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Università Campus Bio-Medico di Roma

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ANTIOXIDANT COMPOUNDS IN DURUM WHEAT: STUDY OF GENETIC, ENVIRONMENTAL AND TECHNOLOGICAL INFLUENCING FACTORS FOR THE DEVELOPMENT OF PASTA WITH HIGH NUTRITIONAL AND HEALTHY POTENTIAL

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ABSTRACT

Aim of the research activity performed during the PhD was to investigate the effects of genetic, environmental (crop year and growing area) and technological factors (both traditional and innovative) on the occurrence of antioxidant compounds and on the total antioxidant activity in durum wheat and pasta. The researches were mainly focused on the occurrence of phenolic acids (PAs), analyzed by RP-HPLC on a semi-micro separation scale. PAs represent the most common form of phenolic compounds in durum wheat whole grain as soluble free, soluble conjugated and as insoluble bound forms acids. Total phenolic compounds, yellow colored pigments and total antioxidant capacity were also investigated.

Results showed that crop year, genotype, growing area and their interactions significantly affected the content of antioxidant compounds and total antioxidant capacity of durum wheat, although to different extents. Regarding the technological processes, traditional milling and pasta-making negatively influence the content of antioxidant compounds and antioxidant activity, while innovative processes like the debranning appeared to be a valuable way for reaching the level representing the best compromise between nutritional, technological, sensory and hygienic-sanitary aspects.

The last part of the research activity was devoted to develop durum wheat pasta with high nutritional, hygienic-sanitary and sensory quality by using durum wheat debranning, and to explore the bioavailability of phenolic compounds when the pasta samples were consumed. The development of innovative pasta obtained by using debranned products demonstrated that this process can be useful to preserve the occurrence of antioxidant compounds with a low impact on the technological quality and a relatively high bioavailability of phenolic compounds.

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In conclusion, the research activity performed during the PhD demonstrated that, besides environmental and genetic factors, innovative technological processes can be usefully applied to obtain high quality pasta which can help the consumers to increase their daily intake of fiber and many other bioactive compounds.

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1. Introduction

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1.1 DURUM WHEAT

1.1.1 OVERVIEW

Cereal can be defined as any grain or edible seed of the grass family (Gramineae) that may be used as food (Bender, 2006). Cultivated cereal species include a range of types differing widely in their environmental adaptation and their characteristics useful for food, feed or other uses (Welch, 2005).

On the whole, the major cereals cropped worldwide are wheat, rice and maize, while less consumed are barley, oats, rye, millet, sorghum (**Fig.1**). The total world cereal production is over 2 billion tonnes (http://www.fao.org/worldfoodsituation/csdb/en/).

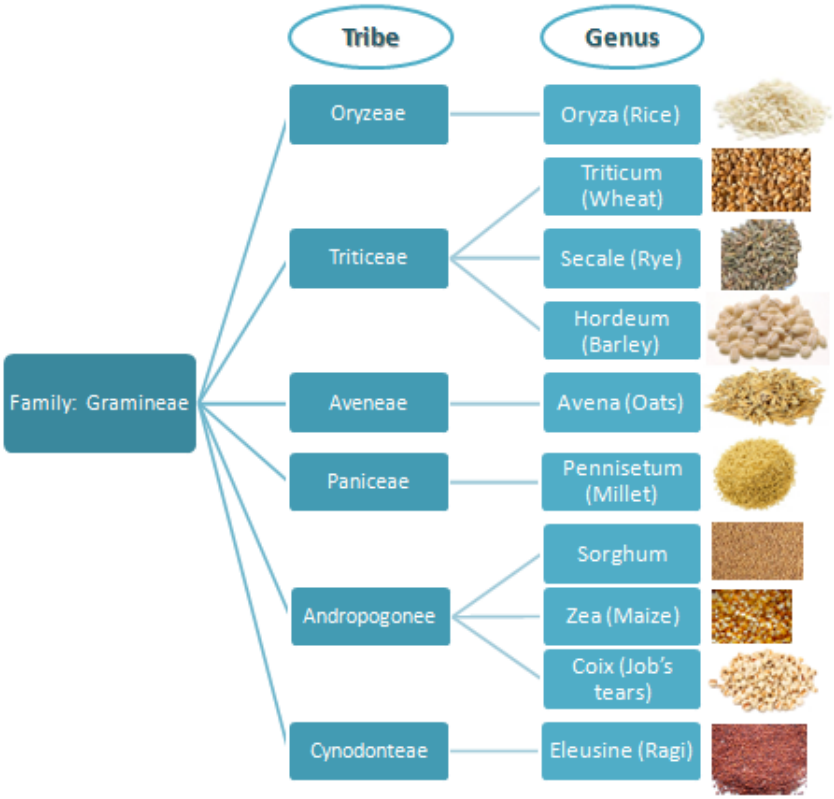

Fig.1 Taxonomy of the Gramineae family (adapted from McKevith, 2004).

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As regards wheat, it is necessary to point out that this term does not refer to a single species. In fact, by the 1920s it was known that cultivated wheat species of the genus *Triticum* may have chromosome numbers of 2n=14, 28 and 42 (Sakamura, 1918); this suggested a basic 1x chromosome number of 7 and the occurrence of diploid (2n=2x=14), tetraploid (2n=4x=28) and hexaploid (2n=6x=42) wheat species (Gill & Friebe, 2002).

Modern wheat cultivars belong primarily to tetraploid wheat (i.e. *Triticum turgidum*, known as durum wheat, genetically describes as AABB plants), and hexaploid wheat (i.e. *Triticum aestivum*, often called common wheat or bread wheat, genetically described as AABBDD). Other species such as *T. monococcum* (AA), *T. dicoccum* (AABB) and *T. spelta* (AABBDD) are indeed less cropped (Peng et al., 2011; Arendt & Zannini, 2013).

Among all cereals, wheat is certainly the leading cereal grain produced, used as human food and traded in the world today.

In detail, about 95% of the wheat cultivated today is grouped under the category of common wheat, while nearly all of the remaining 5% is durum wheat.

Specifically for durum wheat, world production is estimated at over 35 million tonnes/year (**Fig.2**), about 60% of which is concentrated in the Mediterranean area.

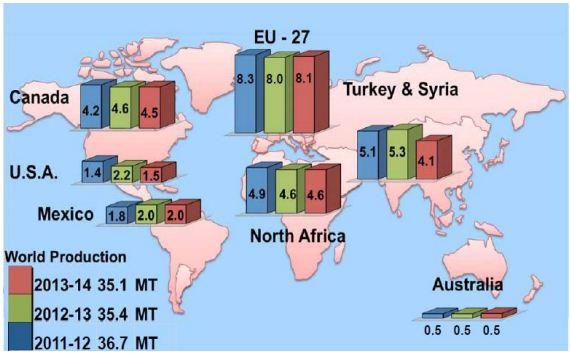

Fig.2 Worldwide production of durum wheat. Source: Canadian Wheat Board.

In Italy, durum wheat is the most cropped cereal with over 1 million hectares cultivated, followed by corn, common wheat, barley and rice (**Fig.3**).

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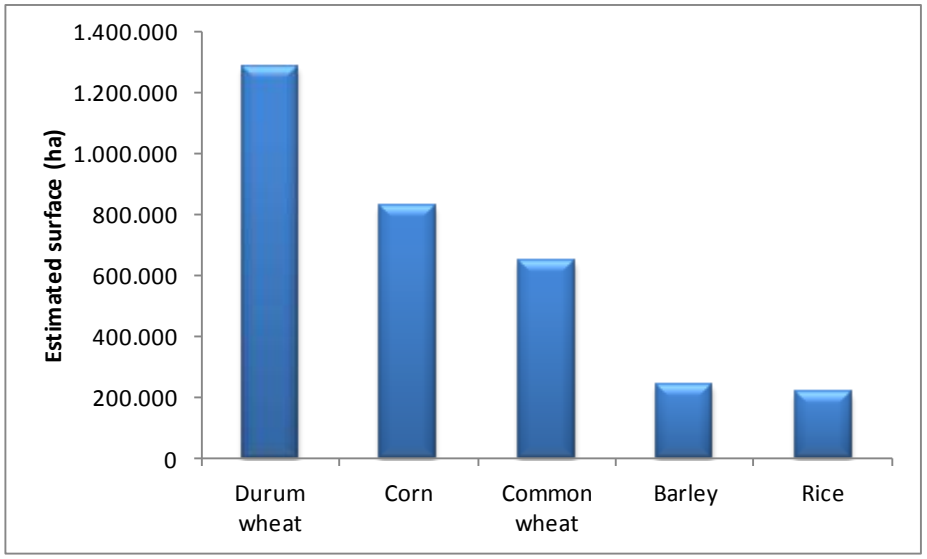

Fig.3 Estimated cereal crop cultivation areas in Italy during the 2014 crop years (Source: elaboration Ismea on data from Istat, 2014; Enterisi, 2014).

1.1.2 CHEMICAL COMPOSITION

From a morphological point of view, durum wheat grains - similarly to the other cereals - consist of three main parts (**Fig.4**):

i) the germ which contains the vital embryo (about 2-3% of total weight of the grain);

ii) the endosperm (about 80-85%), containing the nutritive reserves for the embryo, which consists of the aleurone layer and the mass of starchy endosperm;

iii) the tegumental outer layers (12-18%) which surround the whole seed providing protection. They are composed of several

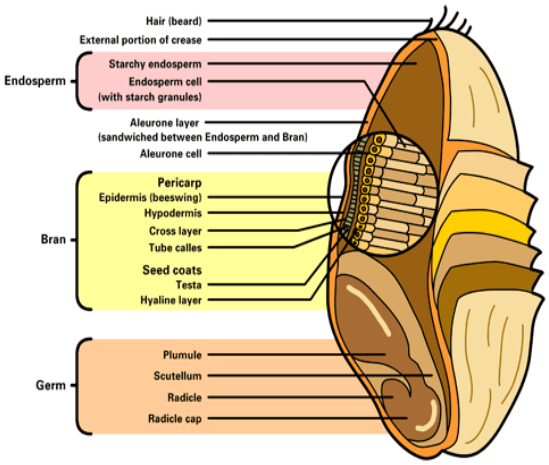

Fig.4 Structure of wheat seed.
Source: www.grainchain.com

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layers, including the outer epidermis (cuticule), hypodermis, cross cells, tube cells, testa and nucellar tissue.

The composition of durum wheat in terms of macronutrients, micronutrients and bioactive compounds is better described in the next paragraphs.

1.1.2.1 Macro and micronutrients

For most people, wheat-based foods are a major source of energy. It has been estimated that wheat, together with rice and maize, provide 60 percent of the world's food energy intake (http://www.fao.org/docrep/u8480e/u8480e07.htm).

In the United States, calories provided by wheat foods account for about 18% of total daily calories, and more than 2% of that are provided by pasta (Ranhotra, 1994). In Italy, where pasta is consumed by over 90% of the population, a mean consumption of 26 kg/pro capita per year has been estimated (IPO, 2012). The energy is mainly provided by carbohydrates but also by protein and least by lipids.

In the next pages, the main macro and micronutrients present in durum wheat will be summarized, also focusing on their impact on different aspects of quality of grains and derived products.

1.1.2.1.1 Starch

Starch is the main constituent of durum wheat, accounting for more than 70% of the dry matter of the endosperm of mature cereal grains. It can be chemically defined as a polymer of α-linked glucose, comprising two polymers of D-glucose: the amylose, a linear polymer with a molecular weight (MW) of 10^5-10^6 which account for 25% of total starch, and the amylopectin, a highly branched polymer with a MW of 10^7-10^8.

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One of the main characteristic of starch is its tendency to absorbs water. Moreover, when heated (58-65°C) in presence of water, starch may gelatinize, leading to granule swelling and bond rupture and irreversibly dissolving the starch granule.

1.1.2.1.2 Proteins

Proteins represent one of the most important quality traits in durum wheat as well as in all cereals (Troccoli et al., 2000), because their content is strongly associated with the final use of cereals and with the quality of derived products. For instance, it has been estimated that protein content can account for 30-40% of pasta cooking quality (Dexter & Matsuo, 1980). The importance of wheat seed protein in determining the technological properties of derived products is demonstrated by the fact that the selection of durum wheat genotypes with an improved protein amount and composition is one of the main objectives in the breeding programs.

Proteins in durum wheat account for about 12% of the dry matter of grains and can be classified in two classes on the basis of biological functions: the cytoplasmic proteins and the storage proteins. The former correspond to the group of albumins and globulins, while the latter comprise prolamins and glutelins.

The major wheat endosperm storage proteins, the gluten proteins, account for about 80% of total grain proteins and comprise two prolamin groups, called gliadin (alcohol-soluble) and glutenin (alcohol-insoluble). Glutenin contributes to the elastic character of gluten while gliadin contributes to extensibility. A balance between elasticity and extensibility is therefore necessary for superior performance in bread and pasta-making.

Cytoplasmic proteins and the storage proteins differ in physical properties and amino acid composition. Generally, the former are easily soluble in water or salt-buffer solution, have

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a small molecular weight and their molecules have globular form. The storage proteins are indeed generally insoluble in water and salt solutions.

The total wheat protein, and especially the storage proteins, are poor in lysine and so their biological value is relatively low. The cytoplasmic proteins have generally a better amino acid composition, due to their higher content of lysine (Lásztity & Hidvégi, 1985).

1.1.2.1.3 Lipids

The lipid content in cereals is highly variable and corn and rye generally displayed the highest content. The content in durum wheat is indeed small (about 2%), nevertheless they play an important role in determining the quality of cereals and derived products.

Lipids are located throughout the kernels as structural components of biomembrans ans organelles as well as in oil-rich tissue like aleurone and scutellum. They can be generally classified as starch and nonstarch lipids (Lafiandra et al., 2012). The latter consist of free lipids mostly belonging to the class of acylglycerols, free fatty acids, free sterols and hydrocarbons which can be extracted with nonpolar organic solvents, and bound lipids which require polar solvents.

Nonpolar lipids comprise many compounds with different physiological functions and represent 60-70% of total lipids. Among these classes, the main components are tryacilglycerols which represent the storage lipids of plants.

Lipids are mainly concentrated in the wheat germ: in fact, germ, endosperm and bran contain 66, 19 and 15% of total lipids, respectively.

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1.1.2.1.4 Mineral and Vitamins

Durum wheat represents a source of various vitamin and minerals, mainly located in bran and germ.

As regards vitamins, it is a source of vitamin B_1, B_2, B_3, B_6, and E (Toepfer et al., 1972), in addition to carotenes, some of which can act as vitamin A precursors. The content of these vitamins is highly variable, depending on genotype, growing area and conditions (Lampi et al., 2010; Shewry et al., 2011).

As regards minerals, durum wheat grains contain magnesium, iron, manganese, copper and molybdenum, beside bioavailable selenium, while it is low in sodium. The content of these trace elements may vary depending on genotype as well as on soil conditions (Zhao et al., 2009).

1.1.2.2 Bioactive compounds

In addition to the macro and micronutrients, durum wheat as well as other cereal grains contain many phytochemicals – defined as "substances naturally occurring in plants and beneficial to human health but not essential for the human body" - which significantly contribute to the health benefits of cereals consumption, particularly as whole grains. In fact, despite concentrations of these substances in foods are usually small, they have attracted attention of researchers because of their biological activities and positive impacts on human health.

These compounds are abundant in the whole grains, with the highest contents in the outermost layers (**Fig.5**). The different groups of health beneficial compounds in whole grains can be classified in fibre and antioxidant compounds, among which phenolic acids, other phenolic compounds and carotenoids.

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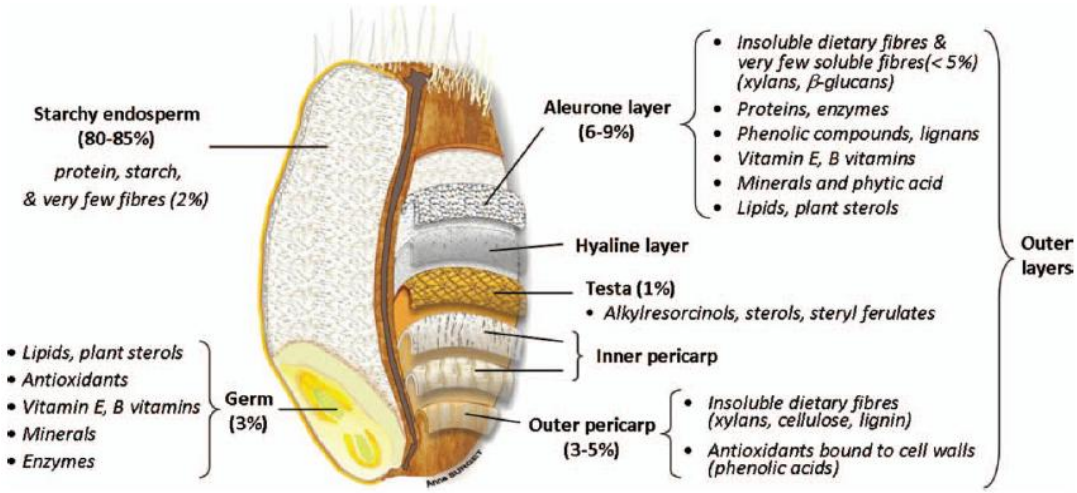

Fig.5 Histological structure of wheat grain and localization of main nutrients and phytochemicals (Brouns et al., 2012).

1.1.2.2.1 Dietary fibre

The Commission Directive 2008/100/EC amending Council Directive 90/496/EEC (Annex II) defined the dietary fibre as "carbohydrate polymers with three or more monomeric units, which are neither digested nor absorbed in the human small intestine and belong to: i) edible carbohydrate polymers naturally occurring in the food as consumed; ii) edible carbohydrate polymers which have been obtained from food raw material by physical, enzymatic, or chemical means and which have a beneficial physiological effect demonstrated by generally accepted scientific evidence; iii) edible synthetic carbohydrate polymers which have a beneficial physiological effect demonstrated by generally accepted scientific evidence."

Dietary fibre are conventionally classified in: i) insoluble dietary fibre (IDF) that is the part of dietary fibre not soluble in water, including cellulose, part of hemicellulose and lignin; ii) soluble dietary fibre (SDF) comprising gums and small oligosaccharides in dietary fibre that are soluble in water, forming viscous gels (Bender, 2006) (**Tab.1**).

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Fibre in whole grain includes both cellulosic (CP) and noncellulosic polysaccharides (NCP) such as lignin, inulin and other constituents mainly present in the bran (Liu, 2007).

Characteristic	Fibre component	Description	Main food sources
Water insoluble/ Less fermented	Cellulose	Main structural component of plant cell wall. Insoluble in concentrated alkali, soluble in concentrated acid.	Plants (vegetables, sugar beet, various brans)
	Hemicellulose	Cell wall polysaccharides, which contain backbone of β-1,4 glucosidic linkages. Soluble in dilute alkali.	Cereal grains
	Lignin	Non-carbohydrate cell wall component. Complex cross-linked phenyl propane polymer. Resists bacterial degradation.	Woody plants
Water soluble/ Well fermented	Pectin	Components of primary cell wall with D-galacturonic acid as principal components. Generally water soluble and gel forming	Fruits, vegetables, legumes, sugar beet, potato
	Gums	Secreted at site of plant injury by specialized secretary cells. Food and pharmaceutical use.	Leguminous seed plants (guar, locust bean), seaweed extracts (carrageenan, alginates), microbial gums (xanthan, gellan)
	Mucilages	Synthesized by plant, prevent desiccation of seed endosperm. Food industry use, hydrophilic, stabilizer.	Plant extracts (gum acacia, gum karaya, gum tragacanth)

Tab.1 Classification of dietary fibre components based on water solubility/fermentability (Dhingra et al., 2012).

Insoluble fibre adds bulk to the stool and appears to help food pass more quickly through the stomach and intestines.

The diets with a high content of fibre have a positive effect on health due to its beneficial effects, including increasing the volume of faecal bulk, decreasing the time of intestinal transit, cholesterol and glycaemic levels, trapping substances that can be dangerous for the human organism such mutagenic and carcinogenic agents, stimulating the proliferation of the intestinal flora and many others (Slavin, 2004; Liu, 2007).

Thanks to these mechanisms, consumption of dietary fibre has been demonstrated to be protective against coronary heart diseases, colorectal and hormone-dependent cancers, diabetes and obesity (Topping & Cobiac, 2005).

The most widely investigated topic related to dietary fibre and human health is the reduction of risk factors for cardiovascular diseases. Many experimental data support that blood cholesterol can be lowered using viscous soluble fibre that produce relatively high

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viscosity in the intestinal tract, by binding bile acids and micelle components, such as free fatty acids and cholesterol, which and increase the faecal excretion of these molecules. For insoluble dietary fibre this reducing effect is rather low compared to viscous soluble dietary fibre (Dello Staffolo et al., 2012).

Among the major constituents of cereal dietary fibre are arabinoxylans (AX), mixed-linked β-glucans, and cellulose.

AX are polymers of pentoses which are the major components of soluble dietary fibre in the cereal grains. AX are distributed over the durum kernel, but the highest content of total AX can be found in bran (Gebruers et al., 2010). AX are the most important structural components of durum wheat kernel cell walls and their contents in durum wheat generally ranges from 4.1 to 7.55% dm; in particular, the water-unextractable ones prevail, generally accounting from 80 to 90% of total AX (Lempereur et al., 1997; Ciccoritti et al., 2011).

Cellulose is a linear polymer of β(1→4) linked D-glucose units and represents an important structural component of the primary cell walls. The cellulose content in durum wheat wholemeal is about 2.7% but the highest concentration are displayed in the bran (9-13%) and the lowest in the endosperm (about 2%).

β-glucans are indeed polysaccharides of D-glucose monomers linked by β-glycosidic bonds and rate among principal fractions of cereal grain dietary fibre, mainly occurring in walls of the subaleuronic layer. β-glucans belong to the class of soluble dietary fibre, and their consumption has been established to be related with the reduction of blood cholesterol concentrations (EFSA, 2011). The β-glucans content in durum wheat is very low compared (0.3-0.6%) compared with that of other cereals such as barley and oat (Lafiandra et al., 2012).

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1.1.2.2.2 Phenolic compounds

Phenolic compounds may be defined as substances that possess an aromatic ring bearing one or more hydroxyl substituents. They are secondary metabolites produced by plants especially under stress condition and act as defence mechanism against pathogens, parasites and predators (Liu, 2007). Most of the literature focused on phenolic compounds in fruit and vegetables, however cereals represent a good source of phenolics, especially considering that a large amount of cereals-based foods is daily consumed.

Among phenolic compounds, the most common ones found in wheat grains of durum wheat and other cereals are phenolic acids (PAs) and flavonoids but also anthocyanidins, quinine, flavonols, flavones, flavanones are present (Liu, 2007) (**Fig.6**).

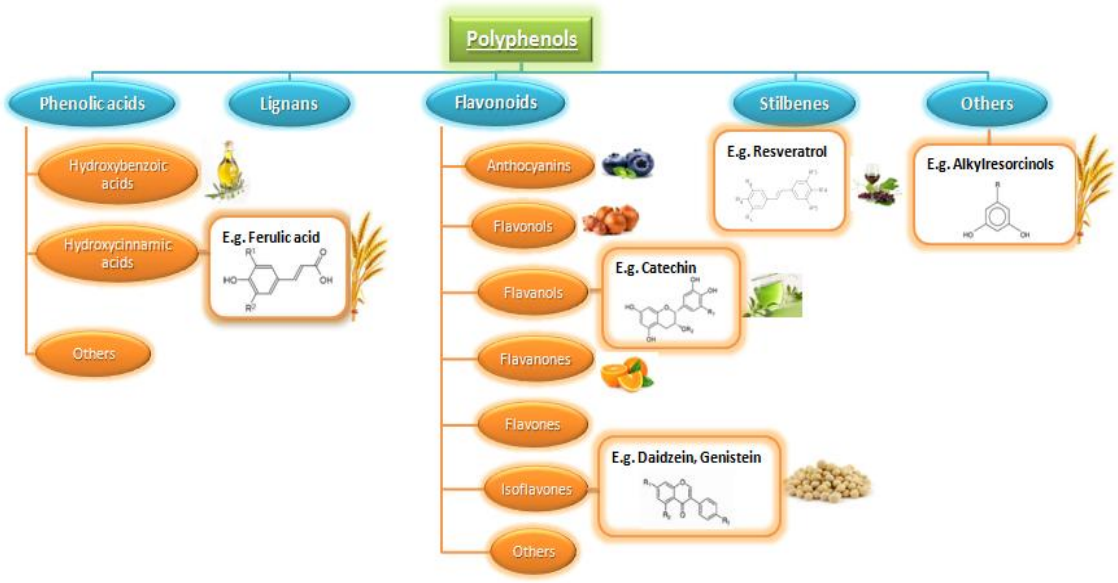

Fig.6 Classification of polyphenols and their main food sources (adapted from Nutrinsight, 2012).

Flavonoids are compounds with a C6-C3-C6 configuration that consists of two aromatic rings joined by a three-carbon link and include anthocyanidins, flavanols, flavonols, flavones and flavanones (Dykes & Rooney, 2007). They have an important antioxidant

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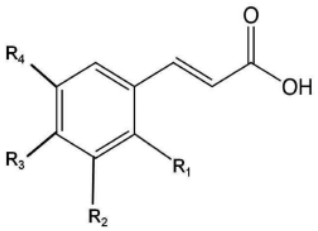
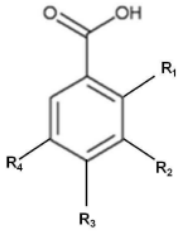
activity which is dependent on the number and location of the hydroxyl group (Hall & Zhao, 2011).

PAs, both derivatives of hydroxybenzoic and hydroxycinnamic acid, possess a common carbon skeleton and differ in the numbers and positions of the hydroxyl group on the aromatic ring (Duodu, 2011). Hydroxybenzoic acid derivatives include p-hydroxybenzoic, vanillic and syringic acid, while hydroxycinnamic acid derivatives include ferulic, coumaric and sinapic acid (**Fig.7**). Ferulic acid is the most abundant PA in cereal grains, which represent its main dietary source (Manach et al., 2004); it is mainly accumulated in the aleurone, nucellar envelope and germ, as other PAs, while lower amounts are present in the endosperm (Lempereur et al., 1997, Žilić et al., 2012).



Cinnamic acid



Benzoic acid

Cinnamic acid derivatives	R1	R2	R3	R4
p-Coumaric acid	H	H	OH	H
Ferulic acid	H	H	OH	OCH_3
Sinapic acid	H	OCH_3	OH	OCH_3

Benzoic acid derivatives	R1	R2	R3	R4
p-Hydroxybenzoic acid	H	H	OH	H
Vanillic acid	H	OCH_3	OH	H
Syringic acid	H	OCH_3	OH	OCH_3

Fig.7 Chemical structure of main phenolic acids present in cereals, both derivatives of cinnamic and benzoic acids (adapted from Duodu, 2011).

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PAs are present in three different forms: soluble free, soluble conjugated (esterified to sugars and other low molecular mass components) and insoluble bound, esterified to cell wall polymers (Li et al., 2008). The latter is the most abundant one (Li et al., 2008; Fernandez-Orozco et al., 2010), differently from fruit and vegetables where the free form of phenolic compounds is generally prevailing (**Fig.8**) (Liu, 2007).

High performance liquid chromatography (HPLC) and gas chromatography (GC), or their combinations, with mass spectrometry are the two most commonly applied methods to quantify phenolic compounds in cereals and other foods.

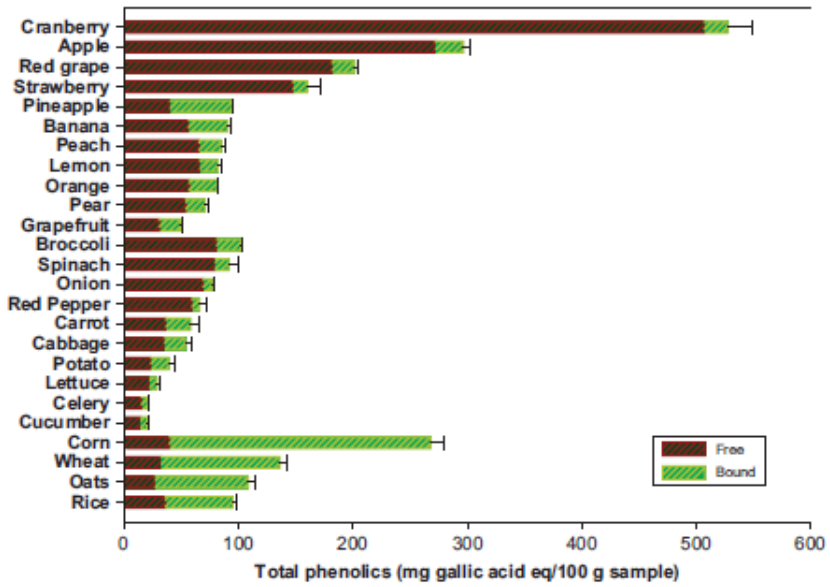

Fig.8 Content of free and bound phenolic compounds of major fruits, vegetables and whole grains (Liu, 2007).

In recent years, there has been a growing interest in understanding the bioavailability and metabolisms of these compounds, essential to evaluate their healthy effects.

In general, bioavailability of polyphenols is affected by three main factors: i) food matrix and other constituents of the diet exerting their effects in the intestinal lumen; ii) genetic aspects of individuals affecting uptake into gut cells and into the bloodstream via a system

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of enzymes and transporters; iii) microbiota metabolism affecting the formation of metabolites in the colon and their subsequent uptake into the bloodstream.

Specifically for PAs, free PAs once ingested are rapidly absorbed from the stomach or the small intestine and conjugated by the intestinal and/or hepatic detoxification enzymes (Lafay et al., 2008; Pekkinen et al., 2014). The esterification of PAs drastically decreases the bioavailability of these compounds in both animal models and human studies (Adam et al., 2002; Kern et al., 2003; Mateo Anson et al., 2009). Nevertheless, in recent years it has been demonstrated that the bound PA form can play an important role because it escapes from upper gastrointestinal digestion along with cell wall materials and are released in the colon where undergo colonic digestion by intestinal microflora, thus providing health benefits through action of scavenging of free radicals and prevention of oxidation of biologically important molecules (Andreasen et al., 2001; Adom & Liu, 2002).

1.1.2.2.3 Carotenoids

The group of carotenoids includes lipid-soluble compounds responsible for yellow, red and orange colours in many fruits and cereals (Tokusoglu & Hall, 2011). Specifically for durum wheat, these compounds have received great attention because yellow colour is a characteristic required by consumers and therefore their concentration is a criterion for the assessment of semolina quality.

Main carotenoids in durum wheat and other cereals include carotenes (α and β) and xanthophylls (β-cryptoxanthin, lutein and zeaxanthin) and their content is generally affected by several conditions, including the cereal species and cultivar, the growing environment and the storage conditions. It has been reported that genetic component is predominant in the carotenoid concentration and so that colour is a highly heritable trait controlled primarily by additive gene effects (Johnston et al., 1983).

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For instance, the total carotenoid concentration in durum wheat is typically at 2–5 mg/g dm, with lutein as the most predominant carotenoid, followed by zeaxanthin, β-cryptoxanthin, α- and β-carotene (Abdel-Aal et al., 2007; Digesù et al., 2009).

In addition to provide colour in semolina and pasta, carotenoids may also act as antioxidants in lipid environments of many biological systems through their ability to react with free radicals and form less reactive free radical products (Liu, 2007).

1.1.2.2.4 Other bioactive compounds

Besides the bioactive compounds previously described, durum wheat and other cereals contain other phytochemicals including among others alchylresorcinols, phytosterols and lignans.

Alkylresorcinols are 1,3-dihydroxybenzene (resorcinol) derivatives with an odd-numbered alkyl chain at position 5 of the benzene ring, which typically has between 17 and 25 carbons (Ross et al., 2004; Mattila et al., 2005). The interest in alkylresorcinols content is linked to their reported anticarcinogenic, antimicrobial and antioxidant properties (Ross et al., 2004; Bartłomiej et al., 2012). However, the anti-cancer activity of these compounds is rather weak, probably because of the lower antioxidant capacity than other compounds.

Plant sterols (also called phytosterols) are secondary metabolites occurring mainly in seeds of oilseed crops and cereals and exist as free sterols, fatty acids and steryl glycosides. The most abundant sterols in durum wheat and other cereal grains are sitosterol, campesterol, stigmasterol and brassicasterol (Piironen et al., 2002; Nystrom et al., 2007). In spite of the low antioxidant activity, phytosterols play an important role in health, for instance through the reduction of blood cholesterol and the enhancement of immune function (Hall & Zhao, 2011).

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Lignans are phytoestrogens compounds which are converted to enterodiol and enterolactone in the human intestine. They have strong antioxidant activity and weak estrogenic activity and may protect against many diseases including heart disease and hormone-related breast and prostate cancers. Lignans are concentrated in the bran and include secoisolariciresinol, matairesinol, lariciresinol, pinoresinol, and syringaresinol (Liu, 2007).

1.1.2.3 Antioxidant activity

Most of bioactive compounds present in durum wheat and other cereals have antioxidant activity which preserves the body from oxidative stress.

Oxidative stress is "the state in which oxidation exceeds the antioxidant systems in the body secondary to a loss of the balance between them" and can be due to inadequate diet-derived antioxidants, excessive production of free radicals like $O_2^{\bullet-}$ and H_2O_2 (Halliwell, 1996). The oxidative stress promotes damaging oxidative changes to important cellular constituents such as DNA, lipids, proteins, and this may lead to cellular dysfunction and, ultimately, to aging, disability, and disease (Benzie & Strain, 2005). Antioxidants, both endogenous or introduced by the diet, prevent the formation and the actions of reactive oxygen and nitrogen species, so helping to maintain the pro-oxidant:antioxidant balance, crucial for optimal health.

The occurrence of antioxidant compounds in cereals contributes to the total antioxidant activity of cereal derived products. The antioxidant health potential of foods is usually expressed as Total Antioxidant Capacity (TAC), normally used to explore the *in vitro* putative role of antioxidant-rich products in the prevention of degenerative diseases (Serafini et al., 2002). The interest in understanding the antioxidant activity of foods is demonstrated by the many analytical methods which have been developed for the analysis,

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which can be classified in: i) methods using different extractions for lipophilic and/or hydrophilic compounds before the analysis (Hirawan et al., 2010; Di Benedetto et al., 2013, Brandolini et al., 2013); ii) direct method which includes the direct contact of the solid pulverized matrix with the radical solution, avoiding the extraction step, as proposed by Serpen et al. (2008). The latter allows to consider the synergistic effect of the various antioxidant compounds that cannot be measured when compounds are extracted and analyzed separately.

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1.2 WHOLE GRAINS

1.2.1 DEFINITION, RECOMMENDATION AND ESTIMATED CONSUMPTION

The HEALTHGRAIN Project indicated that "Whole grains shall consist of the intact, ground, cracked or flaked kernel after the removal of inedible parts such as the hull and husk. The principal anatomical components - the starchy endosperm, germ and bran - are present in the same relative proportions as they exist in the intact kernel. Small losses of components - i.e. less than 2% of the grain/10% of the bran - that occur through processing methods consistent with safety and quality are allowed."

Nutritional recommendations for consumption of whole grain foods vary across countries but normally suggest to eat at least half of cereals as WGs, or more generally to substitute refined foods with the respective WG ones (**Tab**.**2**).

Country	Reference	National recommendations for whole grains and whole grain foods
France	www.mangerbouger.fr	Prefer cereals in whole grain form
United Kingdom	www.eatwell.gov.uk	Prefer cereals in whole grain form
Switzerland	www.sge-ssn.ch	When possible 2 of which in whole grain form
The Netherlands	www.voedingscentrum.nl	Preferably whole grain
Denmark, Sweden	www.food.dtu.dk	The equivalent of 75 g whole grains per 10 MJ (2400 kcal) equivalent to 62 g for a reference 2000 kcal diet
The United States	www.health.gov/dietaryguidelines www.mypyramid.gov	At least half of cereal intake to be consumed in whole grain form, equivalent to at least 3 ounce-equivalent 2 servings of whole grain foods for individuals over 9 years: "Make at least half your grains whole grains".
Australia	www.nhmrc.gov.au	Prefer cereals in whole grain form
Canada	www.hc-sc.gc.ca	Prefer cereals in whole grain form
Italy	http://nut.entecra.it	Prefer cereals in whole grain form

Tab.2 National recommendations for cereals and whole grain consumption (adapted from Nutrinsight, 2011).

In recent years, the growing interest for well-being beside the higher awareness of the association between diet and health, is addressing consumers to rediscover the value of whole grain based products. A recent study performed in the United States by the

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International Food Information Council Foundation reveals that, among people willing to improve at least one aspect of their eating habits, 75% tried to eat more whole grains with respect to the previous year. The same study highlighted that whole grains are becoming a primary motivator in purchases, so much that whole grains content is the second factor influencing consumers to buy a product, just after calories (IFIC, 2012). As a result of this growing interest for less refined foods, the consumption of whole grain products and the supply of whole grains products such as whole wheat bread and whole wheat pasta are raising (Nutrinsight, 2011). Despite the current mentioned and encouraging trends, with a different extent around the world depending on the local habits, levels of consumption generally remain lower than recommendation (Galvin et al., 2001; Krebs-Smith et al., 2010).

An Italian study performed within the INRAN-SCAI 2005-06 survey revealed that mean daily intake dietary fibre of Italian population increased with age (from 8 g per day in infants to 22 g per day in elderly) (Sette et al., 2011). However, in all cases mean daily intake was below recommendation which suggests a minimum intake of 25 g/die (data LARN, 2014).

1.2.2 PROS AND CONS OF WHOLE GRAINS CONSUMPTION

The reasons of low consumption of WG foods are numerous and include: i) tradition of "white" products, ii) taste and texture of whole grain products, iii) higher costs of WGs than of white products; iv) limited positive perception of health benefits about whole grain; v) consumers' mindset and reluctance to change; vi) minor availability of whole grain products on the market (Nutrinsight, 2011).

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1.2.2.1 Hygienic-sanitary aspects

As previously mentioned, the outer layers of kernels are rich in bioactive and antioxidants compounds. However, in the same layers also several contaminants such as mycotoxins, heavy metals and pesticides can be concentrated. In fact, the outer layers of the kernels are the portions with the highest exposure to soil, fungal infections, and pesticides; therefore, the accumulation of natural contaminants such as mycotoxins, as well as of chemical contaminants including heavy and pesticides mainly occurs in this part of the kernel (Cheli et al., 2013). As WGs foods preserve the outer protective layers of the grain, they may have a higher content of toxic compounds in respect to the refined ones (Lancova et al., 2008; Rios et al., 2009).

The ingestions of many of these contaminants may cause serious health problems, so this issue represents a key challenge for public health. For instance, consumption of mycotoxins-contaminated foods may induce teratogenic, cancerogenic and neurotoxic effects, with an extent depending on the levels of contamination. Hence, it is crucial the identification of strategies to prevent the growth of mycotoxigenic fungi as well as of technologies to reduce levels of mycotoxins in grains and final products (Kabak et al., 2006; Pereira et al., 2014).

1.2.2.2 Technological and sensory properties

The presence of kernel outer layers and in particular of fibre is often cause of worse technological and sensory properties of final products. As a matter of fact, the expected or experienced low sensory quality of wholegrain foods represents one of the main barriers to adequate consumption of WGs foods (Kuznesof et al., 2012). The use of bran and whole grain flour also affects dough rheology so that the associated bread usually has increased

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water absorption, decreased bread volume, impaired crumb texture, darker crumb color and reduced softness (Campbell et al., 2008; Alparce et al., 2014).

Therefore, it is essential to develop new strategies to improve the technological properties of wholemeals which could improve the acceptance by consumers and plausibly lead to an increased consumption of WGs.

1.2.2.3 Nutritional aspects: nutrients, phytochemicals and anti-nutrients

As previously cited, independently from the cereal type and regardless from plant type, the basic structure of the grain includes three parts: the endosperm, the outer layers, and the germ. Each layer contains specific nutrients and phytochemicals linked with potential health benefits (Lang & Jebb, 2005). The health-promoting effects of whole-grain consumption have been attributed in part to their unique content of phytochemicals. A large body of evidence, mainly from epidemiological studies, have linked whole grain intake to the prevention of many chronic diseases including obesity, cardiovascular diseases (CVD), type 2 diabetes (T2D) and some types of cancer. Different mechanisms have been proposed to explain this association, including dietary fibre-related mechanisms, antioxidants-related mechanisms, insulin response-related mechanisms and hormone-like related mechanisms (Slavin et al., 2003; Fardet, 2010; Bjorck et al., 2012).

Besides the positive presence of fibre and other bioactive compounds, the WGs may also contain some anti-nutrients, mainly phytic acid (Schlemmer et al., 2009). For long time, phytic acid has been recognized for its negative effect on the mineral bioavailability (Ekholm et al., 2003); however, more recently some beneficial effects have been reported, including a high antioxidant activity (Slavin, 2003).

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1.3 TRADITIONAL AND INNOVATIVE TECHNOLOGIES FOR DURUM WHEAT PROCESSING

In Italy, the majority of durum wheat production is addressed to the pasta industries. The raw material for the pasta production is semolina which consists of granular particles of endosperm having a bright natural yellow colour.

In the next paragraphs, the traditional durum wheat processing (milling and pasta-making) and more innovative technologies will be deepened.

1.3.1 MILLING

Milling process includes three main steps: cleaning, conditioning and milling. Wheat is accurately cleaned for the elimination of foreign weed seeds and dirt through machines which separate by size, specific gravity and shape (Delcour & Hoseney, 2010). Wheat is then tempered through water addition for easier separation of external layers from the endosperm. The water penetrates into the outer layers as well as between the outer layers and endosperm, so preventing the fibre layers from breaking into small pieces which are hardly separated from semolina (Gruber & Sarkar, 2012). The real milling process essentially consists of grinding -done by breaking, sizing and reduction rolls - and separation using sifters and purifiers. The main milling product of durum wheat is semolina, whereas the by-products are coarse bran, fine bran and flour. Italian Law (DPR N°187, 9 February 2001) establishes that semolina alone is allowed for pasta-making. It is defined as "the rough, granular product obtained by grinding and sifting durum wheat, which has had any impurities and extraneous bodies removed" and must have the following characteristics:

- Moisture: maximum 14.50%
- Ash: maximum 0.90% dm
- Protein: minimum 10.50% dm

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1.3.2 PASTA-MAKING

Pasta-making consists of three main operations: i) preparation of a dough by hydrating, mixing and kneading of semolina; ii) extrusion and shaping of dough; iii) drying of pasta.

In the first step, raw materials (semolina and water) are properly dosed in order to obtain a final moisture of the dough of 30-33% dm. The mixing-kneading process allows to form a dough which undergoes to the extrusion stage. At the end of the endless screw, dough is forced through die, to produce a wide range of pasta shapes. The final step of pasta-making is drying, which represents the crucial part of the process aimed to ensure a long time storage of pasta. The drying consists of passing a current of hot air over the fresh pasta that progressively decreases its moisture and includes three main phases: i) predrying, responsible for the majority of pasta moisture loss; ii) real drying; iii) cooling, during which the pasta is acclimatized to a condition in balance with the environment becoming ready for packaging (Gruber & Sarkar, 2012).

The DPR N° 187 defines durum wheat pasta as "the product obtained by drawing, rolling, and drying a dough prepared with durum wheat semolina and water" with maximum moisture of 12.50%, maximum ash of 0.90%, minimum protein content of 10.50% and maximum acidity of 4 degrees.

Besides durum wheat semolina pasta, the DPR n.187 allows also the production of

- low grade durum wheat pasta, prepared with low grade durum wheat semolina and water and with the following characteristics: ash ranging from 0.90% dm to 1.35% dm, minimum protein content of 11.50% dm and maximum acidity of 5 degrees.
- durum wheat whole-meal semolina pasta which is "the product obtained by drawing, rolling, and drying a dough prepared exclusively with durum wholemeal semolina and water" and with the following characteristics: ash ranging from

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1.40% dm to 1.80% dm, minimum protein content of 11.50% dm and maximum acidity of 6 degrees.

In addition, the Italian law permits the manufacture of special pasta which contain other ingredients, such as eggs.

1.3.3 DEBRANNING

In recent years, one of the main process introduced along durum wheat transformation chain is debranning. The debranning, based on the removal of kernel layers through an abrasive scouring, is a well known technology, traditionally used for some hulled cereals such as rice, barley and oat. However, more recently the process is getting more and more accepted as a pre-milling treatment in processing of naked cereals (e.g. durum and common wheat) allowing an easier and more effective removal of the grain's layers (Dexter & Wood, 1996). One of the main target of the milling process is to maximize the separation of the starchy endosperm from the bran and the germ; the debranning is more and more adopted as a pre-step, before the real milling, so that part of the outer layers and of the germ is removed before the conventional milling steps (Ranieri, 2012).

Specifically for durum wheat, main advantages of debranning process include the increase of semolina yield, improvement of semolina quality, reduction of the ash content and of contamination by mycotoxins, pesticides, and heavy metals (Bottega et al., 2009a; Ranieri, 2012). The great potential of debranning process as a tool to produce cereal based foods with a high nutritional, hygienic-sanitary and technological quality, is addressing some researchers to investigate the effect of this process on the content of positive (e.g. nutritional and healthy compounds) and negative (e.g. mycotoxins, heavy metals) compounds in debranned kernels and relative wastes (Liyana-Pathirana et al, 2006; Gamel

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& Abdel-Aal, 2012; Sovrani et al., 2012; Luthria & Liu, 2013). Other studies focused on the production of cereal based foods made with these materials (Fares et al., 2010; Blandino et al., 2013). Most of these studies were performed on common wheat, while durum wheat have been less investigated.

1.3.4 OTHER INNOVATIVE PROCESSES

Some others technological processes can be applied on common and durum wheat for improving its technological and nutritional quality. Micronization, coupled to air fractionation, is considered a useful tool for improving some technology properties and to enrich the flour fractions of nutritional and healthy compounds (Bottega et al., 2009b; Ferrari et al., 2009; Protonotariou et al., 2014). Many authors reported that the reduction of particle size could be a useful strategy to improve its nutritional potential, mainly due to the increased accessible surface (Hemery et al., 2011); different methods for micronization have been proposed, including ball-milling, jet-milling, high pressure micronization, based on impact, shearing, compression, crushing, attrition, infrared heat treatment, etc. (Hemery, et al., 2007 and 2011).

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1.4 FACTORS INFLUENCING THE OCCOURRENCE OF NUTRITIONAL AND HEALTHY COMPOUNDS IN DURUM WHEAT: STATE OF THE ART

The growing interest of consumers for whole grains led many researchers to investigate the factors influencing the occurrence of bioactive compounds in cereals. Three main categories of factors are commonly investigated: genetic and environmental factors (related to agro-climatic conditions) and technological processes.

As regards genetic factors, several studies reported that species, varieties and breeding lines can be crucial determinants in the occurrence of bioactive compounds in cereals. Specifically for wheat, Mpofu et al. (2008) collected data from 11 studies previously performed, showing that genotype significantly affects total phenolic content (TPC) and ferulic acid concentration in wheat, suggesting that it would be possible to select for some quantitative traits in a breeding program. The genetic component is particularly important for specific classes of bioactives, such as carotenoids; different authors (Johnston et al., 1983; Borrelli et al., 1999; Digesù et al., 2009) reported that the genetic factors are predominant in determining the carotenoid concentration in durum.

From the point of view of environmental effects, some studies have investigated the role of specific climate parameters such as rainfall and temperature on the occurrence of bioactive compounds, even though outcomes are often in contrast each other and therefore results are still not conclusive. Specifically for phenolic compounds, some studies have pointed out a negative correlation between high temperature and TPC, others have registered an increase in soluble phenols and anthocyanins in wheat subjected to drought and UV-B stresses (Alexieva et al., 2001; Yu et al., 2004; Moore et al., 2006). A recent study performed within the HEALTHGRAIN Project showed that environment affects content of phenolic acids (especially the free and conjugated forms) in common wheat, suggesting that rainfall

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level in the period before heading may be a more relevant factor than total precipitation (Fernandez-Orozco et al., 2010).

Among studies investigating the effects of genotype and environment on TPC and PAs, only some of them were focused on durum wheat, probably because the interest for this crop is limited in the Mediterranean area. Menga et al. (2010) observed that the phenolic content in durum wheat was mainly influenced by the growing area and secondly by genotype and their interaction.

A larger number of studies investigated the effects of technological processes on the occurrence of various bioactive compounds in wheat. Most of these studies revealed that the milling process is determinant for the content of bioactives in cereal based products, because bioactive compounds are mainly concentrated in the outer layers of kernels and are generally lost during milling when external layers are removed (Adom et al., 2005). Therefore refined products, such as flour and semolina, generally have lower content of bioactives compounds and lower antioxidant activity than the entire kernel (Liyana-Pathirana & Shahidi, 2006 and 2007; Alparce et al., 2014).

Other classes of healthy compounds, such as carotenoids, are preserved during milling, and only some loss of β-carotene may occur (Borrelli et al., 2008) suggesting a different distribution in the kernel.

In spite of the large number of studies performed, no study investigated the effects of the entire durum wheat process (from seed to cooked pasta) on the occurrence of the different classes of bioactive compounds.

As regards the innovative technological processes such as debranning, some authors investigated the effects on specific properties of derived cereal products, such as their content of nutritional compounds or of harmful compounds such as heavy metals,

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pesticides and mycotoxins, finding that because most of both healthy and unhealthy compounds are progressively lost following debranning process being concentrated in the outer layers of kernels (Beta et al., 2005; Liyana-Pathirana et al., 2006; Fares et al., 2010). However, as already observed above, most of the mentioned studies have been performed on common wheat, while just few studies focused on durum wheat (Esposito et al., 2005; Žilić et al., 2012). Moreover, no studies have evaluated the effect of the debranning process on the occurrence of bioactive compounds in both debranning fractions and resulting kernels, considering that both these materials can be used for the production of durum wheat based foods with a high nutritional, technological and hygienic-sanitary quality.

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2. Aim

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In recent years, the awareness of consumers toward the link between the consumption of cereals, especially as whole grains, and the reduced risk of many chronic diseases is continuously increasing. This is mainly due to the presence of bioactive compounds such as fibre and many phytochemicals, mainly concentrated in the outer layers of the kernels, where also contaminants are accumulated threatening the hygienic-sanitary quality of cereals.

In spite of the increased awareness of consumers, the consumption of whole grains remain lower than recommendations, due to many barriers including taste and texture of whole grains based products.

The increased interest for whole grains and their health benefits has addressed many researches to the study of factors influencing the occurrence of beneficial compounds in cereals and to the development/optimization of technological processes aimed to the production of cereal based foods with high nutritional and sensory quality.

Previous studies have shown that the content of bioactive compounds in cereals may have a wide range of variability, due to several factors which can influence their occurrence; moreover, innovative technological processes have been applied for developing cereal based foods with an increased quality. However, just few studies focused on durum wheat, crop of primary interest in the Mediterranean area and most of all in Italy, where it is traditionally used for the pasta production.

On the basis of these considerations, aims of the present research were:

i) To investigate the effects of genetic, environmental (crop year and growing area) and the impact of traditional and innovative technological processes on the occurrence of antioxidant compounds (mainly phenolic acids) and on the total antioxidant activity in durum wheat and pasta;

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ii) To develop durum wheat pasta with high nutritional, hygienic-sanitary, sensory quality by using innovative durum wheat processing;

iii) To explore the bioavailability of phenolic compounds was performed considering this parameter crucial for determining the biological effects of these healthy compounds.

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3. Materials & Methods

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3.1 VARIABLES UNDER STUDY

3.1.1 PHENOLIC ACIDS

The initial research activity was devoted to the optimization of an extraction method of phenolic acids (PAs) and to the development and validation of a separation method for the analysis of these compounds.

In particular, the first step was the development of an extraction method suitable to extract the three PA forms (free, conjugated and bound) in different matrices such as durum wheat and its main products. To this aim, it was crucial to consider the characteristics of the different durum wheat-based matrices under study, mainly in terms of expected PA content.

Moreover, the development and validation of a straightforward HPLC method for the simultaneous identification and quantification of the three PA forms was essential prior the study of different factors influencing the occurrence of PAs in durum wheat and derived products.

3.1.1.1 Extraction

The PA extraction was performed according to the method proposed by Li et al. (2008) with some modifications (**Fig.9).** In particular, the main modifications included:

i) a different ratio weight of sample/ volumes of solvents, particularly important in matrices with high expected PA content in order to avoid saturation;

ii) use of low temperature to reduce the gelatinization process, especially in matrices with high starch content (e.g. semolina, pasta).

Development as well as optimization and validation of the PA extraction method was assessed on selected durum wheat derived products (i.e. wholemeal, semolina, bran and dried pasta) prepared as described in the Section 3.3.1. In detail, to extract free and

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conjugated PAs, a 250 mg portion of sample was weighted and mixed with 1 ml 80:20 (v/v) ethanol-water solution, containing 3,5-dichloro-4-hydroxybenzoic acid (DHB) as internal standard (IS) at a concentration of either 37.5 or 150 μg/ml for the extraction of free and conjugated PAs, respectively.

The resulting mixtures were sonicated for 10 minutes, maintaining the temperature at 4°C to avoid starch gelatinization, and centrifuged for 10 minutes at 10000 rpm at 4°C. The extraction was repeated twice with the 80:20 (v/v) ethanol-water solution, without the incorporation of the IS. The supernatants were reunited in a new vial, half dried with gaseous nitrogen and then lyophilized to avoid the oxidation of the extracted compounds.

For free PAs, a 500 μL volume of 2% aqueous acetic acid solution was added to the lyophilized sample, which was subsequently acidified to pH 2.0 with 12 N HCl (2.5 μL) in order to release PAs from glycosylated forms so allowing the extraction into organic solvent. After mixing, 500 μL ethyl acetate was added and mixed at room temperature. The resulting mixture was centrifuged at 10000 rpm for 2 minutes and the upper organic layer was collected in a clean vial. The extraction with ethyl acetate was repeated twice and the combined supernatants were evaporated to dryness with gaseous nitrogen.

For conjugated PAs, the lyophilized sample was indeed hydrolyzed with 400 μL of NaOH 2 M for 4 h under continuous agitation at 4° C; after acidification to pH 2 with 80 μL of HCl 12 N, conjugated PAs were extracted twice with 500 μL ethyl acetate as described for free PAs.

Finally, for bound PAs, a 250 mg portion of sample was mixed with 1 ml 80:20 (v/v) ethanol-water solution without the incorporation of IS. The resulting mixtures were sonicated for 10 minutes, maintaining the temperature at 4°C to avoid starch gelatinization,

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and then centrifuged for 10 minutes at 10000 rpm at 4°C. The supernatant was discarded and the extraction was repeated twice with the 80:20 (v/v) ethanol-water solution.

The pellet remaining after the extraction with ethanol-water solution was mixed with 20 µL of 7.5 mg/mL of the IS solution (in 80:20 (v/v) ethanol-water) and then hydrolyzed for 4 h under continuous agitation at 4°C by adding 400 µL of 2 M NaOH aqueous solution, with the exception of the coarse bran sample which required the addition of 800 µL of 2 M sodium hydroxide, because this matrix tended to absorb more liquid. After acidification to pH 2 with 12 N HCl (120 µl for all samples and 240 µl for coarse bran sample), bound PAs were extracted in duplicate with 800 µL ethyl acetate (1600 µl for coarse bran) and then centrifuged at 10000 rpm for 10 min. The pooled supernatants were evaporated to dryness with gaseous nitrogen. Once dried, all samples were stored at -20°C and then reconstituted in 100 µL 80:20 (v/v) methanol-water solution, containing 2% (v/v) formic acid, just before HPLC analysis.

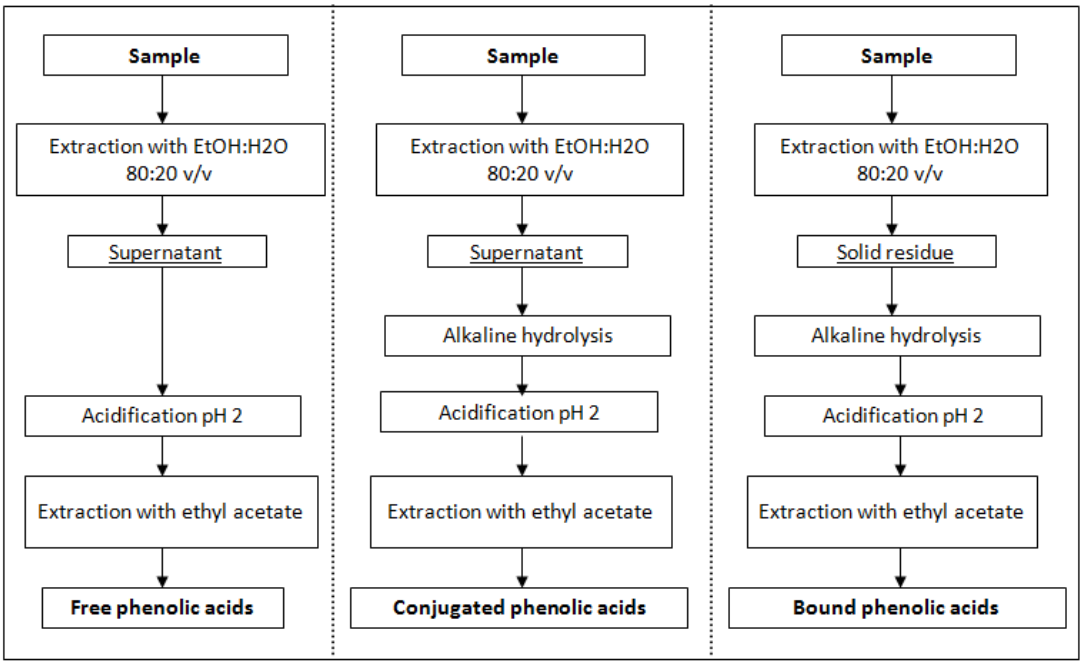

Fig.9 Scheme of free, conjugated and bound PA extraction.

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3.1.1.2 RP-HPLC analysis

3.1.1.2.1 Equipment

Qualitative and quantitative analyses of PAs were carried out by RP-HPLC using a Shimadzu (Milan, Italy) LC-10A_{VP} system consisting of an SCL-10A_{VP} system controller, two LC-10AD_{VP} solvent delivery units, a SPD-M10A spectrophotometric diode array (PDA) detector, a CTO-10AS_{VP} column oven, a DGU-14A on-line vacuum membrane degasser, and a Rheodyne (Cotati, CA, USA) Model 8125 semi-micro injection valve with a 5-µL sample loop. Instrument control and data acquisition and processing was performed by the Shimadzu Class VP 5.6 HPLC data system on a Pentium II 400 PC compatible computer. A Polaris C18A column (150 x 2.0 mm I.D, 5 µm; (Varian Inc. Lake Forest, CA) with a C18 (30 x 2 mm, 5 µm) guard cartridge column was employed at controlled temperature of 30°±1°C.

1.1.1.2.2 Method development

Soluble free PAs were separated by a multi-segments gradient of increasing concentration of acetonitrile in water acidified with 2.0% (v/v) formic acid, at a flow rate of 0.2 mL/min, according to the program described in **Tab.3**. Briefly, a 15 min linear gradient from 5 to 7% (v/v) acetonitrile was followed by 5 min isocratic elution with 7% (v/v) acetonitrile, 10 min linear gradient from 7 to 20% (v/v) acetonitrile, 5 min linear gradient from 20 to 25% (v/v) acetonitrile, 5 min isocratic elution with 25% (v/v) acetonitrile, 1 min steep gradient from 25 to 90% (v/v) acetonitrile and subsequent 5 min isocratic elution with 90% (v/v) acetonitrile to ensure complete elution of any strongly retained components of the extracted samples. The gradient used for the separation of conjugated and bound PAs differed from that described above in the steepness of the last gradient segment (5 min instead of 1 min) and by the introduction of a further steep segment gradient from 90 to 95% (v/v) in 1 min,

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followed by 15 min isocratic elution with 95% (v/v) acetonitrile. At the end of both gradient elution programs described above, the composition of the mobile phase was brought to the initial condition in 1 min, and the column equilibrated for 20 min (first gradient profile) and 23 min (second gradient profile), respectively, before the next injection.

A

T (min)	%B
0	5
15	7
20	7
30	20
35	25
40	25
41	90
45	90
46	5
66	STOP

B

T (min)	%B
0	5
15	7
20	7
30	20
35	25
40	25
45	90
46	95
61	95
62	5
85	STOP

Tab.3 Gradient elution programs used for free (A), conjugated and bound PAs (B) analysis.

UV-Vis spectra were recorded in the 210-400 nm range, and the chromatograms were acquired at 254, 280, and 320 nm corresponding to the maximum absorbance for the different PAs.

To confirm peak identification by electrospray ionization mass spectrometry (ESI-MS) detection in the single ion monitoring (SIM) mode, the above HPLC instrument was hyphenated with a Shimatzu single quadrupole Model LCMS-2010 mass spectrometer. The column effluent was first passed through the PDA detector before being directed to the mass spectrometer with ESI interface. The MS acquisition was performed with the ESI interface in the negative ionization mode at the following conditions: nebulizing gas nitrogen at flow rate of 4.5 L/min; temperature of block heater, 200°C; temperature of the

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curved desolvation line (CDL), 225°C; probe voltage -3.5 V; CDL voltage, 25 V; Q-array voltages, 0, -15, -60 V; Q-array RF, 150. System control and data processing were carried out by the Shimadzu LCMS Solution software running on a Pentium IV personal computer (Gigabyte, Milan, Italy).

The identification of individual PAs was performed on the basis of their retention times and of spectroscopic and mass spectrometric spectra. Libraries comprising retention times, UV-visible and mass spectra for major PAs expected in durum wheat were made by subjecting solutions of each PA standard to RP-HPLC analysis with the optimized multi-segments gradient and both PDA and ESI-MS detection. Stock solutions of each standard were prepared by dissolving weighted amounts in 80% (v/v) methanol-water solution.

Using the Class VP software, a Similarity Index (SI) was calculated to evaluate how closer spectra of standard and corresponding PAs separated in wheat extracts resemble each other. According to the above software, SI closer to unity is indicative of higher similarity. In addition, the use of a Purity Index (PI), based on the comparison of all the spectra to the spectrum at the peak apex, allowed excluding the presence of coeluting substances in the peaks of the PAs separated from the durum wheat extracts.

The quantitative analysis of the identified PAs was based on calibration graphs obtained by plotting the ratio of the analyte peak area to that of the IS as a function of the concentration of the standards; the standards were extracted with the same procedure employed for the real samples in order to account for the losses due to the extraction method. The peak areas of analytes and IS were collected at the wavelength of maximum absorbance of each identified PA, which were determined by the PDA spectra acquired in the wavelength range 210-400 nm. All samples were analyzed in triplicate, and the concentrations of individual PAs were expressed in milligrams per kilogram (mg/kg) of dry matter (dm).

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3.1.1.2.3 Method validation

The RP-HPLC method was validated in terms of linearity, precision and accuracy. The precision of the method was evaluated as intraday and interday repeatability of both retention time and ratio of the analyte response to that of the IS for all PAs considered in the study, which were analyzed by the proposed method in triplicate during the same day and over three consecutive days. Linearity was evaluated by analysing mixtures of PA standard solutions at five concentration levels. Linear least squares regression analysis was employed to calculate slope, intercept and correlation coefficient of the calibration graphs constructed as reported above. Limits of detection (LOD) and quantification (LOQ) were estimated as the concentrations of PAs producing chromatographic peaks with a height at least three times and ten times as high as the baseline, respectively.

The accuracy of the method was evaluated by a recovery study, which was carried out according to the following procedure. Known amounts of the examined PA, corresponding to 80%, 100% and 160% of the values determined in the non-spiked sample, were added to the sample, subjected to the entire analytical method in parallel to a non-spiked sample of the same durum wheat matrix. All samples were injected three times and the recoveries were calculated based on the difference between the total concentration determined in the spiked samples and the concentration observed in the non-spiked samples.

3.1.2 TOTAL PHENOLIC COMPOUNDS

Free, conjugated and bound total phenolic compounds (TPC) were extracted using the same method described above for PAs, without the incorporation of the IS. The extracts were reconstituted in 100 µL of a 80:20 (v/v) methanol-water solution just prior the analysis; the TPC was determined using the Folin-Ciocalteau method as reported by Moore

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and Yu (2008). Briefly, 50 µL of the reconstituted sample were added to 3 mL of water and to 250 µL of Folin-Ciocalteu reagent and then neutralized with 750 µL of sodium carbonate (20%, w/v). After incubation in a dark place at room temperature for 2 h, the absorbance was measured at 765 nm using a spectrophotometer Lambda Bio20 (Perkin Elmer, Monza, Italy). A solution containing all the reagents, but without the samples, was used as blank solution.

TPC was calculated using calibration graphs built by plotting the absorbance as a function of the concentration of standard; ferulic acid was used as standard due to its abundance in cereals, which makes it preferable in respect to other compounds (e.g. gallic acid).

The standard undergone the same extraction method employed for the real samples in order to ensure that losses due to the extraction method were accounted for. Results were expressed as milligrams of ferulic acid equivalents per kilogram of dry matter (mg FAE/kg dm).

3.1.3 YELLOW COLOURED PIGMENTS (YCP)

Yellow coloured pigments (YCP) were determined by the fast method described by Fares et al. (1991). Briefly, 2 g of sample were extracted into glass-stoppered flask with 10 mL of water-saturated 1-butanol for 3 h on continuous shaking at room temperature. Then, the sample extract was filtered and the absorbance read at 435.8 nm by the spectrophotometer Lambda Bio20. The YCP of the extract was calculated directly from absorbance using the conversion factor of 1.6632, and expressed as β-carotene (considering that 1 mg of β-carotene standard in 100 mL water saturated butanol has an optical density of 1.6632 in 1 cm cuvette at 435 wavelenght) (AACC method 14.50).

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3.1.4 TOTAL ANTIOXIDANT CAPACITY

The Total Antioxidant Capacity (TAC) was determined by the "QuENCheR method", which consists of the direct immersion of the pulverized solid sample in a radical solution. The method was firstly described by Serpen et al. (2008) and slightly modified. Briefly, samples were mixed with cellulose powder (1/10 w/w), which was found to be inert toward the assay conditions, and suspended in a previously made radical solution (Re et al., 1999) prepared by dilution of a radical solution (7 mM ABTS radical and 2.45 mM potassium persulfate) in an ethanol 50% solution to reach an absorbance of 0.7 (Re et al., 1999).

Samples were then incubated in an orbital shaker (25°C and 190 rpm) for 50 min and finally centrifuged at 10,500 rpm (10 min). The absorbance of samples was read at 734 nm by the spectrophotometer Lambda Bio2.0. The analyses were performed in triplicate and the total antioxidant capacity was expressed as mmol of trolox equivalent antioxidant capacity per kg of sample on a dry matter basis (mmol TEAC/kg dm) by means of a dose-response curve.

3.1.5 STARCH

Total starch (TS) content was determined according to the AOAC Official Method 996.11 using the Total Starch Assay Kit (Megazyme, Bray, Ireland). Briefly**,** this method included a digestion of 100 mg of milled sample (0.5 mm sieve) firstly with thermostable α-amylase diluted in MOPS (3-[N-morpholino]propanesuHonic acid) buffer and then with amyloglucosidase. The total starch was measured by adding GOPOD (Glucose oxidase/peroxidase) reagent to 100µl of digested sample. The absorbance was set at 510 nm and each sample was read against the blank (100µl distilled water plus 3 ml GOPOD reagent). The D-glucose (100µl of 1 mg/mL D-glucose standard solution) was used as

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control. The analyses were performed in duplicate and data were expressed as starch percentage w/w on a dry matter basis (% dm).

3.1.6 PROTEIN

Protein content was determined by using the Dumas method (AACC 46-30). Briefly, the method was based on the combustion of sample (200 mg) in a high temperature (about 950°C) chamber in the presence of oxygen, leading to the release of carbon dioxide, water and nitrogen. The gases was then passed over special columns that absorb the carbon dioxide and water. A column containing a thermal conductivity detector at the end was used to separate the nitrogen from any residual carbon dioxide and water and the remaining nitrogen content was quantified. The method was performed using the instrument Leco FP 528 (St. Joseph, MI), previously calibrated by analyzing a standard with a known nitrogen concentration (i.e. EDTA 9.57 %N). Proteins were quantified by using the conversion factor Nx5.7.

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3.2 GENETIC AND ENVIRONMENTAL FACTORS

Ten durum wheat genotypes (Achille, Anco Marzio, Claudio, Duilio, Dylan, Iride, Normanno, Saragolla, Simeto and Svevo), grown in Montelibretti (Rome) on 2009/10, 2010/11 and 2011/12 crop years were considered in order to investigate the influence of genotype and crop year on selected antioxidant compounds.

To elucidate effects of growing area and crop year, three of the ten genotypes (Saragolla, Svevo and Simeto) were grown in two additional areas (Jesi, sited in the East part of Central Italy) and Foggia (in the South-East of Italy).

Both genotypes and agro-climatic areas were selected among those included in a national network of experimental trials on durum wheat grown by conventional practices, annually oordinated by C.R.A. Unità per la Valorizzazione Qualitativa dei Cereali (http://qce.entecra.it/frduro/dblist3.asp) (**Fig.10**). In particular, genotype were chosen among the cultivars largely diffused in Italy; their main characteristics are reported in **Tab.4.**

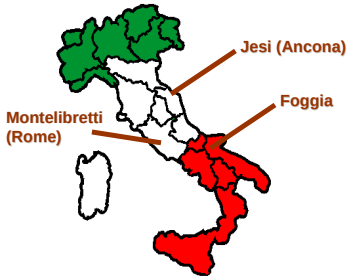

Fig.10 Localization of the three Italian growing areas selected to elucidate effects of grown locations.

The growing areas Foggia and Jesi were indeed selected because representative of the two regions (Puglia and Marche) with the highest durum wheat cultivated surfaces in the South and in the Centre-North of Italy, respectively. During the growing periods, temperature and precipitation data were recorded (**Tab.5**).

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		Test weight (kg/hL)			1000 grain weight (g)			Protein (% dm)			Production (t/ha)		
		2010	2011	2012	2010	2011	2012	2010	2011	2012	2010	2011	2012
ML	AC	79.7	82.7	85.2	41.1	45.0	44.8	12.5	13.2	13.4	2.34	6.53	7.44
	AM	79.0	81.8	84.6	41.4	44.5	45.2	13.2	13.4	14.0	4.48	6.27	6.50
	CL	79.6	82.4	85.1	42.9	47.9	48.3	12.7	13.6	13.6	3.78	6.61	6.84
	DU	75.7	79.4	83.5	40.9	51.6	50.7	13.4	13.7	14.0	3.62	6.87	6.93
	DY	77.8	80.7	83.9	41.7	49.7	48.6	12.4	13.6	13.8	3.57	6.42	8.41
	IR	76.7	80.1	84.0	37.5	46.7	44.3	13.1	13.1	13.3	4.57	6.63	6.88
	NO	76.1	80.0	82.5	40.6	48.7	47.0	12.8	13.6	13.9	4.44	7.13	7.90
	SA	76.2	79.5	82.5	40.6	44.8	43.8	12.9	13.0	13.9	4.88	6.71	7.38
	SI	72.8	78.0	81.6	45.3	55.8	56.3	13.9	14.7	15.2	3.29	4.58	5.79
	SV	78.2	80.4	83.2	41.1	49.1	48.5	13.6	14.6	15.3	4.14	5.24	6.03
JE	SA	78.6	80.8	84.5	40.9	46.4	44.0	13.0	12.9	13.4	5.90	7.94	6.20
	SI	73.5	81.6	83.9	48.0	49.0	56.3	14.9	14.0	15.2	4.19	6.37	7.13
	SV	79.4	80.2	85.1	41.6	54.0	44.0	14.5	14.9	14.7	5.80	7.46	6.14
FG	SA	79.8	79.7	82.3	44.3	46.6	42.6	12.2	12.1	13.1	6.80	5.91	4.33
	SI	78.9	78.7	82.2	52.7	58.4	55.0	12.7	13.9	13.9	5.35	4.83	4.36
	SV	82.0	82.2	82.7	46.4	49.5	45.5	12.8	13.2	14.2	6.90	5.28	4.00

Tab.4 Main quality characteristics of durum wheat genotype included in the study.

Legend: ML, Montelibretti; JE, Jesi; 2010, crop year 2009/10; 11, crop year 2010/11; 12, crop year 2011/12; AC, Achille; AM, Anco Marzio; CL, Claudio; DU, Duilio; DY, Dylan; IR, Iride; NO, Normanno, SI, Simeto; SV, Svevo; dm, dry matter.

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	Minimum temperature (°C)		Maximum temperature (°C)		Rainfall (mm)	
	Oct-Mar	Apr-Jun	Oct-Mar	Apr-Jun	Oct-Mar	Apr-Jun
ML 2010	4.6	9.9	15.7	24.2	621	461
ML 2011	4.5	10.5	16	26.2	713	**150**
ML 2012	3.6	10.6	16.9	25.3	309	217
JE 2010	4.8	11.4	14.1	23.6	498	320
JE 2011	4.3	11.6	13.4	25	552	102
JE 2012	4.5	11.8	14	25.2	325	163
FG 2010	5.1	10.6	14	22.6	416	123
FG 2011	4.7	11.1	14.1	23.7	472	134
FG 2012	0.5	7.6	19.3	30.1	245	99

Tab.5 Observed weather conditions across the growing areas selected in the study (SIAN).
Legend: ML, Montelibretti; JE, Jesi; FG, Foggia; 2010, crop year 2009/10; 11, crop year 2010/11; 12, crop year 2011/12; Oct-Mar, period between October and March; Apr-Jun, period between April and June.

After harvesting, grain samples were stored at 4°C. The grain were ground by a laboratory mill (Cyclotec, PBI, Milan, Italy), using a sieving of 1 mm and immediately stored at -20°C until the analysis. Sample moisture was measured just before the analysis on 3 g of milled sample by a Sartorius MA35 thermobalance (Muggiò, Monza-Brianza, Italy) at 120°C.

PA content was determined as described in the Section 3.1.1.2.2 and the optimal conditions for the extractions of the 3 PA forms in the different matrices were preliminarily evaluated. Following these preliminary tests, bound PAs were extracted from 100 mg of matrix instead of 250 mg, due to the high expected content of this PAs form; moreover, 250 mg was a better solution when a very broad range of content is expected in different matrices (e.g. semolina versus bran); in this study, where only one type of matrix (wholemeal) was considered, 100 mg resulted a more appropriate amount.

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In addition to PAs, the effects of genetic and environmental factors on YCP, TPC and TAC were also evaluated.

All analyses described above were carried out in triplicate and statistical analysis of data was performed.

The whole dataset was subjected to analysis of variance (ANOVA) performed with the MSTATC program (Michigan State University, East Lausing, MI, USA) using two specific factorial models. For the analysis of the genotype effect (10 genotypes grown in a single area), a factorial model (mod.9) with G (genotype) and Y (crop year) and G × Y interaction was used.

For the analysis of environmental factors (growing area and crop year), a factorial model (mod.12) was applied on the dataset related to 3 genotypes, 3 growing areas and 3 crop years, considering the combinations of crop years (2010, 2011 and 2012) and growing areas (Montelibretti, Jesi and Foggia) as nine separate environments and considering as factors G, A, Y (genotype, growing area and year, respectively) and their interactions. The same MSTATC program was used to carry out Duncan's multiple range test ($p \leq 0.05$) in order to highlight differences between genotypes and environments.

Principal component analysis (PCA) and correspondence analysis were performed using MATLAB software (R2010a version, MathWorks Inc., USA) in order to simultaneously evaluate the effects of the different factors on the variables under study. PCA was applied on the dataset related to the 10 genotypes grown in Montelibretti across 3 crop years and used to investigate the influence of genotype on the content of total PAs, total TPC and YCP and on TAC level and their relationships.

In order to evaluate the influence of crop year and environment, correspondence analysis was indeed performed among environmental variables (growing areas and crop years) and

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arbitrary categories of PAs, TPC, YCP and TAC. The arbitrary categories were defined on the basis of the mean values and standard deviations and three categories (low, medium and high) were set for each parameter. Categories were set as follows:

- TPC
    - Free: low <69, medium 69-74, high >75 mg/kg dm;
    - Conjugated: low <180, medium 180-199 and high >200 mg/kg dm;
    - Bound: low <1149, medium 1250-1299, high >1300 mg/kg dm.
- PAs
    - Free: low<4, medium 4.1-4.7, high >4.8 mg/kg dm;
    - Conjugated: low <140, medium 141-150, high >151 mg/kg dm;
    - Bound: low <800, medium 801-899, high >900 mg/kg dm.

The whole data set (3 durum wheat genotypes, 3 growing areas, 3 crop years) for PAs (free, conjugated and bound), TPC (free, conjugated and bound), YCP and TAC was converted to a frequency table with the environment (combination of growing area x crop year) as the first variable and three arbitrary categories for each trait (low, medium, high) as second variable.

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3.3 TECHNOLOGICAL PROCESSES

3.3.1 MILLING AND PASTA-MAKING

The effect of the traditional durum wheat transformation chain (from seed to cooked pasta) on PAs and TAC was studied using an Italian durum wheat cultivar (Duilio), grown in 2010/11 crop year, for which a high amount of samples was available. After harvesting, grain samples were collected and stored at 4°C before the technological processes.

For the milling process, 5 kg of grains were ground by a milling pilot plant (Buhler MLU 202, Uzwill, Switzerland) obtaining semolina and by products (flour, fine bran, coarse bran); coarse and fine bran were remilled on the Cyclotec mill before the chemical analyses to obtain a finer and more homogeneous particle size (maximum 1 mm).

An aliquot (100 g) of the harvested grain samples was ground in the Cyclotec mill (sieving of 1 mm) to obtain wholemeal (W) considered as the reference.

The micronized wholewheat (MW) was indeed obtained by a KMX-500 device (Separ Microsystem, Brescia, Italy), characterized by a 100-200 kg/h capacity with a steel drum containing a rotor operating at various peripheral speeds, and applying a peripheral speed of 120 Hz. The apparatus allowed a particle size reduction due to a mechanical impact against stator and rotor surfaces and to the collisions among turbulent particles.

Samples obtained from both traditional milling (wholemeal, coarse bran, semolina, flour) and micronization process (MW) were stored at -80°C to avoid degradation of bioactive compounds before the analyses.

Pasta was made both using semolina (traditional pasta) and MW (wholewheat pasta). The material (semolina or MW) was mixed with tap water to obtain a total dough water content of 32-33% and processed into spaghetti (diameter 1.65 mm) by an experimental press

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(NAMAD, Rome, Italy) and an experimental drier (AFREM, Lyon, France) applying a low temperature drying cycle (50°C).

Cooking test was performed following the procedure of D'Egidio et al. (1993); 100g of dried pasta were cooked in 1L of boiling tap water applying a standard cooking time of 13 min.

Cooked pasta was frozen, lyophilized and stored at -80°C until analyses.

PAs and TAC analyses were performed as described in the Section 3.1.1 and 3.1.4. statistical analysis (ANOVA and principal component analysis) were performed to analyze the influence of different technological processes on the analytical parameters.

3.3.2 DEBRANNING

3.3.2.1 Laboratory scale

The same Italian durum wheat cultivar (Duilio) selected for the study of milling and pasta-making processes was used to evaluate the influence of debranning process,.

The cleaned grains were firstly hydrated with 3% v/w water for 15 min, because the pre-hydration makes the tegument layers less crumbly, allowing a more regular and homogeneous removal and reducing kernel breakage (Bottega et al., 2009b). Secondly, grains were debranned by a laboratory debranning machine equipped with an abrasive stone element SB-SA (Colombini & Co. S.r.l, Abbiategrasso, Milan, Italy). This instrument allows to carefully check the debranning process and to collect all the debranning products with high accuracy. In detail, 12 debranning steps of 15 seconds each (named from T1 to T12) were applied, reaching the cumulative debranning levels (DL%) reported in **Tab.6**. Following each step, both debranning fractions (DF) and debranned kernels (DK) were obtained. DF and an aliquot of debranned kernels were collected before

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proceeding with the debranning of the remaining kernels; moreover, broken kernels were removed in order to apply the debranning process only on entire kernels.

All DF and DK were milled by the Cyclotec mill (1 mm particle size) and the resulting samples were immediately stored at -80°C until their use for the analyses.

In addition to the samples described above, an aliquot of no debranned grains was used for the analyses as reference (CTR).

All these samples were analyzed for PA content and TAC level in order to elucidate the effect of debranning process on the occurrence of selected antioxidant compounds.

Moreover, the content of total starch was measured in order to point out if the removal of the layers was close to the starchy endosperm.

TAC level, starch and PA contents in DF and DK were compared by analysis of variance (ANOVA); means were separated by Tukey's test at a probability level of 5% when F-values resulted significant. Finally, correlation coefficients among PA content, TAC level and starch content in both DF and DK were calculated.

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Debranning step (code)	Debranning time (sec)	Starting sample mass [a] (g)	Debranned kernels (g)	Debranning fractions (g)	Broken kernels (g)	Debranning level [b] %	Cumulative debranning level [c] %
T1	15	1210.7	1183.0	24.5	26.7	2.0%	2.0%
T2	30	1112.3	1092.1	15.1	13.1	1.4%	3.4%
T3	45	1037.1	1022.5	11.2	10.6	1.1%	4.5%
T4	60	969.8	958.7	9.2	10.7	0.9%	5.4%
T5	75	908.3	898.0	8.1	10.3	0.9%	6.3%
T6	90	849.4	840.7	6.6	9.5	0.8%	7.1%
T7	105	790.5	782.6	6.1	12.2	0.8%	7.9%
T8	120	728.3	721.5	5.7	12.5	0.8%	8.7%
T9	135	670.2	663.2	5.5	13.4	0.8%	9.5%
T10	150	608.6	602.4	5.4	14.3	0.9%	10.4%
T11	165	548.6	542.1	5.0	14.6	0.9%	11.3%
T12	180	487.4	481.3	4.8	14.4	1.0%	12.3%

Tab.6 Experimental conditions of 12 sequential debranning of durum wheat cv Duilio.

[a] Starting sample mass = Kernel mass debranned in each cycle

[b] Debranning level %= (Debranning fractions/ Debranned kernels)*100

[c] Cumulative debranning level % = Debranning level % of current debranning cycle + Debranning level % of previous cycles.

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3.3.2.2 Pilot scale

3.3.2.2.1 Experimental conditions of processing

The last part of the research activity was devoted to the development of durum wheat pasta with higher content of antioxidant compounds and higher antioxidant activity than the traditional one.

To this aim, it was crucial to take into consideration the results obtained from our studies about factors influencing the occurrence of selected antioxidants (PAs, TPC and YCP) and the antioxidant activity.

Two different durum wheat products were tested:

i) a pasta sample produced by enriching semolina with selected durum wheat debranning fractions, obtained by sequential debranning (pasta test 1);

ii) a pasta sample produced by using debranned kernels, whole milled and used in substitution of semolina for the pasta-making process (pasta test 2).

For this study, the durum wheat genotype (Normanno) grown in 2012/13 crop year was employed. This genotype is a recent cultivar, widely used for pasta-making at industrial level; a high amount of seeds (60 kg) was available for the experiments.

Preliminary tests were devoted to detect the optimal debranning level to reach for obtaining a debranning fraction with the highest content of antioxidants and jointly with high hygienic-sanitary quality.

Starting from unprocessed grain, kernels were pearled three times using a debranning pilot plant (NAMAD, Rome, Italy). Both debranning fractions and aliquots of resulting kernels were collected at the end of each step, corresponding to debranning levels (DL) of 2.8 %, 5.1 % and 8.0 %, respectively. The obtained debranning fractions were analyzed for their

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PA content in order to define the optimal debranning level and to select the best DF to be used for enrichment of semolina.

The selected debranning fraction was used to enrich semolina, obtained milling the same durum wheat genotype, for the production of pasta test 1. The ratio of enrichment semolina:debranning fractions was 100:30 w/w.

Pasta test 2 was indeed made by using the kernels debranned at DL 2.8% and micronized by the KMX-500 device, applying a peripheral speed of 120 Hz.

To better evaluate the impact of the enrichment and the use of debranned kernels, the pasta test 1 and 2 were compared to pasta made using semolina (pasta control) (**Fig.11**). The experimental plan of pasta-making process is schematically represented in **Fig.12**.

The three pasta samples were obtained using the respective raw materials (pasta 1: semolina+debranning fractions; pasta 2: debranned kernels; pasta 3: semolina) as previously described (Section 3.3.1). Cooking test was performed adding 100g of dried pasta to 1L of boiling tap water and applying a standard cooking time of 13 min (D'Egidio et al., 1993). Cooked pasta was frozen, lyophilized and stored at -80°C until analyses.

Fig.11 Pasta samples produced using the durum wheat genotype Normanno. Legend: pasta 1, pasta test 1 (semolina+debranning fraction 2; pasta 2, pasta test 2 (debranned kernels); pasta 3, control pasta (semolina).

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All samples were analyzed for their PA content, as well as for their antioxidant capacity and their content of TPC and YCP. The raw materials and the pasta samples were also analyzed for their content of ash, starch and protein.

Finally, the quality of the three pasta samples was assessed by sensory analysis based on three textural characteristics: firmness, stickiness, and bulkiness. Stickiness is the material adhering to the surface of cooked pasta; bulkiness is the adhesion degree of pasta stands to each other; firmness represents the resistance of cooked pasta to chewing by teeth. Each of these three parameters was evaluated by a score ranging from 10 to 100, as reported by Cubadda (1988). For stickiness and bulkiness, <20 = very high, 40 = high, 60 = rare, 80 = almost absent, and 100 = absent. For firmness, <20 = absent, 40 = rare, 60 = sufficient, 80 = good, and 100 = very good. The score of each sensorial component was the arithmetic mean of three assessors.

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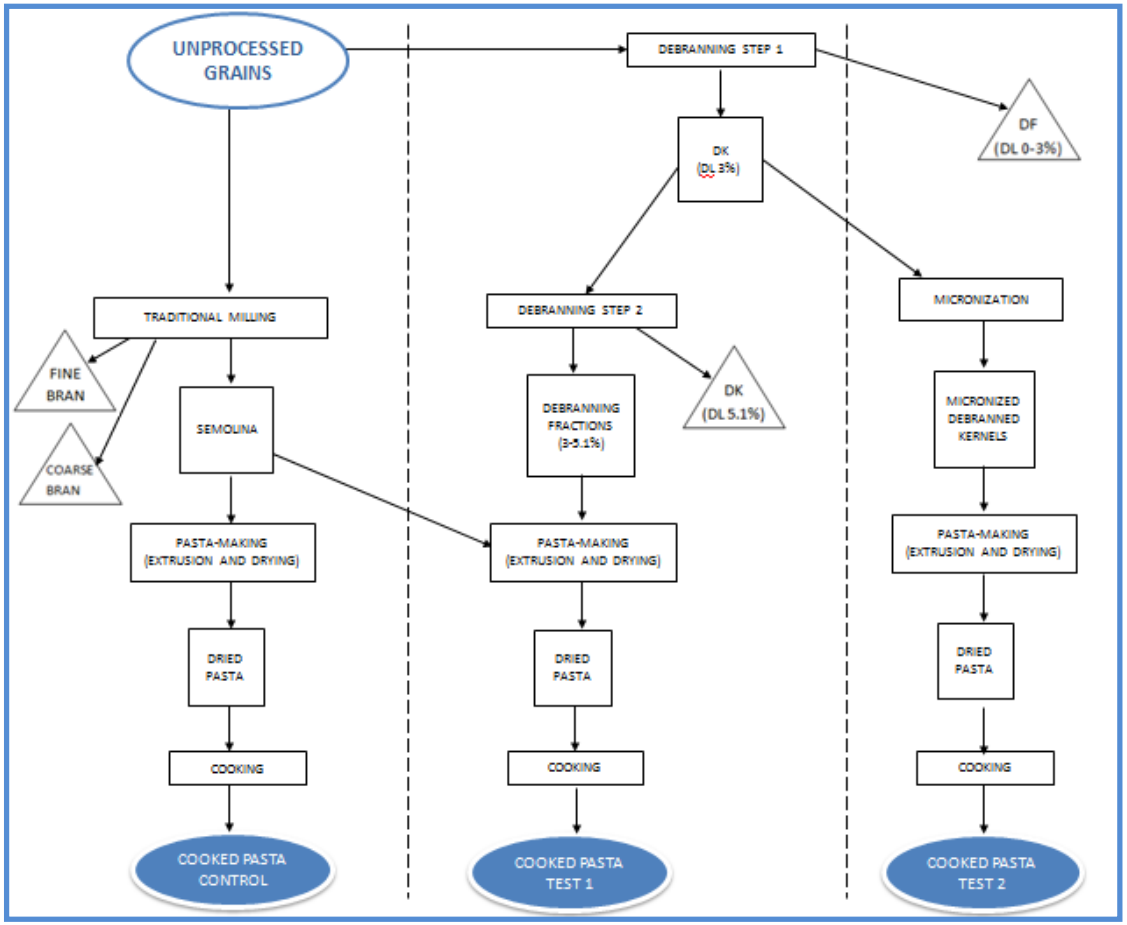

Fig.12 Scheme of pasta-making process for preparation of pasta test samples (1 and 2) and pasta control. Legend: DF, debranning fractions; DK, debranned kernels; DL, debranning level.

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3.3.2.2.2 Bioavailability of phenolic compounds

In order to better comprehend the bioavailability of phenolic compounds present in the three types of pasta, a pilot study was performed. To this aim, three healthy subjects aged 29±3 years (mean BMI 21.5±2.3 kg/m^2) were recruited and enrolled in the study.

The study followed a randomized, cross-over design, with subjects serving as their own controls. Each volunteer received a complete list of foods to be avoided; the list included phenolic-rich foods such as fruit and vegetables, whole grains, tea, coffee, wine and chocolate. Between the 3 periods, there was a washout period of at least 1 week. The subjects were asked to avoid unusually large portions of food on the day preceding each test, and after overnight fasting, the first blood sample was taken in the morning for the baseline measurements (time 0). Directly after that, between 10.00 and 10.30 am, the participants consumed the test meal. It consisted of 100 g of pasta test (1, 2 or 3) dressed with 10 g of sunflower oil. Meal eating time was restricted to 15 minutes. Upon consuming the pasta test, blood was taken after 1, 2, 4, 6, 8 and 24 h (**Fig.13**). During the intervention day, drinks were restricted to water and a standardized lunch, which consisted of ham (50 g) and cheese (50 g). In the evening, a dinner with the exclusion of phenolic-rich food was allowed.

Two aliquots of plasma were obtained by centrifugation for 10 min at 1000×g and samples were stored at −80 ◦C until analysis. Urine was collected during the 24 h after the pasta consumption (urine test) as well as in the 24 h preceding the consumption (urine baseline). For each of the two types of urine (test and baseline) aliquots of 1 mL each were collected and stored at -80°C until analysis.

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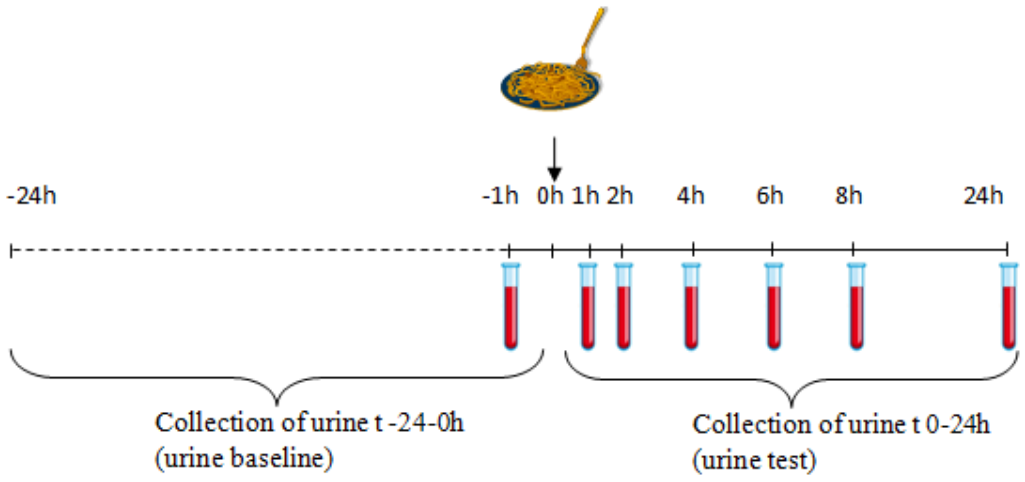

Fig.13 Scheme of the experimental design used to evaluate the bioavailability of phenolic compounds in 3 pasta samples.

An aliquot of 200 µL of plasma and urine for each time was added to 100 µL of buffer A (sodium acetate 0.1 M, pH 4.5), hydrolyzed with 200 U of glucuronidase/sulfatase (by adding 200 µL of a solution containing 1000 U/mL in buffer A) and incubated overnight at 37°C.

The reaction mixture was extracted twice with 600 µl ethylacetate, vortexed and centrifuged at 1000 x g for 1 min. The supernatant fraction (500 µl x 2) was dried using a SpeedVac™ systems (Eppendorf, Hamburg, Germany). Samples were re-dissolved with 200 µl of a methanol:water solution (20:80 v/v) just prior the analysis.

The second aliquot of plasma and urine was indeed treated with acetonitrile in order to allow the protein precipitation. An aliquot of the resulting samples was diluted 1:1 and directly analysed to determine the both free phenolic acids and conjugated with glucuronic acid e/o sulphate.

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The UPLC-HR-MS analysis was carried out on an Acquity UPLC separation module (Waters, Milford, MA, USA) coupled with a mass spectrometry Orbitrap mod. Exactive through an HESI-II probe (Thermo Scientific, San Jose, CA, USA). The ion source and interface conditions were: spray voltage 3.5 kV, sheath gas flow rate 35, auxiliary gas flow rate 15 and temperature 250 °C, capillary temperature 350 °C. Five µL of each sample were separated on a Kinetex C_{18} column (100×2.1 mm, 1.8 µm, Phenomenex) kept at 40 °C. The eluents were 0.1% acetic acid in MilliQ-treated water (solvent A) and acetonitrile (solvent B). The UPLC separation was performed by using the following linear elution gradient: 15% B for 6 min, 15 to 70% B in 6. The flow-rate was 0.5 mL/min. The UPLC eluate was analyzed by MS using Full MS (<5 ppm mass tolerance), and the resolution was set at 50K. The AGC target was 5E5 for Full MS scans, and the maximum ion injection time was 100 ms. The collision energy was 20V to improve fragmentation in the HCD cell. The MS data were processed using Xcalibur software (Thermo Scientific).

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4. Results & Discussion

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4.1 PHENOLIC ACIDS: METHOD DEVELOPMENT AND VALIDATION

4.1.1 OPTIMIZATION OF THE HPLC METHOD

Initial experiments were devoted to optimize the HPLC method employed for the separation, identification and quantification of PAs extracted from wholemeal, its milling main fractions (coarse bran and semolina) and pasta. The PAs were extracted in the three different forms (soluble free, soluble conjugated and insoluble bound) and subsequently analyzed by RP-HPLC, using a narrow bore C-18 column (2.1 mm ID). The selection of a narrow bore column, in combination with a micro-volume (2.5 μL) detection cell, compared to using a conventional analytical size column (4.6 mm ID), provided many advantages including i) the expected higher sensitivity of PDA detection, due to the minor dilution of samples during separation; ii) the sharper peaks, due to minor radial dispersion, minor impact of heat effects on separation performance as a consequence of better heat dissipation and flow rate compatibility with mass spectrometry detection; iii) the reduced consumptions of toxic and expensive organic solvents and of the other reagents employed for elution, with positive impact on the environment and on the cost of the analysis.

As regards the mobile phase employed for the analysis, acetonitrile was preferred over methanol because of its lower viscosity, which makes it more suitable in limiting the back pressure associated with the low permeability of the narrow bore HPLC column and of the capillary tube comprised in the ESI interface employed to hyphenating the HPLC system with the mass spectrometer.

Formic acid was indeed incorporated into the mobile phase to suppress the ionization of PAs and to avoid peak broadening caused by the simultaneous presence of protonated and ionized forms of the phenolic acids. In order to investigate the influence of the concentration of formic acid incorporated in the mobile phase on the separation

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performance of PAs by RP-HPLC, a preliminary study was carried out. Results showed that the retention time of almost all PAs decreased as the concentration of formic acid increased. Besides lowering the pH and thus keeping carboxyl and hydroxyl groups of the analytes in their protonated form, formic acid is believed to interact with these functional groups *via* hydrogen-bonding formation so increasing the virtual polarity of PAs with the consequent reduction of hydrophobic interactions with the octadecyl stationary phase. As a result, the retention time of PAs decreases with increasing the concentration of formic acid. On the other hand, increasing the concentration of formic acid had the effect of raising its efficacy in controlling the protonic equilibrium with consequent decreasing of band broadening and peak tailing. A good compromise between these two opposite effects was obtained incorporating formic acid in the starting eluent at the concentration of 2% (v/v), even though better sensitivity in ESI-MS was obtained with mobile phases containing lower concentration of formic acid.

The use of water-acetonitrile mobile phase containing 2 % (v/v) formic acid and proper selection of a multi-segments gradient of increasing concentration of acetonitrile determined the concomitant resolution, in less than 40 min, of all PAs potentially present in our samples, in addition to the internal standard used for PA quantification (**Fig.14).**

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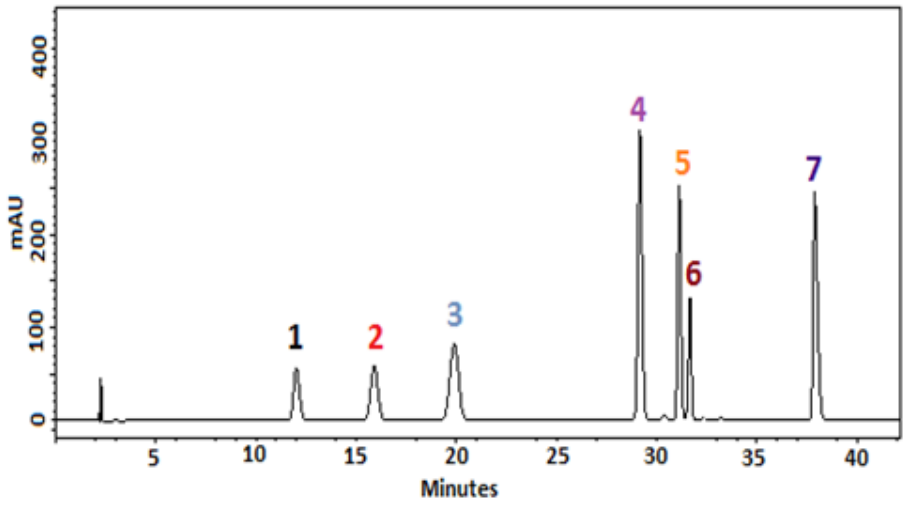

Fig.14 Separation of PA standards and internal standard. Legend: 1: p-hydroxybenzoic acid; 2: vanillic acid; 3: syringic acid; 4: p-coumaric acid; 5: ferulic acid; 6: sinapic acid; 7. dichlorohydroxybenzoic acid (internal standard).

An example of the separations of soluble free, soluble conjugated and insoluble bound PAs in real matrix (i.e. durum wheat coarse bran) is shown in the chromatograms displayed in **Fig.15**. The significant differences in both peak size and values of absorbance units at full scale displayed by the three chromatograms reflected the different content of PAs in the three forms occurring in durum wheat coarse bran.

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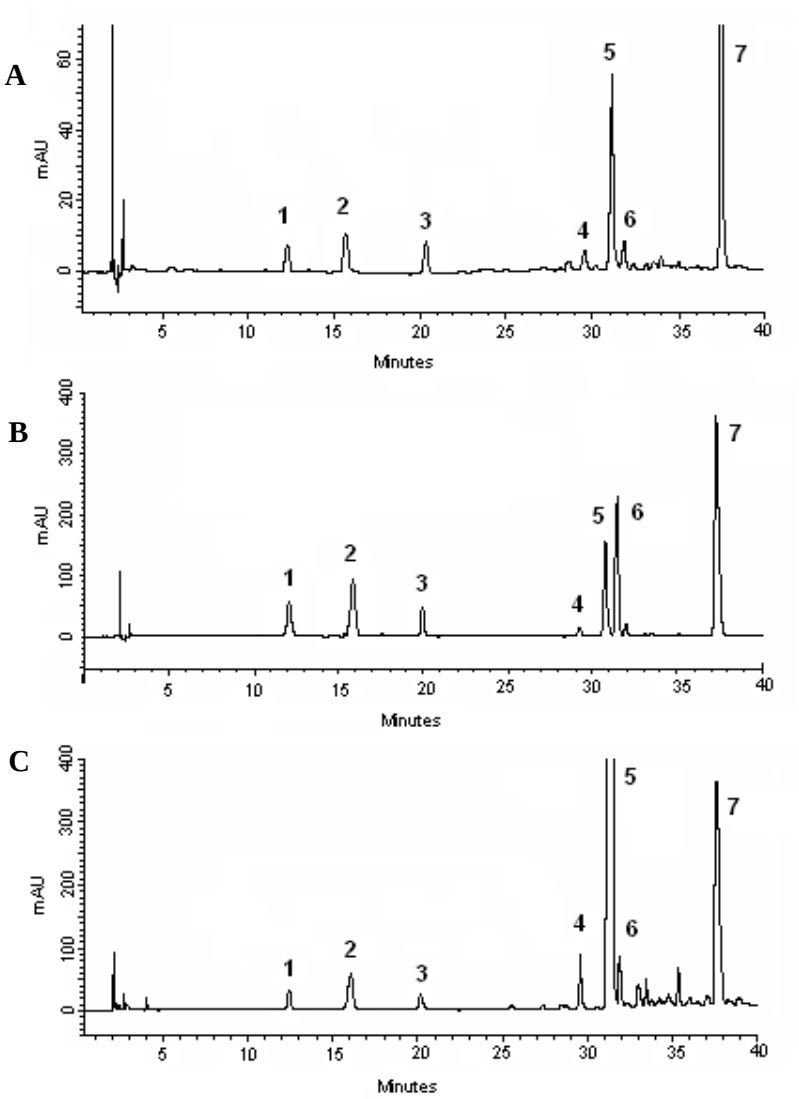

Fig.15 Separation of soluble free (A), soluble conjugated (B) and insoluble bound (C) PAs occurring in durum wheat coarse bran. Legend: 1. p-hydroxybenzoic acid; 2. vanillic acid; 3. syringic acid; 4. p-coumaric acid; 5. ferulic acid; 6. sinapic acid; 7. dichlorohydroxybenzoic acid (internal standard).

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4.1.2 METHOD VALIDATION

4.1.2.1 Linearity

As already described in the Materials & Methods section, linearity was evaluated by the construction of calibration graphs obtained by plotting the ratio of the analyte peak area to that of the IS as a function of the analyte concentration of the standards that had undergone the same three extraction procedures employed for the real samples. In this way, losses due to the extraction method were accounted for . Calibration graphs for the 6 PAs found in durum wheat in the 3 PA forms are shown in **Fig.16-18**.

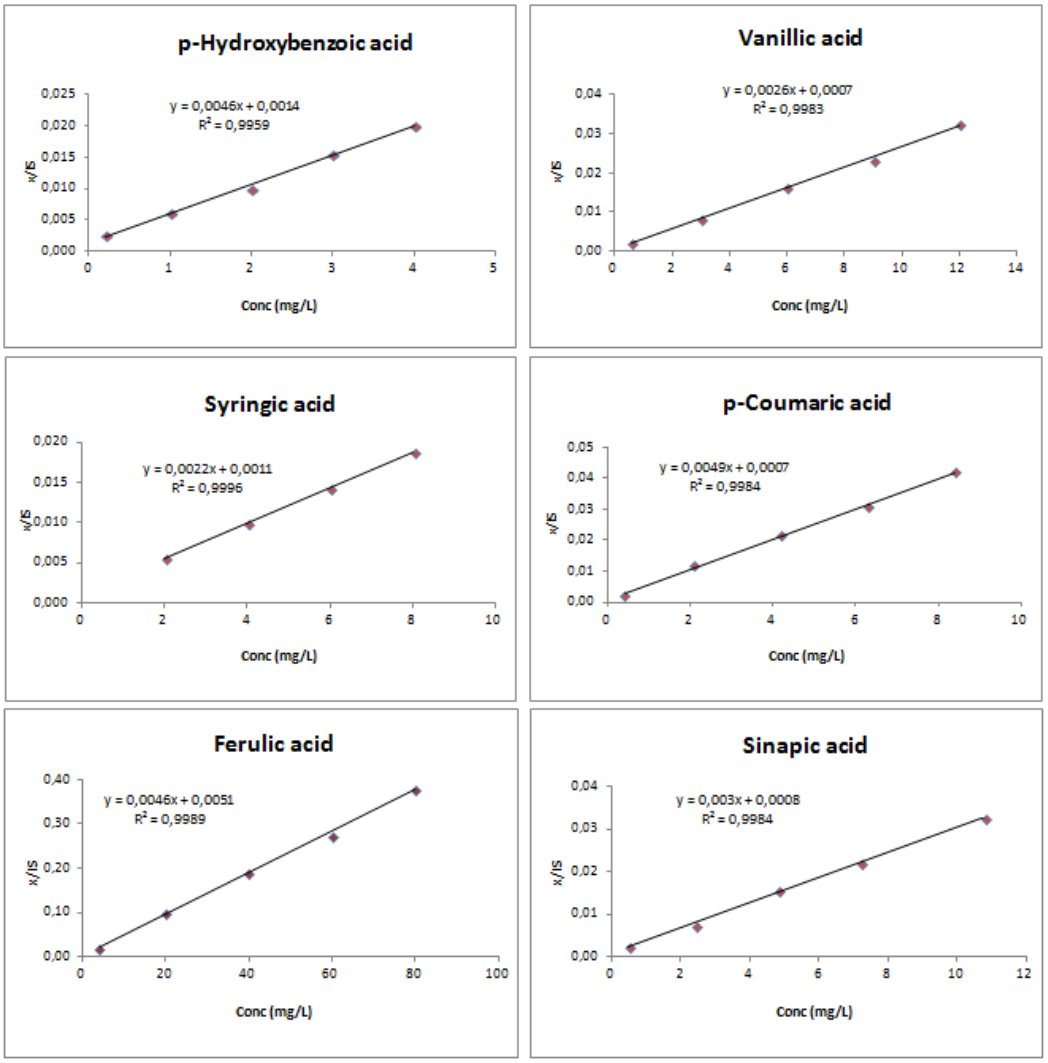

Fig.16 Calibration graphs for free PAs.

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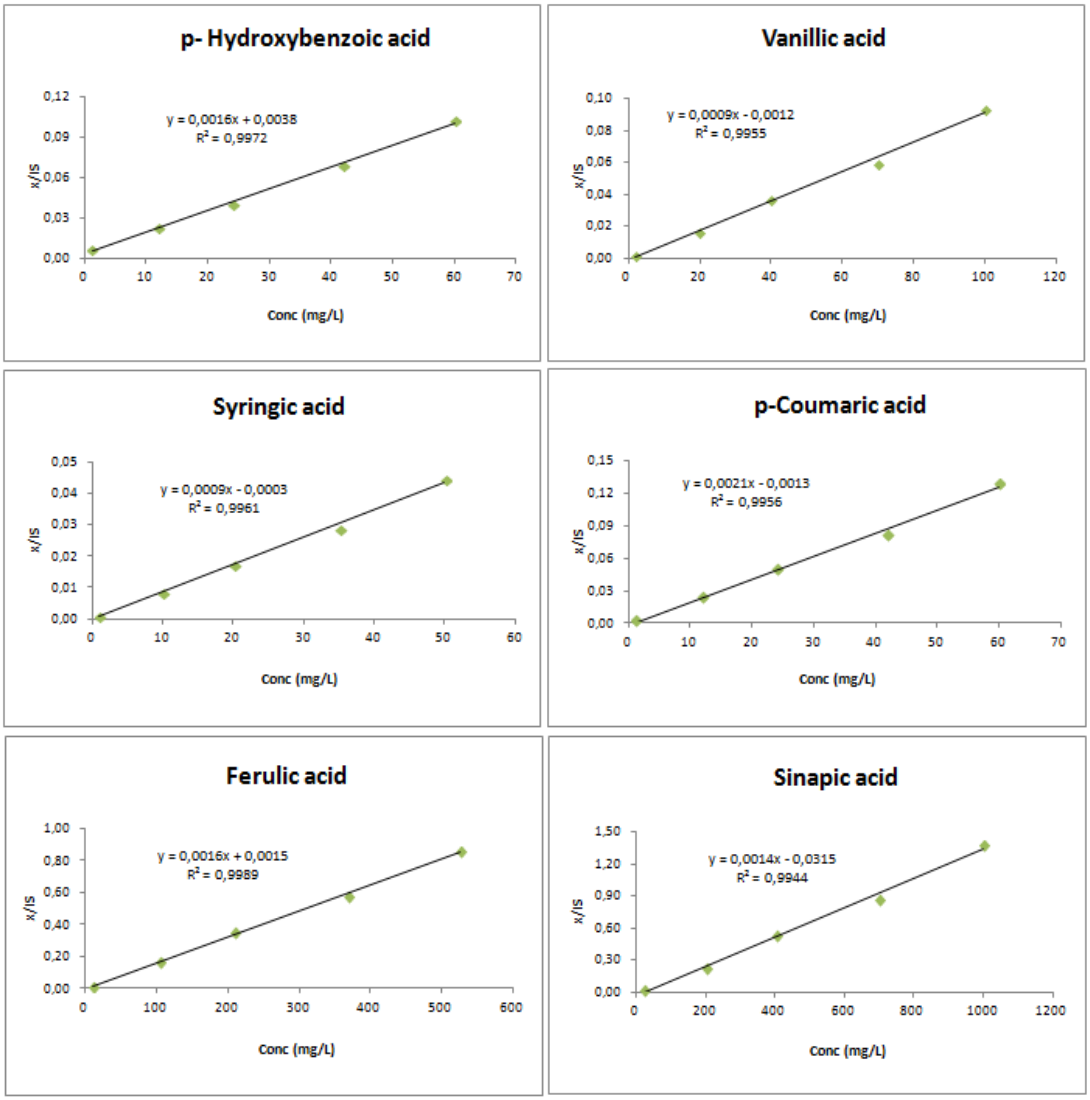

Fig.17 Calibration graphs for conjugated PAs.

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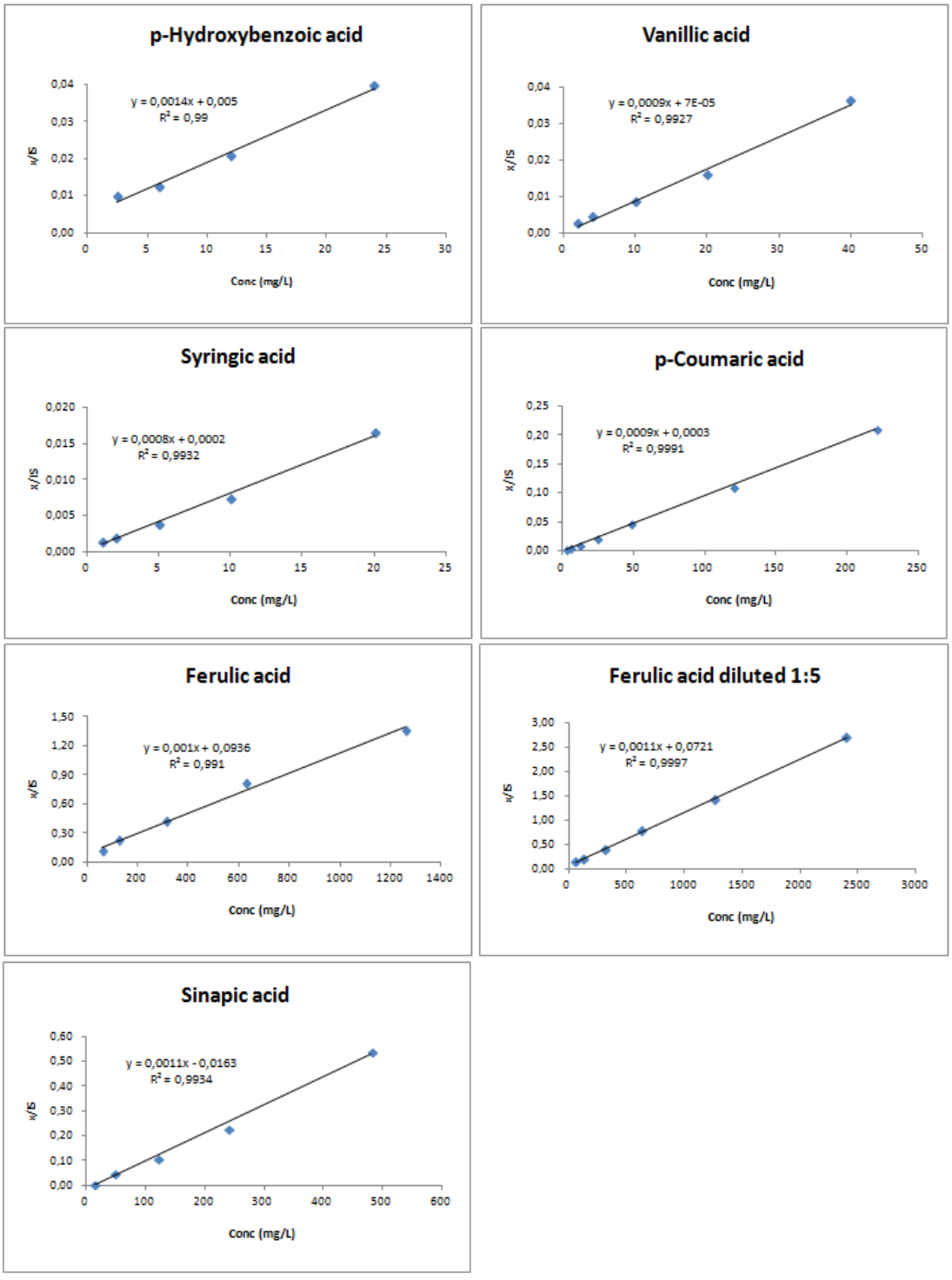

Fig.18 Calibration graphs for bound PAs.

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Limits of detection (LOD) and quantification (LOQ) were considered as the concentrations of PAs that resulted in a peak with a height at least three times and ten times as high as the baseline noise, respectively (**Tab.7**)

	Analyte	λ (nm)	Linear range (mg/L)	Equation*	R	LOD (mg/L)	LOQ (mg/L)
Soluble free PAs	p-Hydroxybenzoic acid	254	0.2-4.0	y=0.0046x+0.0014	0.9959	0.1	0.3
	Vanillic acid	254	0.6-12.0	y=0.0026x+0.00007	0.9983	0.2	0.7
	Syringic acid	280	2.0-8.0	y=0.0022x+0.0011	0.9996	0.7	2
	p-Coumaric acid	320	0.42-8.4	y=0.0049x+0.0007	0.9984	0.07	0.2
	Ferulic acid	320	4.0-80.0	y=0.0046x+0.0051	0.9989	0.06	0.2
	Sinapic acid	320	0.48-10.8	y=0.0030x+0.0008	0.9984	0.2	0.55

	Analyte	λ (nm)	Linear range (mg/L)	Equation*	R	LOD (mg/L)	LOQ (mg/L)
Soluble conjugated PAs	p-Hydroxybenzoic acid	254	1.2-60.0	y=0.0016x+0.0038	0.9972	0.06	0.2
	Vanillic acid	254	2.0-100	y=0.0009x-0.0012	0.9955	0.18	0.6
	Syringic acid	280	1.0-50	y=0.0009x-0.0003	0.9961	0.35	1.0
	p-Coumaric acid	320	1.2-60	y=0.0021x-0.0013	0.9956	0.06	0.2
	Ferulic acid	320	10.5-525	y=0.0016x+0.0015	0.9989	0.1	0.35
	Sinapic acid	320	20.0-1000	y=0.0014x-0.0315	0.9944	0.16	0.48

	Analyte	λ (nm)	Linear range (mg/L)	Equation*	R	LOD (mg/L)	LOQ (mg/L)
Insoluble bound PAs	p-Hydroxybenzoic acid	254	2.4-24	y=0.0014x+0.005	0.9900	0.2	0.5
	Vanillic acid	254	2.0-40	y=0.0009x+0.00007	0.9927	0.4	1.25
	Syringic acid	280	1.0-20	y=0.0008x+0.0002	0.9932	0.2	0.7
	p-Coumaric acid	320	2.4-220	y=0.0009x+0.0003	0.9991	0.3	0.6
	Ferulic acid	320	63-2400	y=0.0010x+0.0936	0.9910	0.08	0.25
	Sinapic acid	320	12.5-480	y=0.0110x-0.0163	0.9934	0.06	0.18

Tab.7 Analysis of Calibration Graphs Based on UV Absorbance at the Reported Wavelength (λ).

*y expresses the detection response (peak area/ IS area in arbitrary units) and x the concentration for phenolic compounds in mg/L.

Legend: LOD=limit of detection; LOQ=limit of quantification; R=coefficient of correlation.

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4.1.2.2 Precision

The precision of the developed method was evaluated as intraday and interday repeatability of retention time and peak area/IS area ratio for the three PAs form. As shown in **Tab.8-10**, the intraday and interday repeatability of peak are/IS area ratio resulted to be lower than 8.4% and 9.9% respectively, whereas the intraday and interday repeatability of retention time was lower than 0.9 and 1.5% respectively.

Repeatability retention time (min)							
		Intraday (*n* = 3)			**Interday (*n* = 3 over 3 days)**		
Peak nr.	**Analyte**	**Average**	**SD**	**RSD (%)**	**Average**	**SD**	**RSD (%)**
1	p-Hydroxybenzoic acid	12.263	0.112	0.9	12.277	0.181	1.5
2	Vanillic acid	16.142	0.125	0.8	16.136	0.164	1.0
3	Syringic acid	20.366	0.151	0.7	20.311	0.169	0.8
4	p-Coumaric acid	29.540	0.089	0.3	29.520	0.123	0.4
5	Ferulic acid	31.342	0.059	0.2	31.320	0.070	0.2
6	Sinapic acid	31.904	0.037	0.1	31.879	0.043	0.1

Repeatability peak area[1] / IS area							
		Intraday (*n* = 3)			**Interday (*n* = 3 over 3 days)**		
Peak nr.	**Analyte**	**Average**	**SD**	**RSD (%)**	**Average**	**SD**	**RSD (%)**
1	p-Hydroxybenzoic acid	0.021	0.001	6.9	0.021	0.001	4.9
2	Vanillic acid	0.033	0.001	2.6	0.031	0.002	4.9
3	Syringic acid	0.019	0.000	0.2	0.019	0.000	2.3
4	p-Coumaric acid	0.029	0.002	7.9	0.027	0.002	8.4
5	Ferulic acid	0.315	0.010	3.0	0.0311	0.008	2.5
6	Sinapic acid	0.007	0.001	8.4	0.007	0.001	9.6

Tab.8 Free PAs: intra-day and inter-day repeatability of retention time and peak area analyte/ peak area IS .
[1]Arbitrary units

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Repeatability retention time (min)							
		Intraday (n = 3)			Interday (n = 3 over 3 days)		
Peak nr.	**Analyte**	**Average**	**SD**	**RSD (%)**	**Average**	**SD**	**RSD (%)**
1	p-Hydroxybenzoic acid	12.018	0.033	0.3	12.153	0.155	1.3
2	Vanillic acid	15.883	0.038	0.2	15.997	0.134	0.8
3	Syringic acid	20.039	0.050	0.3	20.153	0.140	0.7
4	p-Coumaric acid	29.280	0.028	0.1	29.400	0.134	0.5
5	Ferulic acid	31.154	0.022	0.1	31.220	0.078	0.3
6	Sinapic acid	31.755	0.011	0.0	31.791	0.049	0.2

Repeatability peak area[1] / IS area							
		Intraday (n = 3)			Interday (n =3 over 3 days)		
Peak nr.	**Analyte**	**Average**	**SD**	**RSD (%)**	**Average**	**SD**	**RSD (%)**
1	p-Hydroxybenzoic acid	0.019	0.000	0.5	0.021	0.002	9.9
2	Vanillic acid	0.017	0.000	0.6	0.019	0.002	8.4
3	Syringic acid	0.008	0.000	0.4	0.009	0.001	9.6
4	p-Coumaric acid	0.024	0.000	0.6	0.025	0.002	8.5
5	Ferulic acid	0.169	0.001	0.5	0.181	0.013	7.3
6	Sinapic acid	0.215	0.001	0.5	0.229	0.017	7.2

Tab.9 Conjugated PAs: intra-day and inter-day repeatability of retention time and peak area analyte/ peak area IS. [1]Arbitrary units

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Repeatability retention time (min)							
		Intraday (*n* = 3)			Interday (*n* =3 over 3 days)		
Peak nr.	**Analyte**	**Average**	**SD**	**RSD (%)**	**Average**	**SD**	**RSD (%)**
1	p-Hydroxybenzoic acid	12.107	0.060	0.5	12.002	0.113	0.9
2	Vanillic acid	15.982	0.093	0.6	15.848	0.137	0.9
3	Syringic acid	20.141	0.087	0.4	19.982	0.160	0.8
4	p-Coumaric acid	29.365	0.059	0.2	29.264	0.102	0.3
5	Ferulic acid	31.214	0.048	0.2	31.143	0.069	0.2
6	Sinapic acid	31.852	0.097	0.3	31.774	0.079	0.2

Repeatability peak area[1] / IS area							
		Intraday (n = 3)			Interday (n =3 over 3 days)		
Peak nr.	**Analyte**	**Average**	**SD**	**RSD (%)**	**Average**	**SD**	**RSD (%)**
1	p-Hydroxybenzoic acid	0.005	0.000	2.9	0.005	0.000	3.7
2	Vanillic acid	0.004	0.000	6.7	0.004	0.000	6.2
3	Syringic acid	0.222	0.000	1.5	0.002	0.000	5.0
4	p-Coumaric acid	0.005	0.000	2.0	0.005	0.000	2.9
5	Ferulic acid	0.210	0.003	1.4	0.207	0.004	2.0
6	Sinapic acid	0.037	0.002	4.3	0.035	0.002	6.0

Tab.10 Bound PAs: intra-day and inter-day repeatability of retention time and peak area analyte/ peak area IS .
[1]Arbitrary units

4.1.2.3 Accuracy

The accuracy of the method was evaluated by a recovery study, in which known amounts of ferulic acid were added to the sample and extracted and analyzed in parallel to a non-spiked sample of the same wheat. Average recoveries ranging between 90% and 112% (RSD <5.4%) were found for ferulic acid in the soluble conjugated and free form, respectively. On the contrary, the recovery was much lower for the insoluble bound ferulic acid (mean value: 73.3%; RSD: 6.1%), probably due to the oxidative degradation of the added ferulic acid occurred during alkaline hydrolysis.

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4.2 INFLUENCE OF GENETIC AND ENVIRONMENTAL FACTORS

4.2.1 INFLUENCE OF GENOTYPE

4.2.1.1 Phenolic acids

The content of total PAs in 10 durum wheat genotypes grown over 3 consecutive crop years in Montelibretti are shown in **Tab.11**. The mean total content was 1061.4 ± 139.8 mg/kg dm and ranged from 856.6 mg/kg dm (Iride, 2011) to 1464.0 (Achille, 2010). Achille was the genotype displaying the highest content of total PAs (mean content across the 3 crop years:1254.7 ± 205.6 mg/kg dm), while Saragolla showed the lowest one (906.3 ± 42.6 mg/kg dm).

The mean content of PAs in the 3 forms (soluble free, soluble conjugated and insoluble bound) in the 10 genotypes is depicted in **Fig.19 A, B** and **C**, respectively. As expected, bound form was the most abundant one (mean content: 913.8 ± 132.1 mg/kg dm) in all samples, followed by the conjugated form (143.4 ± 24.5 mg/kg dm) and lastly by the free one (4.2 ± 2.4 mg/kg dm), confirming data previously reported in literature (Li et al., 2010).

As underlined for the total PAs, Achille was the genotype with the highest content of bound PAs (mean across 3 crop years: 1131.6 ± 182.0 mg/kg dm) and Saragolla the lowest one (752.9 ± 49.5 mg/kg dm). For the conjugated form, Simeto showed the highest content (169.6 ± 7.2 mg/kg dm) and Iride the lowest one (114.6 ± 12.5 mg/kg dm); lastly, Duilio had the highest content of free PAs (6.7 ± 3.6 mg/kg dm) and Dylan the lowest one (3.1 ± 1.3 mg/kg dm). In agreement with previous data performed on durum wheat (Li et al., 2008; Fernandez-Orozco et al., 2010), the free form showed the highest variability (RSD=56%), followed by the conjugated form (17%), while the bound form was the less variable one (RSD=14%).

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Considering the individual PAs, reported as percentage distribution in **Fig.20**, ferulic acid prevailed in the bound form accounting for almost 90% of total bound PAs, followed by sinapic acid (7.1%), *p*-coumaric acid (2.3%), vanillic acid (0.7%) and lastly by syringic acid (0.2%), whereas *p*-hydroxybenzoic acid was not quantifiable. It could be noted that the percentage distribution of individual PAs in bound form was similar among genotypes: for instance, ferulic acid ranged from 89.0% (Normanno) to 90.9% (Iride).

In the conjugated form, sinapic acid prevailed in all genotypes accounting on average for 65.6% of total conjugated PAs, while ferulic acid only for 19.2%. As already observed for bound PAs, the distribution rate was similar across genotypes; for example, sinapic acid accounted from 62.1% to 70.6% and ferulic acid from 15.8% to 20.7%. These percentage distribution appear highly different to those reported in some other studies (Hernández et al., 2011, Brandolini et al., 2013) and this could be due to the different individual PAs quantified in the previous investigations. For example, the non quantification of sinapic acid might be responsible for differences observed especially in the conjugated forms, where sinapic acid is prevailing on the others.

Greater differences were observed for the free form, plausibly due to the low contents of PAs in this form; vanillic and ferulic acid were the most abundant ones, accounting on average for 35.1% and 33.4% respectively.

4.2.1.2 Total phenolic compounds

The contents of total phenolic compounds for the 10 genotypes grown in Montelibretti are reported in **Tab. 11**. Mean TPC (as sum of free, conjugated and bound TPC) was 1499.1±230.9 mg/kg dm and ranged from 1190.1 mg/kg dm (Anco Marzio, 2011) to 2052.3 mg/kg dm (Svevo, 2010). On average, Simeto showed the highest values (mean across 3 crop years: 1754.8 mg/kg dm), while Iride the lowest ones (1387.0 mg/kg dm).

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These data appear generally higher than those reported in other previous studies: Bellato et al. (2013) reported values ranging from 98.6 to 144.9 mg/kg in 19 varieties of durum wheat, while Menga et al. (2010) reported a mean TPC of 882 mg/kg dm in 30 durum wheat genotypes. These differences could be plausibly mainly due to the different methods used for the extraction, not always including the contribution of the different forms of compounds (free, conjugated and bound) to the total content. Moreover, the different standards used for quantification in some investigation (e.g. gallic acid instead of ferulic acid) might provide an additional explanation for such differences. This hypothesis is supported by the consideration that our data appear comparable to those found by Brandolini et al. (2013) who evaluated both conjugated and bounds forms, reporting a mean TPC of 1374 ±53.6 and 1254 ± 46.5 mg/kg FAE dm in wholemeal of 4 durum wheat genotypes grown in two not consecutive crop years, respectively. Considering the three forms, bound form results the most abundant one, as already observed for PAs: mean values were in fact 1223.3±199.0, 199.7±45.3 and 76.1±14.6 mg/kg dm for bound, conjugated and free TPC, respectively.

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			Total PAs	TPC	YCP	TAC
			mg/kg dm	mg FAE/kg dm	mg/kg dm	mmol TEAC/kg dm
Montelibretti	Achille	2010	1464.0	1961.2	6.00	44.60
		2011	1053.1	1347.1	4.48	41.44
		2012	1246.9	1486.6	4.73	44.72
	Anco Marzio	2010	1211.4	1602.2	5.31	45.39
		2011	1022.1	1190.1	4.89	43.21
		2012	959.5	1374.2	5.46	46.67
	Claudio	2010	1243.1	1645.6	5.75	43.25
		2011	885.7	1248.4	5.70	41.50
		2012	1001.1	1398.3	4.42	45.42
	Duilio	2010	1278.4	1719.3	5.80	46.66
		2011	1098.2	1404.7	4.52	44.64
		2012	1096.3	1314.7	4.05	48.71
	Dylan	2010	1085.1	1463.8	7.47	40.48
		2011	913.8	1324.2	7.13	47.24
		2012	971.6	1471.4	7.27	45.16
	Iride	2010	1088.9	1739.1	5.90	47.77
		2011	856.6	1194.0	5.42	47.40
		2012	974.7	1227.9	4.20	48.56
	Normanno	2010	1148.1	1503.5	6.95	46.58
		2011	998.3	1284.9	6.06	43.65
		2012	1089.6	1398.0	6.47	45.90
	Saragolla	2010	857.1	1796.0	6.14	41.91
		2011	932.6	1314.1	5.12	44.46
		2012	929.1	1363.6	5.53	42.71
	Simeto	2010	943.6	1759.6	4.06	45.47
		2011	1131.9	1614.0	3.91	45.58
		2012	1208.2	1890.8	4.94	43.66
	Svevo	2010	1124.4	2052.3	5.35	51.10
		2011	993.8	1450.3	5.46	47.21
		2012	1035.7	1431.7	5.46	49.26
		mean	**1061.4**	**1499.1**	**5.47**	**45.34**
		sd	**139.8**	**230.9**	**0.97**	**2.51**

Tab.11 Total PAs, TPC and YCP and TAC in 10 genotypes grown in Montelibretti on 3 consecutive crop years.

Legend: PAs, phenolic acids, TPC, Total Phenolic Content, TAC, Total Antioxidant Capacity, TEAC, Trolox Equivalent Antioxidant Capacity, YCP, Yellow Coloured Pigments, FAE, ferulic acid equivalents, dm, dry matter.

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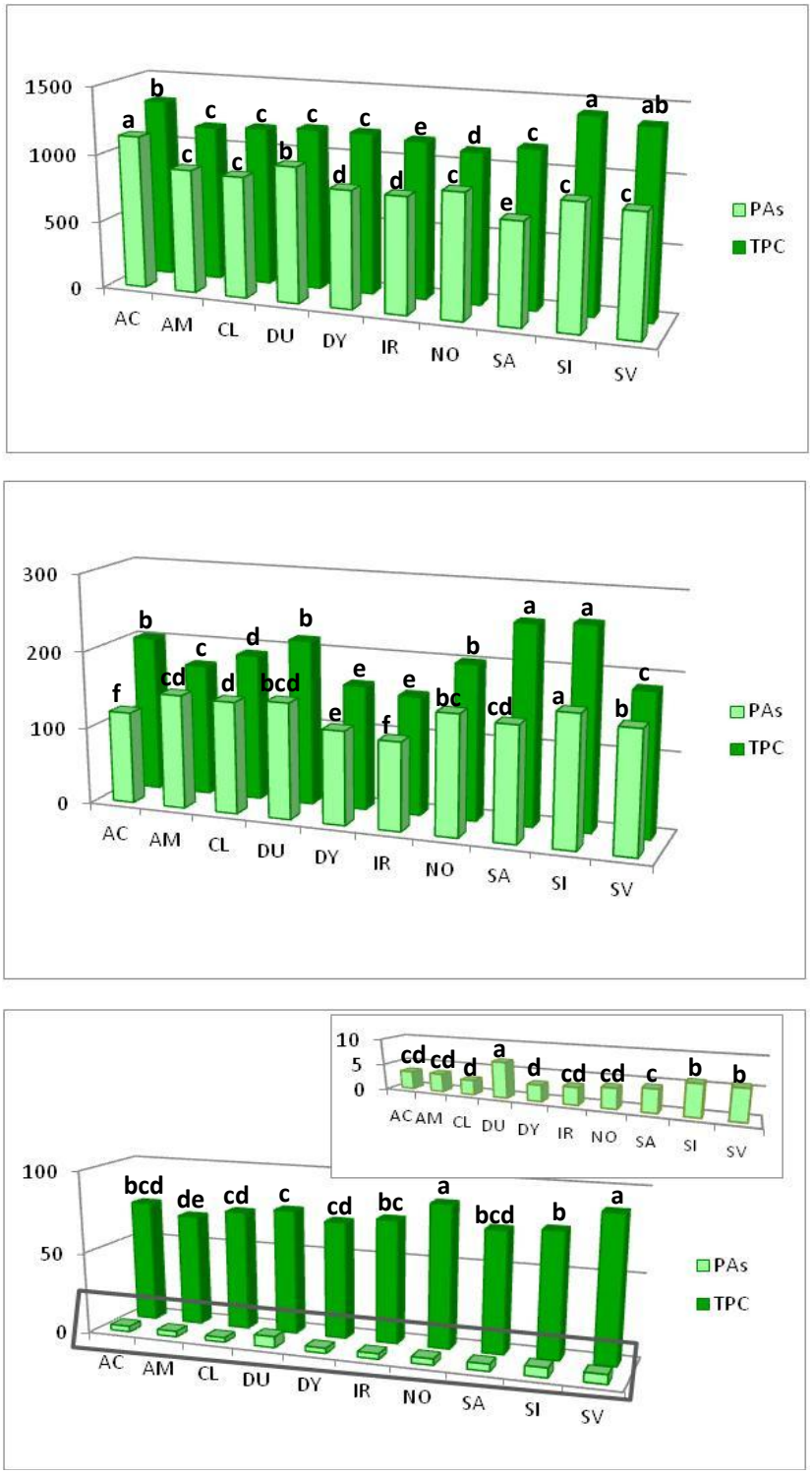

Fig.19 Mean content of bound (A), conjugated (B) and free (C) PAs ( ) and TPC ( ) in 10 durum wheat genotypes grown in Montelibretti in 3 consecutive crop years. Different letters indicate that averages are significantly different from each other ($p < 0.05$). Duncan's test were performed for PAs and TPC separately.

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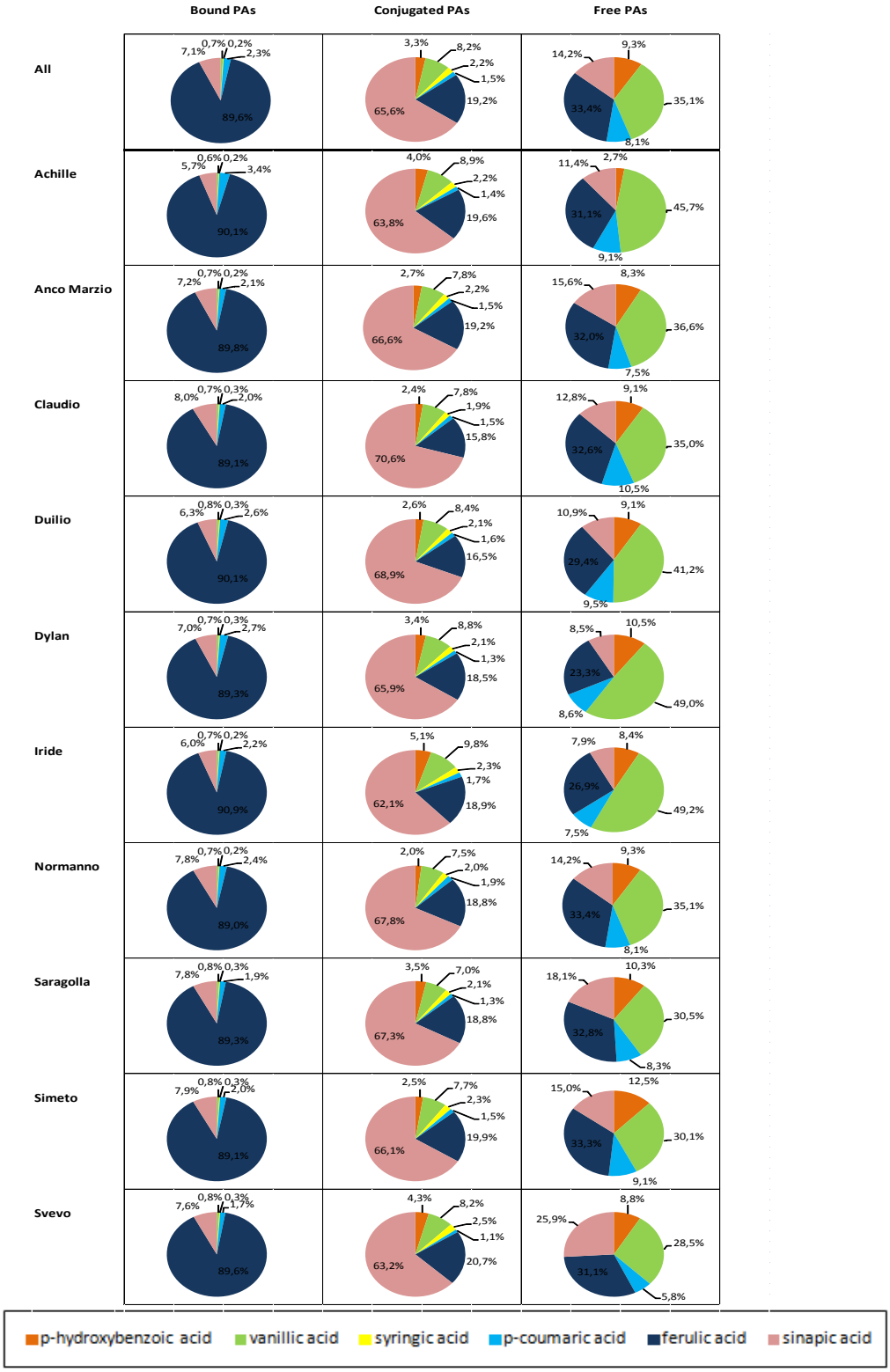

Fig.20 Percentage distribution of individual PAs in 10 durum wheat genotypes grown in Montelibretti over 3 consecutive crop years.

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4.2.1.3 Yellow coloured pigments

Yellow pigments content of 10 genotypes grown in Montelibretti over 3 crop years are reported in **Tab.11**. Mean level was 5.68±0.12 mg/kg dm, ranging from 3.91 (Simeto, 2011) to 7.47 mg/kg dm (Dylan, 2010). Variability among crop years appears modest, confirming that this parameters is mostly affected by genetic factors (Borrelli et al., 1999). In detail, the mean YCP level appeared to be slightly higher in 2010 (5.87±0.92 mg/kg dm) with respect to other two crop years (5.27±0.92 and 5.25±1.02 mg/kg dm for 2011 and 2012, respectively) for all genotypes. Dylan had the highest content of YCP (mean value across 3 crop years: 7.29±0.17 mg/kg dm), whereas Simeto had the lowest one (4.30±0.56 mg/kg dm), confirming that modern genotypes generally have a higher carotenoids content than the old ones (Digesù et al., 2009); this can be due to modern breeding programs which have taken into consideration this parameter because of high interest for pasta industries.

4.2.1.4 Total antioxidant capacity

TAC level determined in 10 genotypes grown in Montelibretti on 2010, 2011 and 2012 crop years is reported in **Tab.11**. The mean value was 45.34±2.51 mmol TEAC/kg dm, ranging from 40.48 (Dylan, 2010) to 51.10 mmol TEAC/kg dm (Svevo, 2010).

TAC levels of 10 genotypes were similar across the three crop years: 45.32±3.03, 44.63±2.24 and 46.08±2.21 mmol TEAC/kg dm in 2010, 2011 and 2012, respectively. Genotype with the highest TAC level was Svevo (mean content across the 3 crop years: 49.19±1.95 mmol TEAC/kg dm), while Saragolla showed the lowest one (43.03± 1.31 mmol TEAC/kg dm). Therefore, genotypes with the highest TAC levels don't coincide with those with the highest content of antioxidants such as YCP. This could be explained considering that different classes of antioxidants contribute to the TAC.

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4.2.1.5 Statistical analysis

Tab.12 shows the results of ANOVA for 10 durum wheat cultivars, grown in Montelibretti for three consecutive years. This statistical analysis highlights that genotype (G), crop year (Y) and their interaction have highly significant effects on PAs and TPC (free, conjugated and bound), YCP and TAC. More specifically, all variables result mostly influenced by crop year with the exception of YCP and TAC which appear mostly affected by genotype ($p<0.001$).

The bi-plot PCA reported in **Fig.21** shows the scores of the 10 genotypes in relations to the different parameters; each loading vector represents one of the parameters under study and the proximity of vectors is indicative of the correlation among parameters. The first component (46.87 % of total variance) seemed to be strongly associated with YCP, while the second one (accounting for 25.24%) was mainly associated with TAC. Dylan genotype scored on the positive side of the first dimension and was characterized by a high YCP content, while Simeto scored on the negative side, due to its high content of PAs and TPC and low of YCP. The bi-plot showed a positive correlation between total TPC and total PAs ($r=0.56$; $p<0.01$), whereas no statistically significant correlations were found among other parameters.

	Factor G	Factor Y	G x Y	Total Error
D.F.	9	2	18	59
Free PAs	10.93***	60.09***	5.97***	0.1
Conjugated PAs	1710.8***	4078.9***	594.5***	236.5
Bound	58383***	77993***	19221***	78.0
Free TPC	224**	2316***	291***	139
Conjugated TPC	7965***	10225***	1602***	197
Bound TPC	72109***	618464***	30699*	30257
YCP	4***	2***	0.5***	0.1
TAC	23***	5*	7**	5

Tab.12 ANOVA: 10 genotypes grown in Montelibretti on 3 crop years. Legend: PAs, phenolic acids; TPC, Total Phenolic Content; TAC, Total Antioxidant Capacity; YCP, Yellow Coloured Pigments; G, genotype; Y, crop year; D.F., degrees of freedom; *, $p<0.01$; **, $p<0.005$; ***, $p<0.001$.

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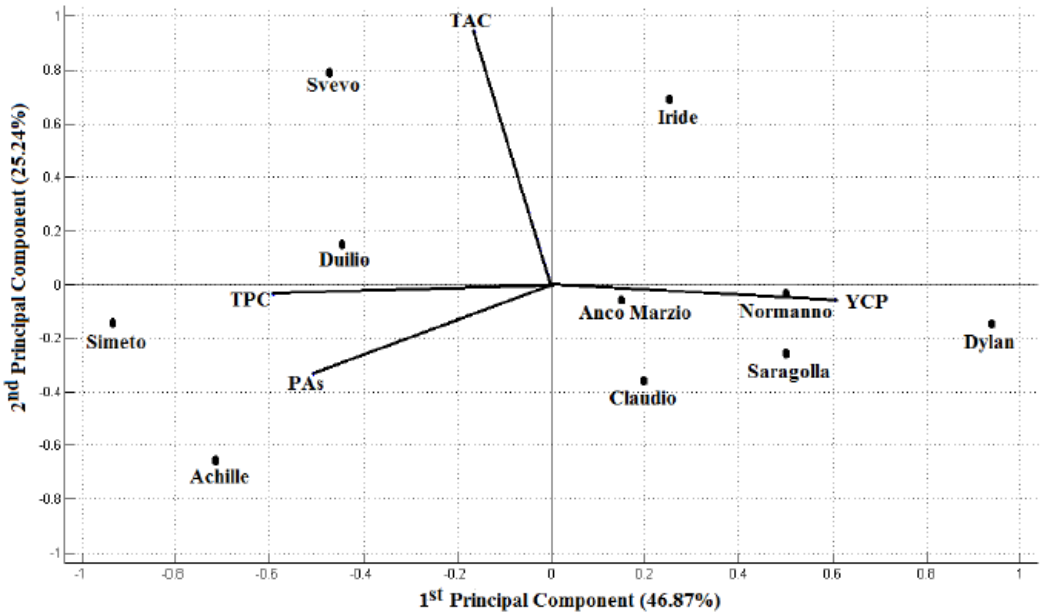

Fig.21 Principal Component Analysis applied to 10 genotypes grown in Montelibretti in 3 crop years. Legend: PAs, phenolic acids, TPC, Total Phenolic Content, TAC, Total Antioxidant Capacity, YCP, Yellow Coloured Pigments.

4.2.2 INFLUENCE OF GROWING AREA AND CROP YEAR

4.2.2.1 Phenolic acids

Total PAs contents in three durum wheat genotypes grown in 3 Italian areas on 3 consecutive crop years are shown in **Tab.13**. Mean total content was 987.3 ± 104.6 mg/kg dm and ranged from 810.9 mg/kg dm (Saragolla, Foggia, 2011) to 1208.2 (Simeto, Montelibretti, 2012). On average, genotypes grown in Montelibretti showed the highest total PA content (1017.4 ± 116.2 mg/kg dm), slightly higher than those grown in Foggia (975.8 ± 106.7 mg/kg dm) and Jesi (968.6 ± 95.4 mg/kg dm). These contents are higher than those observed in literature (Li et al., 2008; Brandolini et al., 2013), partially due to the fact that not all PAs were included in the previous investigations as already mentioned.

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Bound PAs had a similar trend in the 3 growing areas as shown in **Fig.22.** Genotypes grown in 2012 crop year in the 3 environments showed the highest content (mean across the 3 growing areas 929.7 ± 67.6 mg/kg dm) while similar contents were observed in the other two years (782.0 ± 89.9 and 803.7 ± 91.5 mg/kg dm for 2010 and 2011 crop years, respectively). The bound form was also the most stable across growing areas, ranging from a minimum of 825.7 mg/kg dm (Jesi) to a maximum of 853.4 mg/kg dm (Montelibretti).

Differently from the bound form, the content of conjugated PAs showed high variability among years; the content was slightly higher in genotypes grown on 2011 crop year (156.7 ± 19.0 mg/kg dm vs. 145.8 ± 19.7 and 130.2 ± 27.9 mg/kg dm on 2010 and 2012, respectively) and in Montelibretti growing area (158.8 ± 13.8 mg/kg dm vs. 139.0 ± 21.3 and 134.8 ± 30.2 mg/kg dm in Jesi and Foggia, respectively).

Lastly, free PAs showed the highest variability (mean: 4.6 ± 2.0 mg/kg dm; RSD=43%), ranging from 2.3 mg/kg dm (Svevo, Jesi, 2012) to 9.5 mg/kg dm (Simeto, Montelibretti, 2010). The lowest contents were registered in the samples grown in 2012 (mean: 2.9 ± 0.4 mg/kg dm vs. 4.7±1.7 and 6.2±1.8 mg/kg dm for 2010 and 2011 crop years, respectively).

Fig.23 shows the percentage distribution of individual PAs in the 3 forms. The bound one proved to be the most stable form, both across growing area and crop years; for instance, ferulic acid ranged from 89.1% to 90.5% across crop years and from 89.3% to 89.9% across growing areas. On the other hand, the free form showed the highest variability: for example, vanillic acid accounted from 21.5% to 48.6% across crop years and from 29.6% to 39.2% across growing areas, whereas ferulic acid ranged from 26.5% to 47.3% across crop years and from 30.5% to 38.6% across growing areas).

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			Total PAs	TPC	YCP	TAC
			mg/kg dm	mg FAE/kg dm	mg/kg dm	mmol TEAC/kg dm
Montelibretti	Saragolla	2010	857.1	1796.0	6.14	41.91
		2011	932.6	1314.1	5.12	44.46
		2012	929.1	1363.7	5.53	42.71
	Simeto	2010	943.6	1759.6	4.06	45.47
		2011	1131.9	1614.0	3.91	45.58
		2012	1208.2	1890.8	4.94	43.66
	Svevo	2010	1124.4	2052.3	5.35	51.10
		2011	993.8	1450.3	5.46	47.21
		2012	1035.7	1431.7	5.46	49.26
Jesi	Saragolla	2010	832.4	1313.4	5.21	47.25
		2011	901.4	1212.0	6.06	41.46
		2012	1098.4	1478.1	5.04	42.36
	Simeto	2010	1052.0	1515.5	4.07	52.18
		2011	1021.3	1143.0	5.40	46.51
		2012	1009.8	1344.9	4.04	43.43
	Svevo	2010	874.3	1540.7	5.67	51.60
		2011	884.9	1093.6	7.09	44.13
		2012	1042.9	1247.7	6.16	46.92
Foggia	Saragolla	2010	962.5	1808.9	6.51	47.71
		2011	810.9	917.7	6.32	40.45
		2012	1119.1	2050.7	5.58	45.30
	Simeto	2010	927.9	1298.3	4.99	45.13
		2011	1033.4	1663.6	4.64	41.87
		2012	1094.2	2162.7	4.12	42.37
	Svevo	2010	831.6	1761.1	5.68	49.87
		2011	975.5	1524.7	5.80	45.88
		2012	1027.5	1984.4	5.91	48.89
		Mean	**978.3**	**1545.7**	**5.34**	**45.73**
		SD	**104.6**	**320.9**	**0.82**	**3.26**

Tab.13 Total PAs, TPC, YCP and TAC in 3 genotypes grown in 3 Italian locations over 3 consecutive crop years.

Legend: PAs, phenolic acids, TPC, Total Phenolic Content, TAC, Total Antioxidant Capacity, TEAC, Trolox Equivalent Antioxidant Capacity, YCP, Yellow Coloured Pigments, FAE, ferulic acid equivalents, dm, dry matter; SD, standard deviation.

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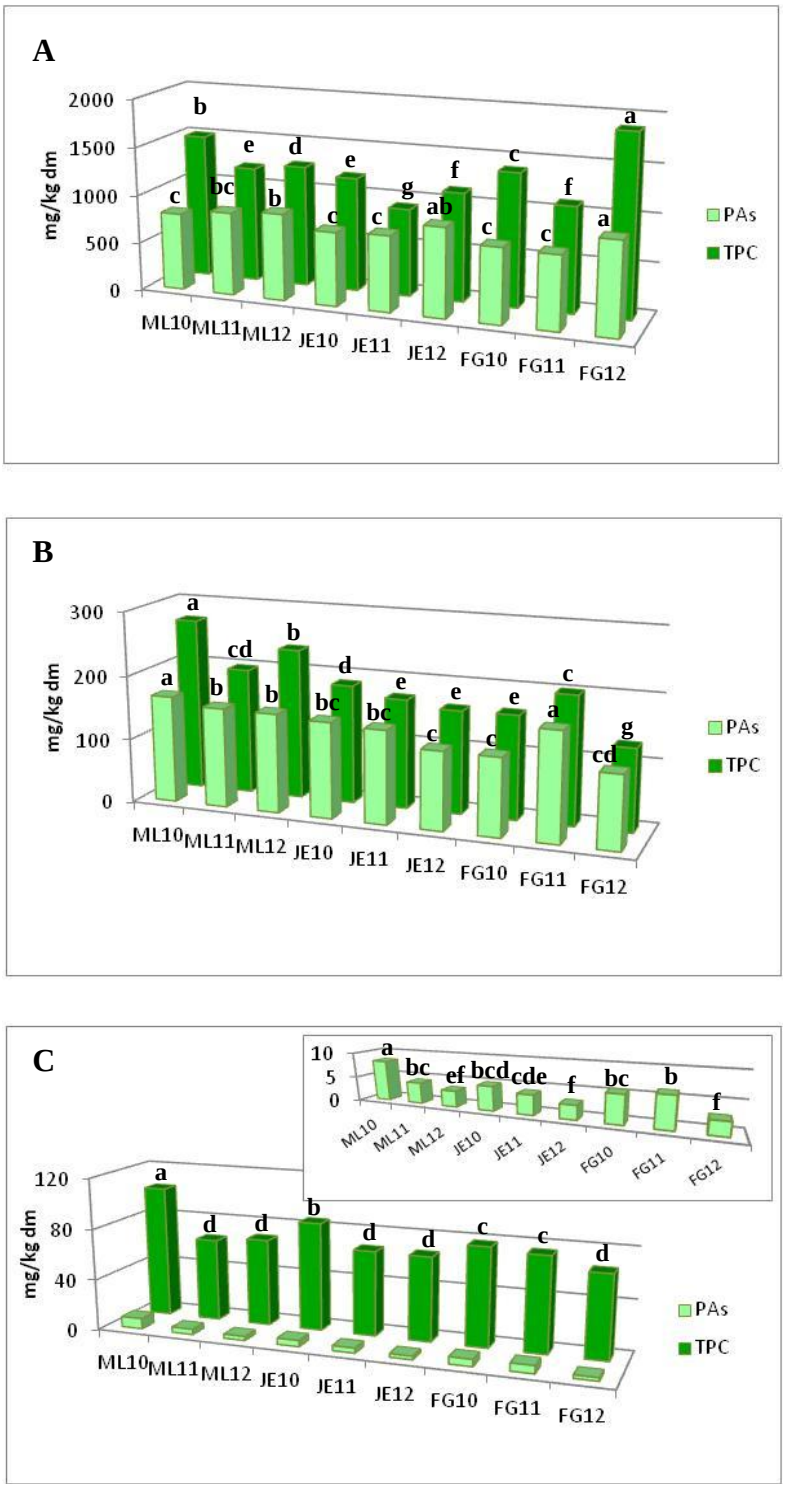

Fig.22 Mean content of bound (A), conjugated (B) and free (C) PAs (and TPC ( ) in 3 durum wheat genotypes grown in 3 Italian areas in 3 consecutive crop years. Different letters indicate that averages are significantly different from each other ($p < 0.05$). Duncan's test were performed for PAs and TPC separately.

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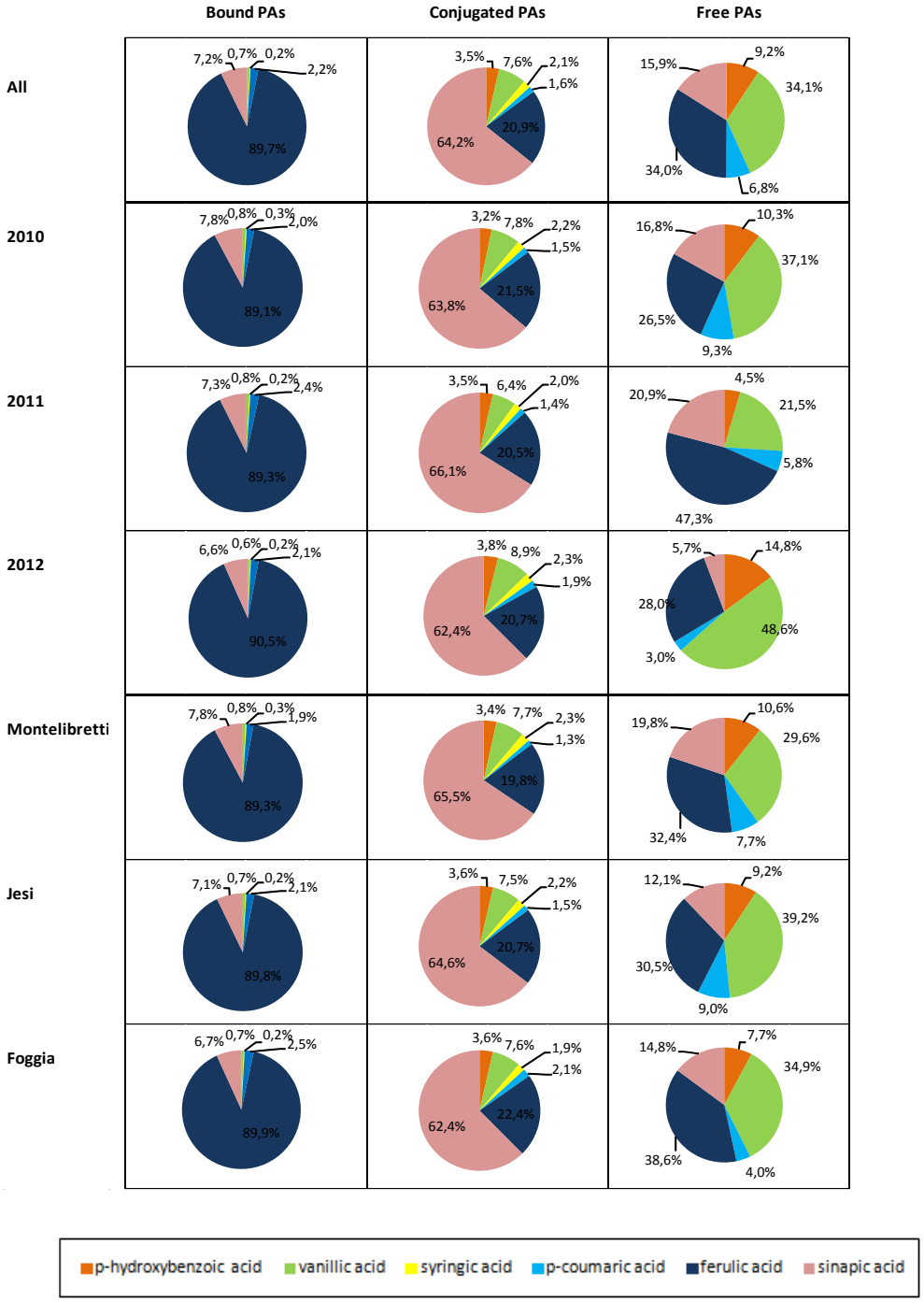

Fig.23 Percentage distribution of individual PAs in 3 durum wheat genotypes grown in 3 Italian areas in 3 consecutive crop years.

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4.2.2.2 Total phenolic compounds

Mean value of TPC (sum of free, conjugate and bound TPC) in the genotypes from 3 locations during 3 crop years was 1545.7±320.9 mg/kg dm and ranged from 917.7 mg/kg dm (Saragolla, Foggia, 2011) to 2162.7 mg/kg dm (Simeto, Foggia, 2012) (**Tab.13**). On average, genotypes grown in Jesi had the lowest TPC (mean across 3 crop years: 1321.0±162.8 mg/kg dm vs. 1630.3±258.2 and 1685.8±393. mg/kg dm observed in Montelibretti and Foggia, respectively).

Considering the three TPC forms, as already observed for PAs, the bound form prevailed on the other two forms, representing over 80% of TPC: mean contents of 1280.7±316.2 mg/kg dm, 190.2±48.8 74.8±15.4 mg/kg dm were found for bound, conjugated and free form, respectively (**Fig.22**). Moreover, the bound form had the lowest variability (CV=11% vs. 25% and 26% for conjugated and free forms, respectively).

4.2.2.3 Yellow coloured pigments

Mean content of yellow coloured pigments was 5.34±0.82 mg/kg dm and the values ranged from 3.91±0.17 mg/kg dm (Simeto, Montelibretti 2011) to 7.09±0.33 mg/kg dm (Svevo, Jesi, 2011) (**Tab.13**). Generally the variability observed among growing areas and crop years was much lower than that observed among genotypes; mean YCP in genotypes grown in Montelibretti, Jesi and Foggia were 5.11±0.72, 5.41±0.98 and 5.50±0.78mg/kg dm respectively.

4.2.2.4 Total antioxidant capacity

Mean total antioxidant capacity was 45.73±3.26 mmol TEAC/kg dm ranging from 40.45 mmol TEAC/kg dm (Saragolla, Foggia, 2011) to 52.17 mmol TEAC/kg dm (Simeto, Jesi, 2010) (**Tab.13**). Levels were similar in the three growing areas: in fact, mean levels across the 3 locations were 45.71±3.02, 46.20±3.80 and 45.27±3.24 mmol TEAC/kg dm for

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Montelibretti, Jesi and Foggia, respectively. The mean TAC was higher in genotypes grown in 2010 (mean across locations: 48.02±3.46 mmol TEAC/kg dm) than in 2011 and in 2012 crop years (44.17±2.40 and 44.99±2.75 mmol TEAC/kg dm, respectively).

4.2.2.5 Statistical analysis

Tab.14 displays the results of ANOVA related to 3 durum wheat genotypes cultivated in 3 Italian growing areas in 3 consecutive crop years. The analysis confirms that genotype (G), growing area (A) and crop year (Y) and their interactions significantly affect all the parameters under study (PAs and TPC, both in the three forms, YCP and TAC). The three PA forms were mostly influenced by Y factor ($p<0.001$) and less by G, A and their interactions. For TPC, bound and conjugated forms were mostly influenced by A factor ($p<0.001$), while the free form by AxY interaction ($p<0.001$). Finally, both YCP and TAC were mostly influenced by G factor ($p<0.001$).

To date, data regarding the influence of the different influencing factors on the content of antioxidants are rather contrasting. Similarly to the present results, Mpofu et al. (2006) reported that both genotype and growing area significantly influences TPC, antioxidant activity and PA composition in common wheat grown in a single crop year. Differently from our findings, Fernandez-Orozco et al. (2010) reported that genotype and secondly environment affects the bound PA content in 26 common wheat genotypes grown over three consecutive years.

For YCP, data are in agreement with literature which generally found that YCP are mainly influenced by genotype. In particular, Borrelli et al. (1999) and Digesù et al. (2009) reported that genetic component is predominant in the carotenoid concentration in durum wheat and so that colour is a highly heritable trait controlled primarily by additive gene effects (Johnston et al., 1983).

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	Factor G	Factor A	G x A	Factor Y	G x Y	A x Y	G x A x Y	Total Error
D.F.	2	2	4	2	4	4	8	53
Free PAs	9.1***	8.6***	1.3***	49.8***	2.3***	9.6***	2.1***	0.1
Conjugated PAs	44***	2953***	997***	3191***	370***	1891***	858***	2.9
Bound PAs	55714***	3503***	10140***	114580***	5065***	9745***	17088***	75
Free TPC	199**	142*	548***	2339*	103*	868***	289***	46
Conjugated TPC	4794***	26345***	4573***	5832***	1927***	5871**	579**	224
Bound TPC	105676**	670025***	48384*	606537**	127354***	272698***	92519***	12893
YCP	10***	1***	0.6**	0.5*	0.25*	1.3***	0.2*	0.2
TAC	128***	2	19**	91**	11*	21**	4	7

Tab.14 ANOVA of the considered parameters in 3 genotypes grown in 3 Italian areas over 3 crop years. Legend: PAs, phenolic acids; TPC, Total Phenolic Content; TAC, Total Antioxidant Capacity; YCP, Yellow Coloured Pigments; G, genotype; A, growing area; Y, crop year; D.F., degrees of freedom; *, $p<0.01$; **, $p<0.005$; ***, $p<0.001$.

The correspondence analysis performed on the whole dataset (PAs, TPC, YCP and TAC) related to 3 genotypes cultivated in 3 Italian areas in 3 crop years is shown in **Fig.24**. Each point of categorical variable 1 represents one of the 9 considered environments (combination of growing area x crop year); the distances between the points are a measure of the similarity in terms of TPC and PA content among the genotypes grown in those environments. Each trait-category point (low, medium or high content of PAs or TPC) is close to the environment for which the trait-category is prominent. In particular, for PAs and TPC the correspondence analysis revealed 3 main groups: samples grown in 2011 and 2012 can be clearly grouped and separated on the basis of crop year, differently from genotypes grown on 2010. These results may be due to different climatic conditions observed during the 3 crop years: in fact, similar rainfall levels were registered during the grain filling period among the three environments in 2011 and 2012, whereas on 2010 a higher variability among the growing areas was observed, as previously reported in the Section 3.2. The closeness between points representing TPC and PAs also suggests a

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correlation between these two parameters, as already shown for the samples grown in a single location (Montelibretti). Correlation was higher for free and conjugated form (r=0.68 and 0.65, respectively) than for the bound one (r=0.57), suggesting that many other phenolic compounds may be included in the class of TPC, mainly in the bound form, and that environment affect the various compounds in a different extent. As regards YCP and TAC, genotypes could not be separated on the basis of crop years and growing areas. Considering that many compounds (e.g. β-carotene, lutein, zeaxanthin) are included in YCP class (Zhou et al., 2004), present findings suggest that these molecules might be individually affected by climatic conditions in a different way. The same observation could be referred to TAC, considering that different classes of phytochemicals contribute to the total antioxidant capacity.

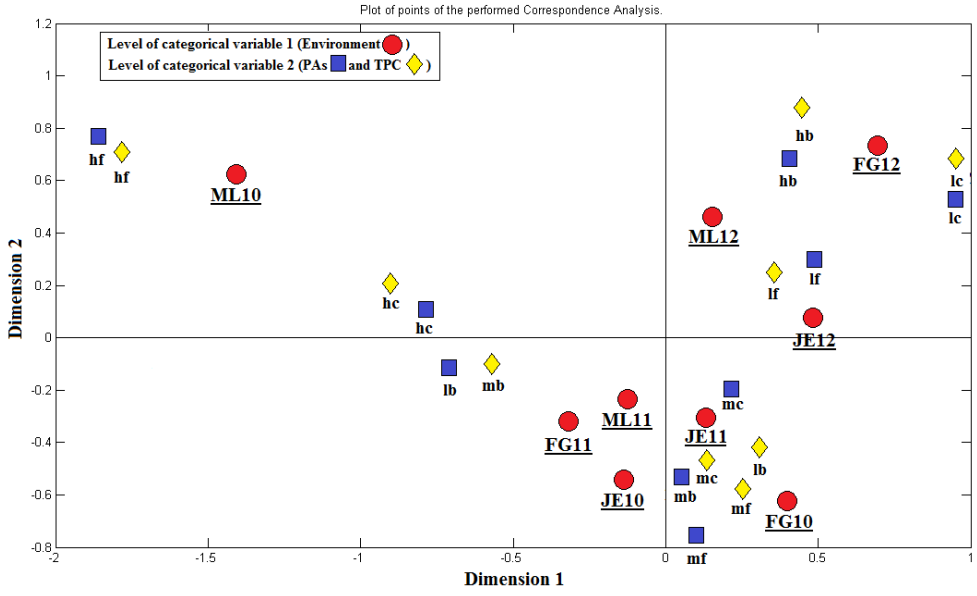

Fig.24 Correspondence Analysis of samples grown in 3 Italian areas in 3 crop years (B). Legend: Variables: ● , growing area x crop year, ■ , phenolic acids (PAs), ◆ , Total Phenolic Content (TPC). Growing areas: ML, Montelibretti, JE, Jesi, FG, Foggia.
Crop years: 10, 2010 crop year, 11, 2011 crop year, 12, 2012 crop year.

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4.3 INFLUENCE OF TECHNOLOGICAL PROCESSES

4.3.1 INFLUENCE OF MILLING AND PASTA-MAKING

4.3.1.1 Phenolic acids

The content of free, conjugated and bound PAs in milling fractions and pasta is shown in **Fig.25**. A significant effect of milling process on PAs was observed; in fact, in respect to the total PA content of the wholemeal (total PA content: 969.9 mg/kg dm), representative of the whole kernel, the main milling product (semolina) had 6.2-fold lower content (154.7 mg/kg dm), corresponding to 85% decrease. This is due to the fact the PAs remain concentrated in the bran displaying the highest content of total PAs (2998.6 mg/kg dm). These results confirm that antioxidant compounds are largely lost during the traditional milling process (Adom et al., 2005) because the outer layers, rich in antioxidant compounds, are removed and recovered as by-products, while semolina is characterized predominantly by chemical components of the endosperm (starch and proteins). Considering the three PA forms, the bound form was more than 3-fold higher in the coarse bran (2658.0 mg/kg dm) and more than 7-fold lower in semolina (106.9 mg/kg dm, respectively) with respect to the wholemeal (810.9 mg/kg dm). Similar results were observed for the conjugated form (320.6, 147.8 and 44.8 mg/kg dm in coarse bran, wholemeal and semolina respectively) and for the free one (20.0, 11.2 and 3.0 mg/kg dm in coarse bran, wholemeal and semolina respectively). The milling process also affected the content of the individual PAs in the three forms. In the bound form, ferulic acid was the most abundant one ranging from 91.9 (semolina) to 2447.5 mg/kg (bran), followed by sinapic (from 10.8 in semolina to 113.16 mg/kg in bran) and p-coumaric acid (from 4.2 in semolina to 73.3 mg/kg in bran). In the conjugated form, sinapic acid was the prevalent, ranging from 26.1 to 198.9 mg/kg in semolina and bran respectively, followed by ferulic

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acid (from 14.6 mg/kg dm in semolina to 64.7 mg/kg dm in bran). In the free form, ferulic acid was generally the most abundant one ranging from 2.4 mg/kg dm (semolina) to 8.1 mg/kg dm (bran) followed by sinapic acid (from 0.4 mg/kg dm in semolina to 3.2 mg/kg dm in bran). However the free form presented the highest variability principally due to the very low amounts, often near to the limit of quantification of the separation system utilized.

In order to compare the traditional milling process with the micronization, the PA content was analyzed also in a micronized wholemeal (MW) made as described in the Section 3.3.1.

Total PAs content showed a small but not significant increase of about 2% in micronized wholemeal in respect to the wholemeal used as reference (992.3 mg/kg dm vs 969.9mg/kg dm respectively). No significant variations were observed for the three forms (bound PAs: 810.9 mg/kg dm and 850.4 mg/kg dm; conjugated PAs; 147.8 and 134.0 mg/kg dm; free PAs: 11.2 and 7.9 mg/kg dm in wholemeal and MW respectively).

As regards the pasta-making process, a significant decrease of PAs was observed comparing the raw materials (semolina and MW) with the end products (traditional pasta and wholemeal pasta). For the traditional pasta-making process, the total PA content decreased from 154.7 mg/kg in semolina to 105.1 mg/kg in dried pasta corresponding to a decrease of 32%. Considering the three forms, a significant decrease was observed in both bound (from 106.9 mg/kg in semolina to 64.5 mg/kg in dried pasta) and conjugated forms (from 44.8 mg/kg in semolina to 27.4 mg/kg in dried pasta); the content of the free form indeed significantly increased from 3.0 mg/kg to 13.2 mg/kg. Considering the individual PAs, in the bound and conjugated forms ferulic acid significantly decreased from 91.88 to 47.55 mg/kg and from 14.64 to 4.49 mg/kg from semolina to dried pasta respectively,

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whereas sinapic acid decreased in the conjugated one (from 26.1 to 17.8 mg/kg dm). In the free form ferulic acid significantly increased from 2.4 to 11.8 mg/kg dm from semolina to dried pasta. The increase of free PA form could be due to the enzymatic action during the formation of the dough that led to a major extractability of this compounds, as already suggested by Fares et al. (2010) who found 1.1 mg/kg ferulic acid in the free form in semolina and 1.9 mg/kg in dried pasta.

As regards MW and the derived pasta, pasta making process caused only a 5% decrease of the total PAs content (992.3 mg/kg dm in MW and 942.8 mg/kg dm in dried MW pasta). The decrease was mainly due to the bound PAs form which moved from 850.4 mg/kg dm in MW to 770.6 mg/kg dm in dried MW pasta; the conjugated one slightly varied from 134.0 to 148.3 mg/kg dm, while the free form increased from 7.9 to 23.9 mg/kg dm. In the bound form, the most representative acids was ferulic acid (moving from 756.17 to 681.19 mg/kg dm), while in the conjugated form sinapic was the most abundant one (from 87.22 to 83.41 mg/kg dm), followed by ferulic acid (from 23.98 to 35.06 mg/kg dm). In the free form, ferulic acid was the most abundant one and its content was three folds higher in dried MW pasta than in MW (5.40 and 14.31 mg/kg dm). These data can be compared with those reported by Hirawan et al. (2010) who analyzed the total ferulic acid content in commercial samples of traditional and wholewheat spaghetti, finding low or absent ferulic acid content in traditional pasta and a mean value of 188.4 mg/kg in wholewheat pasta. However, data by Hirawan et al. (2010) were about three times lower than present results (68.80 mg/kg and 731.06 mg/ kg of ferulic acid in dried pasta traditional and wholewheat respectively), probably for the different extraction and quantification methods applied as well as for the different technological processes utilized to obtain wholewheat pasta. It

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should be noted that in our study PAs were quantified by using the internal standard method, so reducing the effect of complex matrices.

Finally, the impact of cooking process was investigated comparing the dried pasta samples (traditional and wholemeal) with the respective cooked ones; a different impact of cooking process between the two types of pasta was observed. For traditional pasta, cooking did not significantly modify PAs content: total PAs content was 107.57 mg/kg dm, corresponding to a 1.8% increase with respect to dried pasta. Considering the three PA forms, bound PAs increased from 64.48 mg/kg to 72.46 mg/kg, while the conjugated PAs remained almost stable (27.44 *vs* 28.01 mg/kg) and free PAs significantly decreased from 13.15 mg/kg to 7.10 mg/kg corresponding to a reduction of 46%. The high PAs loss found for the free form during the cooking process could be due to the leaching of soluble molecules in the boiling water, as suggested by Fares et al. (2010), who reported an increase of bound PAs in the cooked traditional pasta with respect to the dried one and a slight decrease for free PAs.

For the MW pasta, a small increase for bound PAs, but not for conjugated and free ones, was observed during the cooking process; bound PAs moved from 770.58 in dried MW pasta to 872.84 mg/kg dm in the cooked one, conjugated PAs from 148.32 to 144.17 mg/kg dm and free PAs from 23.88 to 22.76 mg/kg dm. The increase observed for the PA bound form in cooked MW pasta in respect to the dried one was probably due to the higher PAs accessibility to the extraction solvent after the hydrothermal treatment (cooking). Comparing the two types of pasta (traditional and wholewheat), as expected MW pasta had a higher content of PAs with respect to traditional one, confirming that products made by using wholemeal preserve bioactive compound present in the outermost layers of the kernels.

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The effect of milling, pasta-making and cooking processes as well as of micronization on PA content was also investigated analyzing the percentage distribution of individual PAs (**Fig.26**). Considering the three PAs forms, the bound one contributed to most of total PAs in all matrices under study, accounting for more than 80% in bran, wholemeal and micronized wholewheat pasta samples and for more than 60% in semolina and traditional dried pasta samples. Ferulic acid was the most abundant PA in bound form, accounting from 73.7% in traditional dried pasta to 92.8% in the wholemeal, followed by sinapic and p-coumaric acid. Conjugated PAs accounted from 10.7% (in coarse bran) to 28.9% (in semolina) of total PAs; sinapic acid prevailed accounting from 51.8% of total conjugated PAs in cooked micronized wholemeal pasta to 70.1% in cooked traditional pasta, followed by ferulic acid and vanillic acid.

Lastly, the free form represented approximately only 1-2% except for traditional and cooked pasta (12.5 and 6.6 % respectively), scarcely contributing to the total PAs content in all milling fractions and pasta samples; great variability was observed in the free form, probably due to the low content of PAs.

Ferulic acid was the most abundant PA in all matrices ranging from 40.4 % in coarse bran to 89.4% in dried traditional pasta.

Summing up, the technological processes included in this study had a deep impact on the content of PAs in the matrices considered, but not on the percentage contribution of individual PAs in the 3 PA forms.

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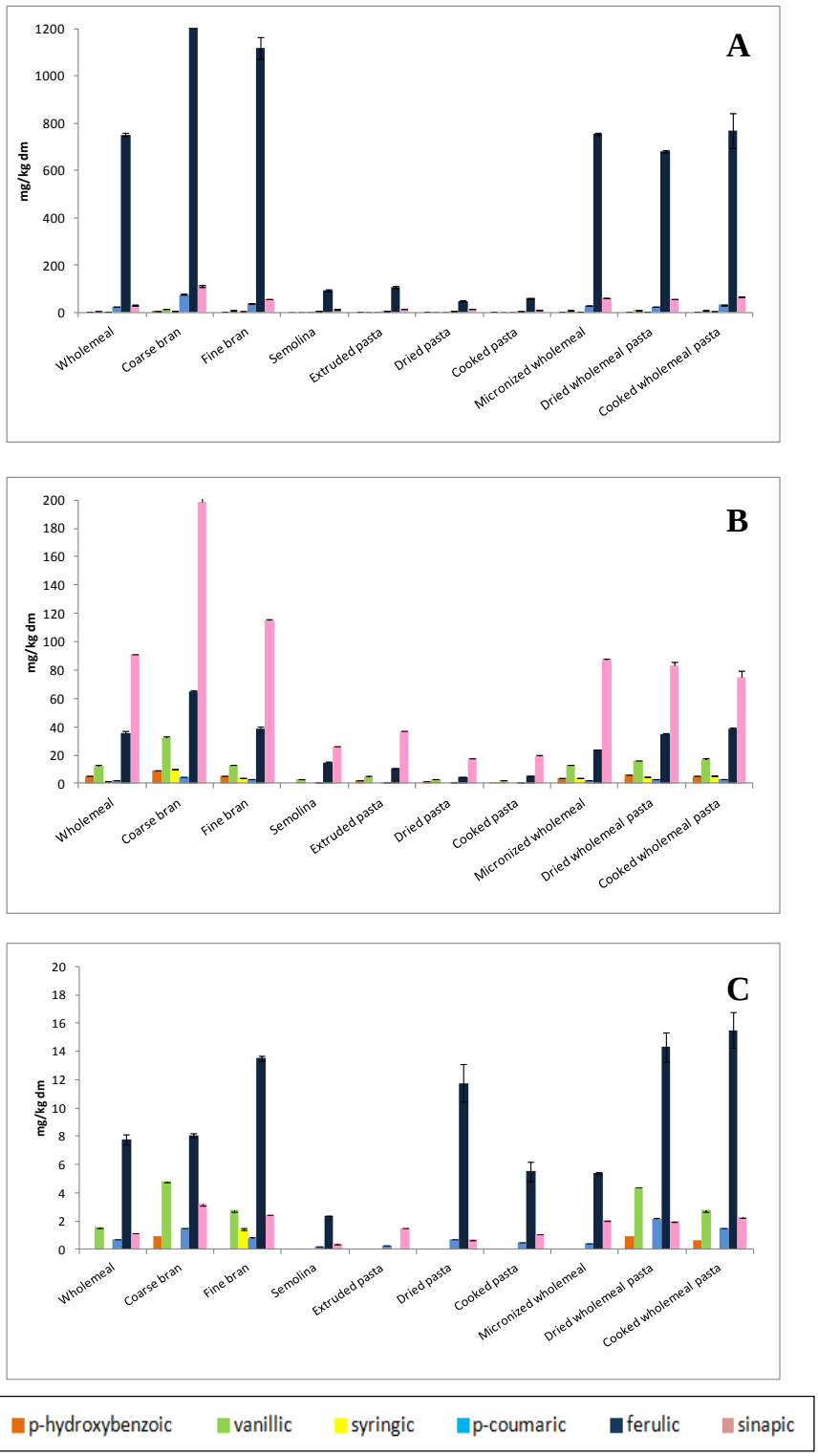

Fig.25 Content of bound (A), conjugated (B) and free (C) PAs in milling fractions and pasta samples obtained by using a durum wheat genotype (Duilio). Bars represent the standard deviation.

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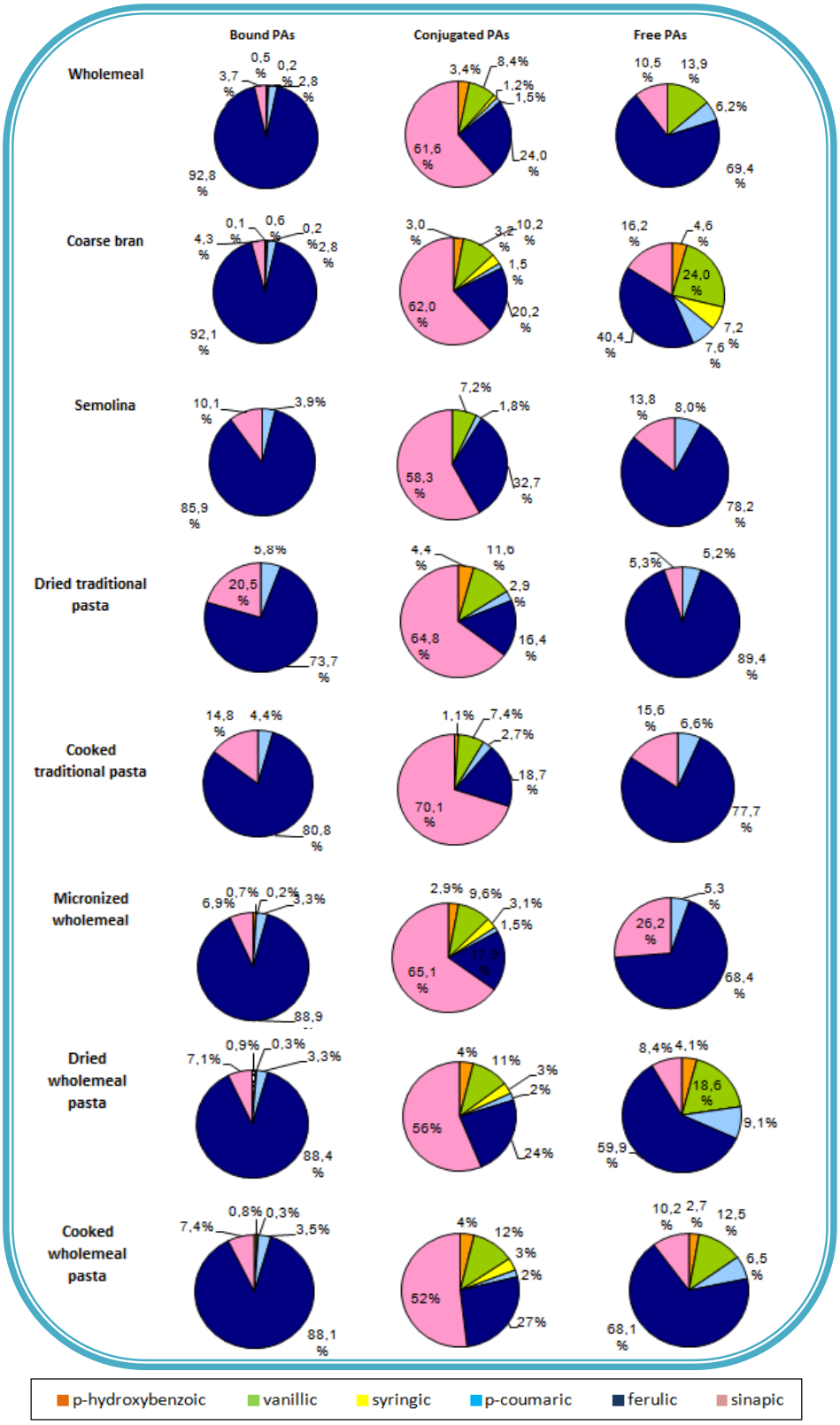

Fig.26. Percentage distribution of individual PAs in selected milling fractions and pasta samples obtained using a durum wheat genotype (Duilio).

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4.3.1.2 Total antioxidant capacity

The milling process deeply influenced the total antioxidant capacity of durum wheat grains, similarly to what observed for PAs. In fact, a significant reduction in semolina (-25%) and an increase in bran (+46%) compared with wholemeal was observed (**Fig.27**). These results confirm that the most part of antioxidant compounds in durum wheat grains are located in the aleurone or external layers (Fincher & Stone, 1986; Pomeranz, 1988; Fulcher & Duke, 2002; Slavin et al., 2003).

As regards the impact of micronization process, it can be observed that TAC in MW was slightly higher than in reference wholemeal (about 4% increase), probably due to the action of ultrafine grinding during the micronization that most likely breaks the cell walls and allows the release of the intra cellular antioxidant compounds. Moreover micronization increases the particle surface area plausibly leading to a higher contact between the sample particles and the radical solution (Zhou et al., 2004; Hemery et al., 2010).

Considering pasta making process, the traditional one caused a 14% increase of the TAC in dried pasta compared with semolina. The observed increase could be due to the rearrangements during the pasta making of some compounds with antioxidant activity or to the higher accessibility of some antioxidants to the ABTS radical in consequence of extrusion and drying of pasta; this hypothesis was already suggested by Di Benedetto et al. (2013) who studied the effects of milling and pasta making processes on ABTS scavenging activity of hydrophilic and lipophilic extracts in 5 durum wheat cultivars and found a mean increase of 15% from semolina to pasta in hydrophilic extracts but not in lipophilic extracts.

Regarding the MW pasta, the pasta making process caused only a 4% decrease in TAC values in respect to the MW. Comparing dried pasta (traditional and wholewheat), the

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latter had TAC values 16% higher than the traditional one. These results are in agreement with those reported by Hirawan et al. (2010), who found on average an increase of 17% (not statistically significant) in TAC values (obtained by the ORAC assay) in commercial wholewheat pasta samples compared with the traditional ones.

Lastly, the cooking process caused a significant decrease of TAC level (from dried traditional pasta to cooked pasta: -30%), plausibly due to the antioxidants leaching in the boiling water as previously suggested by Kalt (2005). On the contrary, for the MW pasta the TAC level raised of about 16% in respect to the dried one, probably attributable to some antioxidants present in the external layers that became more accessible to the radical ABTS after the cooking process.

Considering the entire transformation chain from seed to cooked pasta, the TAC decreased of about 40% from wholemeal to cooked traditional pasta, while increased of about 10% from micronized wholemeal to cooked wholemeal pasta.

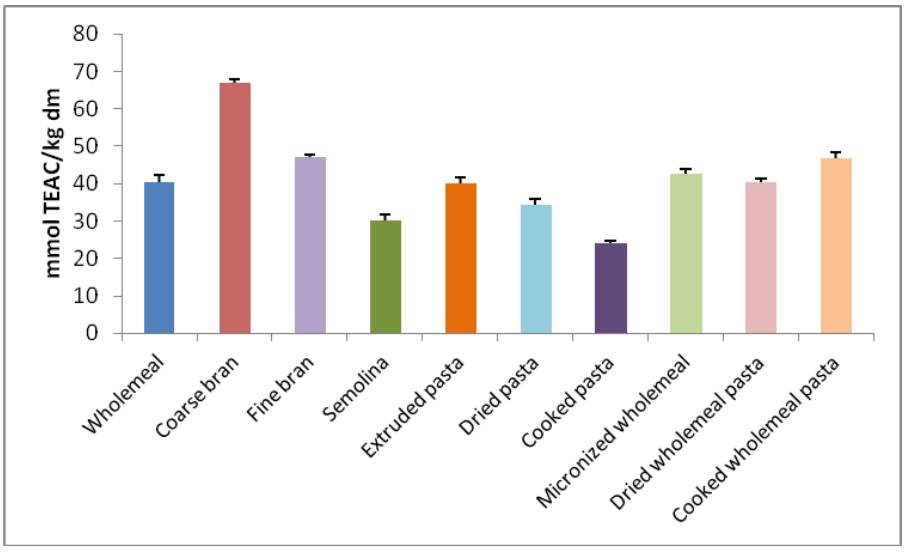

Fig.27 TAC levels in milling fractions and pasta samples obtained with a durum wheat genotype (Duilio). Bars represent the standard deviation.

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4.3.1.3 Statistical analysis

The statistical analysis (ANOVA) performed to investigate the impact of the entire traditional technological process evidenced that, as expected, the total antioxidant capacity is strongly influenced by milling and pasta making processes.

The results of the principal component analysis (PCA) are reported in **Fig.28.** In the bi-plot bi-plot, the first two principal components represented 91.75% of total variance and variance of 1st principal component (PC1) was primarily due to TAC, bound and conjugated PAs, suggesting a positive correlation between these parameters; the 2nd principal component (PC2) was indeed strongly influenced by free PAs. Wholewheat pasta (dried and cooked), micronized wholemeal and coarse bran scored on the positive side of the first dimension and were characterized by a high content of free, conjugated and bound ferulic acid (mean values 10.20±4.42; 39.60±15.08; 1081.30±764.50 mg/kg respectively) and high TAC content (mean value 48.83±10.50 mmol/TEAC/kg). MW pasta (dried and cooked) showed the highest free PAs content, being located on the positive side of PC2. On the contrary, dried and cooked traditional pasta as well as semolina scored on the negative side of the PC1 due to their low PA contents and TAC levels. Therefore, the presence or absence of the tegumental layers, rich in PAs and other antioxidant compounds, prove to discriminate the different grouping of samples.

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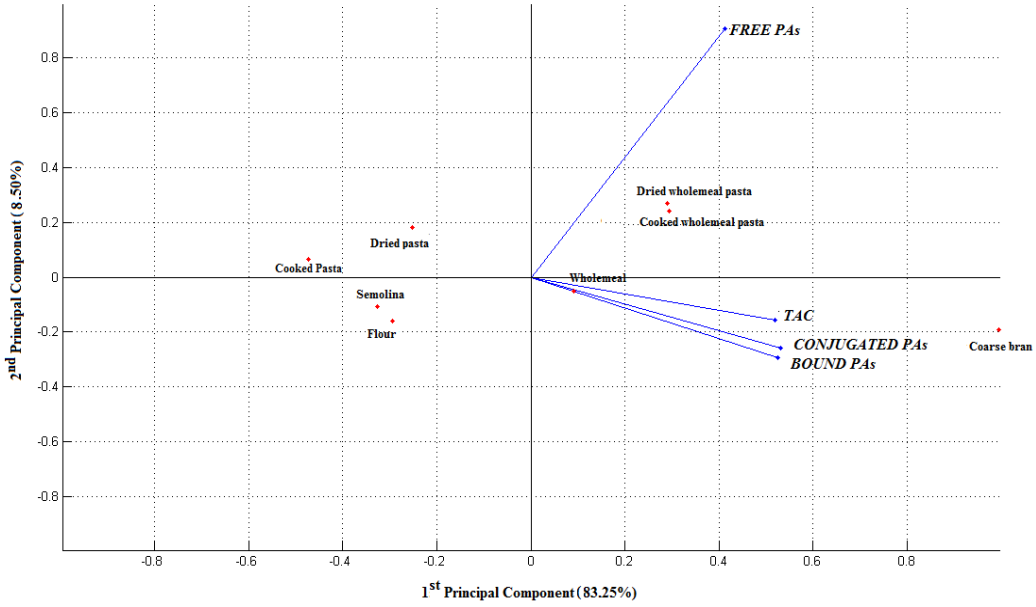

Fig.28 Principal Component Analysis: influence of milling, pasta-making and cooking process on PAs and TAC.

4.3.2 INFLUENCE OF DEBRANNING PROCESS

4.3.2.1 Laboratory scale

4.3.2.1.1 Phenolic acids

Total PA content was much higher in DF (on average 3893.3±835.6 mg/kg dm) than in DK (on average 730.8±75.7 mg/kg dm) in all samples collected during the 12 debranning steps, confirming that these compounds are mainly accumulated in the outer layers (Liyana-Pathirana et al., 2006; Fares et al., 2010; Luthria & Liu 2013).

Considering the 3 PA forms (**Fig. 29)**, the bound form was the most abundant one in DF (mean across the 12 steps: 3441.8±835.8 mg/kg dm *vs* 432.7±75.4 and 18.8±9.2 mg/kg dm for conjugated and free forms, respectively) as well as in DK (on average 631.7±64.6

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mg/kg dm *vs* 94.3±13.8 and 4.9±0.6 mg/kg dm for conjugated and free forms, respectively). The free PAs showed the highest variability in debranning fractions (CV=49%, CV=24% and 17% in free, bound and conjugated forms), while in the resulting kernels a lower variability was observed for all the 3 PA forms (CV=10%, 15% and 12% for bound, conjugated and free form, respectively).

The slight differences in terms of PA content among DK could be explained by the fact that debranning process removes the tegumental layers, representing only the 9-10% of the kernels with respect to the endosperm which accounts for about 70%.

In detail, in DF bound PAs moved from 3736.5 (T1) to 2020.8 mg/kg dm (T12), with the highest contents until T6 corresponding to a debranning level (DL) of 7.1% (mean content from T1 to T6: 4120.0±209.7 mg/kg dm) and a maximum value at T4 (4365.7 mg/kg dm); a gradual decrease was then observed as the debranning proceeded (**Fig.29A**). These results suggest that the most of bound PAs are concentrated in the outer layers of the grain teguments similarly to results obtained by Fares et al. (2010), who found a progressive reduction of bound PAs in debranning fractions in common wheat.

Differently from the bound PAs, conjugated PAs moved from 250.1 (T1) to 421.0 mg/kg dm (T12), gradually increasing until T7 (corresponding to DL=7.9%) which showed the highest content (499.8 mg/kg dm) (**Fig.29B**). This result suggests that this PA form is concentrated in inner layers in respect to the bound ones, so that it can be hypothesized that conjugated PAs, linked to small molecules such as sugars, proportionally increase during debranning due to the progressive removal of external layers where most of bound PAs are present. Lastly, the content of free PAs decreased from 39.6 mg/kg dm in T1 to 8.2 mg/kg dm in T12 (**Fig.29C**); the greatest reduction was between T1 and T2 (39.6 and 27.3 mg/kg

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dm, respectively), so suggesting that free PAs are mainly located in the outermost layers of the kernels.

As regards the DK, bound PAs moved from 707.8 (T1) to 582.7 mg/kg dm (T12), with the highest content at T2 (731.1 mg/kg dm) and the lowest at T9 (558.3 mg/kg dm); a progressive decrease of the content was observed from T4 (corresponding to DL=5.4 %) to T12 (DL=12.3 %) (**Fig.29D**). In the conjugated form, a trend opposed to that of debranning fractions was observed, with content varying from 127.2 mg/kg dm (T1) to 77.5 mg/kg dm (T12) (**Fig.29E**). Finally, the free form showed very similar PA contents along the entire debranning process, varying from 6.1 (T1) to 4.9 mg/kg dm (T12) (**Fig.29F**). This behaviour is plausibly due to the low contents of this PA form which, for some PAs, were close to the limit of quantification.

Considering individual PAs, their percentage distributions in selected DK and DF are reported in **Fig.30** and **Fig.31**. As shown, a very similar percentage distribution among DK and DF in all the three PA forms was observed, so suggesting that durum wheat debranning has a strong impact on the content of PAs, but not on the percentage distribution of individual PAs in debranning fractions as well as in resulting kernels. Ferulic acid was the most abundant one in free and bound PA forms, accounting for about 90% of total free and bound PAs in all the 12 DF and the corresponding DK. In the conjugated form, sinapic acid prevailed, accounting for 60-70% of total conjugated PAs in all the analyzed samples.

Despite some similarities with previous studies, a thorough comparison with data already present in literature appears difficult due to many reasons including: i) just few studies investigated the effects of debranning process on PAs content in cereals, and even less in

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durum wheat; ii) most of those studies applied different conditions (e.g. number of debranning steps, debranning levels.

Fares et al. (2010) evaluated PA content in DF of common wheat debranned for 9 subsequent step of 20 seconds each, reaching a final DL of 40%, deeply different from the present study (DL% at T12= 12.3%). Luthria & Liu (2013) evaluated the effect of 11 sequential debranning on PAs content in sorghum reaching a final DL of about 54%. These differences probably can be attributed to variability among cereals (e.g. texture and size of kernels) and among processes (e.g. time intervals applied, debranning apparatus used and also pre-hydration conditions). Specifically for DK, in addition to the previously listed differences, the comparison appears further difficult because in previous studies DK are collected and analyzed only at the end of the entire debranning process, so impeding to elucidate the effect of debranning process in DK.

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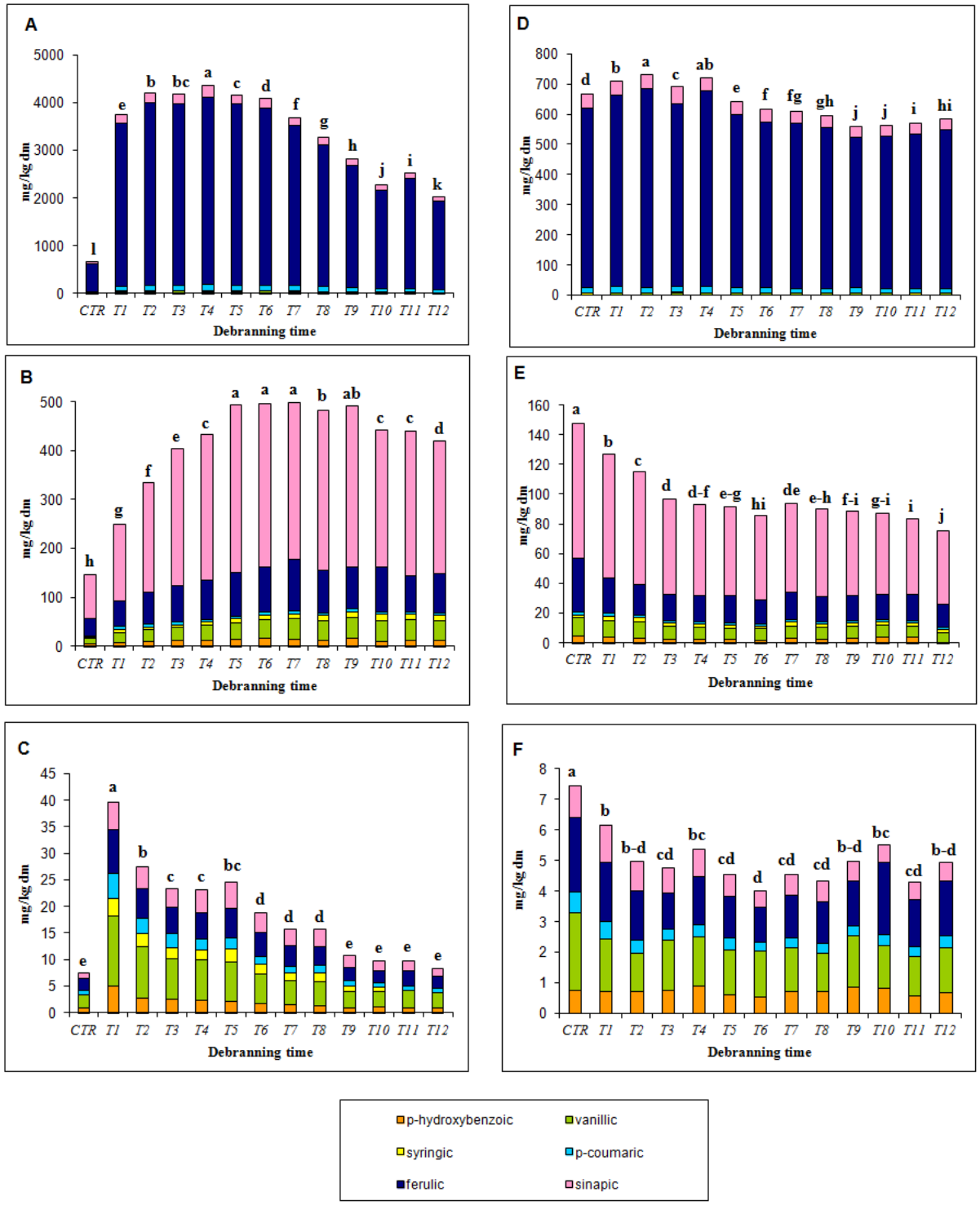

Fig.29 Bound, conjugated and free PAs in debranning fractions (A,B,C) and debranning kernels (D,E,F) of a durum wheat genotype (Duilio). Different letters indicate that averages are significantly different from each other ($p < 0.05$).

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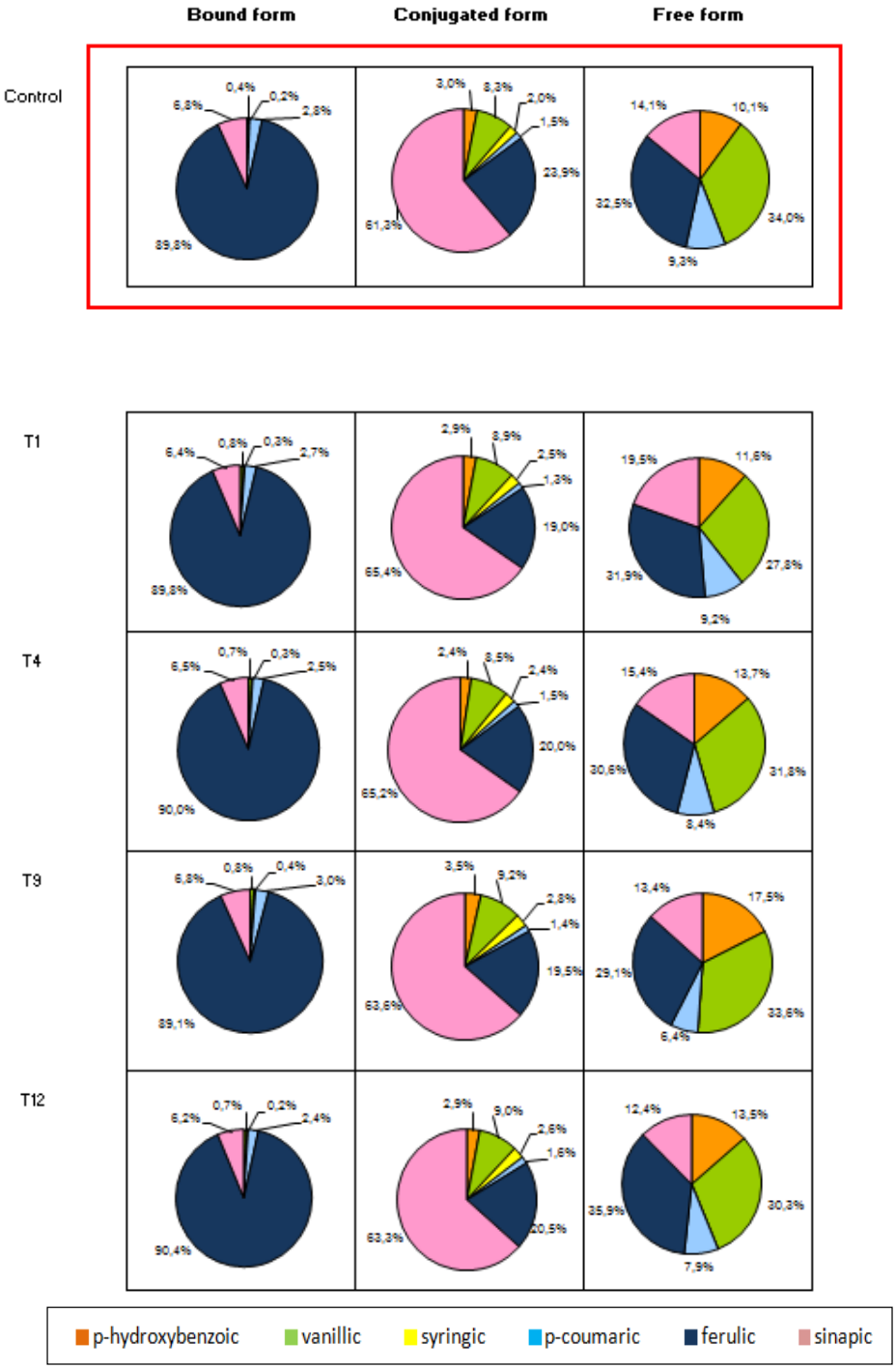

Fig.30 Percentage distribution of individual PAs in selected debranned kernels of a durum wheat genotype (Duilio).

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Fig.31 Percentage distribution of individual PAs in debranning fractions of a durum wheat genotype (Duilio).

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4.3.2.1.2 Total antioxidant capacity

TAC levels of DK and DF, compared to that of the control sample (55.46±0.99 mmol TEAC/kg dm), are shown in **Fig.32**. In debranning fractions, the TAC moved from 44.83 (T1) to 63.64 mmol TEAC/kg dm (T12). TAC significantly increased up to 63.00 mmol TEAC/kg dm (T3), thereafter a plateau was reached (mean level from T4 to T11: 66.86±0.96 mmol TEAC/kg dm); only in the last DF (T12), a slight but significant decrease was observed (63.64 mmol TEAC/kg dm) (**Fig.32A**). This trend suggests that other antioxidant compounds (e.g. carotenoids, tocopherols) present in the inner zone of the kernel progressively contribute to the total antioxidant activity. As regards debranning kernels, mean level of TAC was 49.30±1.43 mmol TEAC/kg dm, showing no significant variability during the debranning process (**Fig.32B)**. This could be explained by a possible compensation between the removal of the outer layers in which PAs are accumulated and the increase of the relative proportion of the endosperm, rich in other compounds with antioxidant activity.

The TAC levels observed in the present study are generally higher than those found in previous studies. In addition to the previously listed reasons, the comparison with results reported in literature appears complicated because many methods and various compounds can be employed for measuring the antioxidant capacity (Beta et al., 2005; Mateo Anson et al., 2008; Luthria & Liu, 2013).

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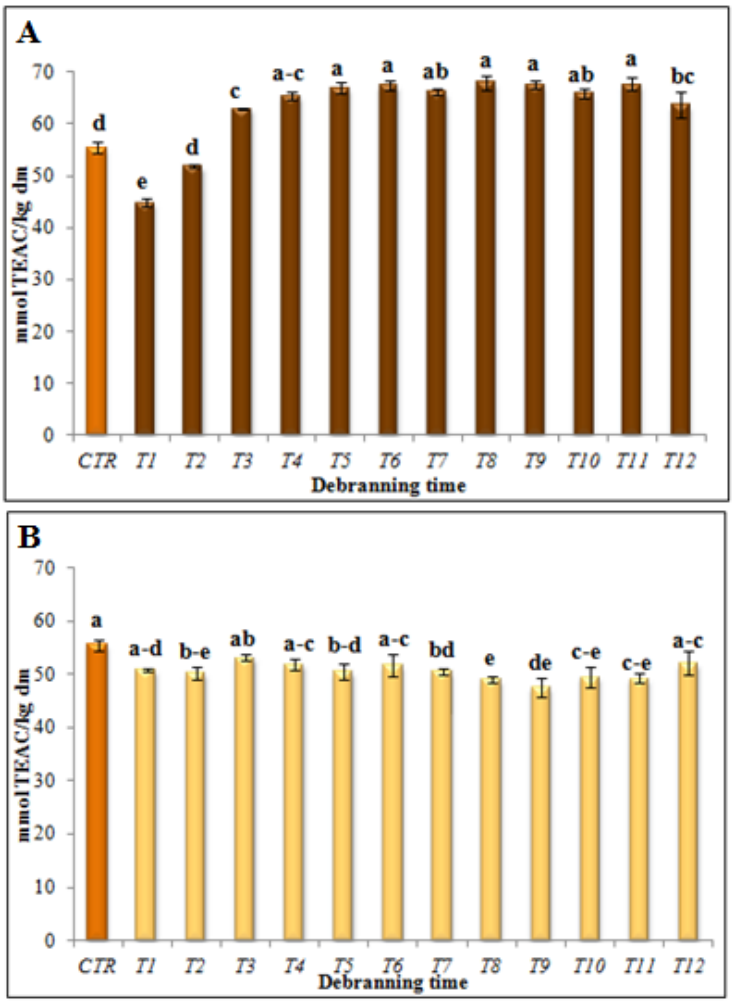

Fig.32 TAC in debranning fractions (A) and debranned kernels (B) compared to wholemeal (CTR). Different letters indicate that averages are significantly different from each other ($p < 0.05$). Bars represent standard deviations.

4.3.2.1.3 Total starch

The content of total starch in debranning fractions and resulting kernels, compared to those of the entire kernel (control, CTR, 62.32% dm), is reported in **Fig.33**.

Regarding the debranning fractions, total starch increased from 9.59% dm (T1) to 43.06% dm (T12). Total starch can be considered an useful indicator of the debranning level achieved during the process, indicating if the progressive removal of the kernel layers also concerns the starchy endosperm (Pagani et al., 2002; Bottega et al., 2009b); in particular,

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considering that starch is mainly present in the endosperm (about 70-80%), these results confirm that starch is gradually accumulated in the DF because inner layers, richer in starch, are progressively removed as the process proceeded.

For the DK debranning kernels, the total starch content slightly increased as the debranning process proceeded (from 65.27% dm in T1 to 67.97% dm in T12). These small variations are due to the fact that the starchy endosperm represents the most preponderant part of the kernels, so the removal of a quote of external layers, characterized by a low starch content, contributes minimally to the TS variations.

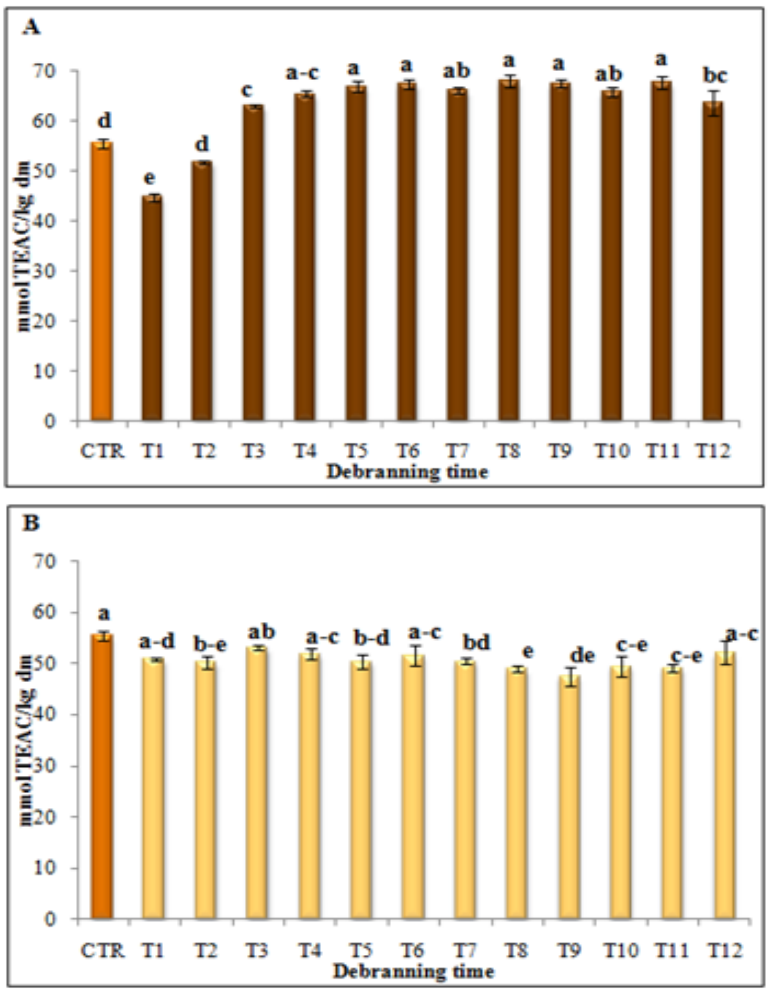

Fig.33 Starch in debranning fractions (A) and debranned kernels (B) compared to wholemeal (CTR). Different letters indicate that averages are significantly different from each other ($p < 0.05$). Bars represent standard deviations.

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4.3.2.1.4 Statistical analysis

The correlations between PA content, TAC and total starch in both debranning fractions and resulting kernels are shown in **Tab.15**. In detail, a significant negative correlation was observed between total starch and free and bound PAs in DF (r= -0.908 and -0.912, respectively) and free and conjugated PAs in DK (r= -0.654 and -0.765, respectively), confirming that small amounts of PAs can be found in the endosperm. Significant correlations between TAC and PA content were also found; a positive correlation for conjugated PAs (r=0.950) and a negative one for the free PAs (r= -0.783) in debranning fractions highlight that these PA forms influence the antioxidant capacity of grains. The correlations between TAC and the 3 PA forms found in the present study are lower than those often reported in previous investigations, plausibly due to the fact that most of the past studies measured TAC in extracts, so without considering the contribution of all compounds with antioxidant activity.

	Debranning fractions					*Debranned kernels*				
	Bound PAs	**Conjugated PAs**	**Free PAs**	**TAC**	**Starch**	**Bound PAs**	**Conjugated PAs**	**Free PAs**	**TAC**	**Starch**
Debranning fractions										
Bound PAs	-	-0.132	0.738**	-0.258	-0.912**	0.758**	0.512	-0.076	0.421	-0.276
Conjugated PAs	-0.132	-	-0.688**	0.950**	0.481	-0.619*	-0.816**	-0.724**	-0.253	0.464
Free PAs	0.738**	-0.688**	-	-0.783**	-0.908**	0.820**	0.893**	0.450	0.343	-0.607*
TAC	-0.258	0.950**	-0.783**	-	0.585*	-0.628*	-0.914**	-0.676*	-0.188	0.601*
Starch	-0.912**	0.481	-0.908**	0.585*	-	-0.867**	-0.776**	-0.262	-0.441	0.409
Debranning kernels										
Bound PAs	0.758**	-0.619*	0.820**	-0.628*	-0.867**	-	0.593*	0.343	0.593*	-0.315
Conjugated PAs	0.512	-0.816**	0.893**	-0.914**	-0.776**	0.593*	-	0.830**	0.543	-0.765**
Free PAs	-0.076	-0.724**	0.450	-0.676*	-0.262	0.343	0.830**	-	0.551	-0.654*
TAC	0.421	-0.253	0.343	-0.188	-0.441	0.593*	0.543	0.551	-	-0.430
Starch	-0.276	0.464	-0.607*	0.601*	0.409	-0.315	-0.765**	-0.654*	-0.430	-

Tab.15 Correlations among phenolic acids (bound, conjugated and free) and other parameters in debranning fractions and corresponding kernels. *p<0.05, **p<0.01.

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4.3.2.2 Pilot scale

4.3.2.2.1 Influence of processing

In **Fig.34** results of bound PAs in DF corresponding to DL of 2.8% (DF1), 5.1% (DF2) and 8.0% (DF3) respectively are reported. Results show that the DF obtained from a three step debranning process (corresponding to debranning levels of 2.8%, 5.1% and 8.0% for DF1, DF2 and DF3, respectively) had higher content of bound PAs than those of coarse and fine bran obtained from the traditional milling process of the same genotype, so confirming that most of these compounds are accumulated in the outermost layers of the kernels (Adom et al., 2005). This is supported by the fact that debranned kernels obtained by a DL=2.8% displayed a lower content of phenolic compounds than the wholemeal. Among the three DF, DF 1 (DL=0-2.8%) and DF 2 (DL=2.8-5.1%) displayed the highest contents of bound PAs; however, DF2 was selected as the optimal debranning level, because the outermost layers are well known to have a higher risk of contamination than the inner ones.

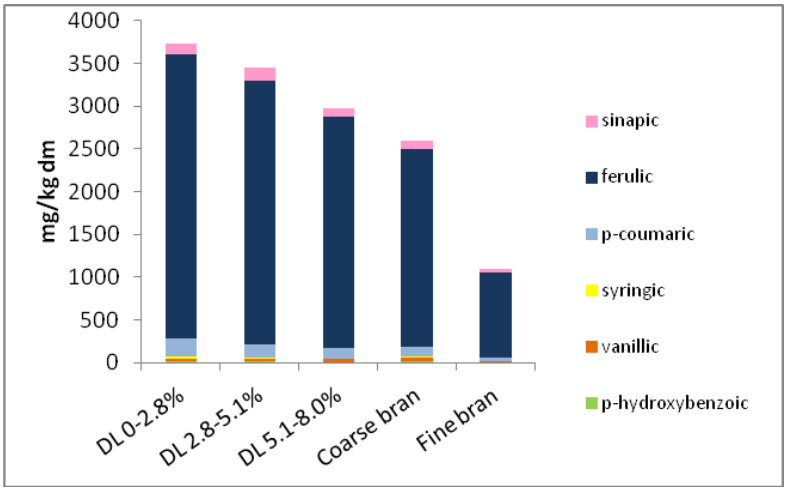

Fig.34 Content of bound PAs in debranning fractions obtained by sequential debranning of durum wheat genotype Normanno (pilot scale).

The results obtained for all the considered parameters (PAs and TPC, both in free, conjugated and bound forms, YCP, starch, protein, ash and the antioxidant capacity) in

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debranning fractions as well as in the other matrices obtained from milling, debranning, pasta-making and cooking processes of Normanno genotype are reported in **Tab.16**.

The progressive increase of starch observed as the debranning proceeded confirms that starch is gradually accumulated in the DF, because the process progressively involves inner parts of the kernel richer in starch; therefore this parameter is confirmed to be useful for indicating if the progressive removal of the kernel layers also concerns the starchy endosperm (Pagani et al., 2002).

Considering the different processes included in the study (debranning, milling, pasta-making, cooking), milling process proved to drastically affect the occurrence of antioxidant compounds in final product (semolina). Total PAs moved from 563.19 mg/kg dm in wholemeal to 166.73 mg/kg dm in semolina (-70%), confirming that antioxidant compounds are mostly lost during milling process (Liyana-Pathirana & Shahidi, 2007). As already observed when the impact of technological processes was investigated (Section 4.3.1), the milling process drastically affected also the TAC level, moving from 49.18 in wholemeal to 36.73 mmol TEAC/kg dm in semolina, whereas higher levels were registered in coarse bran and fine bran (61.44 and 52.97 mmol TEAC/kg dm, respectively) where most of antioxidant compounds are accumulated.

The 3 raw materials used for pasta-making (semolina+DF 2, micronized debranned kernels and semolina for pasta 1, 2 and 3, respectively) displayed highly different contents of nutrients and other compounds (**Fig.35**). Total PA contents were 917.75, 373.01 and 166.73 mg/kg dm respectively, while total TPC were 2007.77, 1055.93 and 577.93 mg FAE/kg dm, respectively. Also TAC level was higher in raw material for pasta 1 and 2 (42.30 and 46.40 mmol TEAC/kg dm, respectively) than in semolina (36.73 mmol TEAC/kg dm)

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The corresponding dried pasta obtained from these 3 raw materials showed total PA contents of 679.66, 415.71 and 70.65 mg/kg dm for pasta 1, 2 and 3 and total TPC contents of 1492.63, 1046.56 and 220.36 mg/kg dm respectively. These results confirm that use of wholemeal or semolina enriched with pearling fractions for pasta-making process allows to obtain pasta samples with a higher content of phenolic compounds, compared to the reference sample (traditional pasta), as already suggested in previous investigations (Hirawan et al., 2010; Fares et al., 2010).

The effect of pasta-making (extrusion and drying) on the occurrence of antioxidants was different for the pasta samples: a reduction of -26% and -58% of PAs was observed for pasta 1 and 3, whereas a small but not significant increase (+11%) for pasta 2 was detected; for TPC, a reduction of -26% and -62% for pasta 1 and 3 and a no variations for pasta 2 was observed.

Considering the 3 PA and TPC forms, it is possible to hypothesize that the decrease observed for pasta 1 and 3 was mainly due to the reduction of the conjugated and bound forms. Conjugated and bound PAs moved respectively from 224.96 and 687.78 mg/kg dm in semolina+DF 2 to 59.44 and 616.12 in dried pasta 1, while and from 39.58 and 126.88 mg/kg dm in semolina to 23.70 and 45.62 mg/kg dm in dried pasta 3. The effect of pasta-making on the free form was indeed less pronounced, plausibly due to the low amounts detected.

As regards TAC, the level among pasta samples were pretty similar, being 38.51, 40,21 and 38.97 mmol TEAC/kg dm in pasta 1, 2 and 3 , respectively.

Finally, the impact of cooking process was investigated comparing the dried pasta samples with the respective cooked ones, revealing a general increase of phenolic compounds in the three types of pasta. In detail, total PAs increased of 95%, 75% and 85% in pasta 1, 2

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and 3 compared to the respective dried pasta samples, while TPC increased of 95%, 87% and 96%, respectively. The increase of both PAs and TPC proved to be mainly due to an increase in the bound form; in fact, bound PAs moved from 616.12 to 1107.22 mg/kg dm in pasta 1, from 374.51 to 717.11 mg/kg dm in pasta 2 and from 45.62 to 102.18 mg/kg dm in the reference pasta (pasta 3). Results were comparable with those obtained by Fares et al. (2010), who investigated the effect of pasta-making and cooking process in pasta enriched with debranning fractions of common wheat, finding that cooking process increased the content of bound PAs, plausibly for the action of boiling water which could enhance the extraction of bound phenolics from the food matrices.

No differences among the occurrence of PAs in the cooked pasta samples were found when the results were expressed as percentage distribution of individual PAs: a very similar percentage distribution among the different cooked pasta samples was observed, mainly for conjugated and bound PAs (**Fig.36**). In the bound form, ferulic acid accounted for over 75 % in almost all matrices, while sinapic acid prevailed in the conjugated form accounting for over 60% in all samples, followed by ferulic acid. As previously observed, a higher variability was indeed found for the free form, plausibly due to the low contents of PAs in this form.

In general, as already observed for dried pasta samples, the use of wholemeal or of enriched semolina is confirmed to be useful for the production of pasta samples with higher content of phenolic compounds than traditional one. However, differently from phenolic compounds, a reduction of TAC level of about -18%, -11% and -17% was observed after cooking process in pasta 1, 2 and 3, if compared to the respective dried pasta. These results are in contrast with those observed in the previously mentioned studies

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by Fares et al. (2010) in which cooking increased the antioxidant properties *in vitro* of pasta samples. This may be partially explained by considering that in our research the antioxidant capacity was determined using the "direct method" and not using the methanolic extracts. In this way, also the contribution of the other compounds with antioxidant activity was accounted for. Moreover, it should be considered that the different compounds may be affected by the various technological processes in a different way.

	Total PAs mg/kg dm	**Total TPC** mg FAE/kg dm	**YCP** mg/kg dm	**TAC** mmol TEAC/kg dm	**Starch** % dm	**Protein** % dm	**Ash** % dm
Wholemeal	563.19	1234.35	7.50	49.18	61.89	12.32	2.21
DF 1 (DL 0-2.8%)	4264.05	7700.91	9.87	60.01	7.74	12.82	7.50
DF 2 (DL 2.8-5.1%)	4040.19	7287.89	8.50	63.57	8.95	13.92	7.88
DF 3 (DL 5-7%)	3540.73	6594.68	10.21	60.63	18.42	15.93	7.26
Coarse bran	2910.79	6295.43	8.08	61.44	24.06	15.25	5.19
Fine bran	1252.61	2307.91	8.65	52.97	49.15	13.71	3.31
Semolina	166.73	577.97	7.77	36.73	73.84	11.26	0.96
Dried traditional pasta	70.65	220.36	6.60	38.97	75.07	10.83	0.97
Cooked traditional pasta	137.63	430.49	6.88	32.09	74.38	11.81	0.65
Semolina + DF 2	917.75	2007.77	8.53	42.30	62.24	11.71	2.40
Dried pasta semolina+DF2	679.66	1492.63	8.04	38.51	64.29	11.57	2.43
Cooked pasta semolina+DF2	1191.71	2788.33	8.47	34.33	62.20	12.46	1.74
Milled DK (DL 2.8%)	457.38	802.04	8.16	44.00	65.65	12.06	1.75
Micronized DK (DL 2.8%)	373.01	1055.93	7.91	46.40	65.53	12.13	1.67
Dried pasta micronized DK	415.71	1046.56	8.85	40.21	62.96	12.29	1.80
Cooked pasta micronized DK	768.51	2049.08	6.89	33.40	66.89	13.05	1.26

Tab.16 Characterization of the different matrices obtained from milling, debranning, pasta-making and cooking processes of Normanno genotype.

Legend: PAs, phenolic acids; TPC, Total Phenolic Content; TAC, Total Antioxidant Capacity; TEAC, Trolox Equivalent Antioxidant Capacity; FAE, ferulic acid equivalents; YCP, Yellow Coloured Pigments; DF, debranning fractions; DK: debranned kernels; DL, debranning level; dm, dry matter.

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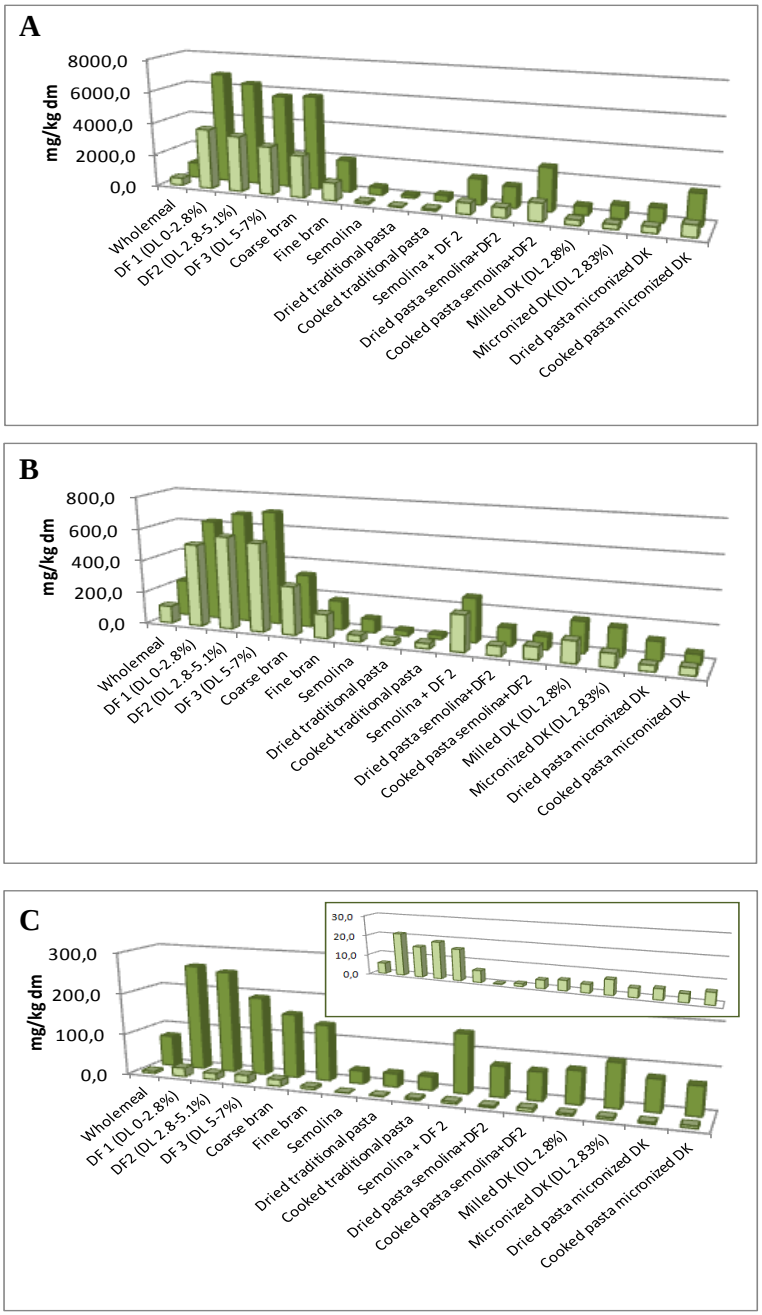

Fig.35 Mean content of bound (A), conjugated (B) and free (C) PAs (□) and TPC (■) and mean percentage distribution of individual PAs (D) in different matrices obtained from milling, debranning, pasta-making and cooking processes of a durum wheat genotype (Normanno).

Legend: DF, debranning fractions; DK: debranned kernels; DL, debranning level; dm, dry matter.

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The statistical analysis on these data (**Tab.17**) revealed high correlations between PAs and TPC in all the three forms, suggesting that PAs are the major contributor to TPC and that the compounds included in the TPC class are localized in the same zones of the kernels. A lower but significant correlation was indeed observed with YCP, suggesting that, as already mentioned above, the compounds included in YCP class (e.g. β-carotene, lutein, zeaxanthin) are not only accumulated in the outermost layers of the kernels, differently from other antioxidants such as PAs and TPC.

Starch was negatively correlated with the other parameters under study (e.g. bound PAs=-0.987; bound TPC=-0.972; $p<0.01$), confirming that just low amount of these antioxidant compounds can be found in the endosperm.

As regards the sensory quality of the pasta samples, the results obtained from pasta cooking evaluation are reported in **Tab.18**. A higher stickiness and bulkiness in traditional pasta compared to the innovative pasta samples was observed, revealing that these new pasta samples can have a great potential also from a sensory point of view.

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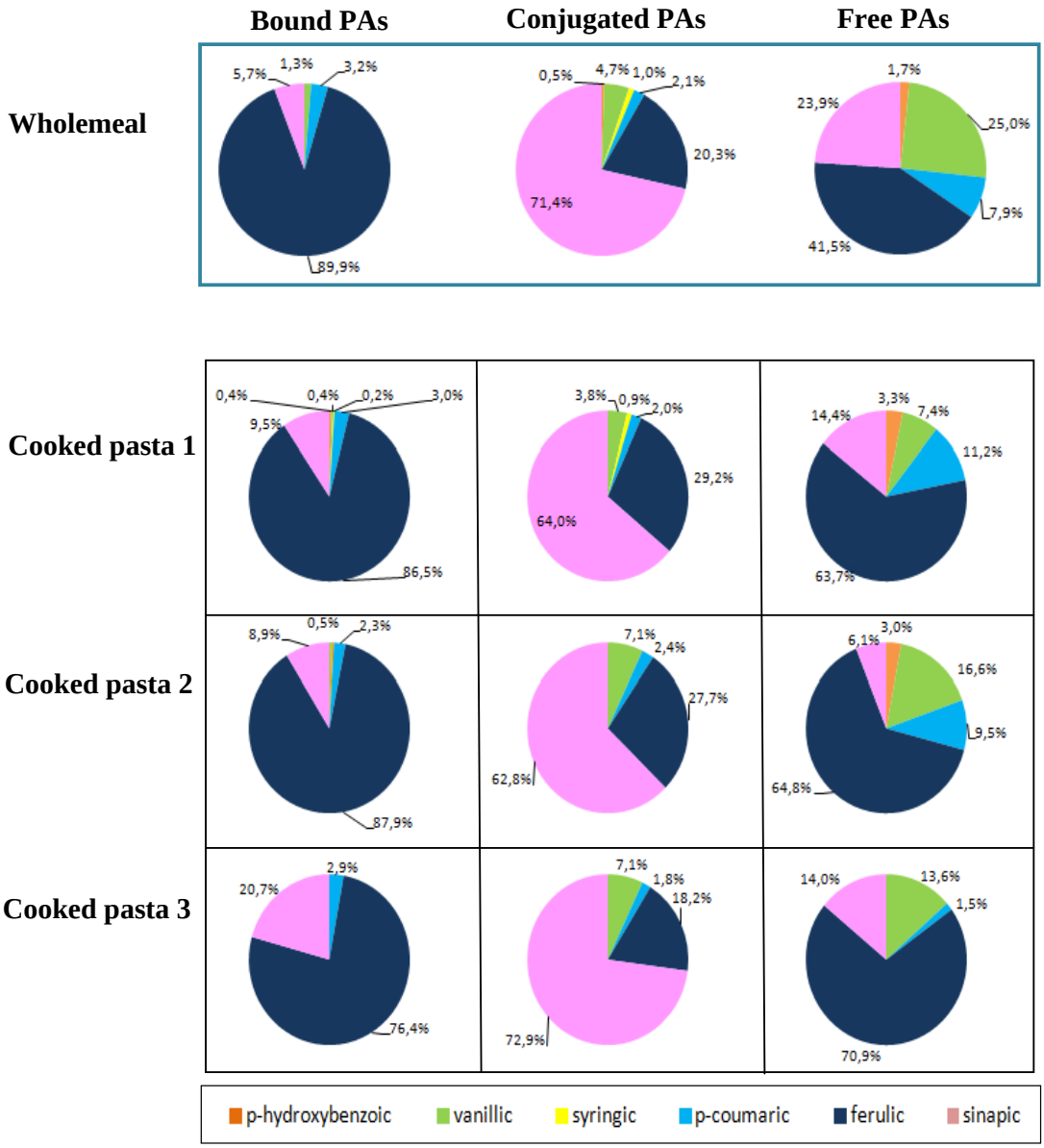

Fig.36 Percentage distribution of individual PAs in pasta samples obtained using the durum wheat genotype Normanno, compared with the wholemeal.

Legend: pasta 1: pasta test 1 (semolina+debranning fraction); pasta 2: pasta test 2 (micronized debranned kernels); pasta 3: pasta control (semolina); PAs: phenolic acids

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	Free PAs	Conjugated PAs	Bound PAs	Free TPC	Conjugated TPC	Bound TPC	YCP	TAC	Starch	Protein	Ash
Free PAs	1.000										
Conjugated PAs	0.916**	1.000									
Bound PAs	0.970**	0.944**	1.000								
Free TPC	0.887**	0.936**	0.920**	1.000							
Conjugated TPC	0.894**	0.988**	0.911**	0.923**	1.000						
Bound TPC	0.968**	0.915**	0.991**	0.890**	0.875**	1.000					
YCP	0.604*	0.567*	0.549*	0.586*	0.604*	0.527*	1.000				
TAC	0.813**	0.869**	0.832**	0.872**	0.883**	0.804**	0.513*	1.000			
Starch	-0.960**	-0.952**	-0.987**	-0.938**	-0.932**	-0.972**	-0.568*	-0.900**	1.000		
Protein	0.773**	0.702**	0.741**	0.637**	0.683**	0.772**	0.486	0.725**	-0.767**	1.000	
Ash	0.939**	0.975**	0.974**	0.942**	0.964**	0.948**	0.585*	0.901**	-0.988**	0.739**	1.000

Tab.17 Correlations between Contents of Phenolic Acids, Other Phytochemicals and other Nutrients and Total Antioxidant Capacity in different matrices obtained from milling, debranning, pasta-making and cooking processes of a durum wheat genotype (Normanno).

Legend: PAs, phenolic acids; TPC, Total Phenolic Content; TAC, Total Antioxidant Capacity; YCP, Yellow Coloured Pigments; *, $p<0.05$; **$p<0.01$.

	Weight after cooking (g)	Stickiness	Firmness	Bulkiness
Pasta 1	251	65	65	80
Pasta 2	263	50	65	75
Pasta 3	281	35	60	40

Tab.18 Cooking quality parameters of three pasta from durum wheat. The score of each sensorial component is the arithmetic mean of three assessors.

Legend: pasta 1: pasta test 1 (semolina+debranning fraction); pasta 2: pasta test 2 (micronized debranned kernels); pasta 3: pasta control (semolina); PAs: phenolic acids

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4.3.2.2.2 Bioavailability of phenolic compounds

The following phenolic compounds were detected and quantified in blood plasma: ferulic acid, and vanillic acid.

The relative bioavailability, i.e. $AUC_{0\text{-}t}$, during the period 0-8 h and 0-24 h are reported in **Tab.19**.

Phenolic acids were present exclusively conjugated with glucuronic acid and / or sulfate.

The maximum plasma concentrations (C_{max}) of ferulic and vanillic acid were significantly higher after the ingestion of pasta 1 and 2 than after the ingestion of pasta 3 (**Fig.37**).

Ferulic acid was the phenolic acid with the highest C_{max} and the largest difference from the baseline and the control (Pasta 3).

	PASTA 1	**PASTA 2**	**PASTA 3**
Ferulic acid			
AUC 0-8h	27.9 (6.8)	20.0 (2.9)	-
AUC 0-24h	38.6 (8.8)	37.4 (8.5)	-
Cmax (ng/mL)	6.9 (1.8)	3.9 (1.1)	-
t max (min)	160	160	
Vanillic acid			
AUC 0-8h	3.4 (1.6)	1.2 (0.8)	-
AUC 0-24h	6.7 (5.8)	2.1 (2.0)	-
Cmax (ng/mL)	0.8 (0.3)	0.3 (0.1)	-
t max (min)	120	120	-

Tab.19 Pharmacokinetics of ferulic and vanillic acid identified in plasma after the ingestion of the pasta 1, 2 and 3. Values are expressed as mean (SD); n=3.
Legend: pasta 1: pasta test 1 (semolina+debranning fraction); pasta 2: pasta test 2 (micronized debranned kernels); pasta 3: pasta control (semolina); PAs: phenolic acids.

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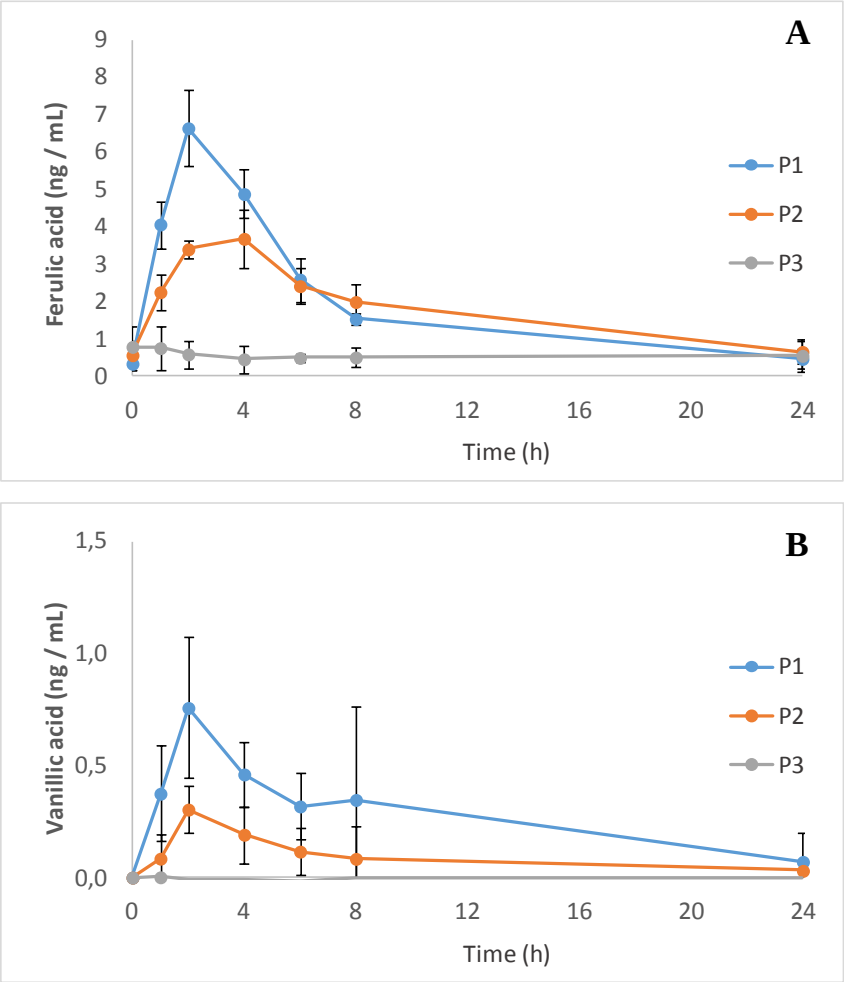

Fig.37 Plasma concentration of ferulic (A) and vanillic (B) acid over time after the ingestion of pasta 1 (P1), 2 (P2) and 3 (P3). Values are expressed as means and bars represent standard deviation; n = 3.

The amounts of ferulic acid, isoferulic acid, vanillic acid and sinapic acid found in urine after the ingestion of the three pasta samples are reported in **Fig.38.**

Increments from the basal excreted amounts of ferulic acid differed among the test meals over 24 hours. After ingestion of pasta 1 and 2, the respective increments in ferulic

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excretion were on average 19 and 3-folds greater as compared to that of pasta 3. However, a great variability in excretion of ferulic acids among subjects was observed.

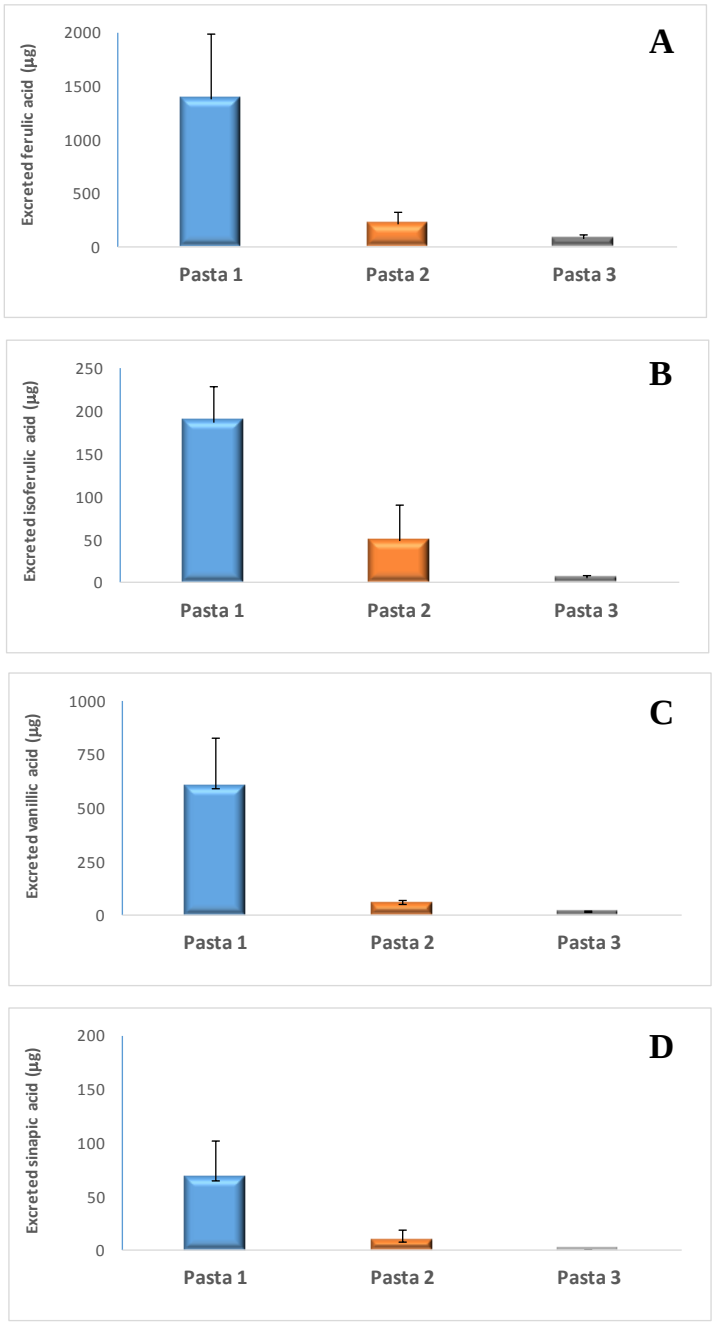

Fig.38 Amount of ferulic (A), isoferulic (B), vanillic (C) and sinapic (D) acid excreted in urine 24 hours after ingestion of pasta 1, 2 and 3. Baseline data were subtracted. Values are expressed as means and bars represent standard deviation; n=3. Legend: pasta 1: pasta test 1 (semolina+debranning fraction); pasta 2: pasta test 2 (micronized debranned kernels); pasta 3: pasta control (semolina); PAs: phenolic acids.

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Considering the PA content in the three cooked pasta samples (Section 4.3.2.2.1), only approximately 1% of the ingested total ferulic acid in the pasta samples was detected as FA and sinapic acid in the excreted urine after ingestion of the meals, in agreement with previous studies (Lappi et al., 2013).

These results suggest that the phenolic acids bound to the dietary fibre complex of cereal are poorly absorbed in the human body but they could be metabolized by microflora.

The consumption of cereal-based foods with high content of phenolic compounds increases the bioavailability of these compounds in absolute terms.

Further investigations with a higher number of subjects are required to better comprehend the bioavailability of these compounds, but also to explore the health benefits following their absorption in the body.

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5. Conclusions

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Summing up the results observed in the different investigations, it is possible to make some remarks about the whole study performed to study the influence of genetic, environmental and technological factors on the occurrence of antioxidants in durum wheat and derived products.

Development of a validated method for PAs extraction and analysis

RP-HPLC on a semimicro separation scale offers both economical and environmental benefits, at the same time maintaining separation performance and reliability of traditional HPLC methods, which use conventional analytical size columns, generally requiring larger amounts of expensive organic solvents and generating greater volumes of hazardous waste. Moreover, the developed HPLC method allows the accurate estimation of the individual PAs occurring as soluble free, soluble conjugated, and insoluble bound compounds in wholemeal of durum wheat and durum wheat products deriving by many technological processes.

Factor influencing the occurrence of antioxidant compounds

Crop year, genotype, growing area and their interactions proved to significantly affected the content of phenolic acids, total phenolic compounds and yellow pigments, as well as the antioxidant capacity of durum wheat, although with different extents. In particular, the content of PAs and TPC were mostly affected by environment, whereas YCP content and TAC level were principally influenced by genetic factors. These findings might be useful for selecting durum wheat genotypes and for developing cereal-based foods particularly rich in bioactive compounds which could lead to health benefits to consumers.

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The technological processes applied on durum wheat, showed that traditional milling and pasta-making deeply influence the content of antioxidant compounds and antioxidant activity. However, the milling process proved to be the most influencing process which drastically reduce the occurrence of healthy compounds from wholemeal to semolina. On the contrary, the process made using wholemeal minimize the loss of these compounds because bran, and so the outermost layers, are preserved.

The debranning process causes a general decrease of PA content and concurrently of TAC level in debranning fractions, because the antioxidant compounds remain accumulated in the outer layers. Specifically for PAs, the process influences the 3 PA forms to a different extent, suggesting that the different forms are mainly located in different layers of the teguments. The process causes a general decrease of PA content in debranned kernels, but less accentuated than in the debranning fractions. TAC appears to be slightly influenced by the process, probably due to the occurrence of other antioxidant compounds different from PAs, also present in the inner layers and in the endosperm. On the basis of these considerations, the multistep debranning process appears a valuable way for detecting the level representing the best compromise between nutritional, technological and hygienic-sanitary aspects. It was highlighted that with this approach it is possible to obtain both debranning fractions which can be re-added for the production of foods with improved nutritional and healthy value and debranned kernels to be milled as a sort of whole grain and used for the production of less refined foods.

Development of high quality and innovative pasta

The development of innovative pasta made by using debranned kernels or semolina enriched with selected debranning fractions demonstrated that these innovative processes could be useful to preserve the occurrence of antioxidant compounds in durum wheat.

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Specifically for phenolic compounds, their bioavailability in innovative pasta is increased in absolute terms in respect to traditional pasta. The pasta samples obtained by these approaches represent less refined product suitable for increasing the consumption of wholegrain foods in order to meet the nutritional recommendations.

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6. References

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7. Appendix

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7.1 DISSEMINATION OF RESULTS

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- Nicoletti, I., Martini, D., De Rossi, A., Taddei, F., D'Egidio, M. G., & Corradini, D. (2013). 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. *Journal of Agricultural and Food Chemistry*, *61*, 11800-11807.

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- D'Egidio, M.G., Martini, D., Taddei, F., De Rossi, A., Nicoletti, I., & Corradini, D. Phenolic acids in durum wheat and derived products: role and perspectives. *Tecnica Molitoria International*, 2013, 62:14/A

CONFERENCE PROCEEDINGS WITH ISBN

- Martini, D., D'Egidio, M.G., Taddei, F., Nicoletti, I., & Corradini, D. Uso di frazioni di decorticazione e di granelle decorticate per la produzione di paste speciali ad elevato potenziale nutrizionale. Incontri di Scienza delle Separazioni. Rome, December 12th 2014. ISBN: 978-88-86208-99-4.
- Nicoletti, I., Martini, D., Taddei, F., Ciccoritti, R., D'Egidio, M.G., & Corradini, D. Determination by RP-HPLC of phenolic acids occurring in durum wheat as soluble free, soluble conjugated and insoluble bound compounds. 20th International Symposium on Separation Science. Prague-Czech Rep., August 30th-September 2nd 2014. ISBN: 978-80-7395-777-3.
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- Taddei, F., Martin,i D., De Rossi, A., Ciccoritti, R., Nicoletti, I., Corradini, D., & D'Egidio, M.G. Influenza del genotipo e dell'ambiente sulla capacità antiossidante totale e sul profilo degli acidi fenolici in frumento duro. AISTEC, Un mondo di cereali – Potenzialità e sfide. Bergamo, Italy, June 12-14 2013.ISBN: 978-88-906680-1-2.
- Martini, D., D'Egidio, M.G., Taddei, F., Nicoletti, I., & Corradini, D. Variations of the content of phenolic acids in durum wheat as a function of genotype and environment.

Tesi di dottorato in Scienze dell'Alimentazione e della Nutrizione, di Daniela Martini,
discussa presso l'Università Campus Bio-Medico di Roma in data 27/04/2014.
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XXIV Congresso della Divisione di Chimica Analitica della Società Chimica Italiana. Sestri Levante (GE), September 15-19 2013. ISBN: 9788890767012.

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- Martini, D. Variazione del contenuto in acidi fenolici e dell'attività antiossidante nei prodotti di decorticazione del frumento duro. Dal seme alla pasta: una tradizione in continua evoluzione. Bologna, November 28-29 2014.
- Nicoletti, I., Martini, D., Taddei, F., Ciccoritti, R., D'Egidio, M.G., & Corradini, D. Identification and quantification of phenolic acids in durum wheat by RP-HPLC on a semimicro separation scale with PDA and ESI-MS detection. 38th International Symposium on Capillary Chromatography (ISCC) and 11th GCxGC Symposium. Riva del Garda (TN), May 18-23 2014.
- Martini, D., Taddei, F., Ciccoritti, R., Nicoletti, I., Corradini, D., & D'Egidio, M.G. Genetic, environmental and technological factors influencing the occurrence of phenolic acids in durum wheat and derived products. 13th European Young Cereal Scientists and Technologists Workshop (EYCSTW). Freising-Germany, May 14-16 2014.
- Martini, D., Taddei, F., Nicoletti, I., D'Egidio, M.G., & Corradini, D. Influenza dei fattori genetici, ambientali e tecnologici sul contenuto e la distribuzione di acidi fenolici nel grano duro e nei prodotti derivati. Incontri di Scienza delle Separazioni. Messina, November 28-29 2013.
- Taddei, F., Martini, D., Nicoletti, I., Corradini, D., & D'Egidio, M.G. Variazione del contenuto in acidi fenolici e dell'attività antiossidante nei prodotti di decorticazione del frumento duro. Incontri di Scienza delle Separazioni. Messina, November 28-29 2013.
- Martini, D., Taddei, F., Nicoletti, I., De Rossi, A., Corradini, D., D'Egidio, M.G. Effects of genotype and environment on phenolic acids content and total antioxidant capacity in durum wheat. Cereals & Europe Spring Meeting 2013. Leuven -BE- May 29-31 2013
- Martini, D., Taddei, F., D'Egidio, M.G., Nicoletti, I., De Rossi, A., & Corradini, D. Extraction, isolation and identification of free, conjugated and bound phenolic acids in milling fractions and pasta obtained from a durum wheat cultivar. International Symposium on Extraction Technologies (ExTech). Messina, September 24-26 2012.

Tesi di dottorato in Scienze dell'Alimentazione e della Nutrizione, di Daniela Martini,
discussa presso l'Università Campus Bio-Medico di Roma in data 27/04/2014.
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7.2. TITLES AND AWARDS

AWARDS

- 2014: Award offered by Fondazione Feltrinelli-Lab.EXPO for the best manuscript edited within the" Expo School: percorso agricoltura e alimentazione". Milan, April 7-11 2014
- 2013: Award offered by Interdivisional Group of Separation Sciences at the "Incontro di Scienza delle Separazioni" Congress. Messina, November 28-29 2013
- 2012: Award offered by Interdivisional Group of Separation Sciences at the International Symposium on Extraction Technologies (ExTech). Messina, September 24-26 2012

TRAINING SCHOOLS

- COST School. Training School "Gastro Intestinal Engineering: The Role Of Material Properties And Microstructure Of Foods In Nutrient Release In The Gastro-Intestinal Tract". Milan, September 8-10 2014
- TRADEIT Entrepreneurial Summer Academy. Tralee - Ireland , June 15-18 2014
- Expo School: percorso agricoltura e alimentazione. Milan, April 7-11 2014

PARTECIPATION TO CONGRESSES

National

- Incontri di Scienza delle Separazioni. Rome, December 12th 2014. Oral Communication.
- Dal seme alla pasta: una tradizione in continua evoluzione. Bologna, November 28-29 2013. Oral Communication.
- Incontri di Scienza delle Separazioni". Messina, November 28-29 2013. Oral Communication.

International

- 13th European Young Cereal Scientists and Technologists Workshop (EYCSTW). Freising-Germany, May 14-16 2014. Oral Communication.
- Bioactives in Cereals and Foods. Wien, Austria, April 24-25 2014. Oral Communication.
- Cereals & Europe Spring Meeting 2013. Leuven, Belgium, May 29-31 2013. Poster Communication.
- International Symposium on Extraction Technologies (ExTech). Messina, September 24-26 2012. Oral Communication.

Tesi di dottorato in Scienze dell'Alimentazione e della Nutrizione, di Daniela Martini,
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8. Acknowledgments

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