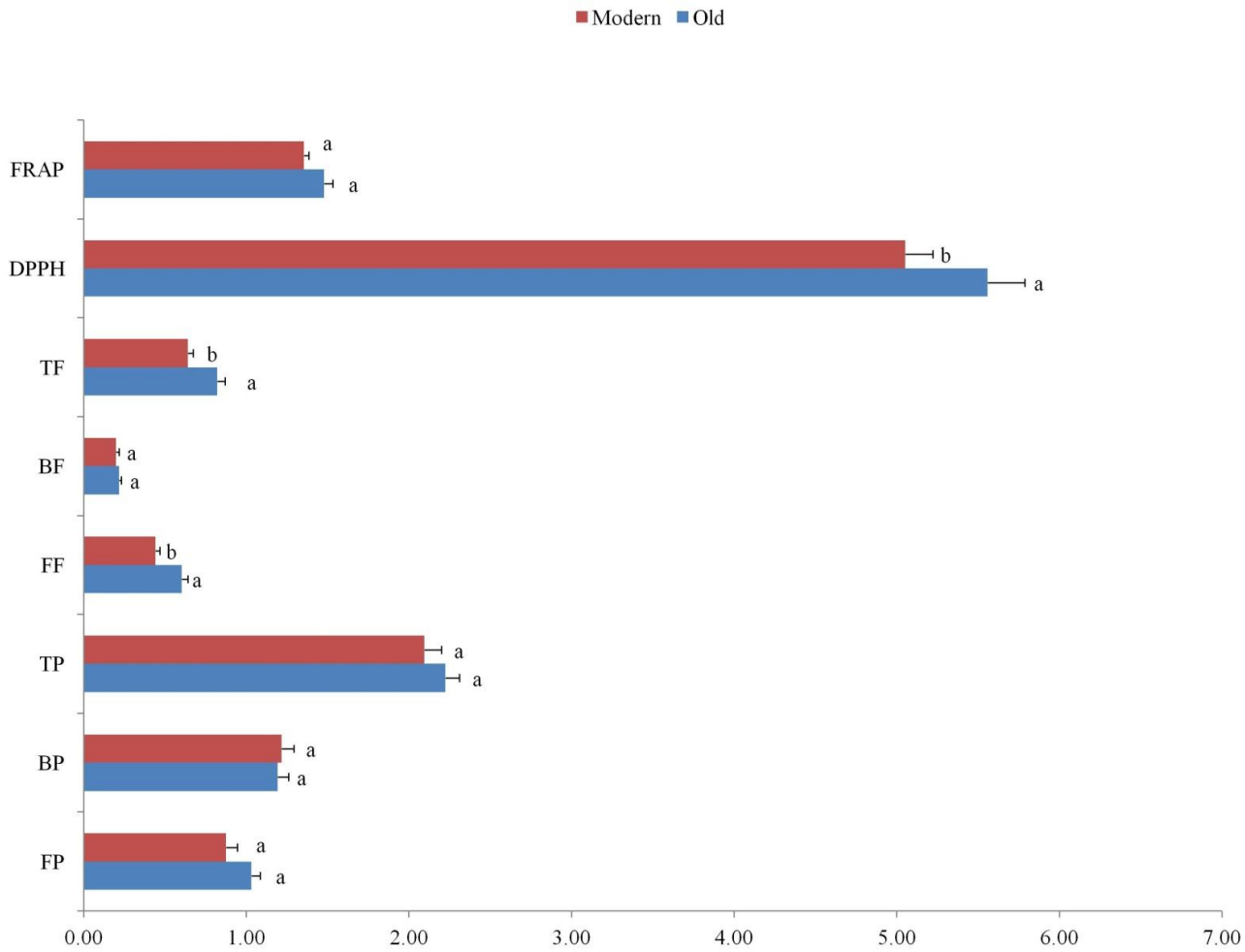

Figure 2: Comparison between the mean values obtained for old and modern wheat varieties. Different letters indicate statistically different values. FP = Free Polyphenols; BP = Bound Polyphenols; TP = Total Polyphenols; FF = Free Flavonoids; BF = Bound Flavonoids; TF = Total Flavonoids.

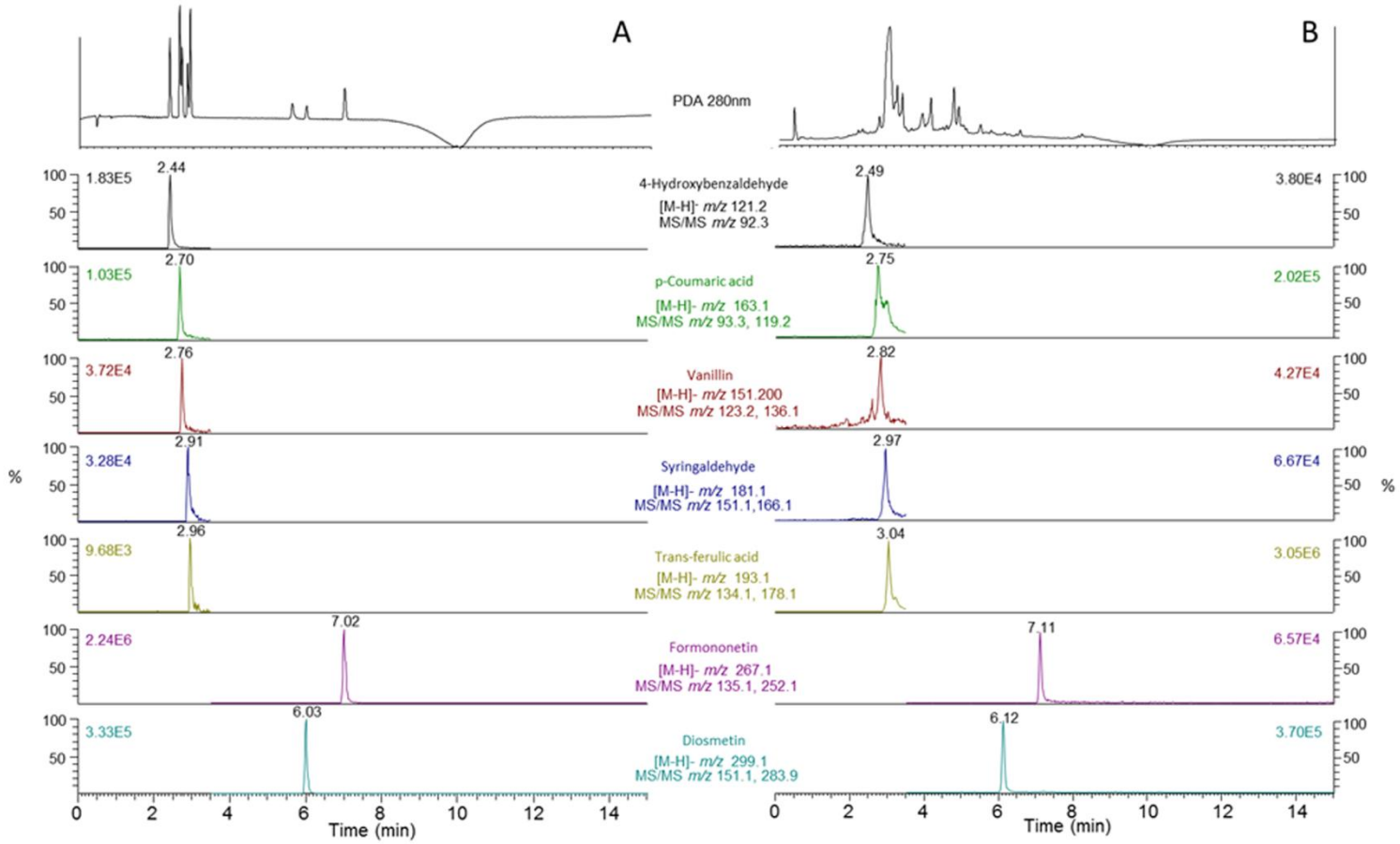

Figure 3: Chromatographic separation (280 nm) and MRM extracted UPLC–MS/MS chromatograms of the quantified compounds (4-hydroxybenzaldehyde, p-coumaric acid, vanillin, syringaldehyde, trans-ferulic acid, and formononetin) and the internal standard (diosmentin) of the mix of standards (A) and of the Senatore Cappelli sample (B).

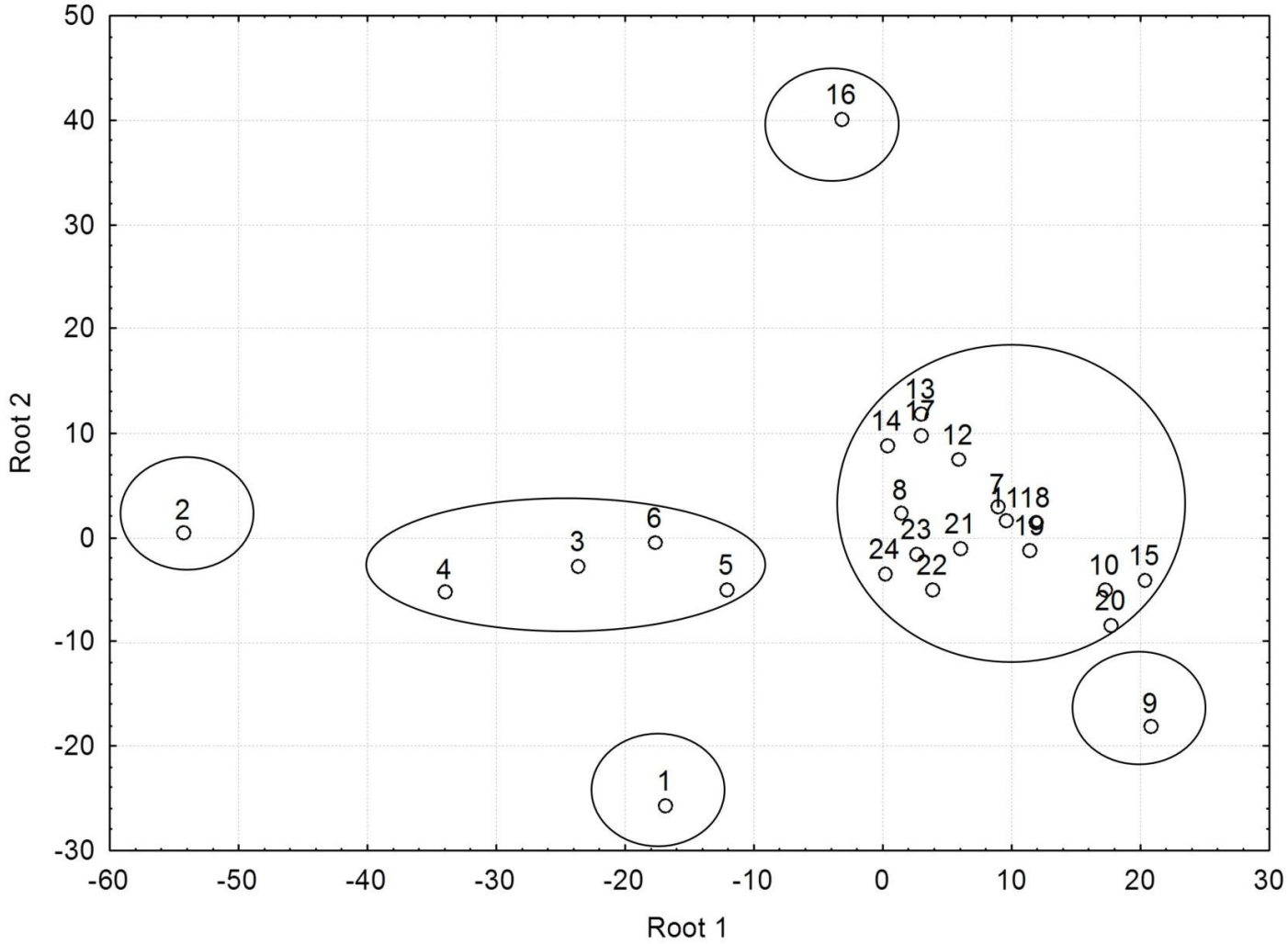

Figure 4: Scatterplot of the 24 investigated wheat varieties: 1, Senatore Cappelli; 2, Urria12; 3, Tripolino; 4, Margherito; 5, Scorsonera; 6, Bidì; 7, Russello; 8, Timilia; 9, Inglesa; 10, Tunisina; 11, Regina; 12, Manto di Maria; 13, Chiattulidda; 14, Ruscia; 15, Anco Marzio; 16, Claudio; 17, Iride; 18, Simeto; 19, Quadrato; 20, Ciccio; 21, Duilio; 22, Alemanno; 23, Creso; 24, KAMUT® khorasan according to the antioxidant composition defined by the first two canonical functions.

CONCLUSION

The work reported in this thesis gives a fundamental understanding of durum wheat grains composition in terms of nutrient and bioactive compounds. Durum wheat was selected for this study because is one of the most important cereal crops used in the Mediterranean-type temperate zones for the production of staple food, such as pasta, couscous, and various types of bread. Among durum type wheat varieties, KAMUT® khorasan raised great interest in the consumers for its nutritional and functional properties (anti-inflammatory, antioxidant and prebiotic).

The first goal of this PhD thesis was the investigation of the nutrient and phytochemical accumulation as a function of changing environmental conditions in a long term experiment. The analyses showed great variability among samples collected in different years of cultivation. In particular, the greater environmental effects were observed for polysaccharides (starch and some dietary fibre components) and for phenolic compounds. More research is needed to investigate the cause of location effects, the correlations with soil samples and previous crops planted in rotation, extend the study to several genotype, and determine the heritability of the antioxidant properties. However, this section has a unique value, because in literature are present very few work in which the same genotype is cultivated during twenty-one cropping years with the same agricultural practice.

This research led to the preparation of one paper:

- "Nutritional and nutraceutical aspects of KAMUT® khorasan wheat grown during the last two decades", Di Loreto et al., (2016) under submission.

The second achievement concern the determination of the productivity, nutrient and nutraceutic composition of KAMUT® grain as a function of the growing location aiming at understanding the environmental dynamics affecting the phytochemical profile. Results were elaborated using Geographic Information System (GIS) to develop *quality maps*. These maps showed the variability of the phytochemical parameters among North American farms and allowed the

identification of areas in which bioactive compounds had accumulated at a higher level in the wheat grains. Results of this section underlined the strong influence of the environment on crop productivity and quality. According to the present results, the choice of a growing location in which the accumulation of health-promoting compounds (i.e. antioxidants, fibres) is stimulated by particular climatic conditions emerges as a key feature for the production of organic wheat foodstuff in the functional food scenario. The study, however, is in progress and results obtained here will be integrated with the third year of KAMUT® khorasan cultivation. Once the study will be completed, a paper will be prepared and submitted.

In the last part of the present research activity for the first time UHPLC coupled to tandem mass spectrometry was used to quantify phenolic compounds in durum and durum-type wheat grains. Remarkable quantitative differences were detected among the 15 old and the 9 modern wheat genotypes investigated in this section of the thesis. Among the single phenolic acids the most abundant in durum wheat was the trans isomer of ferulic acid. All the other identified compounds, even if present at low concentration compared to ferulic acid, showed great variability among the 24 old and modern durum wheat varieties. In addition, this is the first work in which the content of vanillin, 4-hydroxybenzaldehyde and formononetin were studied in durum wheat varieties. Differences were also observed in the amount of polyphenols and flavonoids, considering the free and bound fractions. Comparison between modern and old durum wheat varieties evidenced significant differences in the content of free and total flavonoids and in the antioxidant activity measured by DPPH test. However, further studies are necessary to fully understand the dynamic affecting the concentration of antioxidant compounds in durum wheat caryopsis, including genotypic and environmental influence.
This work led to the preparation of a paper:

- “Quantification of free and bound phenolic compounds in old and modern durum wheat varieties using ultra high performance liquid chromatography coupled to tandem mass spectrometry”, Di Loreto et al.,(2016) under submission.

REFERENCES

Adom, K.K., Sorrells, M.E., Liu, R.H., 2003. Phytochemical Profiles and Antioxidant Activity of Wheat Varieties. J. Agric. Food Chem. 51, 7825–7834. doi:10.1021/jf030404l

Adom, K.K., Sorrells, M.E., Rui, H.L., 2005. Phytochemicals and antioxidant activity of milled fractions of different wheat varieties. J. Agric. Food Chem. 53, 2297–2306. doi:10.1021/jf048456d

Altenbach, S.B., DuPont, F.M., Kothari, K.M., Chan, R., Johnson, E.L., Lieu, D., 2003. Temperature, Water and Fertilizer Influence the Timing of Key Events During Grain Development in a US Spring Wheat. J. Cereal Sci. 37, 9–20. doi:10.1006/jcrs.2002.0483

Ceccarelli, S., Grando, S., Maatougui, M., Michael, M., Slash, M., Haghparast, R., Rahmanian, M., Taheri, a., Al-Yassin, a., Benbelkacem, a., Labdi, M., Mimoun, H., Nachit, M., 2010. Plant breeding and climate changes. J. Agric. Sci. 148, 627–637. doi:10.1017/S0021859610000651

Database - Eurostat. URL: http://ec.europa.eu/eurostat/data/database.

De Vita, P., Di Paolo, E., Fecondo, G., Di Fonzo, N., Pisante, M., 2007. No-tillage and conventional tillage effects on durum wheat yield, grain quality and soil moisture content in southern Italy. Soil Tillage Res. 92, 69–78. doi:10.1016/j.still.2006.01.012

De Vita, P., Nicosia, O.L.D., Nigro, F., Platani, C., Riefolo, C., Di Fonzo, N., Cattivelli, L., 2007. Breeding progress in morpho-physiological, agronomical and qualitative traits of durum wheat cultivars released in Italy during the 20th century. Eur. J. Agron. 26, 39–53. doi:10.1016/j.eja.2006.08.009

Di Silvestro, R., Di Loreto, A., Marotti, I., Bosi, S., Bregola, V., Gianotti, A., Quinn, R., Dinelli, G., 2014. Effects of flour storage and heat generated during milling on starch, dietary fibre and polyphenols in stoneground flours from two durum-type wheats. Int. J. Food Sci. Technol. 49, 2230–2236. doi:10.1111/ijfs.12536

Dinelli, G., Segura-Carretero, A., Di Silvestro, R., Marotti, I., Arráez-Román, D., Benedettelli, S., Ghiselli, L., Fernadez-Gutierrez, A., 2011. Profiles of phenolic compounds in modern and old common wheat varieties

determined by liquid chromatography coupled with time-of-flight mass spectrometry. J. Chromatogr. A 1218, 7670–81. doi:10.1016/j.chroma.2011.05.065

Gebruers, K., Dornez, E., Boros, D., Fraś, A., Dynkowska, W., Bedo, Z., Rakszegi, M., Delcour, J. a., Courtin, C.M., 2008. Variation in the content of dietary fiber and components thereof in wheats in the healthgrain diversity screen. J. Agric. Food Chem. 56, 9740–9749. doi:10.1021/jf800975w

Gil, A., Ortega, R.M., Maldonado, J., 2011. Wholegrain cereals and bread: a duet of the Mediterranean diet for the prevention of chronic diseases. Public Health Nutr. 14, 2316–2322. doi:10.1017/S1368980011002576

Grausgruber, H., Sailer, C., Ruckenbauer, P., 2004. Khorasan wheat, Kamut® and "Pharaonenkorn": Origin, characteristics and potential 75–80.

Graybosch, R. a., Peterson, C.J., Baenziger, P.S., Shelton, D.R., 1995. Environmental modification of hard red winter wheat flour protein composition. J. Cereal Sci. 22, 45–51. doi:10.1016/S0733-5210(05)80006-2

Herencia, J.F., Ruiz, J.C., Melero, S., Garcia Galavís, P. a., Maqueda, C., 2008. A short-term comparison of organic v. conventional agriculture in a silty loam soil using two organic amendments. J. Agric. Sci. 146, 677. doi:10.1017/S0021859608008071

Hirawan, R., Ser, W.Y., Arntfield, S.D., Beta, T., 2010. Antioxidant properties of commercial, regular- and whole-wheat spaghetti. Food Chem. 119, 258–264. doi:10.1016/j.foodchem.2009.06.022

Khlestkina, E.K., Roder, M.S., Grausgruber, H., Borner, A., 2006. A DNA fingerprinting-based taxonomic allocation of Kamut wheat. Plant Genet. Resour. 4, 172-180. doi:10.1079/PGR2006120

Leoncini, E., Prata, C., Malaguti, M., Marotti, I., Segura-Carretero, A., Catizone, P., Dinelli, G., Hrelia, S., 2012. Phytochemical Profile and Nutraceutical Value of Old and Modern Common Wheat Cultivars. PLoS One 7(9): e45997. doi:10.1371/journal.pone.0045997

Liu, R.H., 2004. Potential synergy of phytochemicals in cancer prevention: mechanism of action. J. Nutr. 134, 3479S–3485S. doi:134/12/3479S [pii]

Motzo, R., Fois, S., Giunta, F., 2004. Relationship between grain yield and quality of durum wheats from different eras of breeding. Euphytica 140, 147–154. doi:10.1007/s10681-004-2034-5

Mpofu, A., Sapirstein, H.D., Beta, T., 2006. Genotype and environmental variation in phenolic content, phenolic acid composition, and antioxidant activity of hard spring wheat. J. Agric. Food Chem. 54, 1265–1270. doi:10.1021/jf052683d

Murphy, K.M., Campbell, K.G., Lyon, S.R., Jones, S.S., 2007. Evidence of varietal adaptation to organic farming systems. F. Crop. Res. 102, 172–177. doi:10.1016/j.fcr.2007.03.011

Okarter, N., Liu, C.S., Sorrells, M.E., Liu, R.H., 2010. Phytochemical content and antioxidant activity of six diverse varieties of whole wheat. Food Chem. 119, 249–257. doi:10.1016/j.foodchem.2009.06.021

Pecetti, L., Annicchiarico, P., 1998. Agronomic value and plant type of Italian durum wheat cultivars from different eras of breeding. Euphytica 99, 9–15.

Quinn, R.M., 1999. Kamut: ancient grain, new cereal. Perspect. new Crop. new uses 182–183.

Sahyoun, N.R., Jacques, P.F., Zhang, X.L.L., Juan, W.Y., McKeown, N.M., 2006. Whole-grain intake is inversely associated with the metabolic syndrome and mortality in older adults. Am. J. Clin. Nutr. 83, 124–131.

Shewry, P.R., Piironen, V., Lampi, A.M., Edelmann, M., Kariluoto, S., Nurmi, T., Fernandez-Orozco, R., Ravel, C., Charmet, G., Andersson, A. a M., Åman, P., Boros, D., Gebruers, K., Dornez, E., Courtin, C.M., Delcour, J. a., Rakszegi, M., Bedo, Z., Ward, J.L., 2010. The HEALTHGRAIN wheat diversity screen: Effects of genotype and environment on phytochemicals and dietary fiber components. J. Agric. Food Chem. 58, 9291–9298. doi:10.1021/jf100039b

Slavin, J., 2004. Whole grains and human health. Nutr. Res. Rev. 17, 99–110. doi:10.1079/NRR200374

Sofi, F., Whittaker, A., Cesari, F., Gori, A.M., Fiorillo, C., Becatti, M., Marotti, I., Dinelli, G., Casini, A., Abbate, R., Gensini, G.F., Benedettelli, S., 2013. Characterization of Khorasan wheat (Kamut) and impact of a replacement diet on cardiovascular risk factors: cross-over dietary intervention study.

Eur. J. Clin. Nutr. 67, 190–5. doi:10.1038/ejcn.2012.206

Sofi, F., Whittaker, A., Gori, A.M., Cesari, F., Surrenti, E., Abbate, R., Gensini, G.F., Benedettelli, S., Casini, A., 2014. Effect of Triticum turgidum subsp. turanicum wheat on irritable bowel syndrome: a double-blinded randomised dietary intervention trial. Br. J. Nutr. 1–8. doi:10.1017/S000711451400018X

Truswell, A.S., 2002. Cereal grains and coronary heart disease. Eur. J. Clin. Nutr. 56, 1–14. doi:10.1038/sj.ejcn.1601283

Vincent, L.A., Wang, X.L., Milewska, E.J., Wan, H., Yang, F., Swail, V., 2012. A second generation of homogenized Canadian monthly surface air temperature for climate trend analysis. J. Geophys. Res. Atmos. 117, 1–13. doi:10.1029/2012JD017859

Whittaker, A., Dinu, M., Cesari, F., Gori, A.M., Fiorillo, C., Becatti, M., Casini, A., Marcucci, R., Benedettelli, S., Sofi, F., 2016. A khorasan wheat-based replacement diet improves risk profile of patients with type 2 diabetes mellitus (T2DM): a randomized crossover trial. Eur. J. Nutr. doi:10.1007/s00394-016-1168-2

Whittaker, A., Sofi, F., Luisi, M., Rafanelli, E., Fiorillo, C., Becatti, M., Abbate, R., Casini, A., Gensini, G., Benedettelli, S., 2015. An Organic Khorasan Wheat-Based Replacement Diet Improves Risk Profile of Patients with Acute Coronary Syndrome: A Randomized Crossover Trial. Nutrients 7, 3401–3415. doi:10.3390/nu7053401

Yu, L., Haley, S., Perret, J., 2002. Free radical scavenging properties of wheat extracts. J. Agric. Food Chem. 50, 1619–1624.