

Carotenoids in cereals: an ancient resource with present and future applications

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Abstract Cereals are considered to be a major source of carbohydrates and proteins as well as minor micronutrients such as minerals, vitamins and antioxidants, including carotenoids. Carotenoids are natural lipophilic pigments biosynthesized mainly by plants, and certain bacteria and fungi. Cereals, although having a low carotenoid content when compared with the majority of fruits and vegetables, may have an important impact in the nutritional status of consumers. The daily consumption of cereals, and products derived from them, by a large part of the population, especially in under-developed and developing countries, makes cereals a contributor of carotenoids which should not be overlooked and must be taken into consideration in biofortification strategies. In the present manuscript, we revise the existing information about the composition and distribution of carotenoids in cereals, highlighting factors which alter their profile, such as domestication of wild varieties, genotype, storage, milling and processing techniques. Strategies for stimulating the carotenoid content in cereals, either by means of traditional breeding methods or by genetic manipulation, are also discussed.

Keywords Xanthophylls · Carotenes · Lutein · Wheat · Staple food

Abbreviations

AMD	Age-related macular degeneration
MAS	Marker assisted selection
PSY	Phytoene synthase gene
QTL	Quantitative trait locus
ROS	Reactive oxygen species
XAT	Xanthophyll acyltransferase
YPC	Yellow pigment content

Introduction

Carotenoid pigments are molecules of lipophilic nature and are responsible for some of the yellow, orange and red colour of a wide range of fruits and some animal teguments (Britton and Hornero-Méndez 1997). Animals are not able to synthesize them *de novo* and so they must be acquired through the diet. Carotenoids are located in oil droplets, crystalloids and membrane structures within specialized vegetable organelles, the so-called plastids, being the most common the chloroplasts (in green tissues) and the chromoplasts (yellow, orange and red tissues), and are found in all parts of the plant: roots, leaves, flowers, fruits and seeds (Howitt and Pogson 2006). Carotenoids carry out their most basic functions in vegetables as pigment collectors of light in the photosynthetic apparatus, and preventing oxidative damage acting as antioxidants.

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When carotenoids are ingested, they exert important biological activities; antioxidant, inhibition of carcinogenesis, enhancement of the immune response and cell defence against reactive oxygen species (ROS) and free radicals, and the reduction on the risk of developing cardiovascular and other degenerative diseases (Nishino 1997; recently reviewed by Britton et al. 2009). In addition some carotenoids (β -carotene, α -carotene, β -cryptoxanthin, etc.) have provitamin A activity (Baker and Günter 2004; Olson 1989). Of particular interest are the epidemiological studies showing an inverse correlation between the progression of age-related macular degeneration (AMD) and cataracts and the high intake of lutein and zeaxanthin rich-vegetables, being both pigments present in high concentration at the macula in the retina humans and primates (Ahmed et al. 2005; Alves-Rodrigues and Shao 2004; Calvo 2005; Landrum and Bone 2001).

Cereals are basic food stuffs of vegetable origin, also known as staple food, which are characterised mainly as sources of carbohydrates and proteins, and contain a relatively small amount of carotenoids compared to the majority of fruits and vegetables. However, the daily consumption of cereals and products derived from them by a large part of the population, especially in under-developed and developing countries, makes cereals a contributor of carotenoids which must be taken into consideration (Graham and Rosser 2000). Therefore, cereals can be considered ideal elements for use in biofortification strategies (Bai et al. 2011).

An essential step for the development of carotenoid biofortified crops is the complete characterization of the carotenoid profile contained in the vegetables, allowing this an adequate knowledge of their metabolism, which can be used for the selection and breeding of new cultivars. The success of such processes lies in the existence of a more or less extensive variation of carotenoid contents among different cereals. This variability is mainly due to three factors: (a) the genotype of the cereal, (b) the varietal selection, the pressure exerted by man and its domestication, and (c) the growing conditions and post-harvest storage and processing (industrial and domestic) practices. Regarding the last factor, the evaluation of such changes is an active area of study in food science research, which allows the prediction and selection of the optimal conditions for preserving these phytochemicals, driven by their nutritional properties as well

as the applications derived from their natural role as being responsible for the colour of foods. Thermal treatments and others which include homogenisation and modification of the food matrix are factors which have a beneficial effect on the bioaccessibility of carotenoids since it facilitates their solubility. The term bioaccessibility is used to evaluate the amount of a nutrient that is released from a food during the digestion process. The bioaccessibility of lipophilic compounds, such as carotenoids, in natural foods is frequently fairly low and is constrained by various factors, particularly the degree of food processing and matrix composition. There are evidences suggesting that homogenisation and thermal treatment have positive effects on the bioaccessibility of these compounds. Therefore, foods such as cereals, which are normally consumed as processed foods, could present some advantages in comparison to fresh food.

Up to now, there have been several studies regarding the qualitative and quantitative composition of carotenoids in fruits and vegetables, but very few with respect to cereals and therefore studies which determine the carotenoid profile of these foodstuffs are necessary. Undoubtedly, investigations aimed at improving the bioaccessibility of carotenoids in cereals will benefit from such knowledge and it will aid in the information and recommendation provided to the consumer about the way of processing and consumption of these basic elements.

In the present manuscript, we revise the existing information about the composition and distribution of carotenoids present in cereals, highlighting factors which alter their profile, such as storage and processing. At the same time, the strategies for stimulating the carotenoid content in cereals, either by means of traditional breeding methods or by genetic manipulation, are also discussed.

Composition and distribution of carotenoids in cereals

Cereals and their derivatives are good examples of foods that contain a complex mixture of minor components such as phenols, folates, vitamin E, phytic acid, sterols and carotenoids, some of them with antioxidants properties (McKevith 2004). In general, the content of bioactive compounds in cereals is mainly concentrated in the germ and bran portions and

most of them are located in the aleurone layer. At the same time, the content in phytochemicals is subjected to important variations due to factors such as the type of cereal, the cultivar, cultivation conditions, stage in development, and storage and processing conditions (Fardet et al. 2008; Liu 2007;), with the greatest importance lying in the interactions between the genotype and the environment (Hidalgo et al. 2009; Lv et al. 2013; Van Hung and Hatcher 2011). The carotenoid profile in cereals is mainly composed of xanthophylls, with lutein as the most abundant, followed by zeaxanthin and β -cryptoxanthin in addition to carotenes such as α - and β -carotene in small amounts (Fig. 1). The largest concentration of carotenoids can be found in the embryo, although this part of the seed only represents 3–5 % of its total weight. On the contrary, the contribution of the endosperm, around 80–85 % of the cereal grain, is the most influential in the total content of carotenoids in the grain. The distribution of this carotenoid profile seems to vary among genotypes of the same type of cereal (Siebenhandl et al. 2007) and within the same grain since α -, β -carotene and zeaxanthin are concentrated in the bran and the germ, while lutein is distributed more homogeneously (Borrelli et al. 2008; Konopka et al. 2004; Ndolo and Beta 2013; Panfili et al. 2004). In relation to the composition of carotenoids in cereals, the bibliography is fairly scarce, especially for barley (*Hordeum vulgare*), rye (*Secale cereale*) and millet (*Panicum miliaceum*) (Choi et al. 2007; Kandlakunta et al. 2008; Mamatha et al. 2011).

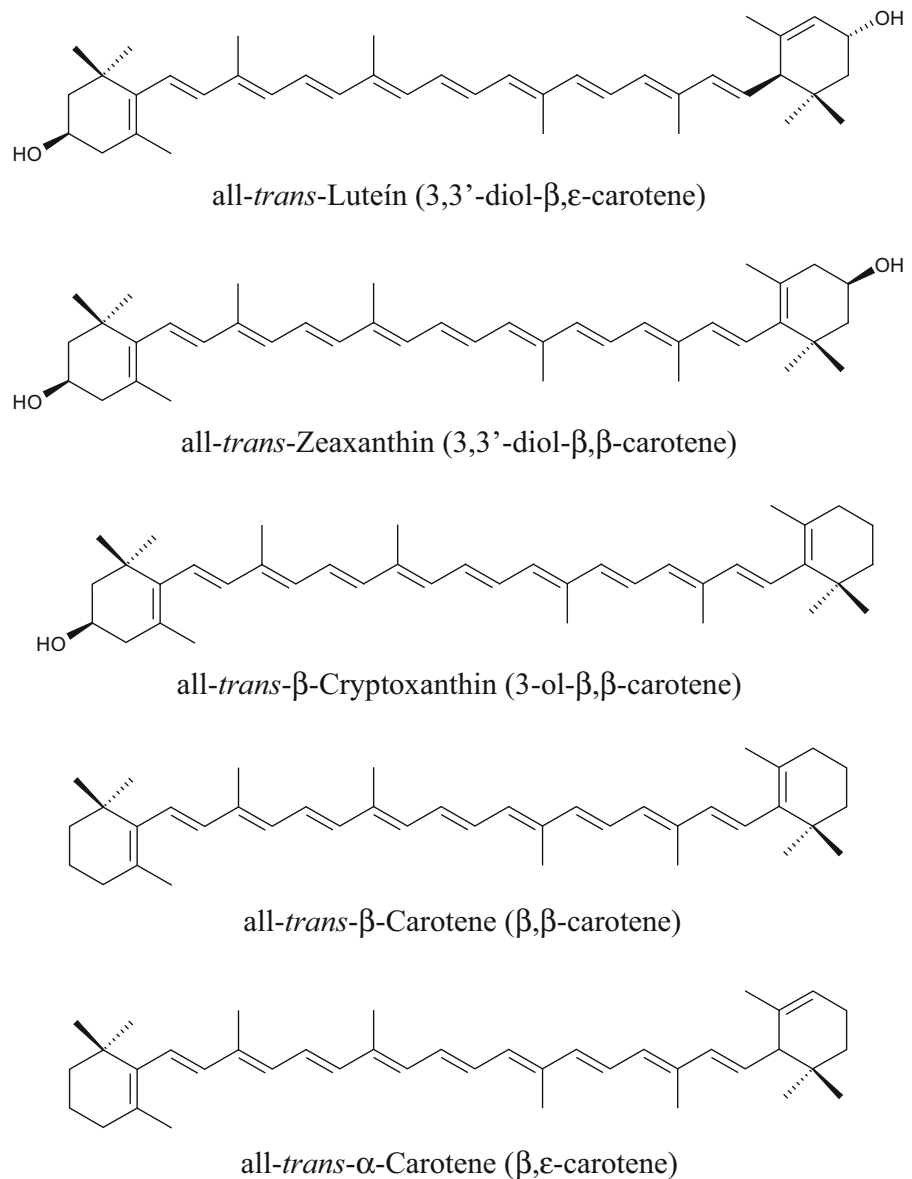
In light of the fact that the level of carotenoids in cereals is low in comparison to fruits and vegetables, corn (*Zea mays*) of the yellow genotype may be cited as the cereal which presents the greatest levels (up to 63 $\mu\text{g/g}$) of these phytochemicals. Most of the cultivars present zeaxanthin as the major pigment but also show small amounts of β -cryptoxanthin α -carotene and β -carotene (Moros et al. 2002; Panfili et al. 2004). From the quantitative studies carried out on this cereal, a large variation in the contents of carotenoids has been determined among varieties (Berardo et al. 2004, 2009; Egesel et al. 2003; Kurilich and Juvik 1999; Quanckenbush et al. 1961), which serves as proof that genotype is the determining factor for this variety (Ibrahim and Juvik 2009; Menkir and Maziya-Dixon 2004), followed by storage and processing conditions (Burt et al. 2010; De Oliveira and Rodriguez-Amaya 2007; Scott and Eldridge 2005).

In the case of rice (*Oryza sativa*), it is obvious that the pigmented varieties are the ones which result in some interest from a quantitative point of view. In the grains of conventional rice the carotenoid content is concentrated almost exclusively in the bran and therefore the common practices of milling and stripping of the grain result in a drastic reduction in this component in the final product (Tan et al. 2005). Similar to most cereals, lutein is the main pigment (Belefant-Miller and Grace 2010), followed by zeaxanthin and β -carotene in crops with dark or black pigmentation (Frei and Becker 2005; Kim et al. 2010; Lamberts and Delcour 2008; Nakornriab et al. 2008). In some varieties of black rice from Thailand, the concentration of β -carotene found in its bran is significant (up to 40 $\mu\text{g/g}$) (Nakornriab et al. 2008).

A recent study carried out on glabrous canary seed (*Phalaris canariensis* L.) has shown a content of β -carotene ranging from 5.2 to 6.3 $\mu\text{g/g}$, which is the major pigment in this cereal (Li and Beta 2012). Although sorghum (*Sorghum bicolor*) has been considered one of the cereals with the least amount of carotenoids, the pioneer findings of Blessin et al. (1958) and Suryanarayana Rao et al. (1968) concerning varieties of yellow endosperm, as well as the recent studies of Kean et al. (2007, 2011), offer better perspectives for this cereal. These authors found a carotenoid content between 0.11 and 0.32 mg/kg, which although lower than the content corresponding to yellow corn, these data must not be overlooked, given the importance of this cereal in the diet of some populations (mainly in Asia and Africa).

Oats (*Avena sativa*) are also cited in the literature as one of the cereals with the lowest level of carotenoids, with contents of lutein and carotenes (α - and β -carotene) in the order of 0.20 mg/kg and 0.01 mg/kg, respectively (Panfili et al. 2004). Wheat (genus *Triticum*), along with corn and rice, are the most popular cereals due to their generalised consumption. This is the most consistently seen aspect in the literature, with special interest being paid to the study of their antioxidant profile and contents of healthy phytochemicals as well as their inter- and intra-varietal distribution (Adom et al. 2003, 2005; Di Silvestro et al. 2012; Lier and Lacroix 1974; Okarter et al. 2010; Velioglu et al. 1998; Zhou et al. 2004a, b; Zhou and Yu 2004a, b). Similar to the case of corn, the genetic variability among wheat genotypes concerning the contents of carotenoids, offers an interesting

Fig. 1 Chemical structures of the most common carotenoid pigment (carotenes and xanthophylls) present in cereal grains



tool which can be effectively used in programs aimed at augmenting the contents of bioactive compounds with properties which are beneficial to health (Digesù et al. 2009; Leenhardt et al. 2006a).

Wheat varieties with diploid genome (AA), in particular the Einkorn wheat (*Triticum monococcum*), are considered those presenting the highest levels, in the order of approximately 2–4 times higher with respect to other wheat varieties. Einkorn also stands out as possessing the greatest antioxidant activity (Lavelli et al. 2009). Followed by these, tetraploid wheats (AABB) can be found and has been determined

to contain the highest levels of carotenoids (around 5–6 $\mu\text{g/g}$) among the durum wheat cultivars (*Triticum turgidum* conv. *durum*), although others have been mentioned such as the ancestral Emmer (*T. turgidum* subsp. *dicoccoides*) with similar levels (approximately 3–5 $\mu\text{g/g}$). Finally, wheat varieties belonging to the hexaploid genome (AABBDD) are considered to have the lowest carotenoid content, with almost exclusive attention paid to the common bread wheat (*T. aestivum*), which shows an average content around 2 $\mu\text{g/g}$ (Abdel-Aal et al. 2007; Hidalgo et al. 2006; Hidalgo and Brandolini 2008a; Serpen et al. 2008).

These quantitative data indicate that the selection and domestication of cereal species carried out by man has derived in a wide variation of their carotenoid levels (both low and high) (Digesù et al. 2009). The case of durum wheat constitutes an example which has been extensively analysed due to the fact that the yellow colour of its semolina (also referred to as *yellow pigment content*; YPC, determined as lutein equivalents by the official method 125 of the International Association for Cereal Science and Technology and method 14–50 of the American Association of Cereal Chemists) is considered one of the principal criteria of quality of processed derived foods, mainly pasta (Blanco et al. 2011; Fratianni et al. 2005; Hentschel et al. 2002; Humphries et al. 2004). An inverse situation occurs with bread wheat destined to flour production, which presents lower levels of carotenoids due to the pressure exerted by industries motivated by a consumer preference for whiter flour for the further preparation of flour-containing products according to market demand.

The *Triticeae* tribe, which includes wheat, barley and rye species, is a series of polyploids which are closely related, being possible to generate fertile hybrid amphiploids among the different cultivated members of this tribe and its wild parents. Among the hybrid cereals, tritordeum, an innovative cereal with interesting properties and applications, must be pointed out. Tritordeum is a cereal which has been obtained from the cross-breeding between a wild barley, *Hordeum chilense*, with diploid genome ($H^{ch}H^{ch}$), and diploid and polyploid wheats (Martín and Chapman 1977). In addition to the development of amphiploids such as tritordeum, the use of *H. chilense* in the enhancement programs has also been focused on the inclusion of wheat with new characteristics of interest. Among the highlighted attributes, a high content of carotenoids, determined by $7H^{ch}$ chromosome, stands out. The first fertile hybrids with the optimal characteristics for becoming a possible crop were only obtained following to the crossing of *H. chilense* with *T. turgidum* (a tetraploid durum wheat, with genomic denotation AABB), generating those denominated hexaploid tritordeums ($2n = 6x = 42$, $H^{ch}H^{ch}AABB$) which showed a low frequency of aneuploids, a wide variation in growth rate and an optimal level of fertility (Martín and Sanchez-Monge Laguna 1982).

Most of the estimations on the carotenoid content made on tritordeum did not provide detailed

information about the individual composition of the pigment profile due to the lack of specificity of the traditional analytical methods employed for the characterisation of carotenoids in cereals. By means of estimations such as YPC, levels of carotenoids in the order of 5–6 times higher (in the range of 11–13 $\mu\text{g/g}$) have been found in tritordeum with respect to common wheat (2 $\mu\text{g/g}$) and 2–3 times higher with respect to durum wheat (5–6 $\mu\text{g/g}$) (Álvarez et al. 1995; Martín et al. 1999), which is comparable to the levels reported for Einkorn wheat. In an extensive study which evaluated a total of 35 primary lines of tritordeum together with their respective parental lineage, 27 *H. chilense* accessions and 19 durum wheat cultivars, the average levels of carotenes ($\mu\text{g/g}$ of β -carotene equivalents, determined as YPC) for the amphiploid continued to be in the order of 2 times higher than durum wheat parent, but 3 times lower than the average of *H. chilense* (Álvarez et al. 1999). Among the advanced lines of tritordeum, HT621 was registered as an elite germplasm line due to its high content of carotenoid pigments (19 μg of β -carotene equivalents/g, YPC) (Ballesteros et al. 2005). Recent studies carried out in our laboratory (Atienza et al. 2007a; Mellado-Ortega and Hornero-Méndez 2012) have contributed substantially to the characterisation and quantification of individual carotenoid pigments in tritordeum. Similar to most cereals, lutein is also the major pigment (>85 %), with a high esterification degree (monoesters and diesters), along with minor quantities of β -carotene (Fig. 2). On average, the total carotenoid content of tritordeum (6.5 $\mu\text{g/g}$ of fresh weight) was significantly higher, approximately eight times higher, when compared to durum wheat (0.7 $\mu\text{g/g}$ of fresh weight). For the first time, the regioisomers of the monoesters and diesters of lutein in a cereals, in this case in tritordeum, have been isolated and characterized (Mellado-Ortega and Hornero-Méndez 2012) (Fig. 3). The fraction of monoesters is made up of the regioisomers, namely lutein-3'-*O*-linoleate, lutein-3-*O*-linoleate, lutein-3'-*O*-palmitate and lutein-3-*O*-palmitate, while the fraction of diesters is composed of two homoesters, lutein dilinoleate and lutein dipalmitate, and by the two regioisomers of a heterodiester, lutein-3'-*O*-linoleate-3-*O*-palmitate and lutein-3'-*O*-palmitate-3-*O*-linoleate. The esterification of lutein with only two different fatty acids (linoleic and palmitic acids) suggests a high-specificity degree for the in vivo process, which should be further investigated. The

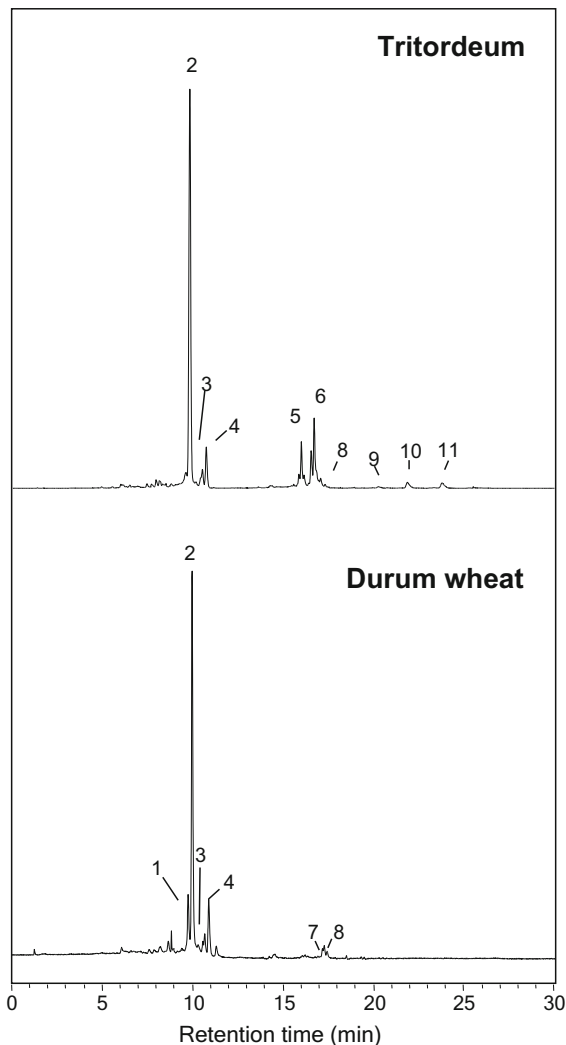


Fig. 2 HPLC chromatograms corresponding to direct carotenoid extracts obtained from tritordeum and durum wheat. Peaks identity: 1 all-*trans*-zeaxanthin, 2 all-*trans*-lutein, 3 9-*cis*-lutein, 4 13-*cis*-lutein, 5 lutein monolinoleate, 6 lutein monopalmitate, 7 all-*trans*- α -carotene, 8 all-*trans*- β -carotene, 9 lutein dilinoleate, 10 lutein linoleatopalmitate, 11 lutein dipalmitate

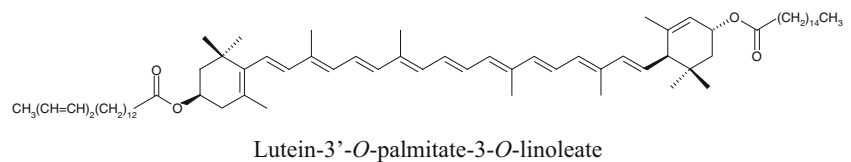
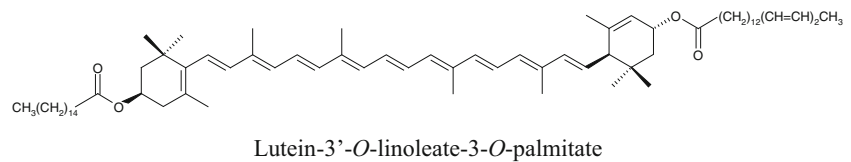
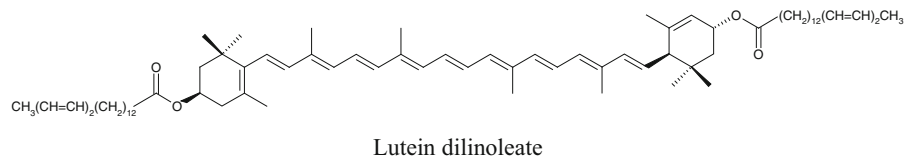
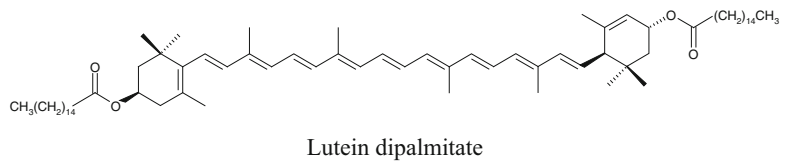
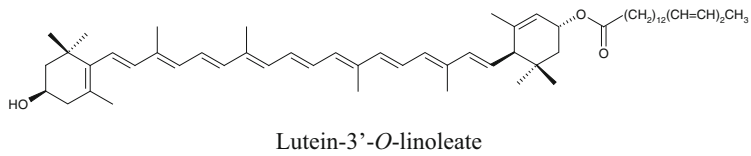
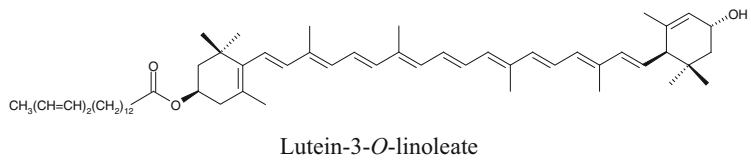
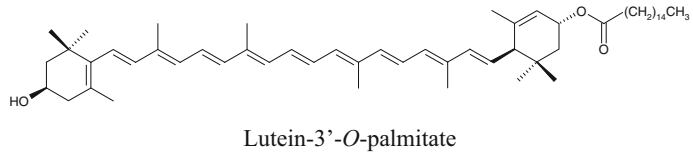
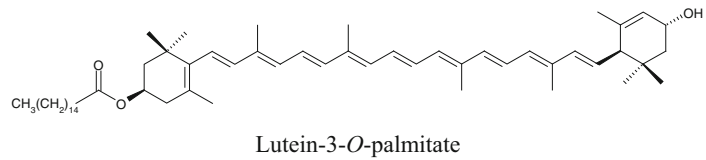
ability to distinguishing the regioisomers of mono- and diesters of lutein provides a powerful tool contributing to a better deciphering of this poorly known biochemical pathway, for which tritordeum grains could be used as an excellent plant model. Evidences for a preferential xanthophyll acyltransferase activity regarding the position (3 or 3') and the acyl moiety are discussed. Further studies should be carried out in order to identify the acyltransferase enzymes (XAT) and the acyl donor molecules involved in the xanthophylls esterification process.

Tritordeum is currently the subject of an intense breeding program being developed at the Institute of Sustainable Agriculture (CSIC; Cordoba, Spain) in order to optimise its use as a new cereal and its incorporation into the formulation of functional foods. Its cultivation areas are concentrated in Spain (Andalusia, Castilla and Catalonia), the south of Italy and the south of Portugal. In 2008 it was registered, under the name Aucan, the first line of tritordeum in the European Union Plant Variety Registration (CPVO), with other advanced lines in their final phases of development and evaluation. As a result of these efforts, at the beginning of 2013, the Spanish company Agrasys (www.agrasys.es) began to commercialise tritordeum flour under the brand name Vivagran[®].

Cereal technology and its influence on the carotenoid contents in grains and derived products

Conservation and storage conditions, as well as different processing treatments, result in overall changes in the composition of foods, being this the main reason for the numerous studies carried out in the field of food technology which are crucial for determining some important sensorial and nutritional quality attributes (Kalt 2005; Mínguez-Mosquera et al. 1997; Nicoli et al. 1999; Rodríguez-Amaya 2003). In this way, it is important to distinguish between the effects of processing itself, and other environmental factors such as partial oxygen pressure, temperature, exposure to light and humidity as well as the interactions with other antioxidant and pro-oxidant molecules which are present (Lindley 1998; Nicoli et al. 1999). In the case of the carotenoids occurring in cereals, the storage of the natural and processed grain normally results in a decrease in the pigment contents, which is directly proportional to the processing time and the increase in the intensity of other variables such as temperature or the degradation process (Cristobal 1965; Weber 1987). It is interesting to note that the literature concerning this aspect is especially scarce since cereals are characterised as undergoing prolonged storage periods as part of their industrial and technological treatments. On the other hand, it is not easy to define a set of general guidelines in the behaviour of pigments in processes such as peeling, grinding, drying, roasting, fermentation, etc.,

Fig. 3 Chemical structure of the monoesters and diesters, including the regioisomers, of lutein with fatty acids identified in grains of *tritordeum* (\times *Tritordeum* Ascherson et Graebner)



due to the fact that positive, negative and even indifferent results can be found in the literature regarding the level of carotenoids present. In general, these processes would affect the internal structure of the cereal matrix and the processed product with substantial implications regarding accessibility and further bio-availability of the carotenoids.

The rate at which these changes occur has been a generalised subject of food science. The bibliography concerning the kinetics of carotenoid degradation and/or retention in foods is abundant as well as the disparity of the results which have often been contradictory (Mínguez-Mosquera et al. 1997). The profusion of such studies reflects the importance of carotenoid pigments for the food industry, not only from a nutritional point of view, but from a technological standpoint, with interest in not just quantifying losses in carotenoids but also analysing the conditions which provide their greater stability and subsequent retention. Colour is considered one of the main consumer acceptance criteria for processed foods. As mentioned before, in the flour and bread as well as the semolina and pasta this factor is important, especially for products derived from durum wheat. Therefore the research efforts in this field try to reproduce the processing and storage conditions of food, including cereals, with the aim of comparing different industrial treatments and monitoring the incidences of individual or joint factors such as water, temperature, light, oxygen and pH activities along with pro-oxidants and antioxidants (Fish and Davis 2003; Mínguez-Mosquera and Gandul-Rojas 1994; Ouchi et al. 2010; Saxena et al. 2012; Selim et al. 2000; Tsimidou 1997), allowing for an estimate of the shelf-life of foods and their bioactive compounds. Most of the studies have been carried out on vegetables such as carrots (Koca et al. 2007; Lemmens et al. 2010; Wagner and Warthesen 1995), tomato (Sharma and Le Maguer 1996; Tonon et al. 2007); peppers and pepper derived products (Carbonell et al. 1986), potatoes (Bechoff et al. 2010), and citrus fruit juices (Dhuique-Mayer et al. 2007; Zepka et al. 2009) where the authors commonly describe a degradation reaction which occurs according to zero or first order kinetics. However, the literature is quite limited in the particular case of cereals, probably due to the low level of pigments that these foods present and there are very few studies which carry out a detailed kinetic evaluation (Guzman-Tello and Cheftel 1990; Hidalgo and

Brandolini 2008b; Mellado-Ortega 2013). Nevertheless, although such alterations have not been quantified, most authors agree in the fact that changes generally occur more rapidly at the beginning of processing and storage and as they progress the rate at which they are occurring diminishes (Burt et al. 2010; Quackenbush 1963).

Stability of carotenoids during the post-harvest storage of grains and flours

The storage process produces a loss in carotenoids mainly caused by oxidation, either of an enzymatic or non-enzymatic nature. The oxygen present in the medium is considered the major factor affecting the stability of carotenoids (Britton and Khachik 2009). Other modifications of the carotenoid molecule associated with these processes are the geometric isomerisation (*cis/trans*) produced by temperature and/or light and cause alterations in the composition rather than net losses in the pigments. The mechanisms of both processes have been extensively studied (El-Agamey and Mcgarvey 2008; Liaaen-Jensen and Lutnaes 2008).

While the oxidation of pigments in the storage of cereal grains comes mainly from oxygen, in processed foods such as flour the alteration of the matrix produces direct oxidative processes as well as those caused by enzymes which come into contact with carotenoids (Doblado-Maldonado et al. 2012). As previously mentioned, the results obtained in these studies depend on the conditions applied, mostly evaluating the effects of time and temperature applied during the treatments. The literature contains examples in which the effects of these two variables are clearly observed, which allow for the prediction of the consequences of these variables in the stability of the carotenoids present (Arya and Parihar 1981; Calucci et al. 2004; Farrington et al. 1981; Hidalgo and Brandolini 2008b; Nghia et al. 2006; Pinzino et al. 1999).

On occasion, in addition to storage, the experimental designs additionally cover another factors corresponding to common practices in cereal technology (such as drying, grinding, shelling, etc.). This widens the study but at the same time difficult the analysis of the obtained results. Belefant-Miller and Grace (2010) evaluate the behaviour of carotenoids during the

prolonged storage of rice which was previously shelled. A common practice in cereals prior to storage is the drying of the grains since the reduction in humidity prevents deterioration due to mould, acarus, etc., as well as a premature germination of the grain (Chelowski 1994; Jood and Kapoor 1994). Some authors, such as Burt et al. (2010) did not obtain a significant reduction in pigment content when comparing drying at a high temperature (90 °C) to the corresponding ambient temperature while maintain the rest of the storage conditions for corn. On the contrary, other studies have found significant differences in the pigment content which are attributed not only to the drying of the grain but also to duration of the process (Quackenbush 1963). In spite of this controversy, the optimal conditions for the preservation of cereals, and particularly to preserve the carotenoids present in them are always reached by employing an exhaustive method of water removal (dehydration) and/or storage at low temperatures.

Studies on the storage of grains and processed grains also allow for the evaluation of aspects relating to the post-carotenogenic metabolism such as esterification with fatty acids which mediate its accumulation and stability in these foods (Kaneko and Oyanagi 1995; Kaneko et al. 1995). The increase in the esterification of lutein as the storage of grain progresses, including storage under controlled temperature, is one aspect that has been characterised by several authors (Ahmad et al. 2013; Mellado-Ortega et al. 2015). This provides information about the ideal conditions for the storage of cereals which preserves the contribution of these phytochemicals. In addition, a specific evaluation has been applied in order to characterise the enzymatic systems involved in esterification of the xanthophylls during the storage of grains and flours of tritordeum and durum wheat. This has resulted in important differences attributed not only to the nature of esterification in each case, but also to the enzymatic regional selectivity of the lutein and fatty acid molecules participating in such reactions (Mellado-Ortega 2013). Regardless of the type of plant material, either grain or flour, the stability of the esterified and free pigment has been studied and it has been found that the degradation rate is always greater for the latter case. This provides valuable information which could be used in crop enhancement programs of cereals. In agreement with these results the studies aimed to increase the cereal contents in carotenoids

should be based on the selection of varieties with greater concentrations of esterified xanthophylls with the objective of increasing their ability to store them within the grain tissues, along with improving their stability in the seeds.

On the other hand, the influence of thermal pre-treatments on the storage of flour has been studied to evaluate the generation of free radicals and their impact on endogenous antioxidants (Andersen et al. 2011), as well as the possible intervention of degrading enzymes (Rodriguez-Amaya 1997).

Effect of processing on the carotenoid contents of cereals

As explained above, the different processing techniques undoubtedly modify the carotenoid content present in the final product of processed cereals, due to, among other factors, an uneven distribution of the pigments in the different parts of the grain (Konopka et al. 2004; Zhou et al. 2004a, b). This has led to the modification and development of new processing techniques with the aim of preserving or stimulating the carotenoid contents along with other phytochemicals of nutritional importance. These emerging techniques are being incorporated into the traditional enhancement programs and genetic manipulation for obtaining bio-fortified grain crops (Fardet 2010; Hemery et al. 2007). Cereals are generally processed in two ways: dry fractioning followed by baking (at different temperature conditions, water contents and pressure) to produce product such as pasta, biscuits, breakfast cereal, etc., and fermentation, which produce alcoholic beverages. The production of the most popular cereal-derived products such as bread and baked goods include both treatments.

The fractionation and grinding processes of the cereal grains have allowed for the detailed analysis of carotenoids in the different layers and industrial fractions of the seeds (Ndolo and Beta 2013). The analyses of whole-grain products and those containing the germ (Kean et al. 2008), flour without bran and its formulations (Žilić et al. 2012), whole and shelled grains (Kean et al. 2011), grains, flour and semolina (Fares et al. 2008; Luterotti and Kljak 2010), are among the most common comparisons. One of the first studies carried out in this way was done by Blessin et al. (1963) on corn. These authors analysed the effect

of manual and industrial processing on the pigment content found in different layers of the grain. Recently, Sellappan et al. (2009) has described the losses in β -carotene, iron and zinc in genetically modified rice varieties subjected to polishing and have estimated them to be higher than 70 %. Polishing is a common industrial practice for rice grains, which involves the near complete elimination of the aleurone layer and the embryo (Juliano 1994). The classification of the genotype of cereals, by means of the characterisation of its pigment contents due to specific formulations of the grains can be a good example of the use of these crop enhancement techniques. Descriptive analyses like those of Siebenhandl et al. (2007) contribute to this aspect, evaluating formulations of different bran particle sizes and flour for the classification and selection of diverse genotypes of wheat and barley. The size of the particle generated in these processes has a great influence from the technical and nutritional viewpoints. A reduction in the particle size facilitates the liberation of vitamins and other compounds from the outer layers of the grain (Fратиanni et al. 2005; Kahlon et al. 1986; Zhou et al. 2004a, b). Zhou et al. (2004a, b) showed that micronizing the aleurone layer resulted in greater antioxidant activity compared to the non-micronized aleurone layers of bran and grain, possibly due to a greater availability of antioxidants.

The diverse thermal treatments which are applied with the objective of prolonging the shelf-life of processed cereal products have also been reproduced in the laboratory to determine their effect on carotenoid contents. Toasting the cereal seems to have a rather pronounced repercussion which causes a significant reduction in pigment contents. This was reported by De Oliveira and Rodriguez-Amaya (2007), who analysed a series of fresh and processed corn products and estimated the loss in zeaxanthin to be about 53 % after toasting the corn flour. Similar results have been described as a result of cooking rice (Lamberts and Delcour 2008). Scott and Eldridge (2005) performed a comparison of diverse thermal treatments and their effects on the carotenoid profiles of fresh, frozen and canned corn and found that the thermal treatments applied to corn prior to canning did not produce a relevant decrease in its carotenoid content. The freezing and scalding pre-treatments applied to cereals and vegetables in general may result in an increase in the bioavailability of carotenoids

(Selman 1994). The heating by microwave of rice bran produced similar results without a reduction in the pigment levels which makes it a good alternative for favouring the stability of this cereal formulation (Abdul-Hamid et al. 2007). A more complex treatment consists of an extrusion, a multi-step treatment which is widely applied in the breakfast cereal industry. The mild conditions applied during the extrusion (high moisture content, short treatment time and low temperature) have a clearly positive effect on the retention of vitamins and carotenoids and the decrease in lipid peroxidation, among others (Cheftel 1986; Singh et al. 2007). Guzman-Tello and Cheftel (1990) studied the changes in the concentration of β -carotene under the most severe extrusion conditions of wheat flour and estimated losses ranging from 38 to 73 % for an applied temperature interval of 125–200 °C. Other treatments such as those involved in the malting process of barley are characterised for generating contradictory results concerning their effect on pigment levels. For example, Goupy et al. (1999) registered losses (of approximately 76 %) as well as gains in carotenoid contents in a study with different varieties of barley.

The analyses carried out during the different steps involved in the processing of semolina and making of pasta indicate that it is during the kneading phase when the greatest decrease in carotenoid content is registered while the subsequent drying and maturation periods turn out to be the least aggressive (Fратиanni et al. 2012; Hidalgo et al. 2010; Panfili et al. 2005). At the same time, important losses (up to 66 %) in carotenoids during the kneading phase are associated with the production of bread, biscuits and baked goods (Leenhardt et al. 2006b). Other authors, however, point out different production phases, especially the baking step as being influential in the reduction in pigments. In a study of the evolution of pigments during the production of bread from wheat flour and einkorn, Hidalgo et al. (2010) registered averaged losses of 47 % for the crust compared to 21 % for the centre of the bread. After comparing the two types of flour, einkorn was found to suffer the greatest loss in pigments. Degradation during kneading is governed by an enzymatic oxidation of the pigments which has led some authors to relate both aspects (degradative enzymatic activity and carotenoid loss) in the enhancement programs for the selection of optimal cereal genotypes for the bread making industry

(Borrelli et al. 2003; Fu et al. 2013; Leenhardt et al. 2006a, b; Trono et al. 1999).

In the case of tritordeum, the properties of this new cereal make it one of the most suitable for bread applications, similar to common wheat, but on the contrary, less appropriate for formulations of semolina. As mentioned before, tritordeum flour has recently began to be commercialised with the aim of several applications such as bread, sandwich bread, biscuits, muffins and other cereal products (www.agrasys.es). In the sense, even though the elaboration process of bread and other products results in losses in carotenoid contents, as in any other cereal, tritordeum bread presents superior levels of lutein (6 times greater) than bread made from traditional wheat (according to the analyses carried out in our laboratory for the Agrasys company).

Applications of the biotechnology of carotenoids to cereal selection and breeding

Biotechnology, as a vegetable enhancement technique, applied to the increase in the carotenoid content in cereals has been used fundamentally on three species: corn, wheat and sorghum. In the case of rice the grains do not produce any carotenoid in the endosperm, however a new rice variety, *Golden Rice*, was developed at the beginning of this century by engineering grains to produce β -carotene (provitamin A) in order to help combating vitamin A deficiency in populations with rice-based traditional diets (this applies to countries such as India, Vietnam, Bangladesh, the Philippines, and Indonesia) (Paine et al. 2005; Ye et al. 2000). The relevance of these cereals and their impact on the nutrition of the populations of under-developed countries is reflected in biofortification programs such as the one of Harvestplus, belonging to the Consultive Group on International Agricultural Research (CGIAR) which is focused on the augmentation of zinc, iron and provitamin A contents in basic crops producing staple foods (<http://www.harvestplus.org/>).

The last developments in the exploration of the genomes of the main cereals such as wheat has been recently reported (Eversole et al. 2014), and has been made thanks to the existence of genomic models such as rice and sorghum (Mace and Jordan 2011; Paterson et al. 2009). A large part of the investigations has been

based, in the first instance, on the study of the variability in the feature of being susceptible to traditional selection and breeding, in this case, for the enhancement of carotenoid content in a given population and in the second place on the ability to inherit such a trait (Clarke et al. 2006; Santra et al. 2005). Rice, sorghum and wheat genomes have shown diverse genetic importance for this characteristic as reflected in the existence of elite germplasm in these species (Chander et al. 2008; Ibrahim and Juvik 2009). Up to now, the study has been focused on the identification of QTLs (Quantitative Trait Loci) whose allelic variation is associated with a variation of quantitative trait such as the carotenoid content, which varies continuously. Numerous studies can be found which describe such locus and their relation to the phenotypic variation in the carotenoid contents of the endosperm of cereals (Chander et al. 2008; Patil et al. 2008; Pozniak et al. 2007; Salas-Fernandez et al. 2008; Wong et al. 2004). Studies at this level go beyond the justification of the overall carotenoid content and identify specific QTLs for certain carotenoids such as lutein and β -carotene. Salas-Fernandez et al. (2008) detected up to five QTLs for β -carotene content in sorghum located at the 1, 2, and 10 chromosomes. Howitt et al. (2009) identified three QTLs for lutein contents in the 3B, 5B and 7A chromosomes of durum wheat. More recently, Blanco et al. (2011) have mapped QTLs for β - and α -carotene in the chromosomes 2A, 3B and 7A.

In wheat, the most determinant QTLs for the pigment contents have been repeatedly mapped onto the 7A and 7B chromosomes, both on durum wheat and common wheat. The PSY1 codifying genes (phytoene synthase, EC 2.5.1.32) generally co-segregate with these QTLs. The allelic variations of these genes are numerous and their continuous appearance is very probable. This demonstrates a large number of genetic polymorphisms as well as many genomes to be explored (Ravel et al. 2013). One of the strategies in crop improvement is the search for new variation sources in related wild species such as *Lophopyrum ponticum* (Zhang et al. 2005) and *H. chilense* (Rodríguez-Suárez et al. 2011; Rodríguez-Suárez and Atienza 2012). The small grains of *H. chilense* are characterised as having a large amount of carotenoids and present at least two loci for the content of pigments located in the 2H^{ch} and 7H^{ch} chromosomes (Álvarez et al. 1998, 1999; Atienza

et al. 2004, 2008). Knowledge about the chromosomal location was first studied by Álvarez et al. (1998) using additional lines of *H. chilense* in wheat. These authors located genes responsible for the level of pigments (determined as YPC) in *H. chilense* at chromosome 7 and mapped this feature onto the α arm of this chromosome. However, the possibility of an interaction between *H. chilense* genes and the durum wheat genes of tritordeum promoted a search for new locus. Subsequently, by means of the construction of the first genetic map of *H. chilense*, a new QTL entitled *carot 1* was identified at chromosome 2 (Atienza et al. 2004; Hernández et al. 2001). Currently, the generation of maps of a wider range for these purposes is now available, and therefore there is a greater chance of finding genomic regions of interest in *H. chilense* (Rodríguez-Suárez et al. 2012).

This information constitute the first steps towards the development of a “Marker Assisted Selection” (MAS) program for the content of carotenoids in tritordeum, which is interesting for the identification and further transference of particular genes related with the carotenoid content from tritordeum to wheat lines subjected to improvement programs. In this way, the amphiploid tritordeum will also become a useful species for the enhancement of cereals acting as a bridge species between barley and wheat (Atienza et al. 2005, 2007a, b; Martín et al. 1999). The tritordeum lines, regardless of their ploidy level, show carotenoid contents which are higher than their wheat relatives (Atienza et al. 2007a). Therefore, the study of the genetic variation of this feature in tritordeum and its relation to the level of pigments of the parental species *H. chilense* and durum wheat has become a very active research topic. From these studies it appears that even though the genome H^{ch} is clearly responsible for the level of pigments in tritordeum there can be interactions between the genetic backgrounds of both parental species that may not be obvious (Álvarez et al. 1999). Recently the first exhaustive analysis of the carotenoid profile of *H. chilense* was carried out, discovering that more than half of the lutein was esterified, with a similar esterification pattern to tritordeum. This corroborates the previous results and indicates that the esterification pattern of tritordeum should result from the genetic background of this wild barley (Mellado-Ortega and Hornero-Méndez 2015). Recent studies have demonstrated that lutein esterification in wheat and *H.*

chilense is controlled by a loci located at the chromosomes 7D and 7H^{ch}, respectively (Ahmad et al. 2015; Mattera et al. 2015).

The utility of the metabolic engineering to improve human nutrition reaches its best objectives in the basic foods field (staple foods) which have an important impact on the nutrition of under-developed populations. The new generation of crops with the *Golden* label such as *Golden Potato* or *Golden Canola* are increasing with time leading up to the well-known *Golden Rice* (Bai et al. 2011; Beyer 2010). The success of last case is encouraging similar studies and improvement programs with other species. The intense investigation from the first attempts made in rice by Burkhardt et al. (1997) to the most recent ones with the *Golden Rice 2* generation (Paine et al. 2005) have provided stimulating ideas for this purpose. Another example is the introduction and expression of the *y1* gene (gene coding for phytoene synthase in yellow endosperm corn) from corn into the hexaploid common wheat and its positive effect on increasing the carotenoid content in the endosperm (Cong et al. 2009). In the case of corn, the improved use of the γ -zein promotor (Marzábal et al. 1998), highly specific for the expression in endospermic tissue (super γ -zein), derived into an increase in carotenoids of up to 34 times with a preferential accumulation of β -carotene (Aluru et al. 2008). A more complex strategy was the one designed by Zhu et al. (2008) with the generation of an entire transgenic corn plant battery that resulted from the multiple combinations possible derived from a multi-gene transformation. More recently, the simultaneous increase in the vitamin contents (vitamins A, B and C) involving the simultaneous manipulation of three different metabolic routes (Naqvi et al. 2009), establishes corn as a likely crop to maximise carotenoid provitamin A contents, showing less restrictions than others such as rice or wheat (Wurtzel et al. 2012). The most recent *Golden Rice* investigations focus now on the study of the stability and transference of the transgenes for the selection and breeding of rice crops with a higher efficiency through introgression and other enhancement techniques (Datta et al. 2006, 2007). Other less studied grains such as sorghum and millet, but having a great impact on under-developed populations such as the African, are increasingly adapting to these techniques making its way into the biofortification mediated by genetic manipulation (O’Kennedy et al. 2006;

www.grandchallenges.org/ImproveNutrition/Challenges/NutrientRichPlants/Pages/Sorghum.aspx).

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