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PhD Thesis

From durum wheat to whole-meal pasta: effects of processing and cooking on physico-chemical and nutritional properties

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Summary

The growing interest in healthy eating behaviors and awareness of the health benefits from the intake of whole grain has resulted in an increase in consumer preferences towards the consumption of whole grain products.

In recent years, preferences for whole-meal pasta have also grown among consumers. In this sense, whole-meal pasta can represent an important vehicle of health-promoting components, including dietary fibre, vitamins, minerals and bioactive compounds. Among vitamins, folates are gaining increasing attention among researchers as they are involved in numerous human metabolic processes and there may be several negative human health implications associated with a folate-deficient diets. At the same time, the sensory consumer acceptability of whole-meal pasta, and in general whole grain products, is a critical point on which industry must confront and/or clash and strive in the direction of developing whole-meal pasta that meet the expectations of consumers during consumption. In this regard, it is important to underline that both the careful selection of the raw materials and the appropriate calibration of the technological process are fundamental prerequisites for obtaining a high quality whole-meal pasta as widely demonstrated by many scientific studies that have investigated the influence of raw materials and processing conditions on the nutritional and sensory quality of whole-meal pasta. Furthermore, since folates are highly unstable compounds it is also appropriate to consider the effects of processing and cooking on folate content in whole-meal pasta given that there are few literature studies available on this point.

On the basis of these remarks, this PhD research work aims at studying the distribution of folates in the debranning and milling fractions of durum wheat and at developing of whole-meal pasta rich in folates, with low levels of furosine and high sensory properties. As part of the National Operational Programme on Research and Innovation 2014-2020, this innovative PhD with Industrial characterization was developed in collaboration between the University of Molise and F.lli De Cecco di Filippo - Fara San Martino S.p.A. In order to achieve the aforementioned objectives, the research work was divided into the following activities:

- the 1st activity concerned the evaluation of total folate content in durum wheat samples of different origins and in the relative debranning and milling fractions obtained with pilot and industrial plants;

- the 2nd activity consisted in assessment the effects of pasta making process and cooking on folate retention in developed whole-meal pasta and its chemical and nutritional characterization;
- the 3rd activity involved the evaluation of heat damage through the quantification of furosine levels in the developed whole-meal pasta and its sensory evaluation by a panel.

The results obtained in this research activity can be used as a starting point for the De Cecco company with a view to a greater qualitative enhancement of whole-meal pasta.

Riassunto

Il crescente interesse verso abitudini alimentari salutari e la consapevolezza degli effetti benefici sulla salute derivanti dall'assunzione di cereali integrali ha portato ad un aumento delle preferenze dei consumatori verso il consumo di prodotti integrali.

Negli ultimi anni sono cresciute anche le preferenze per la pasta integrale tra i consumatori. In questo senso, la pasta integrale può rappresentare un importante veicolo di componenti dalle proprietà salutistiche come la fibra alimentare, le vitamine, i minerali e i composti bioattivi. Tra le vitamine, i folati sono sempre più al centro dell'attenzione tra i ricercatori dal momento che questi sono coinvolti in numerosi processi metabolici umani e considerato che potrebbero esserci diverse implicazioni negative per la salute umana associate a diete carenti di folati.

Allo stesso tempo, l'accettabilità sensoriale della pasta integrale da parte del consumatore, e in generale dei prodotti integrali, è un punto critico su cui l'industria deve confrontarsi e/o scontrarsi e tendere nella direzione di sviluppare una pasta integrale che soddisfi le aspettative dei consumatori al momento del consumo. A tal proposto, è importante sottolineare che sia l'accurata selezione delle materie prime che l'appropriata modulazione del processo tecnologico sono punti di partenza imprescindibili per ottenere una pasta integrale di alta valenza qualitativa come ampiamente dimostrato da numerosi studi scientifici che hanno indagato l'influenza delle materie prime e delle condizioni di lavorazione sulla qualità nutrizionale e sensoriale della pasta integrale. Inoltre, poiché i folati sono composti altamente instabili, è opportuno considerare anche gli effetti della lavorazione e della cottura sul contenuto di folati nella pasta integrale giacché sono limitati gli studi scientifici disponibili in letteratura su questo argomento.

Sulla base di queste considerazioni, la presente attività di ricerca di dottorato si propone di studiare la distribuzione dei folati nelle frazioni di decorticazione e macinazione del grano duro e di sviluppare una pasta integrale ricca in folati, con bassi livelli di furosina ed elevate proprietà sensoriali.

Nell'ambito del Programma Operativo Nazionale Ricerca e Innovazione 2014-2020, questo dottorato di ricerca innovativo con caratterizzazione industriale è stato sviluppato in collaborazione tra l'Università degli Studi del Molise e l'azienda F.lli De Cecco di Filippo - Fara San Martino S.p.A.

Al fine di raggiungere i suddetti obiettivi, il lavoro di ricerca è stato articolato nelle seguenti attività:

- la prima attività ha riguardato la valutazione del contenuto di folati totali in campioni di grano duro di diversa origine e nelle relative frazioni di decorticazione e macinazione ottenute con impianti pilota sperimentale e industriale;
- la seconda attività è consistita nella valutazione degli effetti dei processi di pastificazione e cottura sulla ritenzione di folati nella pasta integrale sviluppata e nella sua caratterizzazione chimico-nutrizionale;
- la terza attività ha riguardato la valutazione del danno termico attraverso la quantificazione dei livelli di furosina nella pasta integrale sviluppata e la sua valutazione sensoriale da parte di un panel.

I risultati ottenuti in questa attività di ricerca possono essere utilizzati come punto di partenza per l'azienda De Cecco nel quadro di una ulteriore valorizzazione qualitativa della pasta integrale.

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Chapter 1

State of the art

1.1 Durum wheat (*Triticum turgidum* subsp. *durum* Desf.)

Durum wheat (*Triticum turgidum* subsp. *durum* Desf.) is a grass plant belonging to the Gramineae or Poaceae family, of which also includes other cereal grains such as barley, oat, rye, corn and rice. As a member of the genus "*Triticum*", the ancestor of durum wheat had seven pairs of chromosomes (*2n* = 14). In fact, durum wheat is a tetraploid species (*2n* = 28, AABB) differentiated from *Triticum turgidum* subsp. *dicoccoides*, which is the first domesticated tetraploid wheat in Fertile Crescent, precisely in the Karakadag Mountains of south-east Turkey about 8,000 B.C.E. result of natural interspecific hybridization between the two wild diploid wheat species *Triticum monococcum* (*2n* = 14; A genome) and *Aegilops speltoides* (*2n* = 14; B genome). Historically, durum wheat evolved in the eastern Mediterranean with the first archeological evidence of its diffusion, as it is known today, dates back to about 7,000 B.C.E. in Egypt; nevertheless, it is necessary to wait until about 2,000 B.C.E., during the Hellenistic period, for its full affirmation as a main crop. Starting from its area of origin, durum wheat extended to all of Europe, the Middle East and North Africa where it has well established at the end of the Roman Empire (Wrigley, 2009; Bozzini *et al.*, 2012; Martínez-Moreno *et al.*, 2020).

Based on the peculiar morphological characteristics and adaptability to the pedoclimatic conditions of the different environments, resulting from many years of durum breeding programs, durum wheat can be classified into the following types:

- European types which grow in environments with continental climates, require a more or less extreme vernalization and the stems reach heights of 150-170 cm;
- Mediterranean and African types which need a medium supply of water during the initial stages of the vegetative cycle and reach heights of 150 cm;
- Syro-Palestinian types which have a low water requirement in all the stages of the vegetative cycle and reach heights of 120 cm;
- Abyssinic types which have diversified morphological characteristics, including thin stems and colored, smaller grains (Bozzini *et al.*, 2012).

1.1.1 Grain anatomy and composition

In the botanical language, the durum wheat grain is a *caryopsis*, that is a single-seeded fruit in which the external tissues, representing the latter the ripened ovary wall, are fused to the seed. It exhibits an oval shape and a deep longitudinal crease on its ventral

side. Moreover, the durum wheat grain is characterized by the presence of a tuft of hairs, or brush, at its apical end and a scar in the attachment region at its basal end (Bechtel *et al.*, 2009; Sapirstein, 2016).

It is possible to recognize three distinct regions in durum wheat grain, namely bran, endosperm and germ, which represent about the 14-16%, 81-84% and 2-3% by weight of the whole grain, respectively (Figure 1) (Stevenson *et al.*, 2012).

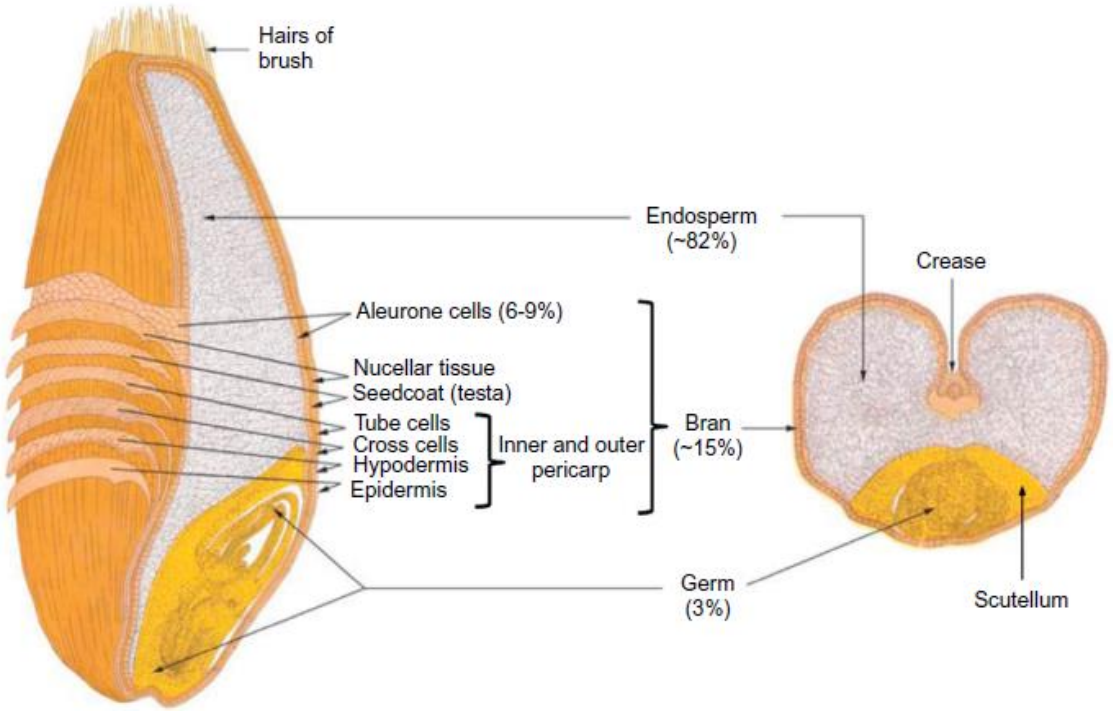


Figure 1. The structure of the wheat grain in its longitudinal (on the left) and transverse (on the right) section. From: Dexter & Sarkar (2004).

From outside to inside the bran consists of an outer epidermis, hypodermis, parenchima, intermediate cells, cross cells and tube cells. These tissue layers constitute the pericarp (or fruit coat) of the durum wheat grain which is tightly adherent to the testa (or seed coat) (Bechtel *et al.*, 2009). The endosperm and the germ are enclosed by the nucellar epidermis (Evers & Millar, 2002). The latter, also known as hyaline layer or perisperm, is strictly united to the testa, on one side, and the aleurone layer, on the other side. The complex tissue mosaic which is the bran explains its different and varied chemical composition which, in turn, reflects the biological functions of this fraction. In fact, the bran is rich in non-starch polysaccharides and lignin in order to protect the seed from microorganisms and insects as well as from adverse meteorological conditions; the bran is also rich in B vitamins, minerals and numerous bioactive compounds (Pagani *et al.*, 2014; Sapirstein,

2016).

Anatomically, the aleurone layer, a single layer of cubic shaped cells, represents the outermost layer of endosperm fraction surrounding the starchy endosperm and part of the germ (Bechtel *et al.*, 2009; Šramková *et al.*, 2009). The aleurone cells are characterized above all by the relatively high amount of proteins and enzymes, that play a crucial role in the germination process, but also of sugars, lipids, vitamins, minerals and phytate. The principal durum wheat grain component, the starchy endosperm, is made up of cells that are differentiated by shapes, sizes and wall thickness. The cell walls of the starchy endosperm contain mostly polysaccharides (about 75%), represented by about 70% of arabinoxylans, 20% of (1→3, 1→4)-β-D-glucan, 7% of glucomannan and 2% of cellulose, and proteins (about 15%). The cells of the starchy endosperm mainly contain starch and proteins, with the starch granules that are packed in a storage protein matrix (Evers & Millar, 2002; Bechtel *et al.*, 2009; Pagani *et al.*, 2014).

The germ (or embryo) is located on the lower dorsal side of the durum wheat grain and derives from the fusion of the egg nucleus and the second sperm nucleus. It includes an embryonic axis and scutellum (Bechtel *et al.*, 2009). The scutellum divides the embryonic axis from the endosperm from which it mobilises food reserves of the grain to support the germination process and the growth of the seedling. The germ has a high content in lipids, lipid-soluble vitamins, minerals, soluble proteins and sugars (Evers & Millar, 2002; Sapirstein, 2016).

1.1.2 Nutritional quality

Generally, durum wheat grain is composed approximately of 70.2% starch, 12.2% proteins, 1.9% lipids, 1.6% fiber, 1.6% minerals and a variable water content (Table 1) (Marcotuli *et al.*, 2020). The nutritional composition of durum wheat can be affected by the genotype, the pedoclimatic and agricultural conditions, but also by the interaction between these individual factors.

Whole durum wheat is an important source of a wide range of bioactive compounds which have peculiar biological functions that positively impact human health. These compounds are concentrated in different anatomical regions of the grain (Table 2) (Marconi, 2004) and include dietary fibre components, oligosaccharides, polyphenols, vitamins, minerals and phytosterols (Figure 2) (Poutanen, 2012).

Table 1. Composition of durum wheat grain. Adapted from: Marcotuli *et al.* (2020).

Nutrients	Durum wheat
Starch (% d.m.)	71
Protein (g/100 g)	14
Lipids (g/100 g)	2.5
Dietary fibre (g/100 g)	1.6
Thiamin B1 (mg/100 g)	0.4
Riboflavin B2 (mg/100 g)	0.1
Sodium (mg/100 g)	3.8
Potassium (mg/100 g)	431
Calcium (mg/100 g)	34
Magnesium (mg/100 g)	42
Iron (mg/100 g)	6.8
Zinc (mg/100 g)	4.2

Table 2. Localization of bioactive compounds in cereal grains. Adapted from: Marconi (2004).

Compound	**Localization**
Non-starch polysaccharides	
Arabinoxylans	pericarp
B-Glucans	starchy endosperm-aleurone layer
Cellulose	pericarp
Lignans	pericarp-aleurone layer
Fructooligosaccharides	grains in the milky phase
Magnesium-chromium	pericarp
Phytate	pericarp
Phenolic acids	pericarp-aleurone layer
Carotenoids	germ
Tocols	germ-pericarp-aleurone layer
Folate	aleurone layer-germ
Alkylresorcinols	pericarp
Phytosterols	germ-aleurone layer

Non-starch polysaccharides (NSPs) are characteristic structural elements in the cell walls of pericarp, starchy endosperm, aleurone, scutellum and embryonic axis in the durum wheat grain (Stone & Morell, 2009). They are mainly arabinoxylans, β-glucans, cellulose and lignans, components of dietary fiber (Grant *et al.*, 2012b). In the context of the EU HEALTHGRAIN project, Gebreus *et al.* (2008) compared 10 durum wheat varieties

finding levels of dietary fiber ranging from 10.7% to 15.5% of dry matter; these are lower than the levels found in winter wheat (11.5%–18.3%) but higher than those found in emmer wheat (7.2%–12.0%). The most important component of dietary fiber of durum wheat grain cell walls is represented by arabinoxylans (AXs), especially water-unextractable arabinoxylans (WU-AX). Arabinoxylans consist of a linear backbone of 1,4-β-linked D-xylopyranose residues to which residues of α-L-arabinofuranose are attached, with arabinose residues linked to O-2 and/or O-3 of xylose (Bartłomiej *et al.*, 2012).

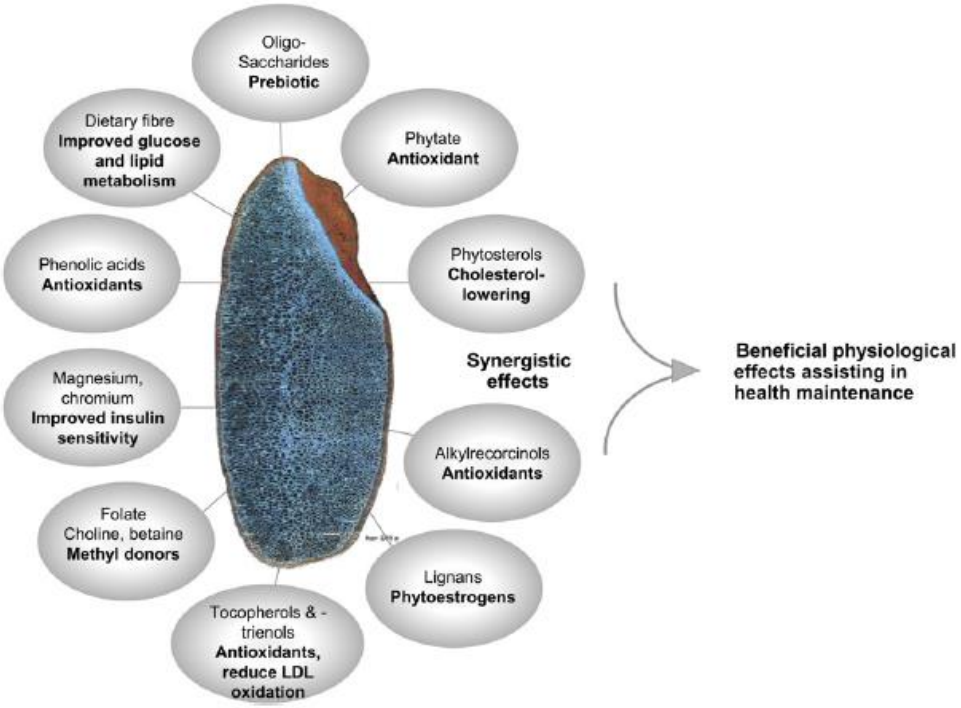


Figure 2. Bioactive compounds in cereal grains and their physiological significance. From: Poutanen (2012).

The total AX content of durum wheat varies between 4.1% to 7.5% of dry matter, which is in line with the results found for durum wheat by Barron *et al.* (2020) equal on average to 5.3 % of dry matter. However, lower and higher values of total arabinoxylans of 2.6% and 12.2%, respectively, were also found in durum wheat (Lafiandra *et al.*, 2012). The water-extractable arabinoxylans (WE-AX) content of durum wheat is low and usually variable from 0.37% to 0.56% of dry matter (Lafiandra *et al.*, 2012). The ratio of arabinose and xylose, which determines the degree of branching, is comparable for all cereals, including durum wheat, and ranging from 0.3 to 1.1 (Bartłomiej *et al.*, 2012).

It is approximately equal to 0.6 in the cell walls of starchy endosperm of wheat grain, while it is lower in those of the outer layers of wheat grain which is close to 1.0 (Barron *et al.*, 2020). The O-5 position of the arabinose residues can form ester bonds with ferulic acid with the content of the latter in durum wheat is higher than that in common wheat (Lafiandra *et al.*, 2012).

Mixed linkage β-(1,3)-(1,4)-D-glucans, commonly named as β-glucans, constitute another fraction of dietary fiber of durum wheat, mostly located both in the cell walls of the starchy endosperm and in those of the aleuronic layer cells of the same durum wheat grain. In durum wheat β-glucans content ranges from 0.25% to 0.45% of dry matter and it is very low when compared to that of other cereals, especially that of barley (3.7%-6.5%) (Gebreus *et al.*, 2008). Chemically, mixed linkage β-(1,3)-(1,4)-D-glucans consist of a linear chain of D-glucose residues linked together by β-(1,4)-glycosidic bonds interrupted by single β-(1,3)-glycosidic bonds. The majority of the β-(1,4)-linked glucose residues consist of blocks of cellotriosyl and cellotetraosyl united by single β-(1,3) linkages with a typical cellotryosyl-to-cellotetraosyl ratio in wheat of 3.7:4.5, higher than that characteristic of β-glucans of barley and oat (Lafiandra *et al.*, 2012). The ratio of β-(1,4) and β-(1,3) linkages in β-glucans, which defines their physico-chemical and functional properties, ranges between 2.5:1 and 3.4:1 in durum wheat (Bartłomiej *et al.*, 2012; Marcotuli *et al.*, 2020).

Cellulose is a homopolymer consisting of β-1,4-linked glucose units and its content in durum wheat is equal to about 2.7%, but it reaches levels of about 9%-13% in durum wheat bran (Lafiandra *et al.*, 2012). Durum wheat also contains small amounts of fructans (1.7%-2.1%) and arabinogalactan peptides (0.27%-0.38%) (Lafiandra *et al.*, 2012), while the lignans content is on average equal to 76 μg/100 g (Durazzo *et al.*, 2013).

Among minerals, magnesium (Mg), manganese (Mn), iron (Fe), zinc (Zn), copper (Cu) and molybdenum (Mo) constitute the most representative minerals found in durum wheat. Potassium (K) is present in high concentrations varying in the range from 3.8 to 5.5 mg/g, followed by phosphorus (1.8-5.2 mg/g), magnesium 81.0-1.5 mg/g) and calcium (0.32-0.47 mg/g). Sodium is present at very low amount (0.01-0.05 mg/g) (Grant *et al.*, 2012b). Potassium is mostly found in the form of phytates (about 80%) bounded to other minerals like Fe, Ca, Zn and Mg, with consequent reduction of the bioavailability of the same minerals (Grant *et al.*, 2012b; Onipe *et al.*, 2015; Brouns, 2022). Durum wheat is also a source of chromium (Cr) which has been found in concentrations ranging from 20.0 to

31.5 ng/g d.m. (Cubadda *et al.*, 2004). Overall, these micronutrients are mostly located in the outermost cell layers of the durum wheat grain; in the bran the ash content is equal to about 50%, while in the germ it is equal to about 40% (Piironen *et al.*, 2009).

Most bioactive compounds are antioxidants which play a crucial role in terms of prevention of diseases such as cardiovascular disease and cancer, by scavenging and preventing the formation of reactive oxygen species and reactive nitrogen species. They include phenolics, carotenoids and tocols (Grant *et al.*, 2012b). Within the phenolic compounds, phenolic acids are the most represented in durum wheat. They are present mostly in bound form and accumulate mainly in the outermost pericarp, testa and aleurone layers of the durum wheat grain (Andersson *et al.*, 2014). Li *et al.* (2008) found a total phenolic acids and a total bound phenolic acids contents varies from 536 to 1086 μg/g of dry matter and from 288 to 832 μg/g of dry matter, respectively, in 10 durum wheats. Bound ferulic acid is the one most present (290-737 μg/g d.m.), followed by conjugated 2,4-dihydroxybenzoic acid (80.5-153.0 μg/g d.m.) and conjugated synaptic acid (55.9–136.6 μg/g d.m.) (Li *et al.*, 2008). Among the compounds with antioxidant activity there are also carotenoids and tocols. In durum wheat total carotenoids are present at levels that range from 277 to 335 μg/100 g of dry matter (Fratianni *et al.*, 2013), with the largest quantities found in the germ followed by the bran and the endosperm fractions (Luthria *et al.*, 2015). The predominant carotenoids of durum wheat are lutein (247.4-300.7 μg/100 g d.m.) and zeaxanthin (19.3-23.2 μg/100 g d.m.); β-carotene is also present in durum wheat (10.3-11.1 μg/100 g d.m., on average) (Fratianni *et al.*, 2013). Tocopherols and tocotrienols, namely tocols, are lipid-soluble compounds also known as vitamin E. The highest concentrations of tocopherols are found in the germ, while tocotrienols are concentrated in the pericarp, aleurone and subaleurone layers and endosperm of the durum wheat grain (Andersson *et al.*, 2014). The average total tocols content (expressed as sum of total tocopherols and total tocotrienols) found by Lampi *et al.* (2008), 48.1 μg/g d.m., are in line with the results found by Fratianni *et al.* (2013) which are equal on average to 3764 μg/100 g d.m. Durum wheat does not contain vitamin C, but it contains other vitamin compounds. The latter are thiamin (3.9-4.8 μg/g d.m.), riboflavin (1.4 μg/g d.m.), niacin (35-76 μg/g d.m.) and pyridoxine (3.7-5.1 μg/g d.m.), B vitamins, mainly present in the germ, pericarp and aleurone layer of the durum wheat (Grant *et al.*, 2012b). Folates (vitamin B9) are discussed in detail in the following chapter.

Other compounds with antioxidant properties are represented by alkylresorcinols (ARs). They are amphiphilic phenolic lipids consisting of an aromatic ring and an odd numbered

alkyl chain attached to carbon C5 of the benzene ring (Bartłomiej *et al.*, 2011). The alkyl chain has a variable length from 13 to 27 carbon atoms depending on the botanical origin, with the ratio of the homologues C17:0 to C21:0 of about 0.01 for durum wheat (Knödler *et al.*, 2010; Bartłomiej *et al.*, 2011; Andersson *et al.*, 2014). ARs are abundant in durum wheat as well as in common wheat and rye, while they are present in small amount in barley and absent in oat, and are mainly concentrated in the hyaline layer, inner pericarp and testa of the grain (Knödler *et al.*, 2010; Andersson *et al.*, 2012; Andersson *et al.*, 2014). In durum wheat the total alkylresorcinols content is variable in the range between 430 and 797 mg/kg d.m. and the relative homologue composition is C17:0, C19:0, C21:0, C23:0, C25:0 at levels of 0.5-1%, 10-15%, 51-60%, 19-25% and 6-13%, respectively (Knödler *et al.*, 2010). Since ARs are compounds present in the outer layers of the wheat and rye grains and thermostable during food processing, their content is used as biomarker of the whole wheat and rye intake in the population (Knödler *et al.*, 2010; Andersson *et al.*, 2012; Andersson *et al.*, 2014).

Plant sterols and stanols, or phytosterols, are secondary metabolites with a structure analogous to that of cholesterol also found in durum wheat grain (Bartłomiej *et al.*, 2011; Grant *et al.*, 2012b). Iafelice *et al.* (2009) took into consideration 5 different cultivars of Italian durum wheat and found a total sterol content ranging from 74 to 84 mg/100 g d.m., higher than that found for hexaploid wheat. The most represented form of phytosterols in durum wheat samples is sitosterol which is present on average in quantities equal to 38.2 mg/100 g d.m., followed by campersterol (13.7 mg/100 g d.m.), sitostanol (12.3 mg/100 g d.m.), campestanol (11.0 mg/100 g d.m.) and stigmasterol (1.5 mg/100 g d.m.) (Iafelice *et al.*, 2009). Plant sterols have been found in different forms in cereal grains as free, steryl esters of fatty acids, hydroxycinnamic acids (usually ferulate), steryl glycosides and acylated sterol glycosides (Lafiandra *et al.*, 2012). Wheat germ as well as the intermediate layers of the bran fraction and the intracellular contents of the aleurone cells have a high content of sterols; the intermediate layers of the bran are also abundant in steryl ferulates (Andersson *et al.*, 2014). Free sterols are the main form of sterols found in durum wheat samples analyzed by Iafelice *et al.* (2009) representing 65% of total sterols, while esterified sterols account for approximately 20% of total sterols.

1.1.3 Economical data and end uses

Durum wheat (*Triticum turgidum* subsp. *durum* Desf.) is an important staple crop ranked 10th among commonly grown cereals globally. However, the production of durum

wheat represents just 5% of the total wheat production equal to about 760 million tonnes on a total of about 219 million hectares of harvested area globally in year 2020 (FAOSTAT, year 2020). The main world producers of durum wheat are the European Union, Canada and the United States. Other important durum wheat producers are North Africa, Turkey, Syria, Australia, Mexico, Kazakhstan and India (Grant *et al.*, 2012a). Within the European Union, Italy is an important producer of durum wheat with an average production of around 4 million tonnes in the year 2020 (Eurostat, year 2020). Italy, together with Spain and Greece, contribute about 80% of European durum wheat production (Grant *et al.*, 2012a). Durum wheat is used for the production of a wide range of end products. Durum wheat and durum wheat semolina represent the raw material of choice for the production not only of pasta but also for the production of typical products of Mediterranean and Asian cuisine such as couscous, different types of unleavened and leavened breads, bourghul, freekeh, yellow alkaline noodles and chapatti (Gruber & Sarkar, 2012; Martínez-Moreno *et al.*, 2020).

1.2 Durum wheat folates

1.2.1 Occurrence and molecular structure

Folate, a water-soluble B vitamin (vitamin B9), is a generic term used to indicate a group of heterocyclic compounds that are based on pteroic acid conjugated with one or more molecules of L-glutamate (Blakley, 1978). These compounds exhibit chemical structures and biological activity similar to folic acid. Folic acid includes a molecule of para-aminobenzoic acid (PABA) joined to both a pteridine ring, fully oxided, through a methylene bridge (C^9–N^{10}) and one molecule of glutamic acid through a γ-peptide bond (Figure 3) (Warzyszynska & Kim, 2014; Saini *et al.*, 2016). Although folic acid is the most stable and readily absorbable form of the vitamin, it is a synthetic form not naturally occurring in significant quantities used in dietary supplements and fortified or enriched foods (Brody, 1999; Arcot & Shrestha, 2005; Saini *et al.*, 2016). There are different forms of folate naturally present in food depending on the oxidation or reduction states of the pteridine ring, the substituent groups attached to N^5 or N^{10} of the pteridine ring and the number of the glutamate residues (Figure 4) (Arcot & Shrestha, 2005; Delchier *et al.*, 2014).

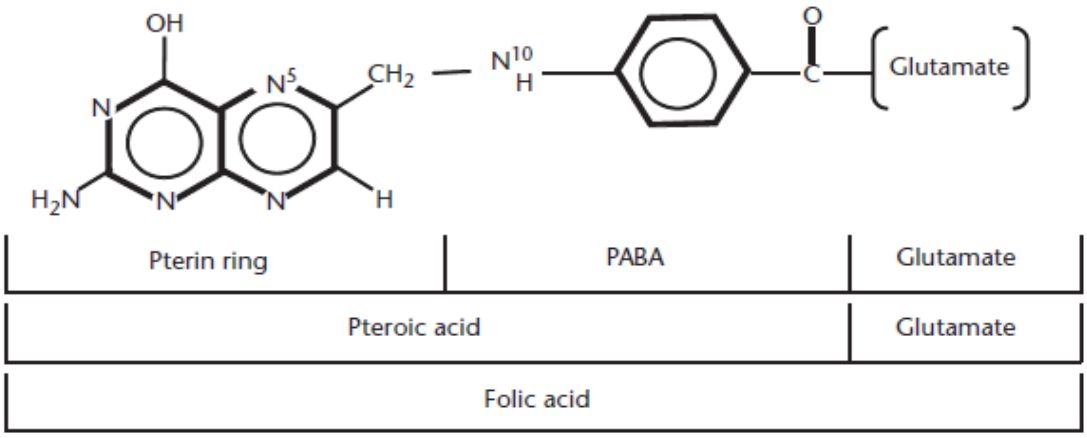
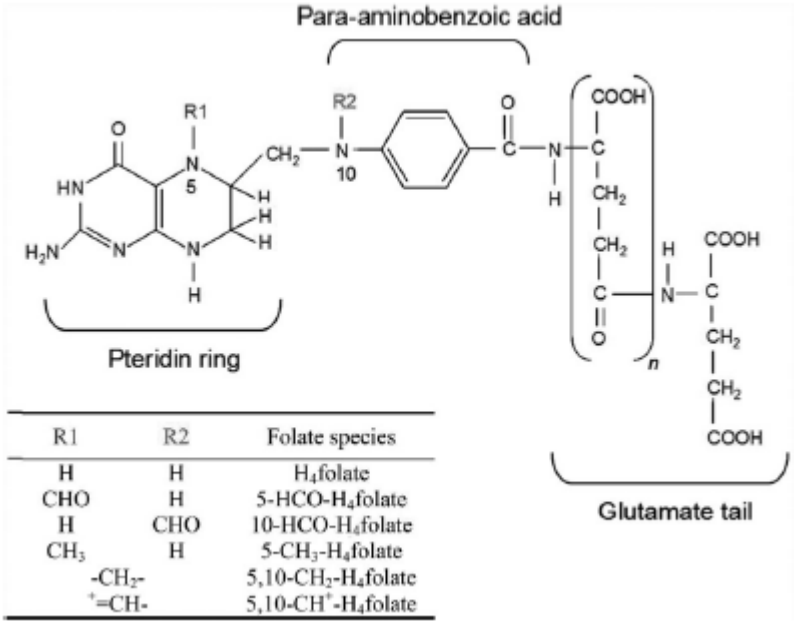


Figure 3. The chemical structure of folic acid. From: Warzyszynska & Kim (2014).



R1	R2	Folate species
H	H	H_4folate
CHO	H	5-HCO-H_4folate
H	CHO	10-HCO-H_4folate
CH_3	H	5-CH_3-H_4folate
-CH_2-		5,10-CH_2-H_4folate
$^+$=CH-		5,10-CH^+-H_4folate

Figure 4. The chemical structure of folates. From: Delchier *et al.* (2014).

Folates can be completely synthesized in higher plants and microorganisms, unlike humans and other vertebrates who lack a complete biosynthetic mechanism, and therefore have to provide the same folate amount with the diet (Strandler *et al.*, 2015). The pteridine ring is synthesized in the cytosol starting from the hydrolysis of guanosine triphosphate (GTP) with the formation of 7,8-dihydroneopterin 3′-triphosphate ($DHNP_3$) in a reaction catalyzed by the enzyme GTP cyclohydrolase I (GTPCHI). Enzymes DHN-P_3 pyrophosphatase and non-specific phosphatase determine the dephosphorylation of 7,8-dihydroneopterin 3′-triphosphate ($DHNP_3$) to 7,8-dihydroneopterin (DHN) in two different steps. In the next step, the lateral side chain of 7,8-dihydroneopterin (DHN) is cleaved to 6-hydroxymethyl-7,8-dihydropterin (HMDHP) and glycolaldehyde by the action of the enzyme dihydroneopterin aldolase.

Para-aminobenzoic acid (PABA) is produced in the plastids from chorismate in two distinct reactions catalyzed by amino deoxychorismate synthase (ADCS) and amino deoxychorismate lyase. The 6-hydroxymethyl-7,8-dihydropterin (HMDHP) and para-aminobenzoic acid (PABA) subunits are assembled to form tetrahydrofolate (THF; H_4folate) in the mitochondria. HMDHP is preliminarily transformed into 6-hydroxymethyldihydropterin pyrophosphate (HMDHP-P_2) by the enzyme HMDHP pyrophosphokinase, before being attached to para-aminobenzoic acid (PABA) to form dihydropteroate (DHP). The bond between dihydropteroate (DHP) and glutamate in a reaction catalyzed by the enzyme dihydrofolate synthase leads to the formation of dihydrofolate (DHF; H_2folate). The reduction of dihydrofolate (DHF; H_2folate) to tetrahydrofolate (THF; H_4folate) is mediated by the enzyme DHF reductase-thymidylate synthase. The polyglutamation of THF-Glu_n occurs by the enzyme folylpolyglutamate synthase (FPGS) which it adds sequentially γ-lined glutamate residues to tetrahydrofolate (THF; H_4folate). Finally, monoglutamate and polyglutamate folates are transported to the vacuoles at which the polyglutamates can be further processed by the enzyme γ-glutamyl hydrolase (GGH) (Saini *et al.*, 2016; Wakeel *et al.*, 2018).

Cereal and cereal-based products represent an important source of folate in the diet both for their folate content and for their frequency of consumption (Boz, 2021). For example, in the IV revision of the Levels of Reference Intake of Nutrients and Energy for the Italian Population (SINU, 2014) it is reported that the group of cereals contributes 29% to the daily intake of total folate in the Italian diet, thus representing an elective source of dietary folate. The folate content in cereals varies according to factors such as genotype and growing conditions, on the one hand, and type of sampling and analytical methods adopted, on the other hand (Table 3) (Piironen *et al.*, 2009; Kariluoto *et al.*, 2010). Piironen *et al.* (2008) studied the variation in total folate contents of a significant number of wheat genotypes, grown in the same location in Hungary in a controlled manner, including 130 winter wheat and 20 spring wheat (*Triticum aestivum* var. *aestivum*), 10 durum wheat (*Triticum turgidum* var. *durum*), 5 diploid einkorn (*Triticum monococcum* var. *monococcum*), 5 tetraploid emmer (*Triticum turgidum* var. *dicoccum*) and 5 spelt cultivars (*Triticum aestivum* var. *spelta*). Durum wheat genotypes are characterized by the highest content in total folate varying in the range from 637 to 891 ng/g d.m., followed by tetraploid emmer (516-937 ng/g d.m.), spelt (505-647 ng/g d.m.), diploid einkorn (429-678 ng/g d.m.), winter wheat (364-774 ng/g d.m.) and spring wheat (323-741 ng/g d.m.). The results found by Piironen *et al.* (2008) regarding folate levels in durum wheat are

lower than those found by Giordano *et al.* (2015) which are on average equal to 1119 ng/g d.m. Piironen *et al.* (2008) also found that the total folate content of winter wheat genotypes was significantly higher with smaller kernel size and higher bran yield. These findings can be linked to the greater presence of the outer cell layers in the smaller kernels. In fact, it is well established that folates are unevenly distributed in wheat kernel, concentrating mainly in the outer layers and germ of the wheat kernel (Piironen *et al.*, 2008; Kariluoto *et al.*, 2010). It was demonstrated that the total folate content of wheat bran, 704-1600 ng/g d.m., was 4-fold higher than that found in flour, when the extraction rate was 74.7%, and 2.5-fold higher than that found in wheat kernel. In the wheat germ, additionally, the total folate content (2400 ng/g d.m.) was more than 3-fold higher than that found in wheat bran (700 ng/g d.m.) (Piironen, 2011).

Table 3. Total folate content in different cereals *(d.m.= dry matter).*

	Total folate content	**References**
Durum wheat (*Triticum turgidum* var. *durum*)	637-891 ng/g d.m. 1119 ng/g d.m.	Piironen *et al.* (2008) Giordano *et al.* (2015)
Tetraploid emmer (*Triticum turgidum* var. *dicoccum*)	516-937 ng/g d.m.	Piironen *et al.* (2008)
Spelt (*Triticum aestivum* var. *spelta*)	505-647 ng/g d.m.	Piironen *et al.* (2008)
Diploid einkorn (*Triticum monococcum* var. *monococcum*)	429-678 ng/g d.m.	Piironen *et al.* (2008)
Winter wheat (*Triticum aestivum* var. *aestivum*)	364-774 ng/g d.m.	Piironen *et al.* (2008)
Spring wheat (*Triticum aestivum* var. *aestivum*)	323-741 ng/g d.m.	Piironen *et al.* (2008)
Barley (*Hordeum vulgare* L.)	518-789 ng/g d.m. 563-773 ng/g d.m.	Andersson *et al.* (2008) Edelmann *et al.* (2013)
Rye (*Secale cereale* L.)	574-775 ng/g d.m.	Nyström *et al.* (2008)
Oat (*Avena sativa* L.)	571-604 ng/g d.m.	Shewry *et al.* (2008)

Mullin & Jui (1986) analyzed the folate content of bran from different wheat classes and found high levels of folate ranging from 1840 to 4140 ng/g d.m. Furthermore, the

application of an innovative milling technology with the isolation of the aleurone cell layer and the splits of the aleurone cell walls led to the obtaining of a novel wheat aleurone flour characterized by a high folate content (5150 ng/g d.m.) (Fenech *et al.*, 1999).

Durum wheat has a higher folate content than that found in other cereal grains. Total folate content in 10 barley cultivars reported by Andersson *et al.* (2008) ranged from 518 to 789 ng/g d.m., in line with the results found by Edelmann *et al.* (2013) in 5 barley cultivars varying on average from 563 to 773 ng/g d.m. Nyström *et al.* (2008) found a total folate content in 10 rye cultivars variable from 574 to 775 ng/g d.m. and close to that found by Andersson *et al.* (2008) and Edelmann *et al.* (2013) in barley cultivars. On the other hand, a total folate content between 571 to 604 ng/g d.m. was found in 5 oat cultivars in a study conducted by Shewry *et al.* (2008).

Although the official method for the analytical determination of total folate is the microbiological one, the chromatographic methods allow the identification and quantification of the individual folate vitamers. Several authors agree that 5-formyltetrahydrofolate (5-HCO-H_4-folate) is the main vitamer present in wheat, accounting for about 50% of all the vitamers (Müller, 1993; Gujska & Kuncewicz, 2005; Piironen *et al.*, 2008). 5-Formyltetrahydrofolate (5-HCO-H_4-folate) was the main vitamer (on average >40%) detected in winter, spring and durum wheat genotypes by Piironen *et al.* 2008, while in spelt, diploid and tetraploid genotypes the same vitamer represented a lower percentage equal to about 34%.

5-Formyltetrahydrofolate (5-HCO-H_4-folate) are followed in lower quantities by 10-formylfolic acid (10-HCO-PGA) and folic acid in wheat kernels (Piironen *et al.*, 2009). However, the distribution of the different folate vitamers differs in quantitative terms in different studies; for example, Gujska & Kuncewicz (2005) found that after 5-HCO-H_4 folate 5-CH_3-H_4 folate and 10-HCO-PGA were the most abundant. 5-CH_3-tetrahydrofolate (5-CH_3-H_4 folate) and tetrahydrofolate (THF) were also detected in wheat kernels (Piironen *et al.*, 2009). 5-CH_3-tetrahydrofolate (5-CH_3-H_4 folate), together with formylated folates, constitute the most representative folate vitamers of the wholemeal rye flour according to the results found by Kariluoto *et al.* (2001). Tetrahydrofolate (H_4) and folic acid are present in small quantities and represent about 4-7% of all vitamers in wholemeal rye flour (Kariluoto *et al.*, 2001). Folates in wheat predominantly occur as polyglutamates (Piironen *et al.*, 2009). The polyglutamate chain of dietary folates is hydrolized by the enzyme glutamate carboxypeptidase II (GCPII) present in the human intestinal brush-border membrane, before absorption in the proximal

small intestine. After hydrolysis, folate enters the cell mainly through the proton-coupled folate transporter (PCFT) expressed on the intestinal apical cellular membrane and, at this level, it is again converted to a polyglutamylated form by the enzyme folylpolyglutamate synthase (FPGS). However, the removal of terminal glutamate residues by the enzyme γ-glutamyl hydrolase (GGH) is necessary to facilitate the passage of folates out of the intracellular environment. In fact, folate circulate through the bloodstream typically as monoglutamate and occur in an unbound form or is bound to albumin or to soluble folate receptors. 5-methyltetrahydrofolic acid (5-CH_3-H_4folate) represents the form of folate most present in the systemic circulation. Folate is selectively conveyed inside the cells of target tissues, through specific membrane transporters such as folate receptors (FRs), reduced folate carrier (RFC) and proton-coupled folate transporter (PCFT), within which it is again subjected to the action of the enzyme folylpolyglutamate synthase (FPGS) and where it can perform its biochemical functions. Folic acid used in supplements and fortified or enriched foods is reduced first to dihydrofolate (H_2folate) and, then, to tetrahydrofolate (H_4folate) by the enzyme dihydrofolate reductase in the passage from the small intestinal mucosa to the liver and, finally, methylated to become metabolically active (Warzyszynska & Kim, 2014; Strandler *et al.*, 2015).

The relative bioavailability, that is defined as *"the proportion of a nutrient ingested that becomes available to the body for metabolic processes or storage"* (Melse-Boonstra *et al.*, 2002), of polyglutamates folates is lower than that of monoglutamates folates and has values between 60% and 80%, while the same relative bioavailability of dietary polyglutamates folates is estimated to be only 50% compared with synthetic folic acid (Iyer & Tomar, 2009). However, it must be considered that the data relating to folate bioavailability are not conclusive given that many dyscrasias emerge in this regard in several studies. Moreover, it is not only the greater presence of polyglutamate forms of folate in wheat and other foods that reduces the bioavailability of the same folates, but also numerous other factors such as the food matrix, the presence specific conjugase inhibitors responsible for intestinal folate absorption, food preparation methods and other factors related to the human genotype (Fenech *et al.*, 1999; Piironen *et al.*, 2009; Istituto Superiore di Sanità https://www.epicentro.iss.it/acido-folico/Biodisponibilita; Zappacosta *et al.*, 2013). However, a study conducted by Fenech *et al.* (1999) showed that endogenous folate in wheat aleurone flour is readily bioavailable and, consequently, the inclusion of foods made with wheat aleurone flour in the diet may represent an important strategy for increasing folate intake levels in the general population.

In order to take into account the differences related to the higher bioavailability of folic acid compared to dietary folate, the Institute of Medicine (IOM, 1988) has developed the definition of "dietary folate equivalents (DFEs)". DFEs are expressed in the following ways:

- 1 μg DFE= 1.0 μg food folate= 0.6 μg folic acid folic acid from fortified food or as a supplement consumed with food= 0.5 μg of a folic acid supplement taken on an empty stomach;
- 1 μg folic acid as a fortificant= 1.7 μg DFE;
- 1 μg folic acid as a supplement= 2.0 μg DFE (European Food Safety Authority, 2014; Ferrari *et al.*, 2015).

Dietary folate equivalents were used to express the quantities for the *Dietary Reference Intakes* (*DRIs*) for folate, the *Recommended Dietary Allowance* (*RDA*) and the *Estimated Average Requirement* (*EAR*), in the American population, in many other European countries such as Belgium, Germany, Ireland, and also by the WHO/Food and Agriculture Organization of the United Nations since 2004. In Italy, neverthless, the *Dietary Reference Intakes* (*DRIs*) for folate are expressed as "total folate" in the IV revision of the Levels of Reference Intake of Nutrients and Energy for the Italian Population, without considering the differences in the bioavailability of the different forms of folate (SINU, 2014; Istituto Superiore di Sanità https://www.epicentro.iss.it/acido-folico/Biodisponibilita).

1.2.2 Health benefits

Folates are compounds of vital importance for human health as they are involved in numerous metabolic and biochemical processes. They act as cofactors in one-carbon transfer reactions, including reactions that lead to the biosynthesis of nucleotides (DNA and RNA biosynthesis), thymidylate, amino acids glycine, histidine, methionine and serine (protein biosynthesis) and some vitamins and homocysteine remethylation (Delchier *et al.*, 2016; Ducker & Rabinowitz, 2017; Azzini *et al.*, 2020). 5-Methyltetrahydrofolate ($5CH_3$-H_4folate), the coenzimatically active form of the vitamin that accepts, transfers and donates C1 groups, acts by transferring a methyl group to homocysteine with consequent production of methionine and tetrahydrofolate (THF) (Warzyszynska & Kim *et al.*, 2014; Strandler *et al.*, 2015; Saini *et al.*, 2016). Methionine, in turn, can be converted to S-adenosylmethionine (SAM) following activation by ATP

and methionine adenosyltransferase (SAM synthase). S-Adenosylmethionine (SAM) acts as a cofactor and main methyl group donor taking part in numerous methylation reactions, including methylation of DNA, RNA and proteins (Warzyszynska & Kim *et al.*, 2014; Bailey *et al.*, 2015). Folates are also involved in the processes of repairing and maintaining of the structural integrity of DNA, regulation of gene expression and are required for normal cell division and tissue development (Warzyszynska & Kim *et al.*, 2014; Gazzali *et al.*, 2016; Saini *et al.*, 2016). Consequently, it is essential for human health to maintain an adequate nutritional folate status since folate deficiency is related to a wide range of health problems, such as megaloblastic anemia, neural tube defects (NTDs) and other congenital disorders, depression and cognitive dysfunction, certain types of cancer (colorectal, breast, and prostate cancer) and cardiovascular diseases (Bailey & Gregory III, 1999; Iyer & Tomar, 2009; Warzyszynska & Kim *et al.*, 2014; Ebara, 2017; Azzini *et al.*, 2020).

The results obtained from randomised controlled trials (RCTs) have shown that there is a positive correlation between folic acid supplementation and a reduced risk of incidence of NTDs during pregnancies (European Food Safety Authority, 2009). In fact, metabolic requirements for folate become particularly high during pregnancy, as well as during lactation, and if this increased demand is not met, a maternal folate deficiency can occur as megaloblastic anemia. A maternal folate deficiency can also lead to premature birth, low birth-weight, including an increased risk of NTDs (especially spina bifida and anencephaly) in the unborn (Iyer & Tomar, 2009; Warzyszynska & Kim *et al.*, 2014). The inclusion in the diet of foods naturally rich in folates together with periconceptional supplementation with folic acid have been shown to be overall effective in reducing the incidence of NTDs and other congenital malformations, as shown by several consolidated scientific evidences (Turascio *et al.*, 2014). Given these evidences many governments have recommended folic acid supplementation for women of childbearing age, during the periconceptional period and for the duration of pregnancy (Buttriss, 2004; Warzyszynska & Kim *et al.*, 2014). The results of a randomised double-blind prevention trial conducted in 33 centers (17 in the UK and 16 in six other countries) and in which a total of 1817 women at high risk of having a pregnancy with a neural tube defect, because of a previous affected pregnancy, were included have highlighted a 72% decreased risk of NTDs in women with a previous NTD affected pregnancy following periconceptional FA supplementation of 4 mg per day; by contrast no significant reduction in risk of NTDs was found following supplementation with a multivitamin lacking folic acid (MRC

Vitamin Study Research Group, 1991). Another randomised double-blind clinical trial conducted in Hungary showed a significant reduction in risk of early cases of NTDs due to the use of a vitamin supplement contained 0.8 mg of folic acid (Czeizel & Dudás, 1992).

This evidence has led to the implementation of mandatory folic acid fortification policies in many countries. In the United States, Canada and Costa Rica mandatory folic acid fortification of white wheat flour, cornmeal and enriched pasta at levels of 140 μg/100 g, 150 μg/100 g and 180 μg/100 g, respectively, was firstly introduced in 1998 (Crider *et al.*, 2011; Warzyszynska & Kim *et al.*, 2014). A greater reduction in NTDs incidence, from 19% to 55% was observed in Canada, South Africa, Costa Rica, Chile, Argentina and Brazil after folic acid fortification; while a reduction in NTDs incidence of between 19%-32% was observed in the United States after folic acid fortification (Crider *et al.*, 2011). Recently, the mandatory folic acid fortification of non-wholemeal wheat flour was also undertaken in the United Kingdom (Wald & Hoffbrand, 2021). In Europe, however, there is only voluntary fortification of food products, that is to say the addition of vitamins or minerals to foods at the discretion of the manufacturer in order to restore micronutrients, ensure the nutritional equivalence of substitute foods, and/or to enhance the nutritive value of a product, which fortification is governed by the European Regulation No 1925/2006 of the European Parliament and of the Council of 20 December 2006 on the addition of vitamins and minerals and of certain other substances to foods and Regulation No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers (Hennessy *et al.*, 2013; Samaniego-Vaesken *et al.*, 2017).

Several studies have pointed out that a low folate status is a risk factor for different types of cancer such as colorectal and pancreatic cancer. A meta-analysis conducted by Kennedy *et al.* (2011) reported that a high folate intake reduces the risk of colorectal cancer by 8-15%. Chittiboyina *et al.* (2018) found that red blood cell (RBC) folate levels, which are indicative of folate levels of tissues, were significantly lower in pancreatic cancer cases (508.4 ± 215.9 ng/mL) if compared with those of unrelated healthy controls (588.3 ± 229.2 ng/mL), on the contrary no significant differences emerged between them with respect to serum folate levels, which are instead indicative of recent folate intake. High intake of folate has been found to reduce the risk for cancers of the head and neck, oral cavity and pharyngeal, esophagus, pancreatic and bladder by about 40%, 35%, 41%, 34% and 16%, respectively. The risk of breast cancer decreased when dietary folate intake

was between 153 and 400 µg, but this protective effect remained unchanged with dietary folate intake >400 µg compared to <153 µg (Pieroth *et al.*, 2018).

Alongside with the aforementioned positive effects on human health deriving from folic acid supplementation, it is necessary to consider that the ingestion of high levels of folic acid has been associated with potential risks of adverse human effects, including the risks of masking the anemia caused by vitamin B12 deficiency, which can cause irreversible neurological damage, and the presence of unmetabolized folic acid in the blood, which could be related to cognitive impairment in aged persons, even if these evidences are not conclusive (EFSA, 2009; Crider *et al.*, 2011).

Likewise, high folic acid intake levels could also be related with the risk of developing various cancers. Results from animal studies seem to suggest a double modulatory effect of folic acid on cancer risk, depending on the timing of exposure to folic acid and dose of the intervention; in normal tissues, high intakes of folic acid would inhibit the development of early neoplastic lesions, while they would accelerate the proliferation and growth of established pre-neoplastic lesions (European Food Safety Authority, 2009; Patel & Sobczyńska-Malefora, 2017). Stolzenberg-Solom *et al.* (2006) conducted an observational study which found that supplements of folic acid of 400 µg or more per day may be associated with an increased risk of breast cancer in postmenopausal women, compared to those who do not take such supplements. Results from a randomized controlled trial showed a 67% increase in the risk of advanced lesions with high malignant potential, high-grade dysplasia or invasive adenocarcinoma in patients, with a history of colorectal adenoma, who have received a daily supplementation of 1 mg of folic acid, compared to patients randomized to the placebo group, after a 6-year follow-up colonoscopy (Cole *et al.*, 2007).

The scientific evidence available to date does not show an association between a high intake of folic acid and the risk of cancer and the European Food Safety Authority, convened a working group at Uppsala in 2009 to review evidence relating to the potential risks of high folic acid intakes, concluded that *"there are currently insufficient data to justify such an assessment and that current evidence does not show an association between high folic acid intakes and cancer risk but neither do they confidently exclude a risk"* (EFSA, 2009).

Other negative effects of high folic acid intake such as the potential acceleration of cognitive decline and the reduction of the efficacy of antifolate drugs have been hypothesized, although other evidence is needed for the latter (EFSA, 2009).

In conclusion, data from epidemiological studies demonstrate the importance of maintaining an adequate intake of folate to counteract the increase in plasma homocysteine levels and, consequently, reduce the risk of cardiovascular diseases (Sauer *et al.*, 2009). However, it has been suggested that there may be a positive correlation between low folate status and an increased risk of coronary heart disease even independently of homocysteine levels (Clarke *et al.*, 2011). The effects of folic acid supplementation on the reduction of plasma homocysteine levels and the incidence of cardiovascular diseases need further studies. The results of a meta-analysis showed how folic acid supplementation reduced homocysteine levels by 23%, or 30% if combined with vitamin B_{12}, in populations without mandatory folic acid fortification and how, at the same time, this reduction effect was less pronounced in populations with the same fortification in force (Homocysteine Lowering Trialists' Collaboration, 2005). Nevertheless, a meta-analysis of 8 large randomized placebo-controlled trials revealed that folic acid supplementation did not have a significant effect on vascular events over a 5-year follow-up period, although a significant 25% reduction in homocysteine levels was observed from the same supplementation (Clarke *et al.*, 2010).

1.2.3 Processing impacts

Given the positive effects on human health deriving from an optimal dietary folate intake and since most cereals are consumed after being processed, it is important to consider the impact of processing on folate content. As mentioned above, the folate content in cereals can vary depending on factors such as genotype and growing conditions (Piironen *et al.*, 2009). However, the degree of milling represents the main factor determining the folate content in flour. Flours with a low extraction rate retain less folate than whole-meal flours. Wheat flour with an extraction rate of 66% had a folate value that is 10% lower than that of whole-meal flour (Hegedüs *et al.*, 1985; Piironen *et al.*, 2011). Liang *et al.* (2020) characterized flours with a 70% extraction rate from six wheat cultivars for folate content and found an average folate loss of 71% due to the milling process. This further confirms the greater localization of these micronutrients in the outermost layers and in the germ of the wheat grain. Fenech *et al.* (1999) studied the folate content of a wheat aleurone flour obtained from an innovative milling process which resulted in the inclusion of both aleurone cells and germ in this flour. Wheat aleurone flour was characterized by a folate content equal to 515 μg/100 g f.w. and higher than that found in wheat bran flour equal to 94 μg/100 g f.w. obtained, the latter, within

the same milling protocol. The folate in wheat aleurone flour was also bioavailable. Hemery *et al.* (2011) showed instead how the use of the electrostatic separation process led to obtaining purer fraction rich in aleurone cells with a high content of folate (on average 119 μg/100 g d.m.), starting from ultrafine wheat bran. Also the debranning or pearling process, before milling, can be used to select intermediate fractions of the cereal grains rich in folates. Giordano *et al.* (2015) studied the distribution of folates in the pearling fractions of durum wheat by observing that the first three fractions (0-5%; 5-10%; 10-15%) had a high folate content equal to about 2.5-fold higher compared to that found in the starting durum wheat. A similar trend was confirmed in the initial pearled fractions of common wheat (Giordano *et al.*, 2015).

The germination process may result in a significant increase in the folate content of cereals. Kariluoto *et al.* (2006) considered two rye cultivars and found a folate content increased up to 3.8-fold that found in the native samples after a 6-day germination period at 25 °C, while Hefni & Witthöft (2012) highlighted an increase in folate content from 45% up to 75% in four cultivars of wheat and six cultivars of rye after a 96 h germination period at 25 °C and found no significant loss of folate due to the oven-drying process (50 °C, 48-72 h) applied after germination.

Folates are extremely sensitive compounds to a wide range of factors, including UV light, oxygen, air, pH, antioxidants and metal ion concentrations, heat treatment, time and product:water ratio, which can occur during food processing and which can lead to their degradation or inter-conversion (McKillop *et al.*, 2002; Strandler *et al.*, 2015; Wusigale & Liang, 2020; Boz, 2021).

Kariluoto *et al.* (2004) noted an increase in folate content during sourdough fermentation used in both rye and wheat bread baking. In fact, the growth of yeast resulted in an increase in the folate content both for the high folate content and for the ability to synthesize folate itself, with consequent mitigation of folate losses during baking. Losses of folate during rye and wheat baking found by Kariluoto *et al.* (2004) in this study were approximately 25%. These results are in agreement with the conclusions reached by other authors who found a loss of folate related to wheat bread baking of about 20-34% (Butterfield & Calloway, 1972; Keagy *et al.*, 1975; Osseyi *et al.*, 2001; Arcot *et al.*, 2002).

Folate losses may also occur during food storage. Liang *et al.* (2020) found a different trend in the loss of folate in six wheat cultivars stored in the forms of grain and powder at room temperature for 2-8 months. A significant loss of 17% was observed in the grains after 6 months of storage, while in the powder of three wheat cultivars it began after only

2 months of storage and was equal to 21%. Similarly, Gujska *et al.* (2009) reported a significant decrease in folate content from a storage period of 5 weeks in both bread leavened with baker's yeast and bread made with sourdough seeds equal to an average of 14%, reaching 25% in bread leavened with baker's yeast and 38% in the other bread type after 6 weeks of storage.

Bui & Small (2007) evaluated the impact of cooking on folate retention in 26 commercial samples of noodles of different brands. What emerged is that the loss of folate was variable in the range from 15% to 30% for white and yellow noodles, while for instant noodles the loss of these nutrients was very low and between 4% and 6%. Overall, these results were in agreement with those of Liang *et al.* (2020) who found an average of 13% folate loss in noodles caused by boiling degradation. The folate losses found for noodles after cooking (Bui & Small, 2007) were comparable to those found for cooked pasta equal to an average of 21% (Ranhotra *et al.*, 1985).

1.2.4 Folates as functional food ingredients

As discussed in the previous paragraph, maintaining an adequate nutritional status of folate is essential in order to prevent a broad spectrum of human health problems. In this sense, folates can represent important ingredients for the development of so-called "functional foods". In Europe, the definition of "functional food" was initially developed within the European Commission Concerted Action on Functional Food Science in Europe (FUFOSE) project with the coordination of the International Life Sciences Institute (ILSI Europe) and published in the *British Journal of Nutrition* in 1999. In the Scientific Concepts of Functional Foods in Europe Consensus Document it is established that *"A food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease"* (ILSI Europe, 1999). Although functional foods do not adhere to a certain European regulatory framework, they can nevertheless convey substances that are, by contrast, regulated in the European Union (Domínguez Díaz *et al.*, 2020). Consequently, functional foods containing specific substances will be able to boast the relative nutrition and health claims on the label and therefore meet the requirements of Regulation (EC) No 1924/2006. In the Article 2.2 of Regulation (EC) No 1924/2006 "nutrition claims", "health claims" and "reduction of disease risk claims" are distinguished. "Nutrition claim" is any claim that states, suggests or implies that a food

has particular beneficial nutritional properties due to its calorific value and the nutrients or other substances that it contains in reduced or increased proportions or that it does not contain. “Health claim” refers to claim that states, suggests or implies that a relationship exists between a food category, a food or one of its constituents and health. The European Commission authorizes health claims using a complex approval process, which includes a careful assessment of the scientific evidence available on the claims for which it is responsible the European Food Safety Authority (EFSA). “Reduction of disease risk claim” refers to health claim that states, suggests or implies that the consumption of a food category, a food or one of its constituents significantly reduces a risk factor in the development of a human disease.

There are currently eight health claims and one reduction of disease risk claim related to folate authorized by the EFSA and listed in the European Register of nutrition and health claims made on foods (European Commission, 2019). While health claims can only be declared in those foods that are at least a “Source of folate”, that is to say a content of at least 15% of the nutrient reference values (NRVs) (200 µg for folic acid) supplied by 100 g or 100 ml in the case of products other than beverages (Regulation (EC) No 1924/2006, Regulation (EU) No 1169/2011 and Regulation (EU) No 432/2012), the reduction of disease risk claim can only be used in the labeling, presentation, and/or advertising of food supplements that provide a daily intake of at least 400 µg of folic acid. As for the reduction of disease risk claim, the target population is women of child-bearing age and the beneficial claimed effect is obtained with a supplemental folic acid intake of 400 µg per day for at least one month before and up to three months after conception (EFSA, 2013; Commission Regulation (EU) No 1135/2014). In US regulation, on the other hand, two health claims related to folate are approved by the Food and Drug Administration (FDA) to appear on the labeling of foods and food supplements, in particular one falling under the “authorized health claims” and the other between the “qualified health claims” (FDA, 2018; FDA, 2022). Health claims for folate approved in the European and American legislation are summarized collectively in Table 4.

Table 4. Health claims for folate in force in EU and US legislation.

Health claims and reduction of disease risk claim in EU	
Health claims	Folate contributes to maternal tissue growth during pregnancy
	Folate contributes to normal amino acid synthesis
	Folate contributes to normal blood formation
	Folate contributes to normal homocysteine metabolism
	Folate contributes to normal psychological function
	Folate contributes to the normal function of the immune system
	Folate contributes to the reduction of tiredness and fatigue
	Folate has a role in the process of cell division
Reduction of disease risk claim	Supplemental folic acid intake increases maternal folate status. Low maternal folate status is a risk factor in the development of neural tube defects in the developing foetus.
Authorized and qualified health claims in US	
Authorized health claim	Healthful diets with adequate folate may reduce a woman's risk of having a child with a brain or spinal cord defect.
Qualified health claim	Folic Acid, Vitamin B6, and Vitamin B12 and Vascular Disease
	Folic Acid and Neural Tube Birth Defects

1.3 Whole-meal pasta

1.3.1 Definition of whole grain and whole grain food

The market for whole grains as food ingredients and whole grain foods is experiencing positive growth worldwide. Despite the ever increasing availability of whole grain products and the positive perception associated with the consumption of the same products by consumers, the inclusion of whole grain foods in the diet remains low and, in any case, such as not to satisfy the dietary recommendations for whole grain intake. An explanation for this could be traced back to the lack and difficulties in establishing a uniform global definition of whole grain, related to issues such as the extent of processing allowed and which cereals should be included in the definition (O'Donovan *et al.*, 2019). Aiming to develop a clear and coherent definition of whole grain globally and the minimum amount of whole grains that a food must contain in order to be labeled as "whole grain" should be a top priority for the many benefits that would ensue,

including which improved diet quality, increased consumer confidence, accuracy of surveillance research to promote improved public health and more precise assessment of whole grains intake aimed at drafting recommendations for whole-grain intake to be included in dietary guidelines (Miller, 2020). A first step in this direction was taken in the United States in 2000 by the American Association of Cereal Chemists (AACC) (now the Cereals & Grains Association) who proposed the following widely cited and used internationally definition of whole grain: *"Whole grains shall consist of the intact, ground, cracked or flaked caryopsis, whose principal anatomical components - the starchy endosperm, germ and bran - are present in the same relative proportions as they exist in the intact caryopsis"* (AACC, 2000). The AACC definition includes "all" cereals from the *Poaceae* family and pseudocereals grains (amaranth, buckwheat and quinoa). In 2013, AACC International also proposed a first definition of "whole grain food", as follows: *"A whole grain food must contain 8 grams or more of whole grain per 30 grams of product"* (AACCI, 2013). The distinction of 8 grams of whole grains per 30 grams of product was made to take into account food products that include refined grains, which currently enjoy higher levels of consumer acceptance. In 2010, in the course of the HEALTHGRAIN European project which saw the participation of a large number of universities and food industries, the HEALTHGRAIN consortium released the following definition of whole grain: *"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 - that is, less than 2% of the grain/10% of the bran - that occur through processing methods consistent with safety and quality are allowed"* (van der Kamp *et al.*, 2014). The development of this definition was conceived in order to have a single definition of whole grain to be used throughout Europe, on the one hand, and to have a definition outside of Europe that can be equivalent with particular reference to the one proposed by AACCI, on the other side (van der Kamp *et al.*, 2014). The definition of whole grain developed by the HEALTHGRAIN consortium includes all cereals and pseudocereals grains included in the AACCI definition of whole grain. The HEALTHGRAIN Forum also suggested that *"whole-grain food products should contain at least 30% whole grain ingredients on a dry-weight basis and more whole-grain ingredients than refined-grain ingredients"* (Ross *et al.*, 2017). By choosing to base this definition on dry weight, it follows that even products with a high moisture content could be defined as whole grain. More recently,

the Definitions Working Group of the Global Whole Grain Initiative (WGI), with gathers experts from academia, government agencies and industry from Asia, Europe, North and Latin America, Oceania and Africa, developed definition of whole grain as food ingredient and definition of whole grain food for global application approved by the leading international scientific associations, the Cereals & Grains Association, the HEALTHGRAIN Forum and the International Association for Cereal Science and Technology (ICC). As stated in van der Kamp *et al.* (2022), *"Whole grains shall consist of the intact, ground, cracked, flaked or otherwise processed kernel after the removal of inedible parts such as the hull and husk. All anatomical components, including the endosperm, germ, and bran must be present in the same relative proportions as in the intact kernel"*. In the latter definition, in addition to being included cereal grains of the *Poaceae* grass family and pseudocereals grains (amaranth, buckwheat and quinoa), the inclusion of newly developed species of cereal grains when they are accepted by the relevant authoritative groups is also envisaged (van der Kamp *et al.*, 2022). For the most part, whole grain products are made from flours obtained through recombination and reconstitution of multiple millstreams. In the recombination process the different grain fractions channeled into separate millstreams are reunited at the mill in the last step of the milling process so that the original proportions of bran, germ and endosperm are respected in the whole grain flour, while in the reconstitution process different grain fractions from milling process are reunited away from the mill by the food manufacturer, but both are allowed in the WGI definition (Jones *et al.*, 2015; van der Kamp *et al.*, 2022). With respect to the definition of whole grain food, the definition proposed by WGI is the following: *"A whole-grain food shall contain at least 50% whole-grain ingredients based on dry weight"*. WGI also suggested that *"Foods containing a minimum of 25% whole-grain ingredients based on dry weight, may make a front-of-pack claim on the presence of whole grain but cannot be designated 'whole grain' in the product name"* (van der Kamp *et al.*, 2022).

Also in Italy the concept of "whole grain" lacks of regulatory clarity as is clearly shown in the Italian Presidential Decree No 187/2001 "Regulation for the revision of laws concerning the production and sale of milling products and pasta, pursuant to Article 50 of Law N° 146, dated 22 February 1994", where there is no match between the definition and the composition of whole grain flour and whole grain semolina. It is desirable to resolve these regulatory inconsistencies since they lead to the use of incorrectly defined, and therefore diversified, whole grain flour and whole grain semolina, with the placing

on the market of whole grain products with different nutritional characteristics and, consequently, confusion among consumers.

1.3.2 Nutritional quality and health benefits

Within the last approximately ten years, the increasingly consolidated scientific evidence about the health benefits of taking whole grains has led governmental authorities and scientific organisations to issue specific recommendations for whole grains (Nugent & Thielecke, 2019; Kyrø & Olsen, 2021). In the 4th edition of the European Code against Cancer where "12 ways to reduce cancer risk" are described it is recommended to have a healthy diet with inclusion of plenty of whole grains, pulses, vegetables and fruits (Schüz *et al.*, 2015). The consumption of whole grains is recommended in the dietary guidelines of many countries, however the intake of whole grains is often below those recommendations. Dietary guidelines vary from being quantitative in the USA, where recommended daily consumption of whole grain is set at least 85 g/d for adults (Reicks *et al.*, 2014) and 42 g/d for children (McGuire, 2011), and Denmark, with a recommended daily consumption of whole grain set at 75 g/10 MJ per day (Frølich *et al.*, 2013), and qualitative in which it is only suggested to regularly consume cereals preferably whole in many other countries including Italy (CREA, 2018). The highest intake of whole grain was recorded in Scandinavia, Germany and Ireland, while it was low in Singapore, France, the Netherlands, the United Kingdom and the United States. Intake of whole grain was highest in adolescents and children in Denmark, Ireland and Germany and was greater than 20 g per day; on the other hand, in the same categories the intake was lower and was equal to or less than 15 g per day in France, the United Kingdom and the United States. Among adults, whole grain intake was highest in Scandinavian countries, while it was low at around 10 g per day in France, the United Kingdom, the United States, the Netherlands and Singapore (Kyrø & Olsen, 2021). Interestingly, the average intake of whole grain has recorded a positive growth trend in both children and adults of approximately 33 g per day in the years 2000-2004 up to 58 g in the years 2011-2013, suggesting how the Danish Whole Grain Campaign has proven very effective in raising consumer awareness of the positive health effects of eating whole grains (Ross *et al.*, 2017; Kyrø & Olsen, 2021). In Italy, the latest data on whole grain consumption derive from the INHES study consisting of a 3-year telephone-based survey on nutrition and health specifically designed to collect information on dietary habits (quality, quantity and meal patterns), food choice determinants, and food health awareness of the Italian

population according to different geographical distribution (Northern, Central and Southern Italy), age, gender and socioeconomic status, which was conducted between 2010 and 2013 and included a sample of 9422 women and men aged ≥ 5 years (Ruggiero *et al.*, 2019). What emerged from this study is that about 22% of children/adolescents and about 27% of adults report regular consumption of whole grain products (≥ 1 times/week), especially bread in both children/adolescents and adults (Ruggiero *et al.*, 2019). These results are in line with those derived from INRAN-SCAI 2005-2006 survey where approximately 24% of the children/adolescents sample and approximately 20% of the adults sample reported consumption of whole grain products during the 3-day registration period, with an average intake of whole grain of 2.1 g per day in children/adolescents and 3.7 g per day in adults (Sette *et al.,* 2017).

Whole grain products are excellent sources of compounds of nutritional interest such as vitamins, minerals, dietary fibre and phytochemicals (Slavin *et al.*, 2013). Among these, pasta can represent an important carrier of bioactive compounds in view of the diffusion, popularity and appreciation among consumers for its versatility, ease of transportation, handling, cooking and storage properties, availability in numerous shapes and sizes, high digestibility, good nutritional qualities and relatively low cost (Padalino *et al.*, 2017). Whole meal pasta, in particular, in recent years has recorded ever increasing numbers of diffusion on the market also due to the beneficial effects on human health widely recognized by consumers. A positive association between the consumption of whole grains and beneficial effects on human health has been widely recognized, even if the physiological mechanisms involved have not yet been precisely determined and the protective effect is generally attributed to the synergistic action of bioactive compounds mainly located in the bran and in the germ of the cereal grains (Fardet, 2010). Meta-analyses from of observational studies, and more precisely among these prospective cohort studies, consistently suggested a protective role of high whole grains consumption on the reduction of mortality from cardiovascular diseases, type 2 diabetes and cancers (Zhang *et al.*, 2018; Tieri *et al.*, 2020). From the analysis of twenty prospective cohort studies carried out by Benisi-Kohansal *et al.* (2016) it emerged that an increase of 3 servings total whole grains per day (90 g/day) was associated with a lower risk of mortality from all causes by 17% and, specifically, with a lower risk of mortality from cardiovascular disease by 25% and a reduced risk of total-cancer mortality by 10%. From a study conducted by Qi *et al.* (2006) aimed at evaluating the effect of whole grains and fibre on inflammatory markers among diabetic patients, it emerged that among women

with type 2 diabetes (T2D) the high intake of whole grains, bran and cereal fibre, resulted in a significant decrease in the levels of markers of inflammation related to T2D. The inclusion in the diet of three or more rations of whole grain products per day resulted in a 20 to 30% decrease in the risk of developing T2D (Gil *et al.*, 2011). It has also been shown that the habitual consumption of whole grain products, including whole-meal pasta, can be a valid strategy to combat obesity through regulation of appetite and satiety control (Costabile *et al.*, 2018). Costabile *et al.* (2018) conducted a study in which 14 health adults (7 males and 7 females, aged 20-50 years) were recruited and randomly assigned into two groups to consume whole-meal wheat pasta (117 g/day) or refined wheat pasta (100 g/day), finding that the consumption of whole-meal pasta significantly reduced the desire to eat by 16%, the feeling of hunger by 23% and the feeling of satiety was found to be 13% higher than that found by consuming refined pasta. Furthermore, two meta-analyses of 24 randomized controlled trials and 21 randomized intervention trials conducted by Ye *et al.* (2012) and Hollænder (2015), respectively, agreed that increased whole grain intake significantly lowered total cholesterol and LDL-cholesterol concentrations.

Although whole-meal pasta is a food with a high nutritional value, it is of fundamental importance to pay attention to a careful selection of raw materials, on the one hand, and to an appropriate calibration of the technological process, on the other hand (Marti *et al.*, 2017). High damage of starch granules and, consequently, of higher susceptibility to amylase activity, typical of whole-meal flours, determine the formation of reducing sugars and, as a direct consequence, of a more intense Maillard reaction (MR). Likewise, the drying phase during the pasta making process can strongly affect the nutritional quality of the final product (De Noni & Pagani, 2010; Marti *et al.*, 2017). The Maillard reaction is a chemical and non-enzymatic browning reaction that initiates through conjugation between carbonyl groups of reducing sugars and amino and imino groups of amino acids, peptides and proteins and that occurs during thermal processing and storage of foods (Hellwig *et al.*, 2018; Nooshkam *et al.*, 2019). Furosine has been widely proposed as a marker of the early stages of MR to evaluate heat damage in pasta and in cereal-based products, but, nevertheless, it cannot be considered a sufficient marker of pasta drying process as when the heat treatment becomes more intense, a progression of MR could occur such as to lead to the consumption of furosine and the consequent formation of other compounds (Cavazza *et al.*, 2013; Marti *et al.*, 2017; Hellwig *et al.*, 2018). In fact, to describe the thermal damage in pasta resulting from the advanced stage

of MR, other compounds are used, such as pyrraline, maltosine, formyline, known as "advanced glycation end products" (AGEs) (Hellwing *et al.*, 2018). AGEs have attracted the attention of many researchers since elevated serum levels of AGEs represent an important risk factor for the development of various metabolic disorders (Gill *et al.*, 2019). High concentrations of AGEs, especially from food sources, are related to oxidant stress and inflammation which, consequently, could lead to diabetes, atherosclerosis and diastolic dysfunction, Alzheimer's and Parkinson's diseases, inflammatory reactions, aging, kidney disease and cancer (Wei *et al.*, 2018; Nooshkam *et al.*, 2019).

In general, whole-meal pasta is characterized by higher furosine levels than those of semolina pasta. Marti *et al.* (2017) from the analysis of 22 samples of whole-meal pasta found variable furosine levels over a wide range ranging from 195 to 836 mg/100 g protein, and, as expected, the average furosine value equal to about 595 mg/100 g protein was higher than the average furosine value equal to about about 400 mg/100 g protein found for semolina pasta samples. The variability found in the furosine levels in the whole-meal pasta samples was due both to the different compositional characteristics of the raw materials and to the adoption of different drying cycles in the drying phase (Marti *et al.*, 2017). Similarly, from the pyrraline analysis conducted by Marti *et al.* (2017) on the whole-meal pasta samples it emerged that the level of pyrraline was on average equal to about 8.5 mg/100 g protein and that the lowest level (0.8 mg/100 g protein) was found in the sample characterized by the lowest furosine level (229 mg/100 g protein) while the highest level (15.8 mg/100 g protein) was found in the sample characterized by the highest furosine level (836 mg/100 g protein), therefore the use of high temperature drying cycles was conceivable for the samples that recorded the highest levels of both furosine and pyrraline. De Noni & Pagani (2010) reported that the highest values of furosine (> 500 mg/100 g protein) and the highest values of pyrraline (> 10 mg/100 g protein) were found when high temperature (> 75 °C) drying conditions applied to pasta with low moisture content (< 15–16%). Overall, the Maillard reaction has an important impact on the nutritional value of the pasta considering that during the early stages a decrease in nutritional availability of essential amino acids, first of all lysine, can be observed and, therefore, a decrease in the biological value of proteins (Nooshkam *et al.*, 2019), on the one hand, while in the advanced stage the formation of advanced glycation end products (AGEs) could negatively influence the digestibility of amino acids and, therefore, reduce the digestibility of proteins (Stuknytė *et al.*, 2014), on the other hand.

The peculiar characteristics of whole-meal pasta, such as darker color, more rough

texture, more bitter and branny flavor with possible formation of off-flavors during storage, compared to semolina pasta can represent important barriers to its consumption by consumers (Steglich *et al.*, 2015; Heiniö *et al.*, 2016). Pasta color results from a desirable yellow component, an undesirable brown component and a red component, under some drying conditions (Feillet *et al.*, 2000). The color characteristics of pasta can be strongly influenced both by the choice of raw materials and by processing conditions. High levels of damaged starch and a high α-amylase activity in semolina, favoring the formation of reducing sugars, also favor the development of the Maillard reaction during pasta drying phase, especially when high and very high temperature drying cycles are used (Sissons *et al.*, 2012). To conclude, in pasta making process the drying phase plays a fundamental role also with respect to the development of specific characteristics of texture and flavor. Boccacci Mariani *et al.* (2018) developed an HS-SPME/GC-MS (headspace solid-phase micro-extraction/gas chromatography-mass spectrometry) method that allowed to carry out an objective evaluation of the flavor of Italian durum wheat pasta by analyzing the volatile fraction in order to discriminate between pasta samples dried at low temperature for long time and pasta samples dried using more drastic drying conditions (high temperature-short time and very high temperature-very short time). This methodological approach was used by Giannetti *et al.* (2021) from the analysis of short-shaped dried pasta samples it emerged that the pasta samples dried rapidly at high or very high temperature compared to those dried at low temperature were more characterized by the presence of volatile compounds deriving from the initial or advanced phase of Maillard reaction, such as maltol, 2-furan-methanol and benzaldehyde, with greater flattening of the flavor. The HS-SPME technique could prove to be very useful for evaluating the flavor of whole-meal pasta. Indeed, West *et al.* (2013) demonstrated that the characterization of taste and flavor attributes of whole-meal pasta by a trained panel was not such as to allow the distinction between whole-meal pasta samples dried at low temperature for long time and whole-meal pasta samples dried at high temperature for short time. On the other hand, West *et al.* (2013) also found that drying had an influence on firmness attribute with whole-meal pasta dried at low temperature which was found to be more firm than whole-meal pasta dried at high temperature.

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Chapter 2

Effects of debranning and milling processes on total folate content of durum wheat

2.1. Introduction and objectives

Debranning technology, which is a process consisting in the removal of the outer grain bran layers by friction or abrasion operations, can be used to select and collect the peripheral layers of wheat grain, with a view to produce new functional food ingredients and/or to enrich specific products in micronutrients of interest (Hemery *et al.*, 2007; Singh & Singh, 2010; Martini *et al.*, 2015).

As extensively discussed in Chapter 1, folates are essential micronutrients for human health belonging to the water-soluble B group of vitamins (B9) which play a central role in numerous human metabolic and biochemical processes (Delchier *et al.*, 2016). It is critically important to maintain an adequate nutritional folate status since folate deficiency is connected with several health disorders, including megaloblastic anemia, neural tube defects (NTDs) in developing fetus, Alzheimer's disease, cardiovascular diseases and some types of cancer (Iyer & Tomar, 2009). Since cereals and cereal-based products, in particular whole-meal products, are an important source of folate in human diet, considering both their folate content and their frequency of consumption (Boz, 2021), the use of the milling and debranning fractions from durum wheat naturally enriched in folate could lead to the obtaining of functional products which can contribute significantly to the daily intake of folate and, above all, represent a valid alternative to fortified products. The development of naturally folate-enriched cereal products is even more important when considering how ingestion of high levels of synthetic folic acid could have negative effects on human health (Crider *et al.*, 2011).

On the other hand, if the demand for whole-meal products among consumers is always greater, the qualitative characteristics of whole-meal products are different because there is no univocal and clear definition of "whole-grain". It follows that it is important to identify "whole-grain" biomarkers which can be used to evaluate grain tissue proportions in grain fractions and, consequently, to judge the quality of whole-meal products (Hemery *et al.*, 2009).

The objective of this part of the research activity was to study the distribution of folates in durum wheat milling and debranning fractions obtained from a milling processing realized with both a pilot plant scale and an industrial scale, in order to select the fractions of flour naturally enriched in folates to be used for the production of whole-meal pasta.

For these purposes, two durum wheat samples of different origin were taken into consideration on which the total folate content was evaluated. The conventional roller

milling process and the debranning tests carried out on a pilot plant scale and with a laboratory debranner, respectively, led to the obtaining of milling and debranning fractions which were also characterized by the total folate content. Finally, two durum wheat grain blends belonging to different batches were used which were subjected to industrial debranning and milling processes and the total folate content was determined on the fractions obtained.

2.2 Experimental

Durum wheat samples

Two durum wheat (*Triticum turgidum* L. subsp. *durum*) grain samples, National (DWGN) and North America (DWGNA) origin, were provided by Casillo Group (Corato, BA, Italy).

The collection of two different batches of durum wheat (*Triticum turgidum* L. subsp. *durum*) grain blends (DWGI) grown at different locations (information not available) was also made at the industrial mill of the F.lli De Cecco di Filippo - Fara San Martino S.p.A. company located in Fara San Martino, Italy.

All grain samples were stored in sealed plastic bags at +4 °C and ground with a refrigerated laboratory mill (model IKA A10-IKA Werke GmbH & Co. KG, Staufen, Germany) before analysis.

Quality characteristics of grain samples

Test weight (kg/hL) was measured with a Shopper chondrometer equipped with a 250 mL cylinder. One thousand kernels weight (g) was determined by weighing 100 kernels and multiplying the result obtained by 10.

Grain samples milling and debranning

Laboratory trials

Aliquots of National and North America grain samples were conditioned to reach a moisture of about 17% and milled by conventional roller milling, without preliminary debranning process, using a hard wheat milling pilot plant “MLU 202” (Bühler, Uzwill, Switzerland) equipped with three break and three reduction rolls and six steel screens, fitted with a small-scale purifier, following the AACC approved methods 26-10A and 26-41 (AACC, 2000). Milling fractions are listed in Table 2.1.

The National and North America grain samples were also debranned by applying different debranning degree (3, 6, 9, 12, and 15%) with a laboratory debranner (Taka Yama mod. TM-05, Taiwan Province, China) obtaining the debranned grain and the debranning by-product. Debranning fractions are listed in Table 2.2.

Industrial trials

The two durum wheat grain blends, on the other hand, were conditioned to about 18% (first sampling) and 16% (second sampling) moisture and debranned before being milled using an industrial plant (Bühler, Uzwill, Switzerland). The debranning process was carried out in two sequential steps. The grains were first debranned at about 2% and, subsequently, the obtained debranned grains were further debranned to remove about 6% of the outer layers of the grains. The debranning by product obtained after the second debranning step was sorted in a plansichter equipped with six sieve stacks of different mesh sizes in order to obtain a fine bran fraction and a coarse bran fraction. A schematic graphic representation of the milling process and the fractions obtained is illustrated in Figure 2.1. Yields of samples were expressed as percentage of weight of the starting sample.

All the samples obtained from the debranning and milling processes were ground, when necessary, with a refrigerated laboratory mill (model IKA A10-IKA Werke GmbH & Co. KG, Staufen, Germany) and stored in sealed plastic bags at +4 °C before analysis.

Table 2.1. Milling fractions and related identification codes of National and North America durum wheat grains.

Milling fractions/Codes			
Break rolls fractions	**B1**	**B2**	**B3**
Reduction rolls fractions	**D1**	**D2**	**D3**
Bran	**B**		
Shorts	**S**		
Semolina	**SEM**		
Purifier by-product	**PP**		

Table 2.2. Debranning fractions and related identification codes of National and North America durum wheat grains.

Debranning fractions/Codes			
3% debranned grain	**3-DG**	3% debranning by-product	**3-DP**
6% debranned grain	**6-DG**	6% debranning by-product	**6-DP**
9% debranned grain	**9-DG**	9% debranning by-product	**9-DP**
12% debranned grain	**12-DG**	12% debranning by-product	**12-DP**
15% debranned grain	**15-DG**	15% debranning by-product	**15-DP**

Physico-chemical analysis

Moisture and ash contents of National and North America durum wheat grain samples and related debranning and milling fractions were determined according to ICC Standard Methods No. 110/1 and No. 104/1, respectively (ICC, 1995). Protein content (N × 5.70) was measured by Dumas combustion method with a Leco nitrogen analyzer, model FP-528 (Leco Corp., St. Joseph, MI, USA), according to AACC method 46-30 (AACC, 2000).

The two batches of durum wheat grain blends were, on the other hand, characterized by their moisture and ash contents according to ICC Standard Methods No. 110/1 (ICC, 1995) and International Standard ISO 2171:2007 (ISO, 2007), respectively. Protein content (N × 5.70) was determined with Kjeldahl method according to ICC Standard Method No. 105/2 (ICC, 1995).

Total folate content of all samples was determined using the microbiological assay kit (VitaFast® Folic Acid- Microbiological microtiter plate test to quantitate Folic Acid) distributed by R-Biopharm AG, Darmstadt, Germany, according to AOAC Official Method 2004.05 (De Vries *et al.*, 2005). Briefly, 1 g sample was extracted in 40 mL phosphate buffer (0.05 mol/L, 0.1% ascorbate, pH 7.2) and 10 mg chicken pancreatin (VitaFast® Chicken Pancreatin (γ-Glutamylhydrolase)), which was once again purchased from R-Biopharm AG, Darmstadt, Germany. The extracts were diluted depending on the concentration range and, then, inoculated into the wells of a 96-wells microtiter plate coated with *Lactobacillus rhamnosus*, together with the culture medium. After an incubation period of 48 hours in the dark at 37 °C, the turbidity was measured with a microtiter plate reader at 620 nm. Total folate content of the samples was calculated by comparison with a calibration curve constructed with the folic acid standard. The certified

reference material BCR - 121 (wholemeal flour; action limit: 50 ± 7 μg/100 g of d.m.), obtained from the Institute for Reference Materials and Measurements, Geel, Belgium, was analyzed in each set of samples as a quality control sample. The average experimental folate content obtained for the certified reference material was 46 ± 7 μg/100 g of d.m.

Statistical analysis

All experiments were performed in triplicate and the data are reported as means ± standard deviation (SD). One-way ANOVA analysis of variance with Scheffé's post-hoc test was conducted to determine significant differences between means ($p < 0.05$) using SPSS software (version 22.0, IBM SPSS Statistics, Armonk, NY, USA). Pearson's correlation was performed to evaluate the relationship between the ash and total folate contents of all the fractions considered.

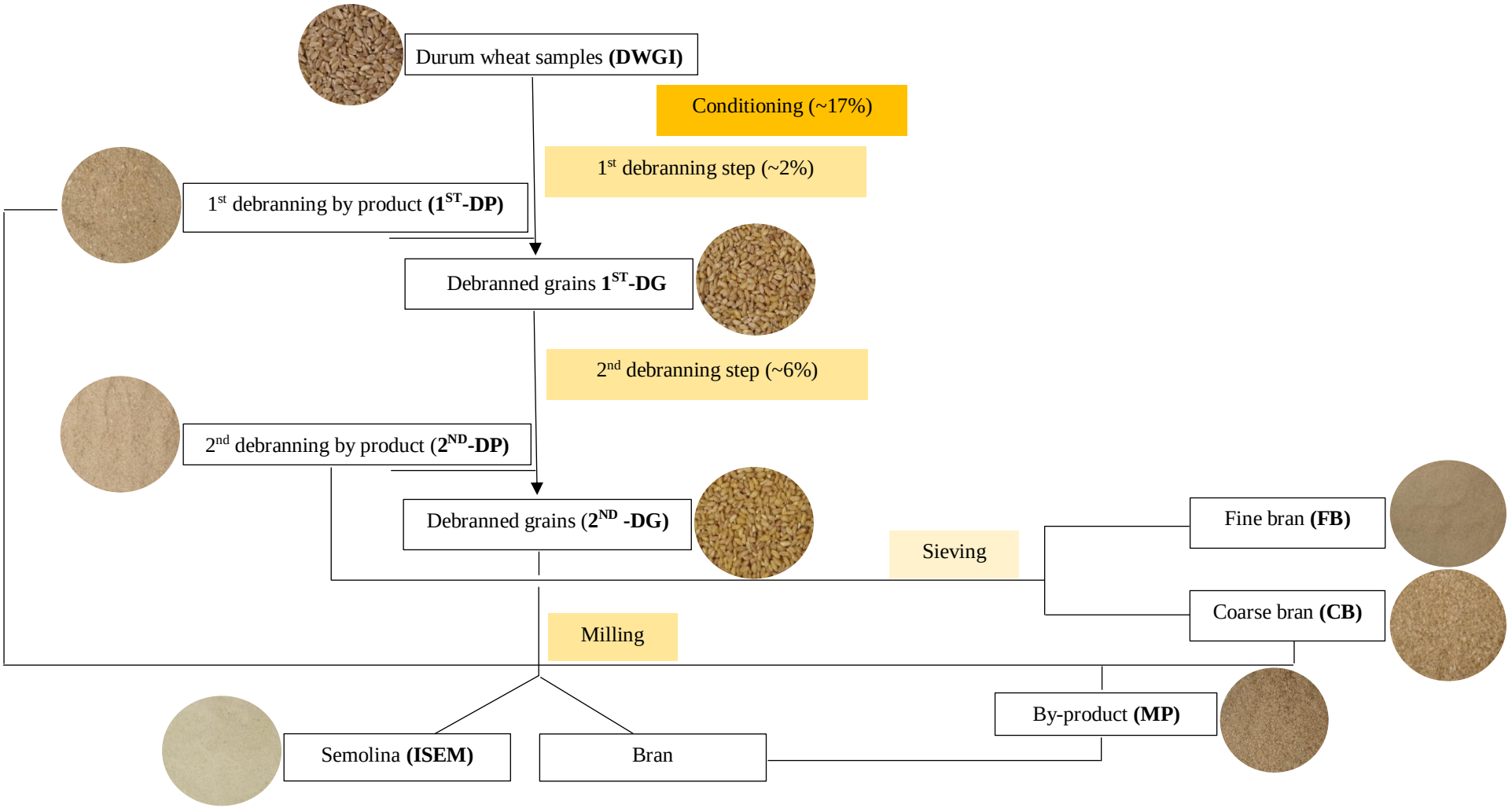

Figure 2.1. Schematic representation of the industrial milling process and the fractions obtained with the identification codes.

2.3 Results and discussion

Chemical-physical qualitative attributes of National and North America durum wheat and their roller milling and debranning fractions

National grain sample was characterized by a test weight, equal to 85.7 kg/hL ± 0.12, and a one thousand kernels weight, equal to 57.9 g ± 2.66, overall higher than those found for North America grain sample equal to 84.8 kg/hL ± 0.12 and 46.0 g ± 0.46, respectively.

Table 2.3 summarizes the moisture, ash and protein content of both the National and North America grain samples considered and of the milling fractions during pilot-scale processing. The National durum wheat grain showed an ash content of 1.75% d.m. and a protein content of 13.5% d.m., while the North America one had a lower ash content of 1.63% d.m. and a higher protein content of 18.4% d.m. The different chemical composition of the starting durum wheat samples reflects the influence that environmental factors have on the moisture, ash and protein content as widely demonstrated in scientific literature (Rharrabti *et al.*, 2003; Li *et al.*, 2013; Rossini *et al.*, 2018). As expected, bran and shorts of both samples had the highest content of both ash and protein compared to the starting grain and the other milling fractions. This is due to the well-known effects of the milling process that separates the outer cell layers from the starchy endosperm, concentrating these nutrients mainly in the bran and shorts fractions. The milling of National grain led to the obtaining of purified semolina characterized by an ash content equal to 0.74% d.m. and a protein content equal to 11.6% d.m., while that obtained from North America grain had a similar ash content equal to 0.73% d.m. and a higher protein content than the National equivalent due to the composition of the starting grain. Looking at the three break and reduction rolls fractions, a comparable trend distinguished both National and North America grains in terms of ash content. The B1 fraction and the by-product of semolina purification, PP, of both durum wheat samples had a comparable ash content equal to 1.09% d.m. for B1 and PP of the National grain and equal to 1.14% d.m. for B1 and 1.11% d.m. for PP of North America grain ($p > 0.05$). There are no significant differences ($p > 0.05$) with respect to both the ash content of D1 and D2 fractions and semolina from National grain and that of D1 and B2 fractions and semolina of North America grain. The D3 fraction of both National and North America grain was characterized by the highest protein content equal to 13.6% d.m. and 19.1% d.m., respectively.

Debranning is a process in which the outer cell layers of wheat grain are removed by friction or abrasion operations (Singh & Singh, 2010). It is now well established that debranning could be a highly advantageous process since it increases the efficiency of the milling process, reduces microbial contamination as well as the concentration of heavy metals and, above all, favors the recovery of intermediate fractions of wheat grain with a high nutritional value (Bottega *et al.*, 2009a; Giordano *et al.*, 2015).

Table 2.3. Chemical composition of National and North America durum wheat and related roller milling fractions.

	National			North America		
Fractions	**Moisture (%)**	**Ash (% d.m.)**	**Protein (% d.m.)**	**Moisture (%)**	**Ash (% d.m.)**	**Protein (% d.m.)**
DWG	10.6±0.03	1.75±0.028	13.5±0.15^{c}	12.1±0.11	1.63±0.021	18.4±0.19
B1	14.2±0.07^{e}	1.09±0.007^{b}	10.9±0.01	15.2±0.01^{c}	1.14±0.011^{d}	16.5±0.00^{a}
B2	14.2±0.05^{e}	0.83±0.000	12.1±0.02^{ab}	15.3±0.01^{c}	0.66±0.020^{a}	17.0±0.00^{b}
B3	14.3±0.07^{e}	0.98±0.014	14.1±0.06^{d}	15.3±0.07^{c}	0.92±0.009	19.7±0.08
D1	14.1±0.04^{de}	0.74±0.007^{a}	11.8±0.01^{ab}	15.1±0.05^{bc}	0.65±0.004^{a}	16.6±0.08^{a}
D2	13.7±0.08^{bc}	0.74±0.014^{a}	12.4±0.01^{b}	14.8±0.07^{ab}	0.76±0.026^{b}	17.5±0.02^{c}
D3	13.6±0.06^{b}	1.21±0.007	13.6±0.06^{cd}	14.8±0.07^{ab}	1.04±0.016^{c}	19.1±0.01
B	12.3±0.03^{a}	4.11±0.035	17.1±0.30^{e}	15.7±0.01^{d}	4.18±0.037	22.6±0.07
S	12.1±0.06^{a}	4.25±0.014	16.5±0.08^{e}	14.2±0.02	4.60±0.021	21.0±0.01
SEM	13.9±0.05^{cd}	0.74±0.007^{a}	11.6±0.06^{a}	15.2±0.04^{c}	0.73±0.013^{ab}	17.4±0.11^{bc}
PP	13.7±0.02^{bc}	1.09±0.000^{b}	12.0±0.06^{ab}	14.8±0.10^{a}	1.11±0.001^{cd}	16.3±0.04^{a}

Mean values ± SD. Mean values within a column lacking the letter or a common superscript letter differ ($p < 0.05$). DWG, durum wheat grain; B1-B2-B2, break rolls fractions; D1-D2-D3, reduction rolls fractions; B, bran; S, shorts; SEM, purified semolina; PP, by-product from semolina purifier.

In the present experimental work five different debranning degrees (3, 6, 9, 12 and 15%) were applied to both National and North America durum wheat and the debranned grains and the debranning by-products obtained were characterized for their moisture, ash and protein content (Table 2.4). As expected, the removal of the outer cell layers of the durum wheat grain led to a progressive reduction of the ash content in the debranned grains of both samples. These results are in accordance with other studies (Fares *et al.*, 1996; Ficco *et al.*, 2020a; Ficco *et al.*, 2020b). Compared to the protein content of debranned grains, this showed a decreasing trend as the percentage of debranning increases in National grain and a first increasing and, then, decreasing trend in North America grain (Bechtel *et al.*, 2009). Among the debranning by-products, proteins tended to progressively increase from 3-DP to 15-DP in both samples and this was consistent with the distribution of these nutrients in the durum wheat grain (Bechtel *et al.*, 2009). The ash content, on the other

hand, first increased from 3-DP to 6-DP ($p < 0.05$) and, then, progressively decreased up to 15-DP in National durum wheat, while the same decreased slightly ($p > 0.05$) from 3-DP to 6-DP and, then, progressively increased to 15-DP in North America durum wheat.

Table 2.4. Chemical composition of National and North America debranned grains and debranning by-products.

	National			North America		
Fractions	**Moisture (%)**	**Ash (% d.m.)**	**Protein (% d.m.)**	**Moisture (%)**	**Ash (% d.m.)**	**Protein (% d.m.)**
Debranned grains						
3-DG	10.6±0.03^{c}	1.53±0.007^{a}	12.8±0.00	12.4±0.11^{b}	1.66±0.016^{c}	16.3±0.02^{b}
6-DG	11.3±0.11^{c}	1.54±0.028^{a}	12.3±0.14^{b}	12.2±0.16^{b}	1.58±0.032^{bc}	18.0±0.17^{c}
9-DG	11.4±0.08^{c}	1.39±0.014	12.3±0.07^{b}	12.4±0.06^{b}	1.45±0.016^{b}	17.6±0.16^{c}
12-DG	11.3±0.04^{c}	1.25±0.028	12.0±0.00^{ab}	12.2±0.03^{b}	1.44±0.028^{ab}	15.8±0.13^{ab}
15-DG	11.3±0.13^{c}	1.08±0.000	11.7±0.00^{a}	12.3±0.09^{b}	1.28±0.064^{a}	15.8±0.06^{ab}
Debranning by-products						
3-DP	9.9±0.18^{a}	3.96±0.035	14.4±0.07	10.4±0.01	3.76±0.016^{d}	15.7±0.23^{a}
6-DP	10.4±0.10^{b}	4.85±0.007^{b}	16.7±0.07	10.9±0.00^{a}	3.71±0.000^{d}	17.5±0.10^{c}
9-DP	10.4±0.00^{b}	4.83±0.000^{b}	17.6±0.07	11.1±0.11^{a}	4.35±0.007^{e}	19.5±0.02
12-DP	10.0±0.09^{ab}	4.76±0.014^{b}	18.1±0.07^{c}	10.9±0.04^{a}	4.36±0.035^{e}	20.2±0.02
15-DP	9.7±0.04^{a}	4.50±0.021	18.3±0.14^{c}	11.2±0.01^{a}	4.22±0.057^{e}	21.1±0.09

Mean values ± SD. Mean values within a column lacking the letter or a common superscript letter differ ($p < 0.05$). DG, debranned grains; DP, debranning by-products.

Total folate content of National and North America durum wheat grains and their roller milling and debranning fractions

Folates are water-soluble vitamins that have gained increasing attention globally due to the countless beneficial effects on human health deriving from their intake with the diet (Kam et al., 2011). In this context, the total folate content was determined by microbiological assay in the starting durum wheat samples, in the milling fractions, in the debranned grains and in the debranning by-products. National and North America durum wheat had an average total folate content of 48.9 μg/100 g d.m. and 44.4 μg/100 g d.m., respectively (Figure 2.2). Both values are lower than those found by Piironen *et al.* (2008) equal to an average of 74.1 μg/100 g d.m. and by Giordano *et al.* (2015) equal to an average of 111.9 μg/100 g d.m. However, Piironen *et al.* (2008), taking into consideration 10 durum wheat genotypes, found total folate values for the latter variables in a wide range from 63.7 to 89.1 μg/100 g d.m. This variability, also found in other wheat

genotypes, is attributable to genetic factors and more strongly to environmental factors (Piironen *et al.*, 2008; Kariluoto *et al.*, 2010). Moreover, the type of sampling and the analytical procedures adopted for the determination of total folate content can contribute significantly to the differentiation of the results found by the various authors (Piironen *et al.*, 2008). In the present study, total folate content was determined through a microbiological assay which provides a preliminary step of monoenzymatic extraction of folates from the sample. Other authors, on the other hand, use trienzymatic extraction for the same purpose and this could justify the higher total folate values by these authors (Schoenlechner *et al.*, 2010); however, still other authors quantifying folates using the microbiological method did not find significant differences between the use of the monoenzymatic extraction compared to the trienzymatic one, although the use of the trienzymatic extraction is to be considered based on the basis of the type of sample (Delchier *et al.*, 2016). Compared to other cereals, total folate content of durum wheat was higher than that found in literature for barley (51.8 - 78.9 μg/100 g d.m.), rye (57.4 - 77.5 μg/100 g d.m.) and oat (57.1 - 60.4 μg/100 g d.m.) (Andersson *et al.*, 2008; Nyström *et al.*, 2008; Shewry *et al.*, 2008). Folates are unevently distributed in the grain concentrating in particular in the outer cell layers, especially the aleurone layer, and in the germ (Kariluoto *et al.*, 2010; Brouns *et al.*, 2012). Wheat bran is characterized by a folate content that varies in the range from 70.4 to 160.0 μg/100 g d.m., while wheat germ is characterized by the high folate content equal to about 240.0 μg/100 g d.m. (Piironen, 2011). Wheat aleurone, on the other hand, has the highest folate content which, however, varies in a wide range between 200 and 800 μg/100 g d.m. (Brouns *et al.*, 2012). Bran and shorts obtained from the conventional roller milling of durum wheat were confirmed to be the richest fractions of folates in both National and North America durum wheat samples. Bran from National grain had an average folate value of 88.6 μg/100 g d.m. and, therefore, approximately 1.8-fold higher than that found in the starting grain; bran from North America grain, on the other hand, was characterized by an average folate value of 109.2 μg/100 g d.m. with was approximately 2.5-fold higher than that found in the starting wholegrain flour. Shorts of both samples exhibited a folate content which was about 2.5-fold higher than that of the starting grain (Figure 2.2). As expected, the main product of durum wheat milling, that means semolina, had on average a low folate value of 20.4 μg/100 g d.m. for National grain and 18.7 μg/100 g d.m. for North America grain (Figure 2.2). As regards the break and reduction rolls fractions, these presented low folate values with a common trend that first saw a decrease from B1 to B2 and an increase from B2 to

B3, and then again a decrease from B3 to D1 for finally return to increase from D1 to D3 (Figure 2.2).

Figure 2.3 shows the total folate content of debranned grains and debranning by-products obtained from the sequential application of debranning starting from both National and North America durum wheat samples. As can be seen in the graph of Figure 2.3, the folate content in debranned grains obtained from National durum wheat showed a non-linear trend from 3-DG to 15-DG, first decreasing from 3-DG to 6-DG, increasing from 6-DG to 9-DG and decreasing from 9-DG to 12-DG to return again to increase from 12-DG to 15-DG. The folate content decreased linearly in the debranned grains obtained from the sequential debranning of North America grain from 3% to 12% and returned to slightly increase in the debranned grain at 15%. Although the folate content in the debranned grains of both samples decreased compared to that of the starting grain, no significant differences were observed ($p > 0.05$). The debranned grains were characterized by lower folate values than those found for debranning by-products, consistent with the greater localization of these nutrients in the outer cell layers of the durum wheat grain.

The debranning by-products at 3% and 6% of National durum wheat had an average folate content of 216.9 μg/100 g d.m. and 203.3 μg/100 g d.m., respectively, significantly higher than the debranning by-products at 9%, 12% and 15% and compared to the starting grain ($p < 0.05$). The folate content drastically decreased in the debranning by-product at 9% (on average 74.7 μg/100 g d.m.), it increased up to 143.8 μg/100 g d.m. in the debranning by-product at 12% and finally decreased again at 97.7 μg/100 g d.m. in the debranning by-product at 15%. The trend found in the debranning by-products obtained from North America durum wheat was different with respect to the folate content and this could be largely explained in light of the great lack of homogeneity that characterizes the debranning by-products with possible repercussions on the analytical variability of the data. The first debranning by-product at 3% had an average folate content of 166.2 μg/100 g d.m., whereas the second one at 6% had a lower folate content on average equal to 129.9 μg/100 g d.m. In the debranning by-product at 9% the folate value increased again (on average 149.4 μg/100 g d.m.) until it reached the highest value in the debranning by-product at 12% (on average 186.1 μg/100 g d.m.). The folate content returned to decrease in the debranning by-product at 15%, settling on the average value of 147.8 μg/100 g d.m. The decrease in folate content observed for both National and North America durum wheat in the debranning by-product at 15% is probably due to the dilution effect given by the presence of starchy endosperm. Since the aleurone layer represents 8-9% of the grain

weight, the application of debranning percentages of 5-10% and 10-15% leads to its complete removal (Bottega *et al.*, 2009a; Pagani et al., 2014; Giordano *et al.*, 2015).

It is also possible to hypothesize that the presence of the germ contributed significantly to the higher folate value found in the debranning by-product at 3% of National durum wheat. Overall, the folate data found for the debranning by-products confirm that these nutrients are mainly localized in the aleuronic layer and in the germ of the wheat grain (Kariluoto *et al.*, 2010; Brouns *et al.*,2012).

These results are in line with findings of other authors although there are few studies available in the scientific literature that have taken into consideration the distribution of folates in the debranning fractions of durum wheat. Giordano *et al.* (2015) studied the distribution of folates in the pearling fractions of durum wheat and found that the first three fractions (0-5%; 5-10%; 10-15%) had a high folate content equal to about 2.5-fold higher compared to that found in the starting grain. Edelmann *et al.* (2013) evaluated the folate content in different scarification fractions obtained starting from two hulled barley cultivars finding that folates are present in greater quantities in the fraction resulting from the removal of approximately 12% of the grain weight reaching values 2.6-3.6-fold higher than native grain.

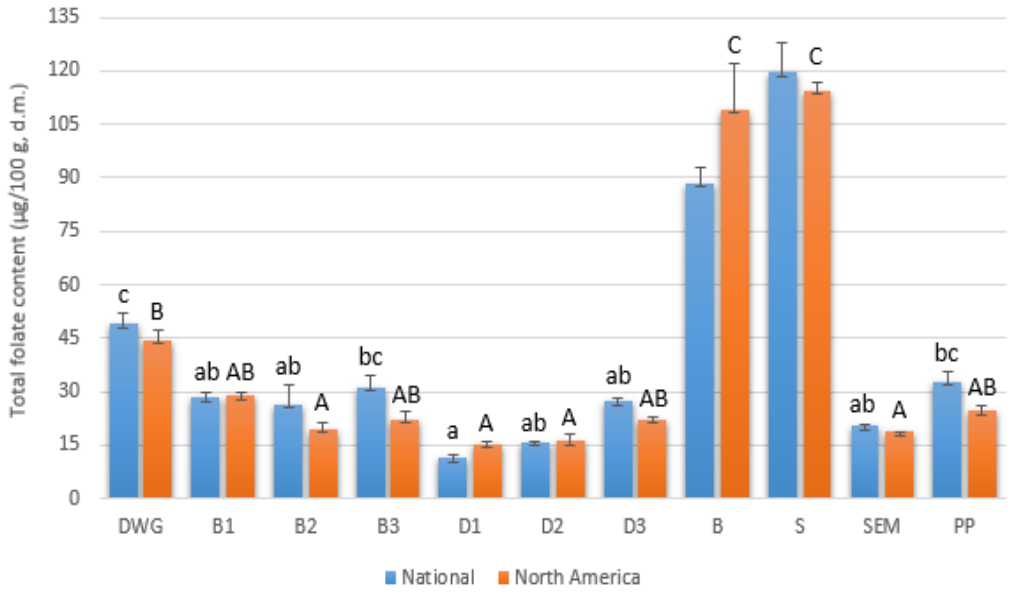

Figure 2.2. Total folate content (µg/100 g, d.m.) of National and North America durum wheat and their milling fractions (mean values ± SD). Error bars refer to standard deviation. Different letters indicate statistically significant differences ($p < 0.05$). Lowercase letters relating to National durum wheat samples and uppercase letters relating to North America durum wheat samples. DWG, durum wheat grain; B1-B2-B2, break rolls fractions; D1-D2-D3, reduction rolls fractions; B, bran; S, shorts; SEM, purified semolina; PP, by-product from semolina purifier.

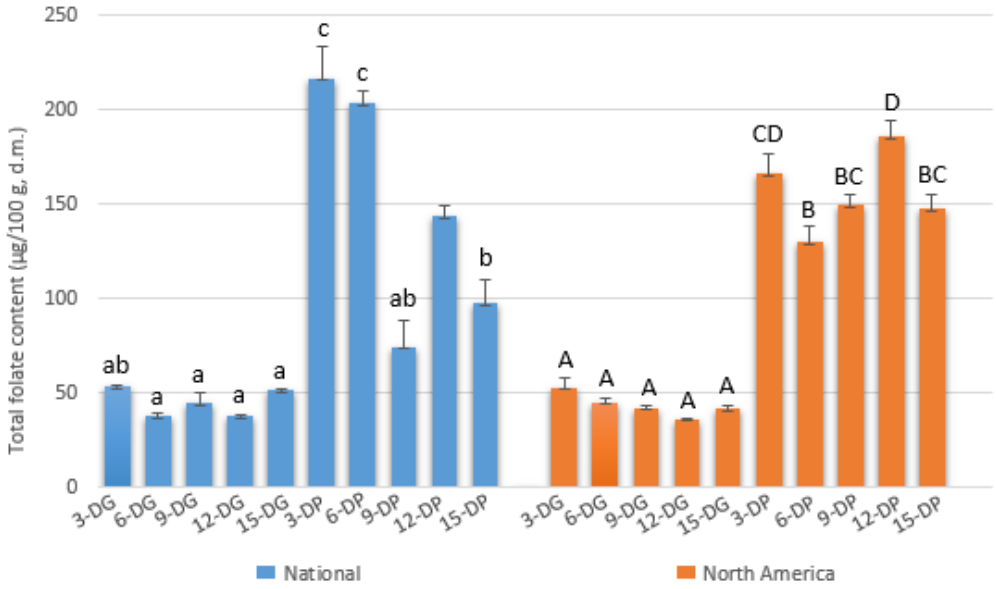

Figure 2.3. Total folate content (µg/100 g, d.m.) of National and North America debranned grains and debranning by-products (mean values ± SD). Error bars refer to standard deviation. Different letters indicate statistically significant differences ($p < 0.05$). Lowercase letters relating to National durum wheat samples and uppercase letters relating to North America durum wheat samples. DG, debranned grains; DP, debranning by-products.

Quality characterization and industrial milling performance of durum wheat samples

The two blends of durum wheat grain intended for industrial milling exhibited both a high test weight and a high one thousand kernels weight, on average equal to 85.2 kg/hL ± 0.16 and 85.2 kg/hL ±0.10, and 55.3 g ± 0.19 and 57.2 g ± 0.93, respectively, demonstrating the absence in significant quantities of shriveled grains.

Results of chemical characteristics of both durum wheat blends and of the relative debranning and milling fractions were divided into first and second industrial sampling and reported in Table 2.5. Moisture content of both grain blends was approximately equal to 8% and reached a final value of about 17% in the first sampling and 16% in the second sampling, after the two conditioning stages, before the debranning and milling processes. The milling process dedicated to obtaining whole-meal semolina was distinguished for its high efficiency, taking into account the milling yield. In fact, yield of whole-meal semolina from the two industrial milling was equal to 83.7% and 86.4%, while that of milling by-product was equal to 14.8% and 12.1%, respectively. Overall, the efficiency of the two milling process was on average 98.5% with no significant loss of initial sample. This efficiency could be related to the preliminary debranning process which involves a gradual removal of the cell layers constituting the bran of durum wheat grain. The high

nutritional value of durum wheat blends in the first sampling is confirmed by the protein content which was equal to 15.9% d.m. on average, while the ash content was equal to 1.98% d.m. on average. In the second sampling, on the other hand, the protein content was lower in the starting durum wheat sample (on average 15.1% d.m.), while the ash content was comparable (on average 1.96% d.m.).

In the present study, the industrial milling process included a preliminary stage of debranning of the two durum wheat blends. This debranning process was divided into two steps and led to the obtaining of debranned grains and debranning by-products. The first debranning step resulted in the removal of about 2% of the grain by weight. In this way a large part of the outer pericarp of durum wheat grain is removed (Hemery *et al.*, 2009). With the second debranning step another 6% of the grain by weight was removed, leading to obtaining a fraction that collects approximately 30-35% of the aleurone layer and 25-30% of the starchy endosperm (Hemery *et al.*, 2009). As reported in the Table 2.5, the ash content was reduced in durum wheat samples as the percentage of debranning increased in line with the removal of the outermost cell layers of the grains, while increased in the relative fractions. In fact, in the first sampling the average ash content of the starting grain, 1.98% d.m., decreased to 1.76% d.m. in the grains debranned at 2% and 1.44% d.m. in the grains debranned at 8%, while it increased consistently up to 7.78% d.m. in the first debranning by-product and slightly less in the second debranning by-product (7.50% d.m.). This decreasing trend of ash content in the grain alongside with the increase of debranning rate was also confirmed for durum wheat blends from the second sampling and was in line with the results found by other authors (Bottega *et al.*, 2009a; Bottega *et al.*, 2009b; Singh & Singh, 2010). However, the ash content in the debranning by-products obtained starting from the blend of grains of the second sampling increased from the debranning by-product at 2% to that at 8%, while in the blend of grains of the first sampling the ash content relating to the same fractions decreased ($p > 0.05$).

The results concerning the protein content also reflect the impact of the debranning process on the nutritional content of the different fractions. The average protein value found for durum wheat samples is 15.9% d.m., 16.3 % d.m. for grains debranned at 2% and 15.5% d.m. for grains debranned at 8% relating to the first sampling. Contrary to what was expected after the first debranning step, the protein content slightly increases, while it decreases after the second debranning step (Table 2.5). Also in the blend of grains of the second sampling the protein content increased from 15.1% d.m. of the starting grain to 15.4% d.m. of the grains debranned at 2% to return to decrease in the debranned one

at 8% up to 15.0% d.m. On the other hand, the average protein content showed an increasing trend from the first debranning by-product, 14.1% d.m. and 13.1% d.m., to the second debranning by-product, 20.2% d.m. and 18.9% d.m., of the durum wheat samples from the first and the second sampling, respectively, in line with the higher concentration in the latter fraction of bran layers, intermediate layer and germ (Hemery *et al.*, 2009). In fact, the protein content increases quantitatively from the center of the endosperm towards the he peripheral layers of the durum wheat grain (Bechtel *et al.*, 2009).

The semolina obtained in the first milling process had an ash and protein content on average equal to 1.29% d.m. and 15.3% d.m., respectively, while that obtained in the second milling process was characterized by an average content in ash and protein of 1.34% d.m. and 15.2% d.m., respectively. The protein content in the semolina was not significantly higher different than that found in the starting grain (Table 2.5).

Starting with the semolina fraction, a reconstituted whole-meal semolina was obtained by adding fine bran in established percentages in order to fall within the legal maximum limit set for ash for durum wheat whole-meal semolina by the Italian Presidential Decree No 187/2001 (Figure 2.1). Fine bran was obtained starting from the second debranning by-product, characterized by high values of both ash and proteins, which was directed to a further sieving step, which also led to the obtaining of coarse bran, normally intended for animal feed. As expected, fine and coarse bran fractions from the first sampling had a high ash and protein content, with fine bran having an average ash and protein value of 8.24% d.m. and 20.7% d.m., respectively, higher than those characterizing coarse bran equal to 5.74% d.m. and 19.2%, respectively, since the milling process concentrates the outermost layers, together with the germ, in these fractions. The same trend was confirmed for the blend of grains of the second sampling with respect to the ash and protein content, although both the bran fractions had lower ash and protein values than the counterparts of the first sampling (Table 2.5).

Table 2.5. Chemical characteristics of industrial durum wheat samples and their debranning and milling fractions.

	First Industrial Sampling			Second Industrial Sampling		
Fractions	**Moisture (%)**	**Ash (% d.m.)**	**Protein (% d.m.)**	**Moisture (%)**	**Ash (% d.m.)**	**Protein (% d.m.)**
DWGI	8.3±0.06	1.98±0.021^{a}	15.9±0.28^{ab}	8.1±0.08	1.96±0.007^{a}	15.1±0.01^{a}
1ST-DG	17.4±0.06^{e}	1.76±0.028^{a}	16.3±0.01^{b}	16.0±0.01^{b}	1.73±0.035^{a}	15.4±0.03^{a}
1ST-DP	15.4±0.08^{cd}	7.78±0.269^{d}	14.1±0.12	14.3±0.04^{a}	7.25±0.042^{c}	13.1±0.11
2ND -DG	17.1±0.01^{e}	1.44±0.050^{a}	15.5±0.01^{ab}	15.8±0.09^{b}	1.34±0.028^{a}	15.0±0.04^{a}
2ND-DP	14.9±0.04^{bc}	7.50±0.013^{cd}	20.2±0.08c	13.6±0.01	7.66±0.007^{c}	18.9±0.06^{bc}
ISEM	14.6±0.06^{ab}	1.29±0.050^{a}	15.3±0.08^{a}	14.3±0.08^{a}	1.34±0.007^{a}	15.2±0.13^{a}
MP	15.4±0.13^{d}	6.41±0.554^{bc}	17.6±0.27	14.4±0.09^{a}	6.05±0.526^{b}	16.7±0.20
FB	14.1±0.10^{a}	8.24±0.071^{d}	20.7±0.04^{c}	13.1±0.01	8.18±0.014^{c}	19.3±0.16^{c}
CB	15.7±0.08^{d}	5.74±0.028^{b}	19.2±0.06	14.7±0.01	5.05±0.057^{b}	18.5±0.05^{b}

Mean values ± SD. *N × 5.70. Mean values within a column lacking the letter or a common superscript letter differ ($p < 0.05$). DWGI: industrial durum wheat grain blends; 1ST-DG: first step debranned grain; 1ST-DP: first debranning by-product; 2ND-DG: second step debranned grain; 2ND-DP: second debranning by-product; ISEM: industrial semolina; MP: milling by-product; FB: fine bran; CB: coarse bran.

Effects of industrial processing on the distribution of total folate content in durum wheat samples and their debranning and milling fractions

The total folate content in the two durum wheat blends and their distribution in the debranning and milling fractions was also studied and the results are illustrated in Figure 2.4. Durum wheat blends from the first and second sampling possessed an average value of total folate of 61.1 μg/100 g d.m. and 48.7 μg/100 g d.m., respectively. These values were lower than that found by other authors in durum wheat (Piironen *et al.*, 2008; Giordano *et al.*, 2015). Folate content found for the durum wheat of the first sampling (61.1 μg/100 g d.m.) was higher than those found for the National (48.9 μg/100 g d.m.) and North America (44.4 μg/100 g d.m.) durum wheat samples, while those found for the durum wheat of the second sampling are comparable with the same National and North America durum wheat.

As mentioned above, folates are mainly localized in the outer cell layers of the wheat grain, especially in the aleurone layer, and in the germ (Kariluoto *et al.*, 2010; Brouns *et al.*,2012). In the first sampling, grains debranned at 2% had an average total folate content of 66.2 μg/100 g d.m. and slightly higher than that found in durum wheat ($p > 0.05$), in contrast to what was expected. In the grains debranned at 8% the total folate content was lowered to an average value of 40.4 μg/100 g d.m. and this can be correlated with the

removal of the more peripheral layers of the grain. The same trend was confirmed for the blend of grains from the second sampling (Figure 2.4). The first and the second debranning by-products, on the other hand, were characterized by a high total folate content equal to 138.5 μg/100 g d.m. and 249.8 μg/100 g d.m on average, respectively, in agreement with findings of other authors (Piironen, 2011; Giordano *et al.*, 2015). The higher total folate content found in the second debranning by-product could be explained in light of the greater presence of the aleurone layer and the germ at this level. These results are also confirmed by the high ash content present in these by-products (Figure 2.4). This trend was also supported by comparable values found for the corresponding fractions of the blend of grains from the second sampling (Figure 2.4).

Semolina fraction had a low average total folate value of 26.6 μg/100 g d.m., in the first sampling, and a higher average total folate value of 35.4 μg/100 g d.m., in the second sampling. The fine bran fraction of both samples showed folate values approximately 3-fold higher than that found in the starting grain and equal on average to those found for the milling by-product (on average 157.2 μg/100 g d.m.). Different folate content was found for coarse bran resulting from the two samplings. Coarse bran from the first sampling is characterized by the higher folate content equal to approximately 4-fold higher than that found in the starting grain, while the corresponding sample of the second sampling showed a lower folate value that was approximately 3.5-fold higher than that found in the starting grain. These differences could be explained in the light of a different folate content in the starting grain, on the one hand, and the dedicated industrial process that could affect the distribution of folates between fine and coarse bran fraction in a different way depending on the starting material, on the other hand. In this regard, it is necessary to consider how the lack of homogeneity of the coarse bran sample could strongly influence the analytical variability of the data. In light of what Hemery *et al.* (2009) found, we would have expected a higher content of total folate in the fine bran since the finer particles are richer in aleurone than the biggest particles.

In the scientific literature there are works that have studied the correlation between total folate content and quality parameters of different wheat genotypes. Piironen *et al.* (2008) found a statistically significant negative correlation both between folate content and kernel weight and between folate and bran yield in winter wheat genotypes. The same correlation was not confirmed in spring wheat genotypes. In this experimental work, Pearson's correlation was carried out in order to study the relationship between the ash and total folate contents of all the fractions considered. A strong positive correlation

(0.878, $p < 0.01$) was found between the two variables analyzed and, considering that the ash content is usually used as a marker of whole grain, it would be open therefore the possibility of an evaluation of the use of folates as biomarkers of whole grain wheat to describe the distribution of these nutrients in the milling fractions of durum wheat.

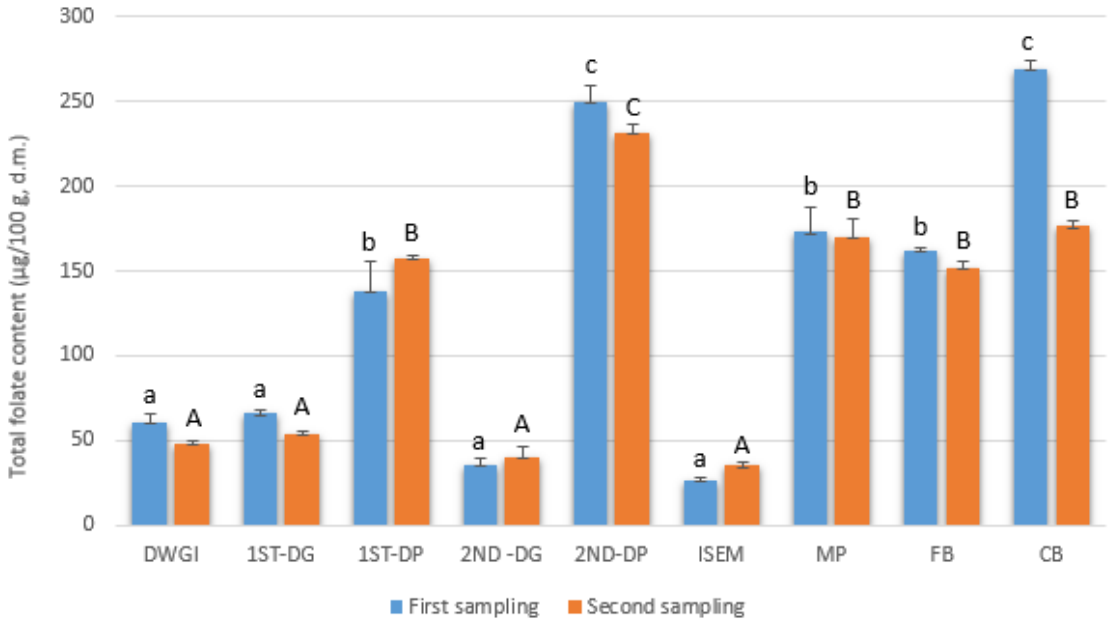

Figure 2.4. Total folate content (µg/100 g, d.m.) of industrial durum wheat and their debranning and milling fractions (mean values ± SD). Error bars refer to standard deviation. Different letters indicate statistically significant differences ($p < 0.05$). Lowercase letters relating to first sampling and uppercase letters relating to second sampling of durum wheat blend samples. DWGI: industrial durum wheat grain blends; 1^{ST}-DG: first step debranned grain; 1^{ST}-DP: first debranning by-product; 2^{ND}-DG: second step debranned grain; 2^{ND}-DP: second debranning by-product; ISEM: industrial semolina. MP: milling by-product; FB: fine bran; CB: coarse bran.

2.4 Conclusion

The results of this study show that durum wheat is a good source of folates, distinguished by an average total folate content ranging from 44.4 to 61.1 µg/100 g d.m. The differences found in the folate content between the different durum wheat samples could be traced to the different origin and, therefore, to the different growing conditions. Both the milling process on a pilot plant scale and the industrial one leads to the obtainment of fractions of flour rich in folates. Bran and shorts obtained from conventional roller milling on a pilot plant scale have the highest folate content which is about 2.5-fold higher than that of the starting grain and confirm the greater localization of these nutrients in the outermost cell layers and in the germ of the durum wheat grain.

Similarly, industrial milling process has also led to the obtaining of fractions where the folate content is approximately 4-fold higher compared to that found in the starting grain. The debranning technology is confirmed to be functional to the collection of fractions rich in folates which can be used alone or mixed with other flours for the development of cereal-based products with an improved folate content, positively contributing to the folate intake with the diet. The debranning trials carried out using a laboratory debranner at different percentages (3, 6, 9, 12, and 15%) resulted in the obtaining of debranning by-products characterized by a folate content up to 4-fold higher than that found in the starting grain. On the other hand, the debranning by-product obtained by removing a quantity of product equal to about 8% of the weight of grain from the industrial-scale milling process has a folate content up to about 4.5-fold higher compared to that of the starting grain. The strong positive correlation found between the ash and total folate variables taking into account the totality of the considered fractions suggests folates as possible biomarkers of whole grain wheat and, at the same time, provides a basis for further studies.

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Chapter 3

Development of whole-meal pasta with high nutritional value and improved sensorial properties

3.1. Introduction and objectives

In recent years, the availability of whole grain foods and ingredients has recorded and continues to record a positive trend of growth at a global level, testifying how consumers are increasingly aware of the health benefits associated with the consumption of whole grain foods (van der Kamp *et al.*, 2022). Indeed, numerous epidemiological studies suggest a clear inverse linear association between whole grain consumption and risk of non-communicable diseases such as cardiovascular diseases, type 2 diabetes (T2D) and colon cancer (Barrett *et al.*, 2020). The beneficial effects are linked to the synergistic action of dietary fibre, micronutrients (minerals and vitamins) and other bioactive compounds that are mostly retained in whole grain products compared to refined counterparts (Barrett *et al.*, 2020). Among cereal-based products, pasta, especially in whole-meal version, may represent an important carrier of beneficial components, including folate, given its popularity, versatility in many preparations, availability in different shapes and sizes, low glycemic index, relatively low cost and long shelf life (Ciccoritti et al., 2017; Padalino *et al.*, 2017; Di Pede *et al.*, 2021). Folates are essential micronutrients that need to be introduced with the diet belonging to the group of water-soluble B vitamins which are gaining more and more attention globally since they play significant roles in several biological functions, including nucleic acids biosynthesis and protein metabolism, with increased risks of defects in the development of the neural system of foetus, megaloblastic anemia, cardiovascular diseases, colorectal cancers and neurological disorders such as Alzheimer's disease in conjunction with a low nutritional folate status (Wusigale & Liang, 2020). Despite the health benefits on human health, dietary folate intake and in general whole grain intake continue not to meet the national recommendations even in Italy (Pounis *et al.*, 2014; Ruggiero *et al.*, 2019; van der Kamp *et al.*, 2022). The reasons for this may depend on the lack of clear and univocal definition of "whole grain" that can be shared globally (van der Kamp *et al.*, 2022), on the one hand, and, in the specific case of whole-meal pasta, on the bitter and branny flavour, inferior texture and color characteristics compared to pasta made from refined flour (Heiniö *et al.*, 2016), on the other hand. Regarding the latter, it is important to pay attention to the selection of raw materials and to the processing conditions used. In fact, due to their qualitative characteristics, whole-meal flours are more susceptible to the development of the Maillard reaction and the drying phase represents a crucial step in the pasta making process that can lead to nutritional impoverishment and deterioration of the sensory

characteristics in pasta (Marti *et al.*, 2017). Furthermore, folates are highly unstable compounds during food processing and their retention in foods depend on various factors such as s UV light, pH, heating, cooking, O_2 content, antioxidant levels and metal ion concentrations (McKillop *et al.*, 2002; Boz, 2021). Nevertheless, the use of peculiar technologies such as the debranning process of durum wheat prior to milling that can lead to obtaining folate-rich flour fractions (Giordano *et al.*, 2015), together with the appropriate calibration of the processing conditions adopted during pasta making process, can represent an important starting point for the development of whole-meal pasta where the nutritional characteristics are preserved and/or improved and the negative sensory attributes are limited.

In light of these considerations, the objectives of this part of the research activity were i) to study the effects of pasta making and cooking processes on folate retention in the whole-meal pasta (spaghetti shape) developed using the folate-rich flour fractions from industrial debranning process of durum wheat and ii) the evaluation of nutritional characteristics, heat damage and sensorial properties of developed whole-meal pasta. For these purposes, two durum wheat grain blends belonging to different batches were debranned and milling using an industrial plant and the obtained folate-rich flour fractions were used together with the semolina in two pasta making trials in which different formulations were tested, using for the initial steps a pilot plant and an industrial plant for the drying phase. Total folate content was determined using a microbiological assay preceded by an enzymatic extraction in durum wheat and debranning and milling fractions, and in the developed uncooked and cooked whole-meal pasta. For comparative purposes, whole-meal pasta of different brands (spaghetti shape) available on the market were also used. In addition, in order to evaluate any differences between pasta shapes with respect to the total folate content, various short whole-meal pasta shapes were also taken into consideration. Lastly, the physico-chemical characterization of the experimental whole-meal pasta samples preceded the characterization of the sensory attributes of the same samples by a semi-trained panel.

3.2 Experimental

Raw material

Semolina ($ISEM_{1-2}$), fine bran (FB_{1-2}) and coarse bran (CB_{1-2}) were obtained from the milling process of two different batches (1,2) of durum wheat blends as previously

described in the "Experimental" section of Chapter 2 and sampled at the industrial mill of the F.lli De Cecco di Filippo - Fara San Martino S.p.A. company located in Fara San Martino, Italy. All samples were stored in sealed plastic bags at +4 °C and ground, when necessary, using a refrigerated laboratory mill (model IKA A10-IKA Werke GmbH & Co. KG, Staufen, Germany) before analysis. Coarse bran sample obtained from the milling process of the second batch of durum wheat blends (CB_2) were milled with a laboratory hammer mill (model 3100, Perten, Milan, Italy) in order to decrease flour particle size (R-CB_2).

Whole-meal pasta preparation

Fine bran, coarse bran and re-milled coarse bran were mixed with semolina following five different formulations as reported in Table 3.1. The formulations were conceived in order to achieve the nutrition and health claims related to folate according to EC Regulation No 1924/2006 and EU Regulation No 432/2012 and to fall within the legal maximum limit set for ash for durum wheat whole-meal semolina pasta by the Italian Presidential Decree No 187/2001. Whole-meal pasta samples (spaghetti shape) were produced in two different pasta making trials through an experimental pasta making pilot plant composed of a pre-mixing chamber and a press (NAMAD, Rome, Italy), while an industrial plant was used for the drying phase. For each pasta making trial the whole-meal pasta produced with the fine bran fraction (WP-FB_{1-2}) was used as a control. Briefly, semolina and the fine bran, coarse bran and re-milled coarse bran fractions were mixed with cold tap water in a pre-mixing chamber from 20 to 30 minutes depending on the fraction used and, then, the doughs were transferred to a vacuum mixing chamber and an extruding system equipped with a bronze die. Fresh spaghetti were dried applying drying cycles at low temperatures (<60 °C). In the second pasta-making trial a low-temperature drying was adopted, usually used for semolina pasta, which involves the use of temperatures a few degrees higher than those used in the first pasta-making trial.

Table 3.1. Formulations of the developed whole-mal pasta samples.

Sample	**Semolina (ISEM) (%)**	**Fine bran (FB) (%)**	**Coarse bran (CB) (%)**	**Re-milled coarse bran (R-CB) (%)**	**Dough moisture content (%)**	**Hydration and mixing duration (min) in pre-mixing chamber**
First pasta making process						
WP-FB₁	94	6	-	-	30	20
WP-CB₁	90	-	10	-	30	20
Second pasta making process						
WP-FB₂	94	6	-	-	31	20
WP-CB₂	88	-	12	-	32	20
WP-RCB₂	88	-	-	12	33	30

Conventional and commercial whole-meal pasta samples

Two whole-meal pasta samples (WSa-WSb) (spaghetti shape) belonging to two different production batches were sampled at the F.lli De Cecco di Filippo - Fara San Martino S.p.A. company (Fara San Martino, Italy). In detail, the latter samples were identified as conventional since they were obtained according to the traditional "De Cecco" production method for whole-meal pasta. Other nine whole-meal pasta samples ($CWP_{1\text{-}2}$) (spaghetti shape) of different brands were purchased in local supermarkets. Two semolina pasta samples ($CSP_{1\text{-}2}$) (spaghetti shape) were also purchased to be used as a control.

Ten samples of short whole-meal pasta of different shapes (Penne Rigate, WPRa-b, Fusilli, WFa-b, Mezzi Ditali Rigati, WMDRa-b, Farfalle, WFAa-b, Grattata, WGa-b**)** belonging to two different production batches called "a" and "b" were also sampled at the F.lli De Cecco di Filippo - Fara San Martino S.p.A. company (Fara San Martino, Italy) at different times of production.

Overall, the types and identification codes of pasta samples are summarized in Table 3.2. These samples were used for comparative purposes with respect to total folate content. All the pasta samples were ground using a refrigerated laboratory mill (model IKA A10-IKA Werke GmbH & Co. KG, Staufen, Germany), when required, and stored at +4 °C until analyzed.

Pasta cooking and freeze-drying process

Pasta samples were cooked in tap water (pasta:water ratio of 1:10) at the optimal cooking time (OCT), the time required for the disappearance of the white core at the centre of the spaghetti strand after squeezing it between a pair of glass plates, following the International Standard ISO 7304-1:2016 (ISO, 2016) (Figure 3.1). The same cooking method was used for short whole-meal pasta samples. Cooked samples were drained and cooled for about 10 minutes at room temperature to recreate the normal conditions of consumption and freeze-dried in a VirTis Genesis 25SES Pilot Lyophilizer (VirTis Co. Inc., Gardiner, NY) for chemical analyses. The freeze-dried cooked samples were ground with a refrigerated laboratory mill (model IKA A10-IKA Werke GmbH & Co. KG, Staufen, Germany), analyzed for their residual moisture content and stored at -20 °C before analysis.

Table 3.2. Types and identification codes of pasta samples for conventional and commercial pasta used in this study.

Samples	**Identification codes**
Conventional whole-meal ***Spaghetti***	WSa-WSb
Commercial whole-meal ***Spaghetti***	CWP_{1-2}
Commercial semolina ***Spaghetti***	CSP_{1-2}
Whole-meal ***Penne Rigate***	WPRa-WPRb
Whole-meal ***Fusilli***	WFa-WFb
Whole-meal ***Mezzi Ditali Rigati***	WMDRa-WMDRb
Whole-meal ***Farfalle***	WFAa-WFAb
Whole-meal ***Grattata***	WGa-WGb

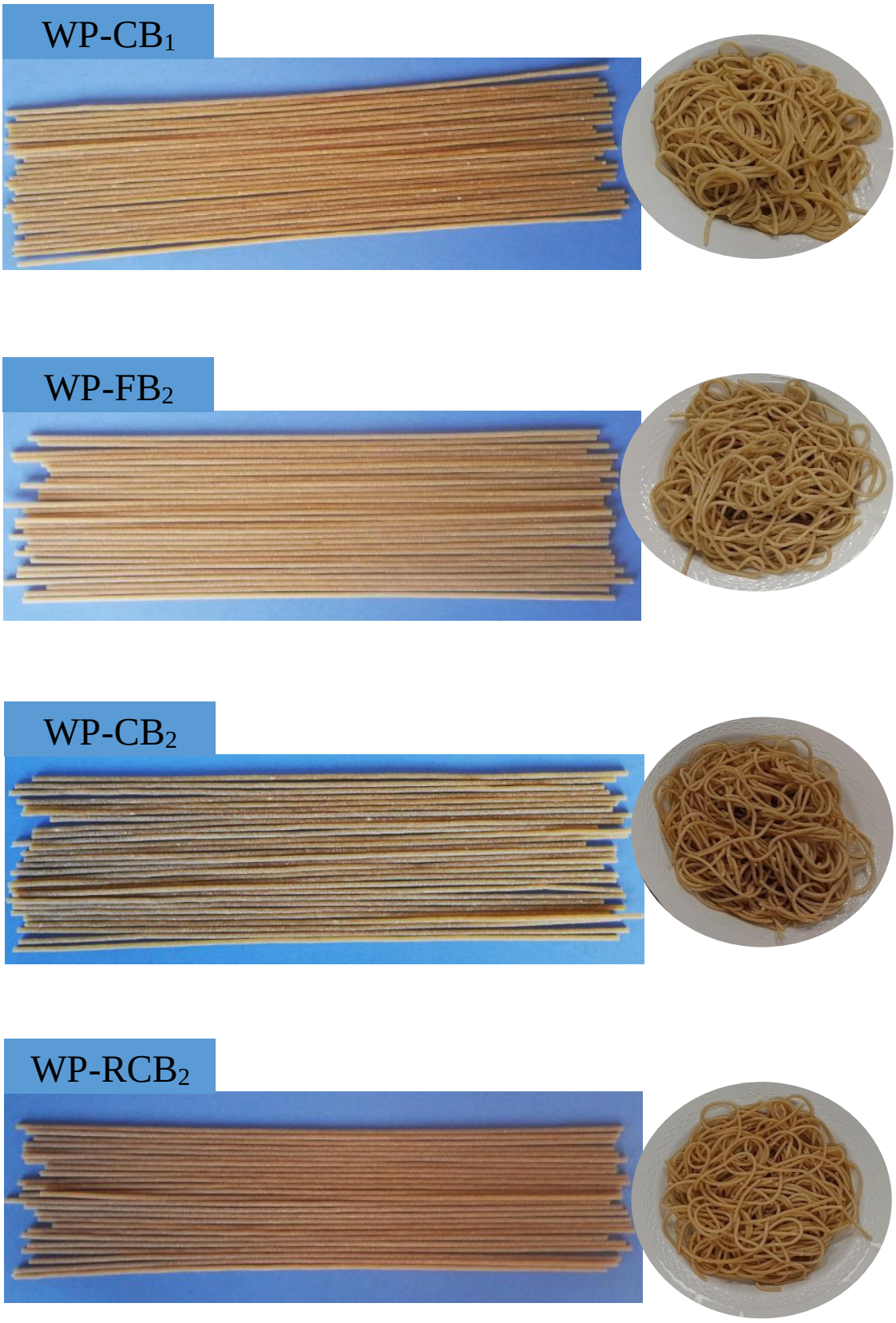

Figure 3.1. Uncooked and cooked experimental whole-mal pasta samples.

Chemico-physical analyses

Particle size distribution

The particle size distribution was assessed according to the Italian standard UNI 10873:2000 (UNI, 2000). Aliquots (100 g) of sample are poured into a sieve shaker composed of a stack of 7 sieves with different size meshes - 630, 560, 400, 355, 250, 180, 100 μm - (Retsch GmbH, Italy) setting a sieving time of 5 minutes.

Color

The color determination is performed in triplicate using a colorimeter model CR300 (Minolta Italia, S.p.A., Milan, Italy). The results are expressed in terms of CIE (Commission Internationale de l'Eclairage) 1976 L* a* b* color space parameters where L* describes lightness, a* is redness and b* is yellowness.

Moisture content

The moisture content was determined according to the ICC Standard Method 110/1 by drying about 10 g of sample in aluminum capsules in a drying over at a temperature of 130 °C until the constant weight is reached (ICC, 1995). The capsules were then left to cool in a desiccator and weighed.

Ash content

The ash content was evaluated following the International Standard ISO 2171:2007 by weighing about 10 g of sample in quartz capsules and incinerating in a muffle at 525 °C until complete combustion of the material and reaching a constant weight (ISO, 2007). The capsules were then left to cool in a desiccator and weighed.

Protein content

The protein content was determined with Kjeldahl method in accordance with the ICC Standard Method 105/2 (ICC, 1995). The total organic nitrogen present in the sample (1 g) is converted into ammonium sulphate, after digestion in concentrated sulfuric acid, in the presence of a catalyst. The ammonium sulphate is treated with alkali and the ammonia that forms is distilled and titrated with a 0.1 N sulfuric acid solution. The percentage of protein present in the sample is calculated from the nitrogen content using a conversion factor of 5.70 for flour samples and of 6.25 for pasta samples.

Lipid content

The lipid content was determined by acidic hydrolysis following the AOAC Official Method 922.06 (AOAC, 2002). About 2 g of sample is hydrolyzed with a dilute solution of hydrochloric acid and released total lipids are extracted with a mixture of equal volumes of diethyl ether and petroleum ether. The ether extracts are then combined in volumetric flasks and evaporated on a steam bath and the lipid residue is dried in an oven at a temperature of 105 °C until the constant weight is reached. The flasks were then left to cool in a desiccator and weighed.

Total dietary fibre content

The total dietary fibre was determined using the enzymatic assay kit distributed by Megazyme International Ltd., Ireland), in accordance with the AOAC Official Method 985.29 (AOAC, 2002). Samples (1 g) weighed in duplicate is subjected to enzymatic digestion with heat stable α-amylase, protease and amyloglucosidase. The soluble fiber is precipitated by adding four volumes of ethanol and the residue is filtered and washed with 78% ethanol, 95% ethanol and acetone. After drying and weighing, one residue is analyzed for proteins and the other is incinerated at 525 °C to determine ash. Total dietary fibre is the weight of the residue less the weight of the protein and ash.

Total folate content

Total folate content was evaluated using the microbiological kit (VitaFast® Folic Acid- Microbiological microtiter plate test to quantitate Folic Acid R-Biopharm AG, Darmstadt, Germany) and the chicken pancreatin enzyme (VitaFast® Chicken Pancreatin (γ-Glutamylhydrolase) R-Biopharm AG, Darmstadt, Germany), for the initial phase of sample extraction, distributed by R-Biopharm AG, Darmstadt, Germany, according to AOAC Official Method 2004.05 (De Vries *et al.*, 2005). Total folate content of the samples was calculated by comparison with a standard curve constructed with the standard folic acid solutions. The certified reference material BCR - 121 (wholemeal flour; action limit: 50 ± 7 μg/100 g of d.m.), obtained from the Institute for Reference Materials and Measurements, Geel, Belgium, was analyzed in each set of samples as a quality control sample. The average experimental folate content obtained for the certified reference material was 49 ± 4 μg/100 g of d.m.

Furosine determination

The determination of furosine was carried out according to the method proposed by Resmini *et al.*, (1990). Briefly, a sample amount equal to about 500 mg was hydrolyzed under nitrogen by adding in the order 2 mL and then 6 mL of 8N HCl at 110 °C for 23 h. The hydrolysate was filtered on a filter paper Whatmann no. 4 and on 2 mL of it the protein content was calculated according to the Kjeldhal method (ICC, 1995), multiplying the determined nitrogen content by the conversion factor 6.25. The filtrate in the amount of 0.5 mL was purified on a Sep-Pak C18 cartridge (Waters Corporation, Milford, MA, USA) and analysed by HPLC (Dionex, Sunnyvale, CA, USA) equipped with an Alltech furosine-dedicated column (250×4.6 mm) (Alltech, Derfield, IL, USA) and with a photodiode array detector (Dionex, Sunnyvale) set at 280 nm wavelength. The purified sample (20 µL) was injected into the column and eluted in 32 minutes at a flow rate of 1.2 mL/min using the following solvents:

- Solvent A= water/0.4% glacial acetic acid;
- Solvent B= solvent A/0.3% potassium chloride.

Table 3.3 shows the elution gradient used. The quantification of furosine was achieved using an external standard of furosine purchased from Neosystem Laboratoire (Strasbourg, France).

Table 3.3. Eluent gradient used for furosine determination.

Time (min)	**A (%)**	**B (%)**
0	100	0
13.5	100	0
20.5	50	50
22.0	50	50
23.0	100	0
32.0	100	0

Cooking quality and sensorial evaluation

Experimental whole-meal pasta samples cooked at the optimal cooking time (OCT) were subjected to sensory analysis according to the International Standard ISO 7304-2:2008 (ISO, 2008) with few modifications. A semi-trained panel of 6 individuals

(four males and two females) aged between 29 and 61 years conducted sensory analysis on cooked whole-meal pasta samples in the sensory room of the F.lli De Cecco di Filippo - Fara San Martino S.p.A. company (Fara San Martino, Italy). The samples were administered provided in white dishes immediately after cooking and in a randomized order. The panelists were asked to judge the following sensory attributes: flavour, colour, taste, firmness, liveliness, starch release and chewiness. The final judgment was expressed on a scale from 1 to 10 and the total score was weighted for the individual sensory attributes considered.

Statistical analysis

All experiments were carried out in triplicate and the data are reported as means ± standard deviation (SD). One-way analysis of variance (ANOVA) and Scheffé's post-hoc test was performed using SPSS software (version 22.0, IBM SPSS Statistics, Armonk, NY, USA) and significant differences (between means) was set at $p < 0.05$. Pearson's correlation was performed to assess the relationship between furosine level and colorimetric indices of the experimental whole-meal pasta.

3.3 Results and discussion

Chemico-physical characterization of raw materials used for the development of experimental whole-meal pasta

As previously reported in Chapter 2, the debranning process before the industrial milling of durum wheat blends led to obtaining a flour fraction richer in folates. In detail, the application of a debranning percentage equal to 8% produced a debranning by-product characterized by a total folate content which was approximately up to 4.5-fold higher than that found in the starting grain. The debranning by-product at 8% was further elaborated in a plansichter equipped with six sieve stacks of different mesh sizes from which a fine bran and a coarse bran were obtained. The coarse bran was re-milled using a laboratory hammer mill in order to reduce the particle size and similarly to that which characterizes the fine bran. The fine bran, the coarse bran and the re-milled coarse bran fractions represented the raw materials used in a mixture with semolina to obtain a "recombined" durum wheat whole-meal semolina to be used for pasta making trials. The chemical composition of these raw materials is shown in Table 3.4. No significant differences were observed for the semolina samples used in the two pasta making trials with respect to the content of moisture, ash, protein and total folate (Table 3.4). The fraction of fine bran

used for the first pasta making trial had an average ash content of 8.30% d.m. slightly higher despite not significant than that found for the corresponding fraction of the second pasta making trial ($p > 0.05$). The average protein content of the fine bran fractions of the two pasta making trials was instead different and higher in the fraction used in the first pasta making trial ($p < 0.05$) (Table 3.4). On the other hand, the coarse bran fraction of the first pasta making trial exhibited an average content of ash and proteins equal to 5.43% d.m. and 19.9% d.m., respectively, higher than that found for the corresponding fraction of the second pasta making trial ($p < 0.05$) (Table 3.4). As already extensively discussed in Chapter 2, folates are micronutrients belonging to the group of water-soluble B vitamins whose distribution in the wheat grain is not uniform but rather concentrates in the outermost cell layers, especially in the aleurone layer, and in the germ of the wheat grain (Kariluoto *et al.*, 2010; Brouns *et al.*, 2012). The average total folate content of the semolina used in the first pasta-making trial was equal to 30.2 μg/100 g d.m. and was slightly lower than that found for the semolina used in the second pasta making trial equal to 35.4 μg/100 g d.m. ($p > 0.05$). However, the average folate content in the fine fraction of the first pasta making trial, 218.9 μg/100 g d.m., was significantly higher than that of the second pasta making trial, 152.2 μg/100 g d.m., but it was also higher than that which characterized both the coarse bran fraction of the first pasta making trial, 204.9 μg/100 g d.m., ($p > 0.05$) and the coarse bran fraction of the second pasta making trial, 176.3 μg/100 g d.m. ($p < 0.05$). No significant differences were observed between the coarse bran fractions of both pasta making trials with respect to the total folate content (Table 3.4). It was found a slightly higher folate content in the fine bran fraction than in the coarse bran fraction in the first pasta making while on the contrary, the coarse bran fraction had a slightly higher folate content than the fine bran fraction both used in the second pasta making trial. This could be explained in light of the fact that the finer particles are richer in aleurone than the biggest particles (Hemery *et al.*, 2009) and in the variegated tissue composition of the coarse bran which it consists of different adhesive tissue layers, including pericarp, trasversal and tubular cells, testa, nucellar tissue and aleurone layer, and this could lead to a higher folate content of this fraction compared to the fine one (Steglich *et al.*, 2015). The re-milled coarse bran fraction of the second pasta making trial, obtained starting from the re-milling of the coarse bran fraction, was characterized by the higher folate content equal to an average of 271.1 μg/100 g d.m. In this regard, it is possible to hypothesize that the re-milling of the bran fraction leading to the obtaining of a finer fraction led to the breakdown of the aleurone cells with consequent release of the

intracellular contents, including folates, and, therefore, to an increase in bio-accessibility of these nutrients, similar to what obtained with the micronization process (Hemery *et al.*, 2010; Ciccoritti *et al.*, 2017). As previously reported in Chapter 2, the variability of the total folate data found in the bran fractions can however be traced back to the lack of homogeneity that strongly characterizes this type of sample, as well as to a different folate content of the starting grain as well as to the peculiar milling diagram adopted.

Table 3.4. Chemical characteristics of raw materials used in pasta making trials.

Sample	Moisture (%)	Ash (% d. m.)	Protein (% d. m.)	Total folate (µg/100 g d.m.)
First pasta making trial				
$ISEM_1$	14.4 ± 0.03^{b}	1.32 ± 0.049^{a}	15.2 ± 0.05^{a}	30.2 ± 2.31^{a}
FB_1	13.0 ± 0.07^{a}	8.30 ± 0.04^{b}	20.3 ± 0.08	218.9 ± 2.65^{d}
CB_1	14.5 ± 0.11^{bc}	5.43 ± 0.064	19.9 ± 0.06	204.9 ± 3.82^{cd}
Second pasta making trial				
$ISEM_2$	14.3 ± 0.08^{b}	1.34 ± 0.007^{a}	15.2 ± 0.13^{a}	35.4 ± 1.94^{a}
FB_2	13.1 ± 0.01^{a}	8.18 ± 0.014^{b}	19.3 ± 0.16	152.2 ± 3.75^{b}
CB_2	14.7 ± 0.01^{c}	5.05 ± 0.057	18.5 ± 0.05	176.3 ± 3.15^{bc}
R-CB_2	10.8 ± 0.06	4.82 ± 0.014	17.9 ± 0.09	271.1 ± 4.70

Mean values ± SD. Mean values within a column lacking the letter or a common superscript letter differ ($p < 0.05$). $ISEM_{1-2}$, semolina; FB_{1-2}, fine bran; CB_{1-2}, coarse bran; R-CB_2, re-milled coarse bran.

The particle size distribution of the fractions of fine bran, coarse bran and re-milled coarse bran from both pasta making trials was measured and the results are shown in Figures 3.2 and 3.3. The fine bran fraction of both pasta making trials had the highest concentration of particles >250 µm, unlike the coarse bran fraction of both pasta making trials in which the particle distribution was concentrated >630 µm. On the other hand, the particle size distribution of the re-milled coarse bran fraction of the second pasta making trial was similar to that of both fine bran fractions with a higher concentration of the particle distribution between 355 and 180 µm (Figure 3.3).

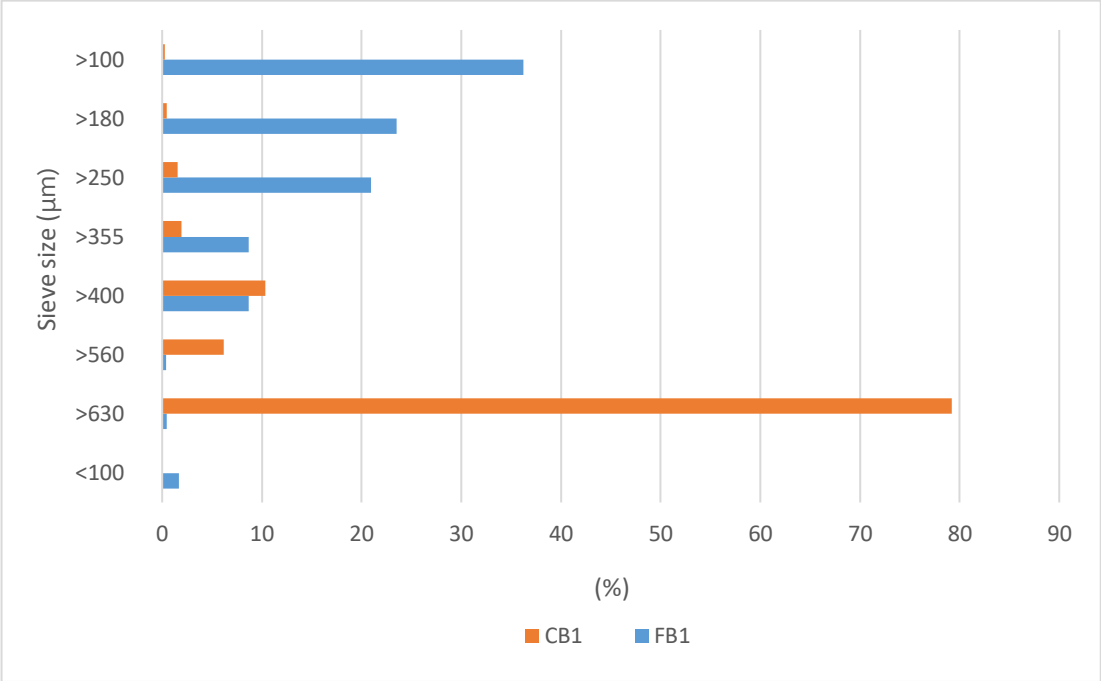

Figure 3.2. Average particle size distribution of bran fractions of first pasta making trial. CB_1, coarse bran; FB_1, fine bran.

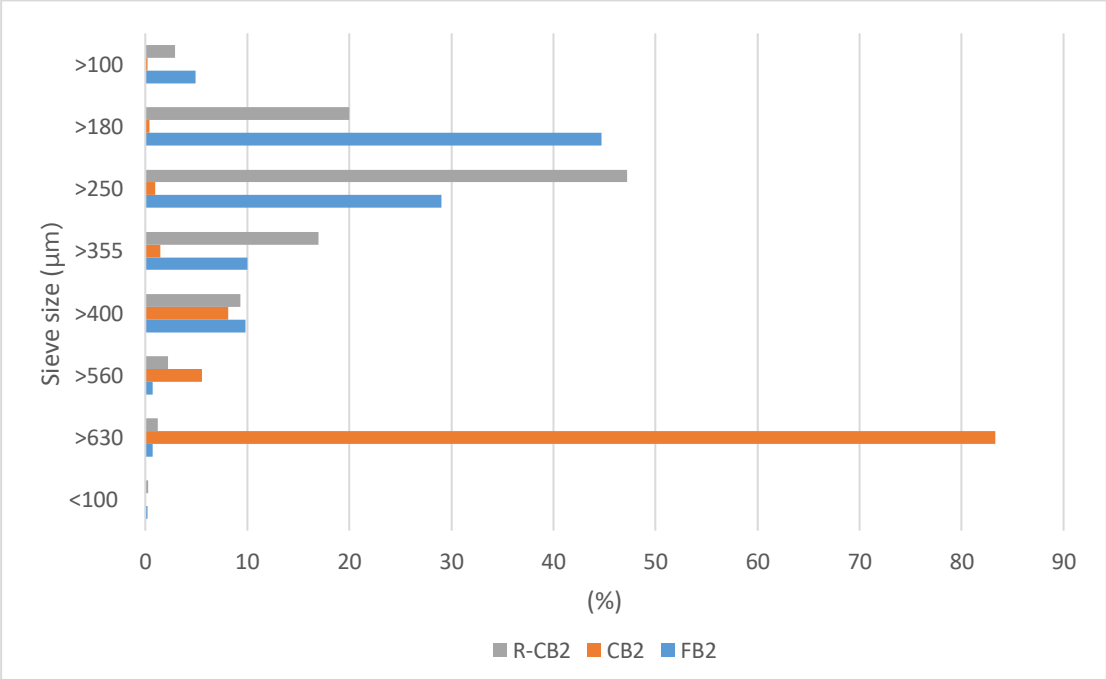

Figure 3.3. Average particle size distribution of bran fractions of second pasta making trial. R-CB_2, re-milled coarse bran; CB_2, coarse bran; FB_2, fine bran.

Chemical and nutritional characteristics of experimental whole-meal pasta

Starting from the fine bran, coarse bran, re-milled coarse bran and semolina fractions obtained from the industrial debranning and milling of durum wheat blends, different formulations have been studied in order to obtain a whole-meal pasta characterized by a total folate content capable of achieving the nutritional and health claims envisaged for folate (EC Reg. No 1924/2006 and EU Reg. No 432/2012), and an ash content complying with the legal limits (1.40-1.80% d.m.) set for ash for durum wheat whole-meal semolina pasta by the Italian Presidential Decree No 187/2001. For each pasta making trial, a whole-meal pasta using fine bran was produced to be used as control. The formulation of the standard whole-meal pasta of both pasta making trials is the same and corresponding to 94% of semolina and 6% of fine bran. Proximate composition of experimental whole-meal pasta is shown in the Table 3.5. As can be seen from the table, all the experimental whole-meal pasta samples had an ash content such as to respect the maximum limit of 1.80% d.m. set for whole-meal pasta by the Italian Presidential Decree No 187/2001 (data expressed on dry matter basis not shown). No significant differences were observed with respect to the mean protein content between the WP-CB_1 and WP-CB_2 samples and the WP-FB_1 and WP-FB_2 samples, equal to 15.2% and 15.1% for the former and 15.0% and 15.2% for the latter ($p > 0.05$). WP-CB_2 exhibited the highest total lipid content, equal to 2.8%, compared to that found for the other whole-meal pasta samples ($p > 0.05$). The average total lipid content in the experimental whole-meal pasta samples was 2.6% and was in line with the typical lipid level of whole wheat grain (Lafiandra *et al.*, 2012). The inclusion of coarse bran in the formulation of the WP-CB_1 and WP-CB_2 samples determined a higher total fibre content of the former compared to WP-FB_1 and WP-FB_2, consistently with the high fibre content of the bran fraction of wheat kernel (Onipe *et al.*, 2015). Overall, all the whole-pasta samples had a fibre content greater than 6 g per 100 g and, therefore, could boast the claim "High fibre" according to the European Regulation No 1924/2006 on nutrition and health claims on food. Regarding the total folate content, WP-CB_1 and WP-FB_1 showed the highest values of 43.1 and 37.8 μg/100 g, respectively, compared to the other experimental whole-meal pasta. However, it is important to underline that the total folate values found for the samples of the second pasta making trial (29.6 μg/100 g for WP-FB_2; 36.9 μg/100 g for WP-CB_1 and 35.1 μg/100 g for WP-RCB_2) are all lower than the expected values based on the formulations considered (36.5 μg/100 g for WP-FB_2; 44.7 μg/100 g for WP-CB_1; 55.7 μg/100 g for WP-

RCB_2). This could be explained in the light of the analytical variability linked to the method of analysis rather than to losses linked to the pasta making process, considering that for the samples of the first pasta making trial the theoretical and found values of folate with respect to the formulations considered are in full agreement. In any case, it is also possible to hypothesize that the differences linked to the drying cycle negatively influenced the folate content of the pasta samples in the second pasta making trial. Although there are no studies in the literature on the effects of processing on folate retention in pasta, there are studies that have evaluated the retention of other vitamins belonging to the water-soluble B vitamin group. Dexter *et al.* (1982) considered the effects of processing conditions on riboflavin, thiamine and niacin retention in enriched spaghetti by noting that substantial riboflavin losses occurred when high-temperature drying cycles were applied, while no riboflavin losses were observed during extrusion and when a conventional low-temperature drying cycle was applied. On the contrary, no negative changes were observed in either thiamine or niacin content which remained stable in all three drying cycles. On the other hand, Watanabe & Ciacco (1990) recorded not only losses in riboflavin but also losses in thiamine and niacin linked to the drying phase, with the greatest losses linked to the adoption of high-temperature drying cycles. Nevertheless, all the experimental whole-meal pasta samples, except for WP-FB_2, had a folate content greater than 30 μg/100 g and, therefore, capable of covering more than 15% of the nutrient reference values (200 μg) (EU Reg. No 1169/2011), being able to boast the relative nutrition and health claims "Source of folate" and "Folate contributes to maternal tissue growth during pregnancy", "Folate contributes to normal amino acid synthesis", "Folate contributes to normal blood formation", "Folate contributes to normal homocysteine metabolism", Folate contributes to normal psychological function", "Folate contributes to the normal function of the immune system", "Folate contributes to the reduction of tiredness and fatigue" and "Folate has a role in the process of cell division" according to the European Regulation No 1924/2006 and European Regulation No 432/2012). If we consider the tolerance values applicable to the nutrition declaration of foods other than food supplements for foods other than food supplements reported in the "Guidance Document for the control of compliance with EU legislation on Regulation (EU) 1169/2011, Council Directive 90/496/EEC and Directive 2002/46/EC with regard to the setting of tolerances for nutrient values declared on a label" on the values declared on the label with respect to vitamins, which means + 50% and − 35%, even the WP-FB_2 sample can boast of the aforementioned nutrition and health claims for folate (European

Commission, Health and Consumers Directorate-General, 2012). The slight differences found with respect to the compositional characteristics of the standard whole-meal pasta samples, despite these having the same formulation, were due to the differences in the chemical composition of the raw materials.

Table 3.5. Proximate composition (g/100 g, f.w.) and folate content of experimental whole-meal pasta.

Pasta sample	Moisture	Ash	Protein	Fat	Total Fibre	Carbohydrate*	Total Folate (µg/100 g)
WP-FB₁	10.3±0.04^{a}	1.52±0.014^{a}	15.0±0.08^{a}	2.2±0.01^{a}	6.5±0.29^{a}	64.4±0.43^{c}	37.8±1.80^{ab}
WP-CB₁	10.0±0.07	1.53±0.057^{a}	15.2±0.09^{a}	2.4±0.01^{ab}	8.3±0.06^{bc}	62.5±0.29^{bc}	43.1±0.74^{b}
WP-FB₂	10.6±0.10^{b}	1.55±0.007^{a}	15.2±0.00^{a}	2.7±0.05^{ab}	7.5±0.58^{ab}	62.5±0.74^{bc}	29.6±2.72^{a}
WP-CB₂	10.4±0.00^{ab}	1.59±0.007^{a}	15.1±0.05^{a}	2.8±0.24^{b}	9.2±0.11^{c}	61.0±0.40^{ab}	36.9±1.17^{ab}
WP-RCB₂	10.5±0.11^{b}	1.60±0.014^{a}	15.2±0.04^{a}	2.7±0.00^{ab}	9.6±0.03^{c}	60.3±0.10^{a}	35.1±5.37^{ab}

Mean values ± SD. Mean values within a column lacking the letter or a common superscript letter differ ($p < 0.05$). WP-FB_1, whole-meal pasta 94% semolina and 6% fine bran; WP-CB_1, whole-meal pasta 90% semolina and 10% coarse bran; WP-FB_2, whole-meal pasta 94% semolina and 6% fine bran; WP-CB_2, whole-meal pasta 88% semolina and 12% coarse bran; WP-RCB_2, whole-meal pasta 88% semolina and 12% re-milled coarse bran. *Calculated by difference.

Total folate content in uncooked and cooked experimental, conventional and commercial whole-meal pasta and semolina pasta

Considering the positive implications of folate on human health and the various health claims envisaged for folate, which can add value to foods that are "Source of folate", the total folate content of experimental whole-meal pasta samples was evaluated in this work compared to total folate content of conventional whole-meal pasta samples and other commercially available whole-meal pasta samples. The data are shown overall in the Table 3.6.

Experimental whole-meal pasta samples had the highest average folate content of 36.5 μg/100 g compared with that of conventional whole-meal pasta samples of 28.8 μg/100 g and with that of commercial whole-meal pasta samples of 27.4 μg/100 g. Among the commercial samples only samples CWP_6 and CWP_9 are "Source of folate" and can boast the health claims provided for folate on the label (EC Reg. No 1924/2006, EU Reg. No 1169/2011 and EU Reg. No 432/2012). The mean total folate content of commercial samples varied over a wide range between 20.7 μg/100 g and 34.9 μg/100 g. The variability found at this level could be ascribed to differences in the raw materials used and, therefore, to differences in the milling techniques of obtaining whole-meal flours (Jones *et al.*, 2015) which, consequently, determine the placing on the market of whole-meal products with different chemico-nutritional characteristics. It is also possible to hypothesize that this variability is to be ascribed to a different method of analysis for the analytical determination of folate. Furthermore, the total folate values found for all whole-meal pasta samples were lower than that reported by Hirawan & Beta (2014) for whole-meal pasta equal to 57 μg/100 g. This discrepancy between the data could be explained once again in light of a different analytical method for determining total folate in these samples. As expected, the whole-meal pasta samples had the highest total folate content compared to that found for the corresponding semolina pasta samples characterized by an average total folate content of 11.4 μg/100 g, consistent with the greater localization of these micronutrients in the outer cell layers and in the germ of wheat grain.

Given the water solubility of folates, losses of these nutrients during boiling may be due to the combination of thermal degradation and leaching into the cooking water (Lešková et al., 2006). In order to evaluate the impact of the cooking process on folate retention, the data relating to the folate content in both uncooked and cooked whole-meal pasta samples were expressed on a dry matter basis (Figure 3.4). In this way it was possible to

calculate the folate retention values in different samples using the method of apparent retention (AR) as defined by Murphy *et al.* (1975). Total folate content did not vary between uncooked and cooked whole-meal pasta samples, except for WP-FB_1, WP-CB_1 and WP-CB_2 samples where there was a decrease in total folate content between uncooked and cooked ($p < 0.05$). The commercial sample CWP_4 had a higher folate content after cooking and this was in contrast to what was observed in the other whole-meal pasta samples and was probably due to analytical variability of the method of analysis. What emerged in this study was that cooking retained folate in the average measure of 70% in the experimental whole-meal pasta samples, with peaks of 87% in the WP-FB_2 sample, and in the average measure slightly higher and equal to 78% in the commercial samples. However, the highest folate retention was found in semolina pasta samples where it averaged 89%.

Table 3.6. Total folate content (μg/100 g, f.w.) of uncooked experimental, conventional and commercial whole-meal pasta samples and semolina pasta samples.

Pasta sample	**Total Folate (μg/100 g, f.w.)**
Long whole-meal pasta shape (spaghetti)	
Experimental whole-meal pasta	
WP-FB1	37.8 ± 1.80^{ef}
WP-CB₁	43.1 ± 0.74^{f}
WP-FB₂	29.6 ± 2.72^{bcde}
WP-CB₂	36.9 ± 1.17^{def}
WP-RCB₂	35.1 ± 5.37^{cdef}
Conventional whole-meal pasta	
WSa	28.7 ± 1.10^{bcde}
WSb	28.9 ± 0.28^{bcde}
Commercial whole-meal pasta	
CWP₁	27.2 ± 0.67^{bcd}
CWP₂	28.5 ± 1.10^{bcde}
CWP₃	22.5 ± 4.77^{b}
CWP₄	20.7 ± 0.71^{ab}
CWP₅	25.7 ± 2.02^{bc}
CWP₆	30.3 ± 0.53^{bcde}
CWP₇	28.8 ± 0.21^{bcde}
CWP₈	28.3 ± 0.28^{bcde}
CWP₉	34.9 ± 0.64^{cdef}
Commercial semolina pasta	
CSP₁	11.1 ± 0.21^{a}
CSP₂	11.7 ± 0.00^{a}

Mean values ± SD. Mean values within a column lacking a common superscript letter differ ($p < 0.05$).

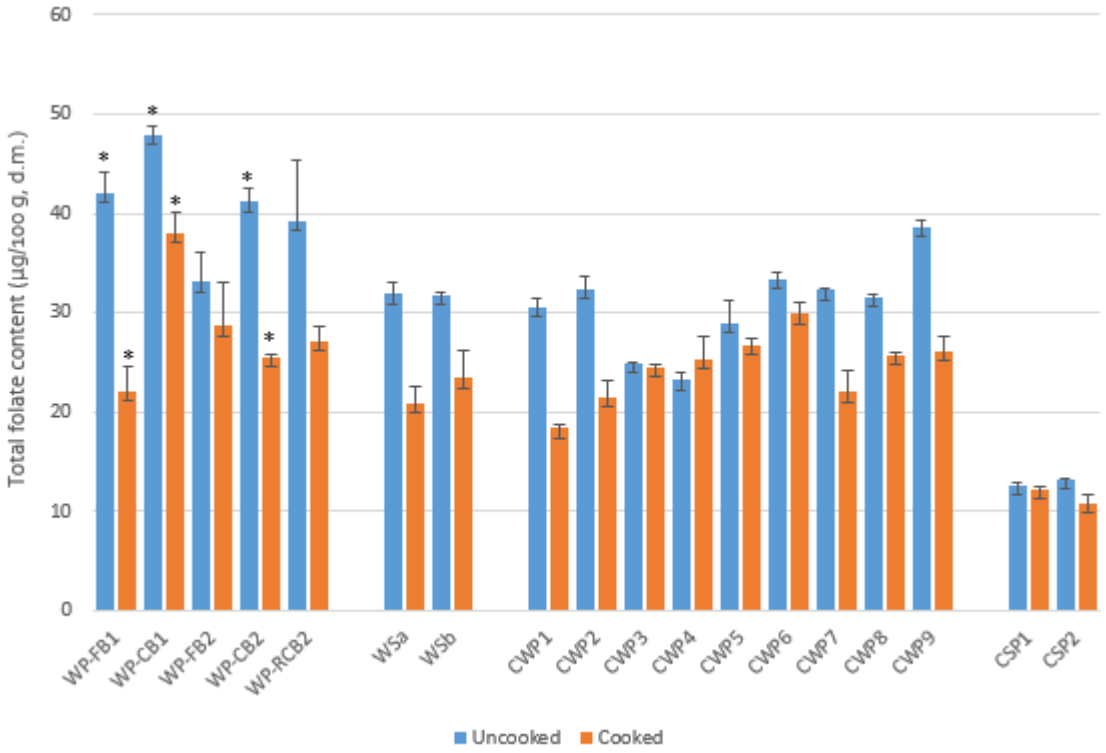

Figure 3.4. Total folate content (µg/100 g, d.m.) of uncooked and cooked experimental, conventional and commercial whole-meal pasta samples and semolina pasta samples (mean values ± SD). Error bars refer to standard deviation. The asterisk represents statistically significant differences ($p < 0.05$) between uncooked and cooked samples. WP-FB_1, WP-CB_1, WP-FB_2, WP-CB_2, WP-RCB_2, experimental whole-meal pasta; WSa, WSb, conventional whole-meal pasta; CWP_1-CWP_9, commercial whole-meal pasta; CSP_1, CSP_2, commercial semolina pasta.

In the scientific literature there are few studies that have evaluated the effect of cooking process on folate retention in pasta. Ranhotra *et al.* (1985) calculated the "true" retention of folate in the semolina pasta samples taking into account, therefore, also the loss of solids during cooking and found that the cooked samples retained a significant amount of folate on average equal to 79% for spaghetti and 77% for macaroni. These results are lower than what was found in this work for the corresponding samples of semolina spaghetti. Liang *et al.* (2020) found a high folate retention in noodles samples after cooking equal to an average of 78% and an average loss of folate of 13% caused by boiling degradation; similarly, Bui & Small (2006) found that boiling the noodles resulted in an average loss of folate ranging from 13% to 30% for white and yellow noodles, while for instant noodles the loss of these nutrients was very low and between 4% and 6%. The retention of folate in both whole-meal and semolina pasta samples is higher than that found in boiled vegetables and legumes. In fact, folate content of boiled spinach and

broccoli was reduced by 51% and 56%, respectively, compared to their corresponding uncooked contents (McKillop *et al.*, 2002), while Dang *et al.* (2000) found a folate retention of 45% for boiled peas and of 52.6% for boiled chickpeas.

Total folate content in whole-meal pasta before and after cooking: long and short pasta shapes compared

In order to evaluate the differences in total folate content between long and short whole-meal pasta shapes, total folate content was also determined in short whole-meal pasta samples of different shapes and the results are shown in the Table 3.7. Total folate content was on average 31.1 μg/100 g with WPRa sample having the lowest total folate content (28.6 μg/100 g on average) and WFa sample having the highest total folate content (34.9 μg/100 g on average). Even with respect to the total folate content, no significant differences were found between different production batches of the samples considered. Overall, the results for the total folate content in the uncooked whole-meal pasta samples of both long (experimental, conventional and commercial) and short pasta shapes are shown in Figure 3.5. Short whole-meal pasta of different shapes had an average total folate content that was slightly higher than both that found for conventional long whole-meal pasta (spaghetti shape) (28.8 μg/100 g on average) and that found for commercial long whole-meal pasta (spaghetti shape) (27.4 μg/100 g), but on the other hand part, it was lower when compared to that found in experimental long whole-meal pasta (spaghetti shape) (36.5 μg/100 g).

Total folate content was also evaluated after cooking and the data relating to the folate content in both uncooked and cooked short whole-meal pasta samples were expressed on a dry matter basis in order to calculate folate retention (Figure 3.6). Compared to what was observed in the long whole-meal pasta samples, total folate content in short whole-meal pasta samples varied between uncooked and cooked except in the WPRb and WMDRa samples where no significant differences were observed between uncooked and cooked ($p > 0.05$). The results for the total folate content in the raw and cooked whole-meal pasta samples are also shown in comparison between the long and short pasta shapes in Figure 3.7 and are expressed on a dry matter basis. Moreover, even for short whole-meal pasta samples there was a high retention of folate after cooking equal to an average of 73%, in line with the retention rates found for long whole-meal pasta samples equal to an average of 74%.

Table 3.7. Total folate content (µg/100 g, f.w.) of uncooked short whole-meal pasta samples.

Pasta sample	**Total Folate (µg/100 g, f.w.)**
Short whole-meal pasta shapes	
WPRa	28.6±0.53[a]
WPRb	29.5±3.22[a]
WFa	34.9±1.45[b]
WFb	31.9±1.98[ab]
WMDRa	31.1±3.71[ab]
WMDRb	31.3±1.24[ab]
WFAa	31.0±0.21[ab]
WFAb	33.0±2.30[ab]
WGa	30.6±0.42[ab]
WGb	28.8±2.23[a]

Mean values ± SD. Mean values within a column lacking a common superscript letter differ ($p < 0.05$). WPR, whole-meal Penne Rigate; WF, whole-meal Fusilli; WMDR, whole-meal Mezzi Ditali Rigati; WFA, whole-meal Farfalle; WG, whole-meal Grattata.

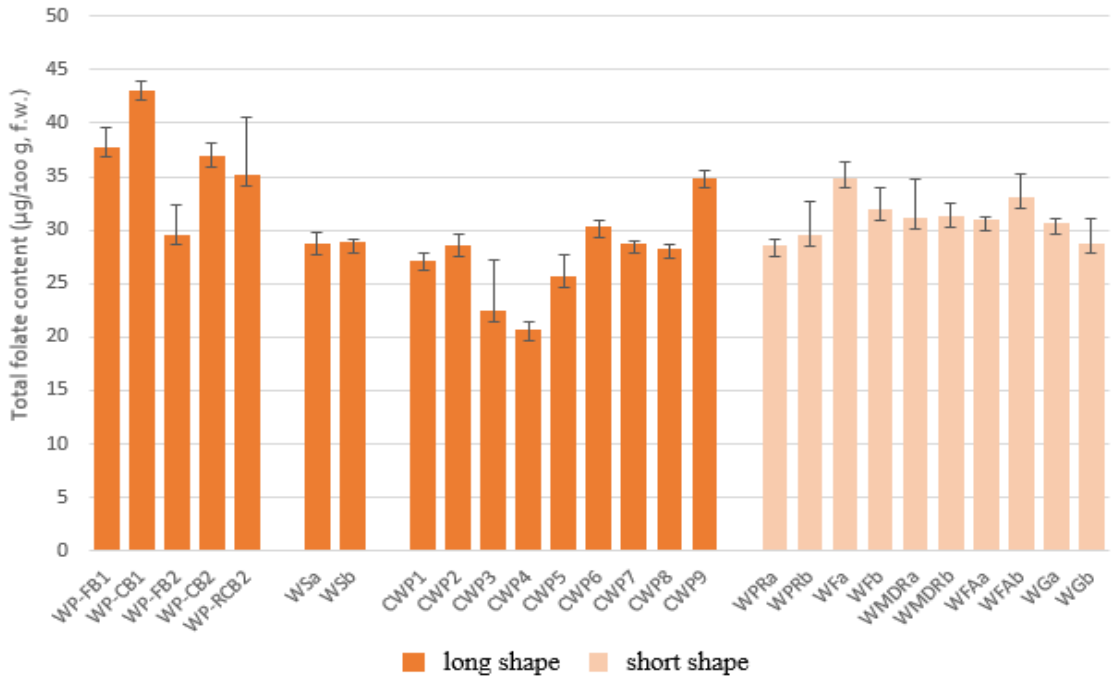

Figure 3.5. Total folate content (µg/100 g, f.w.) of uncooked long and short whole-meal pasta samples (mean values ± SD). Error bars refer to standard deviation. WP-FB_1, WP-CB_1, WP-FB_2, WP-CB_2, WP-RCB_2, experimental whole-meal Spaghetti; WSa, WSb, conventional whole-meal Spaghetti; CWP_1-CWP_9, commercial whole-meal Spaghetti; CSP_1, WPR, whole-meal Penne Rigate; WF, whole-meal Fusilli; WMDR, whole-meal Mezzi Ditali Rigati; WFA, whole-meal Farfalle; WG, whole-meal Grattata.

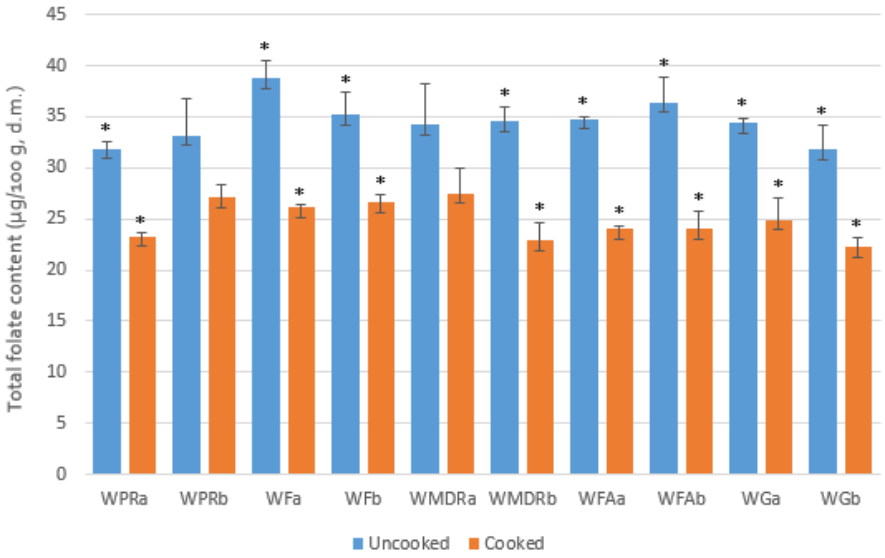

Figure 3.6. Total folate content (µg/100 g, d.m.) of uncooked and cooked short whole-meal pasta samples (mean values ± SD). Error bars refer to standard deviation. The asterisk represents statistically significant differences ($p < 0.05$) between uncooked and cooked samples. WPR, whole-meal Penne Rigate; WF, whole-meal Fusilli; WMDR, whole-meal Mezzi Ditali Rigati; WFA, whole-meal Farfalle; WG, whole-meal Grattata.

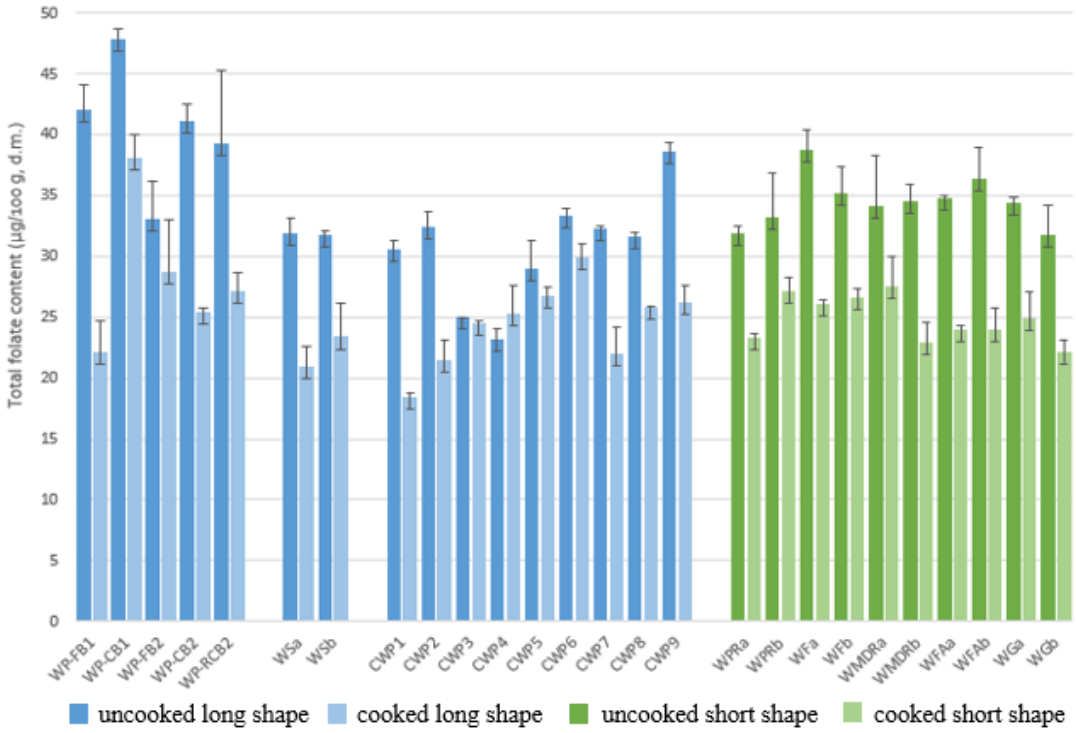

Figure 3.7. Total folate content (µg/100 g, f.w.) of uncooked and cooked long and short whole-meal pasta samples (mean values ± SD). Error bars refer to standard deviation. WP-FB_1, WP-CB_1, WP-FB_2, WP-CB_2, WP-RCB_2, experimental whole-meal Spaghetti; WSa, WSb, conventional whole-meal Spaghetti; CWP_1-CWP_9, commercial whole-meal Spaghetti; CSP_1, WPR, whole-meal Penne Rigate; WF, whole-meal Fusilli; WMDR, whole-meal Mezzi Ditali Rigati; WFA, whole-meal Farfalle; WG, whole-meal Grattata.

Evaluation of heat damage and color characteristics of experimental whole-meal pasta

The evaluation of the nutritional quality of pasta also passes through the evaluation of the intensity of heat damage attributable to the development of the Maillard reaction (MR) or "non-enzymatic browning". The MR occurs when reducing sugars (e.g., glucose, fructose, lactose, maltose) interact with available amino groups of proteins or amino acids. The key factors influencing the development of the Maillard reaction include high protein content, high reducing sugars content, intermediate, moisture content, temperature, pH and water activity (a_w) (Giannetti *et al.*, 2021). During the drying phase of pasta processing, the MR occurs easily (Acquistucci, 2000; Hellwing *et al.*, 2018). In the early stage of the MR, Amadori rearrangement products (ARP) are formed which can be quantified as furosine after acid hydrolysis (Hellwing *et al.*, 2018). Furosine (FUR) is the marker of the early phase of the MR most used to describe heat damage in pasta and cereal products (Marti *et al.*, 2017). In order to assess the extent of the Maillard reaction in the experimental whole-meal pasta, the furosine level in these samples was determined. As shown in Table 3.8, the furosine level varies from 224 mg/100 g protein, in the WP-FB_1 sample, to 360 mg/100 g, in both the WP-CB_2 and WP-RCB_2 samples. The furosine values found are related to the adoption of drying cycles at low temperature. De Noni & Pagani (2010) reported furosine levels between 400 mg/100 g protein and 700 mg/100 g protein for pasta samples with low moisture (<15-16%) dried at high temperatures (>75 °C). Higher furosine values result from the application of high temperature or very high temperature drying cycles, while lower furosine values are indicative of the adoption of the mild conditions for drying phase (Giannetti *et al.*, 2021). Marti *et al.* (2017) found furosine values for whole-meal pasta samples varying in a wide range between 229 mg/100 g protein and 836 mg/100 g protein. This variability is due to the peculiar characteristics of the raw materials used as well as to the drying cycles adopted. Furthermore, it is widely known that whole-meal pasta is characterized by a higher furosine level than semolina pasta, which has furosine values between 107 mg/100 g protein and 506 mg/100 g protein (Giannetti *et al.*, 2013). These differences are due to the presence of damaged starch, the intense amylase activity and the high content in reducing sugars and proteins typical of whole-meal semolina, which make whole-meal pasta more susceptible to the MR (De Noni & Pagani, 2010; Marti *et al.*, 2017). However, it should be emphasized that differences in furosine levels were found between the experimental whole-meal pasta samples from the first and second pasta making trials,

with the former having lower furosine values than the latter. This could be explained in light of the use of a low temperature drying cycle usually dedicated to the drying of semolina pasta and not to that of whole-meal pasta.

Pasta color, especially in the whole-meal version, is another important parameter that allows us to judge the quality of pasta and that plays a fundamental role in consumer acceptability (Cavazza *et al.*, 2013). The colorimetric indices, L* (lightness), a* (redness) and b* (yellowness) were determined on the experimental whole-meal pasta samples and the results are summarized in Table 3.9. The lightness (L*) and yellowness (b*) values which is associated with the pigment content of raw materials and the enzymatic reactions (Acquistucci, 2000; Feillet *et al.*, 2000) were variable between 48.3 to 55.3 and 19.4 to 22.3, respectively. On the other hand, red (a*) index is strictly correlated to the development of Maillard reaction and showed values ranging from 5.2 to 7.3 (Acquistucci, 2000; Feillet *et al.*, 2000). As expected, the highest values of a* equal on average to 7.0 and 7.3 were found in WP-RCB_2 and WP-CB_2 samples, respectively, characterized by the highest levels of furosine. This positive correlation, however, was not confirmed in the WP-FB_2 sample which had values of a* that did not differ from that found in the WP-FB_1 sample ($p > 0.05$), although the two samples had a different furosine level averaging 308 mg/100 g protein and 224 mg/100 g protein, respectively. Deng *et al.* (2017) found higher values of a* (11.91 on average) in whole-meal spaghetti samples than those found in this work and this is to be correlated with the adoption of high temperature drying cycle used for the former compared to the experimental whole-meal spaghetti samples which were dried with a low temperature drying cycle.

Table 3.8. Furosine level and colorimetric indices of experimental whole-meal pasta samples.

Pasta sample	**Furosine (mg/100 g protein)**	**Color (as is)**		
		L*(lightness)	**a*(redness)**	**b*(yellowness)**
WP-FB₁	224±5	52.8±1.12^{b}	5.2±0.13^{a}	21.5±0.29^{bc}
WP-CB₁	245±4	51.6±1.09^{b}	6.0±0.27	20.7±0.56^{ab}
WP-FB₂	308±17	55.3±0.29	5.3±0.07^{a}	20.5±0.68^{a}
WP-CB₂	360±1^{a}	48.3±0.29^{a}	7.3±0.20^{b}	22.3±0.27^{c}
WP-RCB₂	360±0^{a}	49.5±0.10^{a}	7.0±0.16^{b}	19.4±0.23

Mean values ± SD. Mean values within a column lacking the letter or a common superscript letter differ ($p < 0.05$). WP-FB_1, whole-meal pasta 94% semolina and 6% fine bran; WP-CB_1, whole-meal pasta 90% semolina and 10% coarse bran; WP-FB_2, whole-meal pasta 94% semolina and 6% fine bran; WP-CB_2, whole-meal pasta 88% semolina and 12% coarse bran; WP-RCB_2, whole-meal pasta 88% semolina and 12% re-milled coarse bran.

In order to better understand the relationship between furosine level and colorimetric indices in the experimental whole-meal pasta, Pearson's correlation analysis was carried out. A good positive correlation (0.756, $p < 0.01$) was found between the furosine level and the redness index (a*), suggesting how these variables can be used in combination with each other to discriminate between samples dried using different drying cycles and, in general, to evaluate the heat damage of dried pasta during processing.

Sensorial evaluation of experimental whole-meal pasta

The appearance and cooking quality are fundamental attributes that discriminate the acceptability of whole-meal pasta by consumers. With respect to the optimal cooking time shown in Table 3.10, no variations were observed in the experimental control whole-meal pasta samples (WP-FB_1 and WP-FB_2) of both pasta making trials. The use of coarse bran in the formulation of experimental whole-meal pasta samples (WP-CB_1 and WP-CB_2) resulted in a decrease in the optimal cooking time in these samples compared to that of the samples obtained using fine bran and this was probably due to a greater physical disruption of the gluten matrix by of coarser bran particles resulting in increased water absorption which reduces the optimal cooking time (Manthey & Schorno, 2002). The higher percentage of coarse bran used for the production of WP-CB_2 sample in the second pasta-making trial compared to that of the first pasta-making trial (WP-CB_1) resulted in a shorter optimal cooking time of the first than in the second. WP-RCB_2 sample obtained from the use of re-milled coarse bran in the formulation had an optimal cooking time that was between those found for control samples and those found for WP-CB_1 and WP-CB_2 samples.

Experimental whole-meal pasta samples were then evaluated by a semi-trained panel who were asked to express a judgment on the sensory attributes of flavor, color, taste, firmness, liveliness, starch release and chewability. Total score and judgment are summarized in Table 3.9. In general, what emerged was that the use of fine bran led to obtaining a whole-meal pasta with better sensorial characteristics compared to those of whole-meal pasta obtained using coarse bran both in the first and in the second pasta making trial. Although with a slightly different total score, WP-FB_1 and WP-FB_2 samples had an intense and fragrant flavor of whole-meal semolina, a light brownish color with yellow reflections and a sweet taste with a clear reference to the raw material. Firmness was judged very positively with a clean cut under the teeth, but a slight tendency to disintegration typical of whole-meal shapes was noted. Both WP-FB_1 and WP-FB_2 samples presented with

strands of pasta separated from each other on the plate and the starchy residues on the fork were absent. On the other hand, WP-CB_1 and WP-CB_2 samples shared an intense and fragrant flavor of whole-meal semolina, an intense brown color characterized by small bran pieces more evident in WP-CB_1 sample than in WP-CB_2 sample and by a taste very characterized by the bran, slightly woody. Firmness was judged positively even if a strong tendency to disintegrate was perceived, with small bran pieces under the teeth detectable. Also WP-CB_1 and WP-CB_2 samples presented with strands of pasta separated from each other on the plate and the starchy residues on the fork were absent. WP-RCB_2 sample had an intense and fragrant flavor of whole-meal semolina, an intense brown color and a taste very characterized by bran, slightly woody, although to a slightly less accentuated extent than that perceived in WP-CB_1 and WP-CB_2 samples. Firmness was judged positively, the cut under the teeth was clean and the tendency to disintegrate was slight. In WP-RCB_2 sample presented with strands of pasta separated from each other on the plate and the starchy residues were absent. These results are broadly consistent with those found by Steglich *et al.* (2015) who found that bran particle size did not significantly influence the sensory attributes liveliness and firmness on the one hand, and that whole-meal spaghetti with larger bran particles had a more intense flavor than whole-meal spaghetti with smaller bran particles, on the other side.

Table 3.9. Optimal cooking time (OCT) and cooking quality of experimental whole-meal pasta samples.

Optimal cooking time (min)	***Pasta sample***				
	WP-FB₁	***WP-CB₁***	***WP-FB₂***	***WP-CB₂***	***WP-RCB₂***
	15:00	14:09	14:59	13:20	14:13
Total score	8.75	8.20	8.60	8.00	8.40
Total judgement	More than good	Good	More than good	Good	Good

WP-FB_1, whole-meal pasta 94% semolina and 6% fine bran; WP-CB_1, whole-meal pasta 90% semolina and 10% coarse bran; WP-FB_2, whole-meal pasta 94% semolina and 6% fine bran; WP-CB_2, whole-meal pasta 88% semolina and 12% coarse bran; WP-RCB_2, whole-meal pasta 88% semolina and 12% re-milled coarse bran.

3.4 Conclusion

The results obtained in this study indicate that whole-meal pasta (spaghetti shape) obtained both from the recombination between semolina and fine and coarse bran fractions, the latter derived from the industrial debranning by-product at 8% of durum wheat blends and subsequent sieving operated by a plansichter, shows a good total folate

content equal to an average of 36.5 μg/100 g. All the samples of whole-meal pasta developed, with the exception of WP-FB_2, have folate values such as to be able to boast the nutrition claim "Source of Folate" and the numerous health claims envisaged for folate on the label (EU Reg. No. 1924/2006; EU Reg. No 1169/2011; EU Reg. No 432/2012). There are few scientific studies that have taken into consideration the effects of pasta making process and cooking on folate retention in whole-meal pasta. The pasta-making process turned out to have a negligible effect on the total folate content in the whole-meal pasta samples of the first pasta-making trial compared to those of the second pasta-making trial, and no significant differences emerged with respect to the total folate content in the whole-pasta samples developed using both the fine and the coarse bran fractions in the formulations. The lowest retention in total folate was observed in the whole-meal pasta sample, WP-RCB_2, obtained from the recombination between semolina and re-milled coarse bran fraction, which showed a total folate value of 35.1 μg/100 g compared to the expected value of 55.7 μg/100 g. The developed whole-meal pasta samples have an average total folate content, 36.5 μg/100 g, higher than both that found for conventional De Cecco whole-meal pasta samples (spaghetti shape), 28.8 μg/100 g, and that found for commercial whole-meal pasta samples (spaghetti shape), 27.4 μg/100 g, while all whole-meal pasta samples have a higher average total folate content than that found for semolina pasta samples (spaghetti shape), 11.4 μg/100 g, as expected. No significant differences emerged even with respect to the total folate content between long and short whole-meal pasta shapes. After cooking, the average folate retention is high in both developed whole-meal pasta (70%) and commercial whole-meal pasta (78%) samples, although the highest average folate retention was found in semolina pasta samples (89%). Even short whole-meal pasta shapes show a high average folate retention of 73%.

Overall, all the developed whole-meal pasta samples are distinguished by a high nutritional value, showing an average content in proteins, fat, total fibre and carbohydrates of 15.1 g/100 g, 2.6 g/100 g, 8.2 g/100 g and 62.1 g/100 g, respectively.

The use of low-temperature drying cycles explains the low furosine levels found for whole-meal pasta samples from the first pasta making trial, where WP-FB_1 has an average furosine level of 224 mg/100 g protein and WP-CB_1 has an average furosine level of 245 mg/100 g protein. On the other hand, the use of a drying cycle equally at low temperature but usually used for semolina pasta leads to higher, despite contained, levels of furosine in the whole-wheat pasta samples of the second pasta making trial. In fact, the highest mean values of furosine equal to 360 mg/100 g protein were found in WP-RCB_2 and WP-

CB_2 samples, which also had the highest values of a* equal on average to 7.0 for WP-RCB_2 and 7.3 for WP-CB_2 samples.

Whole-meal pasta developed using the fine bran fraction has the greatest sensory acceptability when compared with that developed using the coarse bran fraction which shows a darker color and a more bitter taste, even if the use of the re-milled coarse bran fraction leads to obtain a whole-meal pasta with sensory attributes closest to those found for whole-meal pasta produced with the fine bran fraction.

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Chapter 4

General conclusions

4.1 Overall conclusions and future perspectives

Over the last few years, whole-meal pasta has registered an unprecedented spread on the Italian market thanks to the numerous advantages associated with it such as ease of use, affordable price and beneficial effects on health widely recognized and appreciated by consumers. It follows that the pasta industries are called to respond to the growing interest of consumers in whole-meal pasta by providing them with healthy and quality products that meet their expectations. The "whole grain" challenge is even more important if we consider the importance of the pasta industry in Italy. In fact, Italy still continues to be not only the main world producer with an annual production of about 4 tons in 2020, but also the main world consumer of pasta with an average consumption of 23-24 kg/per capita per year. De Cecco company has accepted this challenge by committing itself to the production of a whole-meal pasta with distinctive qualitative, nutritional and sensory attributes, producing whole-meal pasta from durum wheat whole-meal semolina processed in its own mill and offering consumers a wide range of long and short shapes.

Whole grains provide a wide range of nutrients and phytochemicals, including dietary fibre, vitamins and minerals, from whose synergistic action derive positive effects on human health. Among the vitamins, folates (vitamin B9) are gaining increasing attention becoming the subject of studies among food chemists and nutrition researchers given the importance of the functions they are called to perform in human metabolism and, above all, because folate deficiency it is associated with the onset of a wide range of health problems, such as megaloblastic anemia, neural tube defects (NTDs), depression and cognitive dysfunction, certain types of cancer and cardiovascular diseases. Folates are also an object of interest among the pasta industries, considering the nutrition and numerous health claims envisaged for folate pursuant to European Regulations No 1924/2006 and No 432/2012, representing an added value in the label of whole-meal pasta. However, food processing can increase or decrease folate content in durum wheat and whole-meal pasta with consequent more or less positive human health implications deriving from their consumption.

The overall aim of this PhD thesis was to evaluate the effects of debranning, milling, pasta making and cooking processes from durum wheat to whole-meal pasta and to develop a whole-meal pasta with a high nutritional and sensorial value.

The first part of this PhD thesis was to study the distribution of folates in the debranning

and milling fractions of durum wheat samples of different origin using both pilot and industrial plants in order to obtain folate-rich flour fractions to be used in the production of a "functional" whole-wheat pasta. The highest average total folate content was found in the durum wheat blends of the first industrial sampling where it was equal to 61.7 μg/100 g d.m., confirming that the origin also influences the folate content in durum wheat. Both conventional roller milling process of durum wheat on a pilot plant scale and milling process, preceded by preliminary debranning, on an industrial plant scale have led to the obtainment of fractions rich in folates. In the first case the bran and shorts fractions had the highest average total folate content equal to 109.2 μg/100 g d.m. and 114.5 μg/100 g d.m. in North America durum wheat, respectively, as well as a higher average protein and ash contents compared to that found in the starting durum wheat and in the other milling fractions. In the second case, milling by-product obtained from both industrial sampling was characterized by the highest average total folate content equal to 171.7 μg/100 g d.m. Overall, the application of debranning technology led to the recovery of folate-rich flour fractions. Starting from the durum wheat samples debranned with a laboratory debranner, debranned grains and debranning by-products were obtained, the latter characterized by the highest folate levels. Debranning by-product at 3% obtained from National durum wheat had a higher folate content of about 4-fold compared to that found in the starting grain, while in the case of North American durum wheat it was the debranning by-product at 12% that collected the largest amount of folate to the extent of about 3.5-fold higher than that found in the starting grain. The analytical variability found can be attributed to the lack of homogeneity that strongly characterizes the debranning by-products of durum wheat. Similarly, the application of debranning prior to industrial milling led to obtaining a debranning by-product at 8% which was characterized by the highest average total folate content equal to 240.6 μg/100 g d.m. The subsequent sieving at the industrial mill of the debranning by-product at 8% in a plansichter made it possible to recover a fraction of fine bran and a fraction of coarse bran; the coarse fraction obtained from the first industrial sampling was distinguished by an average total folate content that was 4.5-fold higher than that of the starting grain. The strong positive correlation found between folate and ash in the considered samples opens up the possibility of considering the use of folate as possible biomarker of whole grain wheat although further studies are needed. The results found confirm the greater localization of these micronutrients in the outermost cell layers, in particular in the aleurone layer, and in the germ of durum wheat grain, together with the possibility given by debranning technology to recover folate-rich

fractions to be used as functional ingredients for the production of functional foods naturally enriched in folate.

The second part of this PhD thesis was to consider the use of folate-rich flour fractions, such as fine bran and coarse bran, obtained from the industrial debranning and milling process as raw materials to be used for the production of whole-meal pasta enriched in folates (spaghetti shape), together with the evaluation of the impact of the pasta making and cooking process on folate retention. For this purpose, the fine bran and coarse bran fractions were mixed with the semolina according to different formulations in two different pasta making trials. The pasta making process had a negligible effect on the total folate content in the first pasta making trial and both the whole-meal pasta obtained with the fine bran fraction (WP-FB_1) and that obtained with the coarse bran fraction (WP-CB_1) have high folate values equal to 37.8 μg/100 g and 43.1 μg/100 g, respectively. In the second pasta-making trial, the pasta-making process had a greater negative effect on folate retention in whole-meal pasta and this was probably due to the drying cycle used dedicated to semolina pasta and not for whole-meal pasta. Overall, all the whole-meal pasta samples developed, except for WP-FB_2, had folate values higher than 30 μg/100 g (EU Reg. No 1169/2011) so that they can be considered "Source of folate" and the health claims intended for folate can be adopted (EU Reg. No 1924/2006; EU Reg. No 432/2012). However, the developed whole-meal pasta samples had a higher average total folate content (36.5 μg/100 g) than that characterizing both De Cecco conventional whole-meal pasta samples (spaghetti shape) (28.8 μg/100 g) and commercial whole-meal pasta samples (spaghetti shape) (27.4 μg/100 g). No significant differences were found in the total folate content between long and short whole-meal pasta shapes. Interestingly, the developed whole-meal pasta samples after cooking also showed a high average folate retention (70%), becoming an important vehicle of folate in the human diet and an alternative to fortified products with folic acid, in line with the mean folate retention values found in the conventional whole-meal pasta samples (70%) and slightly lower than the mean folate retention values found in the commercial whole-meal pasta samples (78%). All the whole-meal pasta samples developed were characterized by a high nutritional value and could also boast the nutrition claim "High fibre" (EU Reg. No 1924/2006).

The last part of this PhD thesis was to assess the heat damage of the developed whole-meal pasta through the quantification of furosine and the evaluation of the overall sensory quality. The use of the low-temperature drying cycle dedicated to whole-meal pasta

resulted in a low heat damage in the whole-meal pasta samples as confirmed by the low levels of furosine found WP-FB_1 (224 mg/100 g protein) and WP-CB_1 (245 mg/100 g protein). On the other hand, the low-temperature drying cycle dedicated to semolina pasta used in the second pasta making trial turned out to be unsuitable for whole-meal pasta since the Maillard reaction was more intense, as evidenced by the furosine levels found in WP-FB_2 (308 mg/100 g protein), WP-CB_2 (308 mg/100 g protein) and WP-RCB_2 (360 mg/100 g protein). The greater heat damage characterizing the whole-meal samples of the second pasta making trial was further explained also in the light of the higher values of a* in the sample characterized by the highest furosine levels. With respect to the characterization of sensory attributes, whole-meal pasta produced using the coarse bran fraction exhibited a darker color and a more bitter flavor than that produced using the fine bran fraction which boasted the best sensory profile. Interestingly, the re-milling of the coarse bran fraction led to obtaining a whole-meal pasta with improved sensory attributes compared to that produced using the coarse bran fraction as-is.

On the basis of the overall results obtained, it is possible to state how the use of debranning technology before the milling applied to durum wheat in the industrial field favorably impacts folates concentration in derived products. Whole-meal pasta developed using the debranning and milling fractions of durum wheat is a good source of folate and the use of the low-temperature drying cycle dedicated to whole-meal pasta enhances its nutritional and sensorial profile.The use of this technology applied to durum wheat, but also to other cereals, could be used as a starting point to obtain flours rich in folates to be used for the production of further semolina-based products and other cereal products naturally enriched in folates, so as to significantly contribute to the increase of folate intake with the diet.

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