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Review

The colours of durum wheat: a review

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Abstract. Pigments are essential to the life of all living organisms. Animals and plants have been the subjects of basic and applied research with the aim of determining the basis of the accumulation and physiological roles of pigments. In crop species, the edible organs show large variations in colour. In durum wheat grain, which is a staple food for humans, the colour is mainly due to two natural classes of pigment: carotenoids and anthocyanins. The carotenoids provide the yellow pigmentation of the durum wheat endosperm, and consequently of the semolina, which has important implications for the marketing of end products based on durum wheat. Anthocyanins accumulate in the aleurone or pericarp of durum wheat and provide the blue, purple and red colours of the grain. Both the carotenoids and the anthocyanins are known to provide benefits for human health, in terms of decreased risks of certain diseases. Therefore, accumulation of these pigments in the grain represents an important trait in breeding programs aimed at improving the nutritional value of durum wheat grain and its end products. This review focuses on the biochemical and genetic bases of pigment accumulation in durum wheat grain, and on the breeding strategies aimed at modifying grain colour.

Additional keywords: durum wheat, pigment accumulation, pigment oxidation, pasta processing, marker-assisted selection, pigment analytical methods.

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Introduction

Wheat is one of the most widely grown grain crops in the world, and durum and bread wheat represent staple foods for human nutrition, especially in the Mediterranean area. Durum wheat [*Triticum turgidum* (L.) subsp. *turgidum* (L.) convar. *durum* (Desf.)] is a tetraploid wheat that comprises the A and B genomes, and it is the main source of semolina for the production of pasta, couscous and burghul.

Over the last few decades, the yellow-amber colour of semolina has become an important quality trait for durum wheat end products. The yellow colour is due to the carotenoid (yellow) pigment content (YPC) in the whole kernel, and is commercially identified as the yellow index (YI) in semolina (CIE 1986). In addition to their role as an important aesthetic parameter, the carotenoids have important nutritional and health roles. Some carotenoids have provitamin A activity, which provides protection from ocular diseases (Ribaya-Mercado and Blumberg 2004), and all of the carotenoids show antioxidant capacity, which reduces the risk of chronic degenerative diseases (Abdel-Aal *et al*. 2007; Nishino *et al*. 2009).

The other class of pigments, which characterises the aleurone or pericarp of the majority of cereals including durum wheat, is the anthocyanins. The anthocyanins give rise to the so-called pigmented grains, the colour of which can range from blue to purple, and to red. The anthocyanins also have therapeutic roles for humans, against tissue inflammation, capillary fragility, cardiovascular disease, cancer, hyperglycaemia, and oxidative liver damage (Mazza 2000; Stintzing *et al*. 2002; Galvano *et al*. 2007; Ghosh and Konishi 2007; Guo *et al*. 2007; Abdel-Aal *et al*. 2008). Unlike the carotenoids, for which plant breeding is mainly in response to the needs of the pasta producers, the anthocyanins represent a new target for genetic improvement due to consumer demand for foods with greater health benefits.

The colour of the grain and the end products arises from phenotypic variations in the pigments present in the grain, which depend on genetic factors, growing conditions, and technological processes. In particular, in terms of the genetic control, the genes involved in pigment accumulation code both for enzymes involved in pigment biosynthesis and pigment degradation (e.g. enzymes with oxidase activity), and for proteins with regulatory roles (e.g. transcription factors). Information on these genes can be exploited in genetic improvement programs aimed at modifying the contents of these pigments in the processed products.

This review focuses on: (*i*) current knowledge of the biochemical and genetic bases of accumulation and degradation of these pigments in durum wheat grain and pasta products; (*ii*) breeding strategies that are aimed at modifying the accumulation of these pigments in durum wheat grain; and (*iii*) analytical techniques for rapid and simple screening of

advanced durum wheat genotypes characterised by higher pigment contents in the grain.

Biochemical aspects of durum wheat grain colour

Carotenoid pigments: composition and distribution along the kernel

The carotenoids are a group of yellow-orange pigments that are found in many biological systems (Goodwin 1980; Krinsky 1993). The carotenoid pigments include two chemical classes: the carotenes, which are unsaturated hydrocarbons; and the xanthophylls, which are hydroxylated derivatives of the carotenes that have one or more oxygenated groups (Fig. 1). In durum wheat, the endosperm carotenoids are the main colour components, and contribute substantially to the YI of semolina. The YPC in whole kernel is highly correlated with the YI in the semolina, with correlation coefficients >0.94 reported (Fratianni *et al*. 2005; Abdel-Aal *et al*. 2007; Digesù *et al*. 2009).

The YPC reported for durum wheat is higher in the cultivated modern varieties than in the older varieties, landraces and wild populations (Digesù *et al*. 2009). This arises because of the more recent, intense breeding activities towards higher grain pigment concentrations. These activities have been facilitated by the high heritability of this trait, which is largely controlled by additive genetic effects and which has a strong genotypic component and low genotype × environment interaction (Elouafi *et al*. 2001; Clarke *et al*. 2006; Van Hung and Hatcher 2011). Nevertheless, despite this relatively high genetic weighting, some environmental factors can influence the final YI of semolina. Indeed, the YPC has been shown to increase in durum wheat grown under adverse environmental conditions, such as cool and wet conditions (Clarke *et al*. 2006), and salt and water stress (Katerji *et al*. 2005; Borrelli *et al*. 2011; Van Hung and Hatcher 2011; Fratianni *et al*. 2013). This might be due to an increase in the production of components of the plant defence machinery under stress conditions, which includes antioxidant molecules.

The major carotenoid in durum wheat grain is the xanthophyll lutein, which can represent 86–94% of the total carotenoids (Abdel-Aal *et al*. 2007; Digesù *et al*. 2009). For the other carotenoids, such as zeaxanthin, esterified lutein, *Z*-isomers of lutein and zeaxanthin, and the carotenes (e.g. α-carotene, β-carotene, β-cryptoxanthin), these are present in very low amounts that range from 3% to 5% (Panfili *et al*. 2004; Fratianni *et al*. 2005; Abdel-Aal *et al*. 2007; Digesù *et al*. 2009).

With regard to carotenoid distribution throughout the durum wheat kernel, it has been reported that the endosperm has the highest lutein and total carotenoid contents (Hentschel *et al*. 2002; Abdel-Aal *et al*. 2007; Borrelli *et al*. 2008). During milling, lutein is highly preserved, but there might be some loss of β-carotene, although at relatively low levels (Borrelli *et al*. 2008).

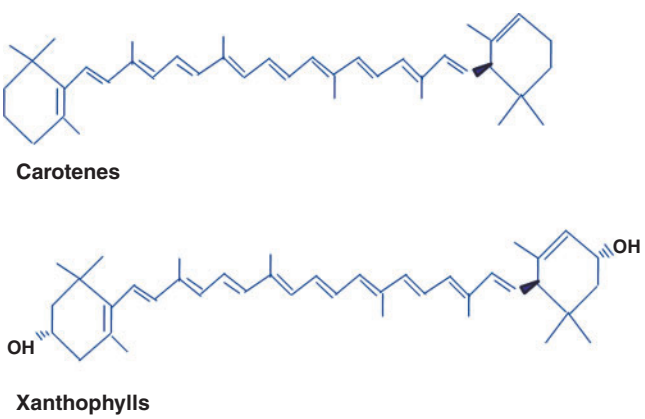

Fig. 1. Basic structure of carotenes and the xanthophylls.

Carotenoid biosynthesis and degradation pathways and their contribution to the yellow colour of semolina and pasta products

The degree of yellowness in durum wheat grain and its end products is affected not only by carotenoid biosynthesis in the grain (Hentschel *et al*. 2002; Panfili *et al*. 2004), but also by carotenoid degradation during processing. This degradation has been mainly ascribed to oxidative enzymes that are responsible for the discoloration and darkening processes that can occur during pasta making (Borrelli *et al*. 1999; Trono *et al*. 1999; Dexter and Marchylo 2000; Feillet *et al*. 2000; Hessler *et al*. 2002).

The first reaction in the carotenoid biosynthesis pathway is the condensation of two molecules of geranylgeranyl pyrophosphate to form phytoene, which is catalysed by phytoene synthase (PSY) (Fig. 2). From this point, a subsequent series of cascade reactions provides increases in the numbers of conjugated double bonds from three in phytoene to eleven in lycopene; this process is catalysed by phytoene desaturase and ζ-carotene desaturase (ZDS), respectively. Lycopene cyclisation then occurs at both ends of the molecule, to generate β-carotene or α-carotene, through the activity of lycopene cyclase (ε-LCY). The formation of the xanthophylls lutein and zeaxanthin arises through the carotenoid hydroxylases. Zeaxanthin can undergo reversible double epoxidation of the rings, which is mediated by zeaxanthin epoxidase, to form violaxanthin, a precursor to abscisic acid. Zeaxanthin, antheraxanthin and violaxanthin (the xanthophyll cycle pool) are involved in the dissipation of light energy in the green tissues of plants. This pathway has at least three rate-limiting steps (Fig. 2): (*i*) early in the pathway, for the synthesis of phytoene; (*ii*) for lycopene cyclisation; and (*iii*) for carotene hydroxylation. Since in wheat the PSY-catalysed reaction has been reported as the rate-limiting step of the biosynthesis pathway, it is feasible that this reaction has a role in the regulation of carotenoid accumulation (Lindgren *et al*. 2003; Cong *et al*. 2009).

For carotenoid degradation, an important family of oxidative enzymes is responsible for the loss of the yellow colour during pasta making—the lipoxygenases (LOXs) (McDonald 1979; Trono *et al*. 1999; Pastore *et al*. 2000; De Simone *et al*. 2010; Verlotta *et al*. 2010). The LOXs are a class of non-heme iron enzymes containing dioxygenase activities and catalysing the positional and specific dioxygenation of polyunsaturated fatty acids with 1,4-*cis*,*cis* pentadiene structures, to produce the corresponding hydroperoxides. The radicals produced during the intermediate states of linoleate hydroperoxidation can cause oxidation of carotenoid pigments and, consequently, a

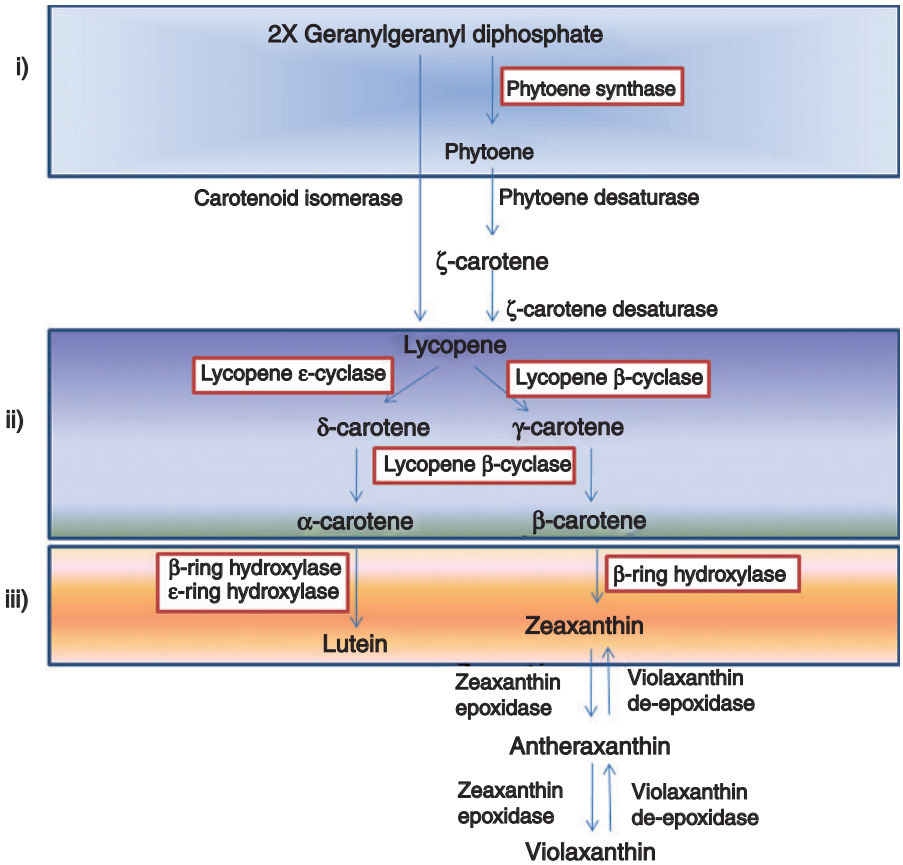

Fig. 2. Carotenoid biosynthesis pathway. Coloured boxes are the three limiting key steps in the carotenoid metabolism.

loss of the yellow colour in pasta products (Siedow 1991). In durum wheat, partial purification of this endosperm protein has led to the definition of at least two typical LOX isoforms (Hsieh and McDonald 1989; Pastore *et al*. 2000). A third, atypical LOX isoform has also been reported, which also shows peroxidase activity (Hsieh and McDonald 1989). The LOX reaction has been shown to be inhibited by carotenoid compounds, and in fact, the percentage of carotenoid loss during pasta processing is inversely related to the initial carotenoid content in the semolina (Trono *et al*. 1999). The LOX-catalysed carotenoid degradation can also be limited by α-tocopherol (Pastore *et al*. 2000). Unfortunately, the content of α-tocopherol in semolina is very low because the germ, where the α-tocopherol is more concentrated (Lintas 1988), is the first portion to be removed during the milling (Fares *et al*. 2006).

Carotenoid pigment degradation is also affected by the peroxidases (PERs), a class of enzymes that can oxidise a large number of compounds at the expense of hydrogen peroxide (Fraignier *et al*. 2000). Fortunately, PERs do not show activity during pasta processing, probably because of the lack of availability of hydrogen peroxide (Icard-Vernière and Feillet1999; Feillet *et al*. 2000).

Finally, the polyphenol oxidases (PPOs) catalyse the oxidation of several phenols that occur naturally in wheat plants and grain. The PPO activities produce quinones, thus generating brown polymers (Sullivan 1946; Mayer and Harel 1979). The brown colour generated in this way tends to mask the yellow colour when it reaches sufficient levels. Nevertheless, the localisation of the PPOs in the aleurone layers indicates that they are unlikely to have a role in the enzymatic browning of the end products derived from semolina.

Pasta processing conditions that affect the yellow pigment degradation

Milling and pasta making (i.e. mixing, kneading, extruding, drying) are the processes involved in the preparation of the durum wheat end products. Milling is based on the opening of the tempered grain (at 16.0–16.5% humidity) and the recovery of the endosperm, step-by-step, going gradually from the inner to the outer part of the grain.

The desirable characteristics of semolina, the main product of durum milling, are a yellow-amber colour, minimum bran specks, and low oxidative enzyme activities, which are responsible for the loss of yellow pigments, and which, together with the low ash content, are involved in the browning of the semolina (Taha and Sagi 1987; Feillet *et al*. 2000).

These oxidative enzymes are not homogeneously distributed in the kernel; rather, there are decreasing amounts in the embryo, bran and endosperm (Rani *et al*. 2001; Borrelli *et al*. 2003, 2008).

It is therefore important to consider the extraction rate, as the milling products that are richer in bran fractions might also include increased enzymatic activities (Hatcher and Kruger 1993; Okot-Kotber *et al*. 2001). The loss of carotenoid pigments that occurs during milling was calculated by Borrelli *et al*. (1999) to be ~8%, based on a laboratory semolina mill with three breaking and three sizing passages. Moreover, it has been reported that high levels of ash in the semolina, together with PERs and PPOs, can lead to an increased brown hue, which reduces the semolina and pasta yellowness (Kobrehel *et al*. 1974; Matsuo and Dexter 1980; Taha and Sagi 1987; Borrelli *et al*. 1999). Therefore, there is need for a balance between the demand of the miller for higher semolina yield and the requirement to have higher YPC and lower ash and oxidative enzyme levels.

In addition to traditional milling, the debranning process has been studied recently, and this involves the removal of the peripheral layers of the grain from the outermost to the internal regions. The debranning process has been shown to favourably affect not only the yield of semolina and the technological properties of the dough (by increasing the alveographic parameters and decreasing the α-amylase activity and microbial contamination; Dexter and Wood 1996; Gys *et al*. 2004), but also the yellow colour (by lowering the oxidative activities and the ash content; Fares *et al*. 1996; Borrelli *et al*. 2008).

The critical point for the final colour is the pasta processing. At the beginning of the process, kneading leads to incorporation of water and oxygen into the dough, which promotes LOX-mediated oxidation of polyunsaturated fatty acids, and which accordingly starts the oxidation of the carotenoids (Delcros *et al*. 1998; Borrelli *et al*. 2003). The next step is the pasta extrusion, during which there is further stimulation of the reduction of the total carotenoids. This decrease has been more evident in pilot laboratory pasta than in industrial processes, because the industrial kneading–extrusion operates under vacuum to limit the presence of oxygen and its oxidative effects on the dough lipid fraction and to reduce the englobing of small bubbles into the dough, which is detrimental for the final pasta structure (Hidalgo *et al*. 2010). In this way, the semolina particles can be hydrated more rapidly and more thoroughly.

Drying is the end phase of pasta processing, and this can also influence the final cooking quality of the pasta (Cubadda *et al*. 2007). Pasta yellowness is the result of concurrent chemical and physical factors. The involvement of the drying process in carotenoid losses can be considered negligible, although different time and temperature processing conditions can affect the final pasta colour in other ways (de Stefanis and Sgrulletta 1990; Borrelli *et al*. 2003). In particular, high or ultra-high temperatures favour the Maillard reaction, which leads to the formation of brown 'melanoidin' pigments. At high concentrations, these pigments can cause browning of pasta products, with consequent masking of the yellow colour (Marchylo and Dexter 1989).

Throughout pasta processing, the percentage of carotenoid loss has been shown to range from 4% for pasta produced from semolina with high YI and low LOX activity, to 20% for pasta produced from semolina with low YI and high LOX activity (Borrelli *et al*. 1999; De Simone *et al*. 2010).

Anthocyanin pigment: composition and distribution along the kernel

The anthocyanins are secondary plant products of flavonoid metabolism, and they have long sparked the interest of biologists. Chemically, the anthocyanins are based on anthocyanidin (aglycone), with sugar saccharide residues bound at different hydroxylated positions on the basic structure (Fig. 3). Individual anthocyanins differ in the numbers of hydroxyl groups and sugars, and in the aliphatic aromatic acids attached to the sugars, and they are also affected by pH, temperature, solvent and presence of co-pigments (Mazza 2007). The anthocyanins contribute almost all of the blue, purple and red colours to many fruits, vegetables and flowers (Delgado-Vargas *et al*. 2000; Winkel-Shirley 2001).

Among the cereals, many studies have investigated pigmented rice (Ryu *et al*. 1998; Abdel-Aal *et al*. 2006; Sompong *et al*. 2011) and maize (Moreno *et al*. 2005; Del Pozo-Insfran *et al*. 2006). In contrast, information in the literature relating to pigmented durum wheat is still lacking. In bread wheat, the anthocyanins are located in the grain and in other organs such as the culm, coleoptile, anthers and glumes (Khlestkina *et al*. 2010). For the kernel, the anthocyanins are predominantly in the external layers (Adom *et al*. 2005), similar to the other antioxidant phytochemicals. The blue wheat pigments are in the aleurone layer, whereas the purple is in the pericarp layers (Zeven 1991; Abdel-Aal and Hucl 1999).

Few studies have been conducted to evaluate the effects of the environment on the expression of anthocyanin content in the wheat kernel. Abdel-Aal and Hucl (2003) evaluated the effects of different growing seasons on the anthocyanin content of the wheat grain, and showed greater effects in a blue aleurone spring wheat line than in two commercial red and purple wheat cultivars. These effects are probably related to the different localisation of the pigments inside the wheat kernels.

Purple grain has been identified for several tetraploid wheats including *Triticum dicoccum*, which originates from Ethiopia and which was then introgressed into hexaploid wheats, where this trait has been widely investigated (Zeven 1991; Eticha *et al*. 2011). The gene for blue aleurone was transferred from the tall wheatgrass *Agropyron elongatum* to bread wheat (*Triticum aestivum* L.) (Zeller *et al*. 1991; Morrison *et al*. 2004). Similarly, translocation lines of the tall wheatgrass *Thinopyrum ponticum*

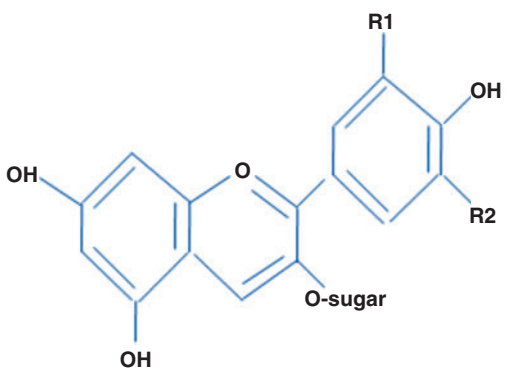

Fig. 3. Basic structure of anthocyanins, which are formed by two benzene rings and an oxygen-containing pyran ring.

with the blue aleurone gene(s) have also been characterised (Zheng *et al*. 2006).

All of the published data report greater total anthocyanin content in blue wheats and in purple wheats than in red wheat (Abdel-Aal *et al*. 2006, 2008; Eticha *et al*. 2011). With respect to anthocyanin composition, Hu *et al*. (2007) reported that in blue-coloured bread wheats, cyanidin-3-glucoside is the main component, and that pelargonidin-3-glucoside and cyanidin-3-galactoside are also present. Abdel-Aal and Hucl (2003) and Abdel-Aal *et al*. (2006) reported the first component as delphinidin-3-glucoside, followed by delphinidin-3-rutinoside, with trace levels of cyanidin-3-glucoside and peonidin-3-glucoside. For the purple-coloured bread wheats, cyanidin-3-glucoside, peonidin-3-glucoside and cyanidin-3-galactoside are the main anthocyanins found, while 10 other compounds have been seen at trace levels (Abdel-Aal and Hucl 2003; Abdel-Aal *et al*. 2006).

The anthocyanin biosynthesis pathway shows two main parts (Fig. 4): the general phenylpropanoid pathway, and the specific steps towards flavonoid biosynthesis. In the first of these pathways, phenylalanine is converted to 4-coumaryl-CoA through different steps. The second committed step in anthocyanin biosynthesis is catalysed by chalcone synthase, which uses 3-malonyl-CoA, the main precursor of the flavonoids, and 4-coumaroyl-CoA as substrates to produce chalcone. Chalcone isomerase then catalyses the stereospecific isomerisation of the yellow-coloured chalcone to the colourless naringenin. Naringenin is converted by flavanone 3-hydroxylase into dihydroflavonols. The conversion to the coloured anthocyanins then initially requires the reduction of the dihydroflavonols to leucoanthocyanidins by dihydroflavonol-4-reductase. Further oxidation, dehydration and glycosylation of the different leucoanthocyanidins can then produce the corresponding pigments: orange-red pelargonidin, red cyanidin, and blue delphinidin. The differences in the types of glycosides and acyl groups attached are both species- and variety-dependent.

For wheat, it has been reported that expression of the gene for flavanone 3-hydroxylase is the pivotal point in the regulation of anthocyanin biosynthesis (Tereshchenko *et al*. 2013), as previously seen in other plant species (Pelletier and Shirley 1996).

Applications to the food and colorant industry

Anthocyanin-pigmented grains can be useful for the production of foods, either from the whole grain, or following the extraction of the natural colourants from the anthocyanin-rich grain fractions, as an alternative to artificial colorants. The location of the anthocyanins in the outer layers of the kernel also facilitates their extraction.

To date, among the cereals, only pigmented rice and maize have been used for the production of foods for human consumption. In particular, blue and purple maize grains are used for blue and pink tortillas, while red rice is used in the manufacture of commercial infant cereals (Hirawan *et al*. 2011). For pigmented wheat, use in the food industry has been more limited; in particular, purple bread wheat is crushed into large pieces that are spread over the outside of bread, whereas blue bread wheat does not appear to have any food applications at present (see Abdel-Aal *et al*. 2006, and references therein). Although it has been predicted that the production and addition of anthocyanins as natural food colourants will steadily increase, particularly following the current trend away from synthetic colourants (Horbowicz *et al*. 2008), at present there are only a few applications regarding anthocyanin pigments extracted from red rice (Ma *et al*. 2000; Shipp and Abdel-Aal 2010).

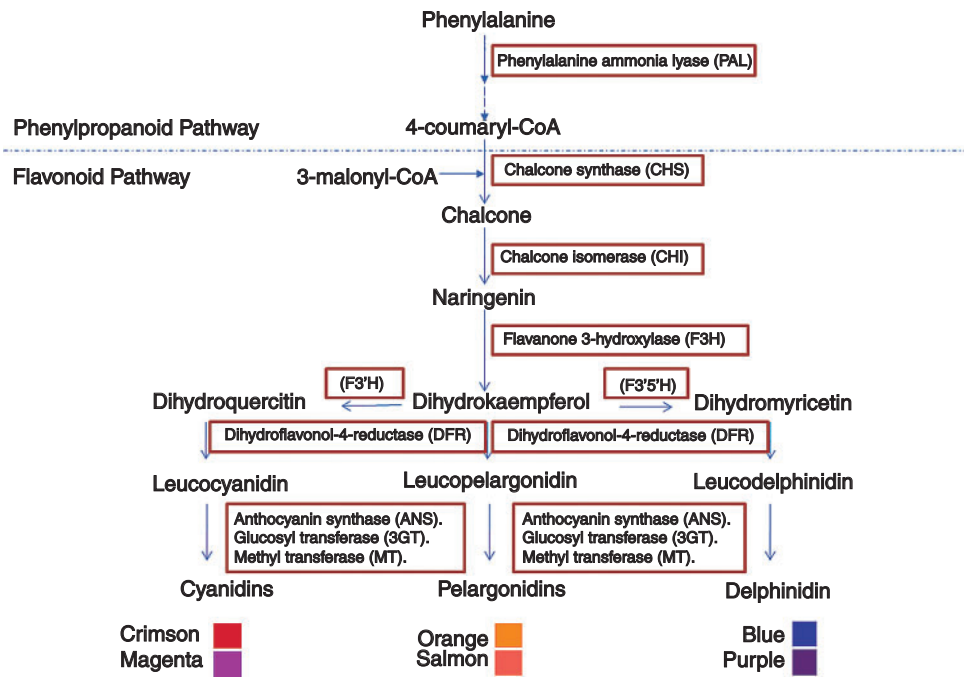

Fig. 4. General phenylpropanoid and flavonoid pathways.

In this regard, investigations aimed at better defining the total and specific contents and the distribution of the anthocyanins in pigmented durum wheat accessions and varieties will be useful.

Genetic aspects of durum wheat grain colours, and breeding strategies for their improvement

The long-term history of durum breeding has been characterised by genetic progress in terms of yield, plant height reduction, and lowering of the straw yield, which has resulted in increased kernel size. This was followed only at the end of the last Century by improvements to end-use quality attributes such as gluten strength and endosperm carotenoids (Clarke *et al*. 2010). In Italy, until the mid-1990s, selection for YPC did not receive particular attention from breeders, as demonstrated by the results of De Vita *et al*. (2007) and as previously reported by Digesù *et al*. (2009). However, the most recent durum wheat varieties released in the last decade show significantly higher YPC than the old varieties released before the 1970s (Digesù *et al*. 2009). This observation suggests that grain and semolina colour has become a particular sign of quality in durum wheat, and that in the last two decades, breeders have focussed attention on high YPC during the selection of new cultivars.

The selection process has been expedited due to the integration of traditional plant breeding methods with modern molecular marker technologies, and also due to the widespread use of non-destructive colourimetric reflectance measurements according to the Commission Internationale de l'Eclairage (CIE) scale and using near-infrared reflectance (NIR) spectroscopy. Genetic analyses based on molecular markers have allowed the identification of genomic regions involved in the accumulation of pigments in wheat grain. Moreover, the finding of molecular markers that are closely linked to genes and/or quantitative trait loci (QTL) that control the colour trait opened the way for increasing the pigment content of élite cultivars using of marker-assisted selection.

On the other hand, it must be considered that improvement of final colour will be due to the important roles of both the biosynthesis and degradation pathways in the determination of colour of the grain and end products. Therefore, molecular studies have been directed towards the genes that encode the key enzymes that regulate both of these metabolic pathways.

Genetic control of carotenoid biosynthesis

Studies using linkage and association mapping have led to the demonstration that the QTLs involved in the control of YPC in wheat grain are numerous and spread across many chromosomes of the wheat genome. Not all QTLs identified have the same importance in terms of their influence on this colour trait. The major QTLs were mapped to telomeric regions of the long arm of chromosomes of the homeologous group 7, for both durum and bread wheat. The QTLs that mapped to chromosomes 7A (Mares and Campbell 2001; Patil *et al*. 2008; Zhang and Dubcovsky 2008; Zhang *et al*. 2008, 2009; Howitt *et al*. 2009; Blanco *et al*. 2011) and 7B (Kuchel *et al*. 2006; Pozniak *et al*. 2007; Zhang and Dubcovsky 2008; Zhang *et al*. 2008) have been shown to explain in many cases the highest percentages of observed phenotypic variability (>50%) (Parker *et al*. 1998; Elouafi *et al*. 2001). Recent findings have shown that as well as the QTLs mapped at the telomeric region of the long arms of these chromosomes, there are other QTLs that have minor effects on colour traits on both arms of chromosomes 7A (Singh *et al*. 2009; Blanco *et al*. 2011; Roncallo *et al*. 2012) and 7B (Zhang and Dubcovsky 2008; Blanco *et al*. 2011; Roncallo *et al*. 2012).

The inheritance of YPC has been shown to be relatively complex, as many minor QTLs have been found on all of the chromosomes of durum wheat through different approaches. Significant marker-trait associations for YPC have been detected on all of the chromosomes by linkage mapping (Parker *et al*. 1998; Hessler *et al*. 2002; Pozniak *et al*. 2007; Patil *et al*. 2008; Zhang *et al*. 2008, 2009; Howitt *et al*. 2009; Blanco *et al*. 2011; Roncallo *et al*. 2012) and by association mapping (Reimer *et al*. 2008).

Most studies of QTLs for YPC have been based on the evaluation of total YPC. Recently, Blanco *et al*. (2011) analysed a segregating population for the accumulation of individual carotenoid components. Some of the QTLs identified explained both the individual and total carotenoids. This is in agreement with significant positive correlations among the individual carotenoids, and between the individual carotenoids and the total carotenoids and the YI within the segregating population. The QTLs have been found that explain the accumulation of more than one of these compounds, as previously shown for maize (Wong *et al*. 2004; Chander *et al*. 2008), chickpea (Abbo *et al*. 2005) and carrot (Just *et al*. 2009). Since the examined carotenoid traits represent compounds that are synthesised at different steps in the same biochemical pathway in several plant species (Just *et al*. 2009), the clustering of several QTLs for various carotenoid traits may be due to pleiotropy. The identification of QTLs specifically controlling the accumulation of α-carotene and β-carotene (chromosomes 2A, 3B, 7A; Blanco *et al*. 2011) represents a valuable tool to increase the levels of provitamin A activity of durum and bread wheat grain and, therefore, the nutritional value.

Several studies have been dedicated to the identification of candidate genes involved in the control of YPC. Two main approaches have been used in this regard: identification of significant associations between particular allelic forms of a gene and the phenotypic expression of the trait, and demonstration of co-localisation between a candidate gene and a QTL that explains the YPC. One of the genes best studied is the gene that directs the metabolism towards carotenoid synthesis, which codes for PSY. The PSY are also considered as the rate-limiting enzymes for this pathway, and the PSY1 enzymes, in particular, are required for carotenoid accumulation by the endosperm (Gallagher *et al*. 2004; Li *et al*. 2008). Almost 50 different alleles have been identified at the *Psy-A1* and *Psy-B1* loci in different species of wheat (He *et al*. 2008, 2009*a*; Howitt *et al*. 2009; Singh *et al*. 2009; Wang *et al*. 2009; Crawford *et al*. 2011; Ravel *et al*. 2013) (Table 1). The genes coding for PSY were mapped to the chromosome 7 groups by Pozniak *et al*. (2007); in particular, the *Psy-B1* locus co-segregated with a QTL on chromosome 7B, which demonstrated an association between the position of this gene and part of the phenotypic variation for endosperm colour (Pozniak *et al*. 2007; Roncallo *et al*. 2012). Similarly, *Psy-A1*, located on chromosome 7A, showed a co-dominant marker based on polymorphisms between two *Psy-A1* haplotypes that explained 20–28% of the phenotypic

Table 1. Markers for grain colour and related traits on the A and B wheat genomes
Bold text indicates that marker is correlated positively to the colour trait. SNP, single nucleotide polymorphism

Locus	Marker	Allele	Chromosome	Reference
		Yellow pigment content (YPC)		
Psy-A1	YP7A	*PsyA1a*, *PsyA1b*	7AL	He *et al*. 2008; Ravel *et al*. 2013
	YP7A-2	*PsyA1a*, *PsyA1b*, *PsyA1c*	7A	He *et al*. 2009*b*; Ravel *et al*. 2013; Crawford *et al*. 2011
	Psy-A1_STS	***Psy1-A1o***		Singh *et al*. 2009
		Psy-A1e, ***Psy-A1p***, *Psy-A1a Psy-A1c*, ***Psy-A1t***	7A	Crawford *et al*. 2011
	Psy-A1_R_49	*Psy-A1a*, *Psy-A1k*, *Psy-A1ka*, *Psy-A1kb*, *Psy-A1e*, *Psy-A1ca*, *Psy-A1cb*, *Psy-A1p*,*Psy-A1ra*, *Psy-A1rb*, *Psy-A1j*, *Psy-A1t*	7A	Ravel *et al*. 2013
Psy-B1	YP7B-1	*Psy-B1a*, *Psy-B1b*	7BL	He *et al*. 2009*a*; Ravel *et al*. 2013
	YP7B-2	*Psy-B1c*		He *et al*. 2009*b*
	YP7B-3	*Psy-B1d*		He *et al*. 2009*b*
	YP7B-4	*Psy-B1e*		He *et al*. 2009*b*
		Psy-B1a, *Psy-B1aa*,*Psy-B1b*, *Psy-B1ca*,*Psy-B1m*, *Psy-B1d*		Ravel *et al*. 2013
Zds-A1	YP2A-1	*Zds-A1a, Zds-A1b*	2AL	Dong *et al*. 2012
ε-*LCY* (lutein)		***e-LYC3Aa, e-LYC3Ab***	3A	Crawford and Francki 2013
		Lipoxygenase activity (LOX)		
Lpx-B1.1c			4BS	De Simone *et al*. 2010;Verlotta *et al*. 2010
Lox-B1	LOX16	*Lox-B1a*	4BS	Geng *et al*. 2012
	LOX18	*Lox-B1b*		
		Polyphenol oxidase activity (PPO)		
	wPt-7024		2AL	Sadeque and Turner 2010
Ppo-A1	PPO18, PPO33	*Ppo-A1a*, *Ppo-A1b*	2AL	Sun *et al*. 2005; He *et al*. 2007; Nilthong *et al*. 2012
Ppo-B1	F-8	*Ppo-B1a*, *Ppo-B1b*	2AL	Si *et al*. 2012*a*, 2012*b*
	PPO-A2	Closely linked to SNP markers Xcfa2058 and Xiwa174	2AL	Beecher and Skinner 2011; Beecher *et al*. 2012
	PPO-B2	Closely linked to SNP markers Xiwa175 and Xiwa4866	2BL	Beecher and Skinner 2011; Beecher *et al*. 2012
		Purple grain colour (Pp)		
Pp3	Xgwm328 and Xgwm817	*Pp3a*, *Pp3b*	2A	Dobrovolskaya *et al*. 2006
Pp1	Xgwm983 and Xgwm676	–	7BL bread wheat	Dobrovolskaya *et al*. 2006
Pp1	Xgwm0573	–	7BS durum wheat	Khlestkina *et al*. 2010; Tereshchenko *et al*. 2013
Pp	Xgwm47	–	2AS	Li *et al*. 2010
Pp	Xgwm155	–	3AL	Li *et al*. 2010

variance for YPC across three environments (He *et al*. 2008). Positive associations were found between YPC and the *Psy1-A1o* allele in a collection of 93 cultivars of durum wheat and in a recombinant inbred line population (Singh *et al*. 2009), with the *Psy-A1t* allele in a panel of 30 Australian lines of bread wheat and *Psy-A1p* allele in the segregating population Ajana × WAWHT2074, in which the locus controls 32–36% of the observed variability for the trait (Howitt *et al*. 2009; Crawford *et al*. 2011). Seven new alleles for *Psy-A1* and two new alleles for *Psy-B1* were recently identified in bread wheat by Ravel *et al*. (2013). Moreover, the marker *Psy-A1_R_49* was shown to be associated with high YPC in a core collection of 372 genotypes.

The *Psy* genes are the most studied genes in terms of their association with the YPC phenotype, although recent evidence has shown that other genes are involved in the control of this trait. One study reported that *ZDS* is highly associated with yellow pigment (Crawford and Francki 2013), where a co-dominant functional marker *YP2A-1* showed polymorphisms of the two alleles *Zds-A1a* and *Zds-A1b*. A new QTL for yellow pigment was detected on chromosome 2A that co-segregated with the marker *YP2A-1* and explained 11.3% of the phenotypic variance in a doubled haploid population. Moreover, an association between the ε-*LCY* gene on chromosome 3A and a QTL that explains the lutein content in seeds was described by Howitt *et al*. (2009) in bread wheat. More recently, a highly significant association was shown between the homologous copy of a gene on chromosome 3A (ε-*LCY3A*) and a QTL for b* colour in two segregating populations (Crawford and Francki 2013).

Little is known about the causal modifications that determine high YPC in associated alleles. Crawford *et al*. (2011) identified unique phosphorylation sites in the *Psy-A1t* allele. The production of alternatively spliced transcripts of *Psy-A1* determines the translation of enzymatically inactive proteins

(Howitt *et al*. 2009). Point mutations in genes coding for ε-LCY result in the substitution of a conserved amino acid in high-lutein alleles (Howitt *et al*. 2009; Crawford and Francki 2013).

Genetic control of carotenoid degradation during pasta processing: lipoxygenases, polyphenol oxidases and peroxidases

Lipoxygenases

The wheat genes that encode the LOX isoforms are named *Lpx*. The *Lpx-1* and *Lpx-3* genes encode the LOX-1 and LOX-2 isoforms, respectively, and they are located on chromosome 4, whereas the *Lpx-2* gene that encodes the LOX-3 isoform is on chromosome 5 (Manna *et al*. 1998; Carrera *et al*. 2007; Zhang *et al*. 2008; Garbus *et al*. 2009; De Simone *et al*. 2010; Feng *et al*. 2010; Verlotta *et al*. 2010). Molecular studies of *Lpx* in developing durum wheat kernels have shown different transcript levels, with *Lpx-1* transcripts being the most abundant in mature grain (De Simone *et al*. 2010). This suggests that the LOX-1 isoform might have a major role in oxidation of carotenoid pigments during pasta processing. This hypothesis is supported by evidence highlighting the existence of a major QTL for total LOX activity on chromosome 4BS, where three copies of the *Lpx-1* gene, *Lpx-B1.1*, *Lpx-B1.2* and *Lpx-B1.3*, along with three different *Lpx-B1.1* alleles, *Lpx-B1.1a*, *Lpx-B1.1b* and *Lpx-B1.1c*, have been mapped (Nachit *et al*. 2001; Hessler *et al*. 2002; Carrera *et al*. 2007; Zhang *et al*. 2008; Verlotta *et al*. 2010). The *Lpx-B1* genes/alleles comprise seven exons and six introns, with the exception of the *Lpx-B1.1c* allele, which has a large central deletion and most probably encodes a non-functional LOX-1 isoform (Verlotta *et al*. 2010).

Screening of a large collection of germplasm has revealed that all of the durum wheat genotypes that carry the *Lpx-B1.1c* allele also have very low LOX activity in the grain (Verlotta *et al*. 2010). Therefore, selection and fixing of this allele (Table 1) in all breeding lines will contribute to significantly reduced pigment loss during pasta processing and, consequently, to improvement of the aesthetic and nutritional qualities of the pasta products.

Recently, in hexaploid wheat, Geng *et al*. (2012) characterised the full-length genomic DNA sequence of a *LOX* gene (designated as *Lox-B1*) (Table 1) that is located on chromosome 4BS and shows high identity (98.6%) with the durum wheat *Lpx-B1.2* gene, previously reported by Verlotta *et al*. (2010). Two complementary, dominant sequence-tagged site markers, *LOX16* and *LOX18*, were developed based on the single nucleotide polymorphism of two alleles at the *Lox-B1* locus, with the amplification of 489-bp and 791-bp fragments in cultivars with higher and lower LOX activities, respectively.

Polyphenol oxidases and peroxidases

A major genetic effect on PPO activity has been reported to be located on the long arm of chromosomes 2A and 2D in hexaploid wheat (Jimenez and Dubcovsky 1999; Raman *et al*. 2005; Sun *et al*. 2005; He *et al*. 2007), and several markers have been identified (Table 1). Sadeque and Turner (2010) reported that PPO activity in wheat is mainly controlled by a locus on the long arm of chromosome 2A that has strong genetic association with the diversity arrays technology marker *wPt-7024*. Recently, other paralogous *PPO* genes were identified on group 2, *PPO-A2*, *PPO-B2* and *PPO-D2* (Beecher and Skinner 2011). Genes *PPO-A2* and *PPO-D2* were shown to be expressed at high levels in developing wheat kernel. Beecher and Skinner (2011) localised the new *PPO-2* gene family members to group 2 homeologous chromosomes (long arm); *PPO-A2* was shown to be located 8.9 cM proximal to *PPO-A1* on the long arm of chromosome 2A. Similarly, *PPO-D1* and *PPO-D2* are separated by 10.7 cM on the long arm of chromosome 2D, while *PPO-B2* was mapped to the long arm of chromosome 2B and is the site of a novel QTL for PPO activity (Beecher *et al*. 2012). Due to the high similarity between bread and durum wheat genomes, the results obtained in bread wheat can be used to accelerate breeding for this trait in durum wheat. Indeed, the QTL analysis on PPO activity in durum wheat carried out by Watanabe *et al*. (2006) and Simeone *et al*. (2002) reported a major genetic effect on the long arm of chromosome 2A. For PERs, several homologous sets of loci that control the peroxidases have been identified independently in different studies (Asins and Perez de la Vega 1985; Bosch *et al*. 1986; Liu *et al*. 1990). In particular, Asins and Perez de la Vega (1985) described several seed peroxidase loci in tetraploid wheat on the short arms of homeologous groups 1, 2, and 3: *Per-1* is active in coleoptile tissue; *Per-2* shows some polymorphism and is most active in root tissue; *Per-3* is highly variable and most active in embryo tissue; and *Per-4* is carried on chromosome arms 7AS, 4AL and 7DS, and is relatively variable and most active in endosperm tissue.

Genetic control of anthocyanins

The *Pp3* genes that influence the purple grain colour trait were mapped to the centromeric region of chromosomes 2A and 7BL (*Pp1*, at ~24 cM distal to the centromere) (Dobrovolskaya *et al*. 2006). The *Pp1* (pericarp) gene was shown to be non-allelic to the *Rc-1* (red coleoptiles) and *Pc* (purple culm) genes. Similar results have been found for durum wheat, except that the *Pp* gene maps to the short arm of chromosome 7B, instead of the long arm, as found in bread wheat (Khlestkina *et al*. 2010).

Using bulked segregant analysis, Li *et al*. (2010) detected associations of this trait with two microsatellite markers, *Xgwm47and Xgwm155* (Table 1). Based on the positions of the molecular markers in bread wheat maps, the gene region indicated by the markers might correspond to that of *Pp3* (Röder *et al*. 1998). Further studies are needed to clone and characterise the genes responsible for this trait.

In a recent study, transcriptional analysis of the five anthocyanin biosynthesis structural genes was carried out in near-isogenic lines that differed in the allelic state of loci involved in the purple colour of different organs in wheat, comprising *Pp1* (Tereshchenko *et al*. 2013). The alleles that confer strong pigmentation promoted higher transcription levels of the structural genes, which, for *Pp1* and the other genes, suggests roles as transcriptional factors in the anthocyanin biosynthesis network (Tereshchenko *et al*. 2013).

Analytical methods for pigment determination: classical and rapid methods

In breeding programs, there is often a need to evaluate grain quality on large numbers of plants or families, often using only small amounts of grain for each selection. In such situations, the

availability of rapid, small-scale analytical methods provides opportunities to improve efficiency and complements the use of classical analytical methods. Even when molecular markers are used early in plant development to impose selection for grain-quality traits, it is still necessary to confirm the quality of the grain harvested from selected plants.

Two different approaches for durum wheat colour measurements are described below that have found widespread acceptance: one that considers the individual pigment components using sensitive techniques, and the other as a rapid method for screening purposes of total pigments.

Analytical methods for individual pigment compounds

The literature reports different solvents and extraction procedures (Konopka *et al*. 2006; Digesù *et al*. 2009) for the identification of carotenoids in grain and in various food matrices used as food additives (Breithaupt 2004), and also for the anthocyanins in grain (Abdel-Aal *et al*. 2006; Hosseinian *et al*. 2008).

The direct extraction of carotenoids with organic solvents is based on the use of water-saturated 1-butanol, ethanol, butylated hydroxytoluene, and methanol/tetrahydrofuran (Konopka *et al*. 2006; Digesù *et al*. 2009). Some of these solvents have also been used in aqueous solution for anthocyanin extraction, including ethanol, methanol and acetone (Castañeda-Ovando *et al*. 2009) and, as most widely used in wheat, acidified methanol (Abdel-Aal *et al*. 2006; Hosseinian *et al*. 2008).The most sensitive, although expensive, technique used for the identification and quantification of the carotenoids is based on high-performance liquid chromatography (HPLC) with a photodiode array detector system. The chromatographic separation is achieved using either normal or reverse-phase HPLC (Panfili *et al*. 2004; Burkhardt and Böhm 2007; Digesù *et al*. 2009; Van Hung and Hatcher 2011).

Analysis with HPLC was used for the quantitative determination of the main *Z*/*E* carotenoid stereoisomers, while for unequivocal structural determination of all of the main stereoisomers, HPLC-atmospheric pressure chemical ionisation mass spectrometry (HPLC-APCI-MS) and HPLC-nuclear magnetic resonance (HPLC-NMR) coupling have been used (van Breemen *et al*. 1996; Byrdwell 2001; Putzbach *et al*. 2005). Such HPLC techniques provide selective methods for the identification and quantification of novel genetic sources aimed at increasing β-carotene levels. These techniques are also useful to distinguish the two provitamin A components, α-carotene and β-cryptoxanthin, attributable to a single provitamin A structure (Wong *et al*. 2004; Blanco *et al*. 2011).

Quantification of the anthocyanins has been achieved using HPLC with anthocyanin reference standards. However, some peaks have remained undefined due to unavailability of all standards. These non-identified peaks were shown to be anthocyanins using mass spectrometry.

Indeed recently, techniques such as HPLC coupled mass spectrometry (HPLC-MS), APCI-MS and electrospray ionisation mass spectrometry (ESI-MS) have become powerful tools for anthocyanin identification in different matrices (Castañeda-Ovando *et al*. 2009), including cereals (Escribano-Bailón *et al*. 2004; Abdel-Aal *et al*. 2006; Hu *et al*. 2007; Hosseinian *et al*. 2008; Hirawan *et al*. 2011; Žilić *et al*. 2012). As a complement to the more widely used methods described, NMR remains a powerful method for the structural definition of the anthocyanins, as reported in a study by Aoki *et al*. (2002) on glycosylated derivatives in the seed of purple corn.

Rapid analytical methods for total pigment concentration

Several analytical procedures have been developed to evaluate the carotenoids and anthocyanins in durum wheat. Early methods that were designed to quantify total colour and are today still in use are based on light reflectance and spectrophotometric measures.

Methods to evaluate the yellow pigment as a colourimetric index have commonly used semolina colour, and are based on light reflectance measurements. Reflectance measurements are obtained using a Minolta CR-300 Chroma Meter (Konica Minolta Pty Ltd, Macquarie Park, NSW) equipped with a pulsed xenon arc lamp, for absolute measurements of the L* (lightness), a* (red-green chromaticity), and b* (yellow-blue chromaticity) (CIE 1986) coordinates in the Munsell colour system, using D65 lightning. The *b* value represents the variation in the yellow intensity.

In the same way, for the anthocyanins, the Hunter Laboratory colourimeter has been used to evaluate the L*, a* and b* colour values, as reported in a study of anthocyanin colour development in spring wheat by Knievel *et al*. (2009).

The yellow pigments of durum wheat flour, semolina and pasta are analysed according to the International Association for Cereal Science and Technology (ICC) Standard Method 152 (ICC 1990), or the AACC International Official Method 14-50.01 (AACC International 2013). Both are based on the extraction of durum wheat total pigments from samples (100 or 8 g, respectively) using water-saturated 1-butanol, followed by spectrophotometric evaluation of the optical density of the clear filtrate at an absorbance of 435.8 nm, and they are reported in terms of the β-carotene content. These official methods have been revised and adapted to breeding programs in which the limiting factor is the low amount of material.

In this context, Fu *et al*. (2013) proposed a rapid, micro-scale pigment extraction procedure for semolina that reduces the sample size to 200 mg and the volume of water-saturated 1-butanol to 1 mL. This method quantifies the pigment loss in the first step of processing due to LOX degradation, although the limiting step is the availability of durum kernels to make the semolina.

Beleggia *et al*. (2010) set up a micro-method on a single wheat kernel for the determination of YPC in durum wheat. This method is based on micro-sample amounts (10–100 mg, corresponding to a single wheat kernel) with micro-extraction volumes of water-saturated 1-butanol (250–500 µL) and a short extraction time (15 min, with an ultrasonic bath). The results obtained are not significantly different from those obtained with the Official AACC International Method 14-50.01 (AACC International 2013). Moreover, Beleggia *et al*. (2011) also applied the same micro-method to determine the difference in YPC of single kernels taken from different positions along a single ear of durum wheat.

As with the carotenoids, at present spectroscopy is the main technique used for the anthocyanins. In particular, this method is based on spectrophotometric determination at two pH levels (i.e. 4.5, 1.0) of 500-mg samples, with the reading at the maximum absorption wavelength of 520–540 nm (Abdel-Aal and Hucl

1999; Escribano-Bailón *et al*. 2004; Abdel-Aal *et al*. 2006; Hosseinian*et al*. 2008).

The most used methodology is based on the NIR application, as a rapid, non-destructive method with no environmental hazards; NIR does not include organic solvent extractions and is cost-effective for large-scale programs (Osborne *et al*. 1982; Shenk *et al*. 1990; Zandomeneghi *et al*. 2000). McCaig *et al*. (1992) used NIR/visible spectroscopy to estimate yellow durum pigment, where they showed a high association with a reference method based on solvent extraction ($r^2 = 0.94$).

Recently, Sissons *et al*. (2006) used NIR spectra to assess the quality of Australian breeding lines of durum wheat grain, to predict kernel, flour and dough qualitative parameters such as test weight, 1000-kernel weight, grain hardness, semolina yield, semolina yellow colour, semolina browning and cooked pasta firmness. Dowell (2000) also used NIR spectroscopy to determine the vitreousness of durum wheat kernels and to predict many of their other parameters, including their b* colour value, with accuracies that are suitable for screening (Dowell *et al*. 2006).

Although some aspects of NIR spectroscopy are indeed advantageous for all the reasons mentioned above, this remains a comparative technique that relies on multivariate calibration of sample spectra and accurate reference analysis. It needs to take into account a sample set that covers most of the variability reported in the literature for colour of durum wheat grain. Moreover, it requires ~4 g of each sample to be packed into a black aluminium cup with a quartz window, which is not always available (Brenna and Berardo 2004). However, if properly calibrated, NIR spectroscopy can be exploited not only to measure the total quantity of pigments, but also to quantify the individual components. Indeed, over the past 25 years, the number of applications of NIR spectroscopy has grown rapidly, as demonstrated by the number of dedicated journals and large international conferences; therefore, it has the potential to be exploited as a rapid analytical method for many qualitative characters. The prediction of total anthocyanin concentrations with the NIR technique has been used for wine quality assessment (Urbano-Cuadrado *et al*. 2004; Janik *et al*. 2007), although it has not yet been applied in cereal quantification. Therefore, this technique could also be developed in durum wheat for prediction curves for beneficial health parameters.

Conclusions

The information in the literature indicates the great advances that have been made in our understanding of the biochemical and molecular basis of durum wheat colours. This has enabled the setting up of successful breeding programs. Added to this, upgraded analytical methods will help in the manipulation of the grain and the end product colour.

The combination of a high colour concentration with the presence of specific, highly nutritional coloured compounds should contribute further to the development of functional food for improved nutritional and health qualities.

The knowledge that has been obtained on the biochemistry and genetics of pigment accumulation in durum wheat grains will open the way for further studies aimed at the identification of the genes and proteins that act on the biochemical pathways, from the structural and regulatory points of view. The cloning of the genes within the major QTLs that control the accumulation of the pigments will further increase the speed and effectiveness of breeding programs, in which the selection can be based on genotype rather than phenotype.

Moreover, understanding the mechanisms by which these pigments exert their beneficial effects for humans, in terms of intestinal absorption *in vivo* and optimum dosage, should accelerate ongoing improvements to wheat programs and investment.

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