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Effect of long-term storage on the free and esterified carotenoids in durum wheat (*Triticum turgidum* conv. *durum*) and tritordeum (×*Tritordeum* Ascherson et Graebner) grains

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Abstract

The effect of long-term storage on the carotenoid composition in durum wheat and tritordeum grains was studied. Total carotenoid (mainly lutein) content decreased according to a temperature dependent first-order degradative kinetic model. The carotenoid retention was similar in both genotypes at the lower temperatures (71-73% at -32ºC, 70% at 6ºC and 55-56% at 20ºC), whereas at the higher temperatures the pigment retention was higher in tritordeum (42% at 37ºC; 10% at 50ºC) than durum wheat (23% at 37ºC; 1% at 50ºC), probably due to the greater proportion of esterified xanthophylls. A clear difference between free and esterified pigments was observed, with smaller losses and slower degradation (higher stability) observed for the latter. The xanthophyll esterification process was highly specific and had a significant effect on the carotenoid stability according to the fatty acids involved in the esterification and their position on the lutein molecule. The results were consistent with a degradation process in which the carotenoid behavior is influenced by the chemical structure. Xanthophyll esterification can be promoted by environmental conditions, regardless of the cereal genotype, and is a powerful tool to modulate the carotenoid profile in cereals.

Keywords

Carotenoids; lutein esters; tritordeum; *Triticum turgidum* conv. *durum*; kinetics; long-term storage

Chemical compounds studied in this article

β-Carotene (PubChem CID: 5280489); Zeaxanthin (PubChem CID: 5280899); Lutein (PubChem CID: 5368396)

1. Introduction

Wheat is among the most important staple food crops in the world, accounting for one sixth of crop acreage worldwide and feeding about 40 percent of the world population (Reynolds, Nagarajan, Razzaque, & Ageeb, 2001). Wheat grains are considered an outstanding dietary source for three major nutritional components: starch, protein, and cell wall polysaccharides (dietary fiber). Moreover, a wide range of minor components, including tocopherols, phytosterols and carotenoids, confer wheat additional health benefits for humans. Carotenoid pigments are tetraterpenoids and are present in many parts of plants, especially in fruits, flowers, roots, leaves and seeds (Britton & Hornero–Méndez, 1997). They provide vegetables with their distinctive red, orange and yellow colors as well as a number of aroma-derived compounds, which make carotenoids commercially important for the agri-food, cosmetic and nutraceutical industries (Cazonelli, 2011). Carotenoids are synthesized and accumulated in the plastids and participate in essential processes such as light harvesting and photo-protection processes; regulation of plant growth, photomorphogenesis and development; and in attracting animals for pollination and seed dispersal (Cuttriss, Mimica, Howitt, & Pogson, 2007). In non-green plastids, and especially in chromoplasts, carotenoid accumulation relies on the presence of structures capable of storing them, such as lipid-rich bodies named plastoglobules (Vishnevetsky, Ovadis, & Vainstein, 1999). The accumulation and stability of the carotenoids within these structures is much higher than in the chloroplast membranes, indicating that pigments are better protected from photooxidative degradation in the plastoglobule. This aspect seems to be facilitated by the esterification of most hydroxylated carotenoids (xanthophylls) with fatty acids. The majority of studies about carotenoids have focused on chloroplasts and chromoplasts,

but there are other plastid types that can contain carotenoids (Cuttriss et al., 2007). The amyloplasts present in seeds, such as cereal grains, are a good example, although the carotenoid content is much lower than in chromoplasts and chloroplasts. Although carotenoids are minor constituents, their presence in the seed is essential for abscisic acid (ABA) production and the dormancy process (Maluf, Saab, Wurtzel, & Sachs, 1997) as well as to limit free radical production (antioxidant action) involved in membrane deterioration and seed ageing (Calucci et al., 2004).

Animals are unable to biosynthesize carotenoids and consequently need a dietary intake of plant products to meet daily health and other physiological demands (Cazonelli, 2011). For many years, there has been a considerable interest in the dietary carotenoids due to the provitamin A activity of some of them (Olson & Hayaishi, 1965) and their ability to prevent the onset of certain cancers and eye diseases (Landrum & Bone, 2001). The beneficial role of carotenoids in maintaining human health makes them potential candidates for enhancement and manipulation of their natural sources (Graham & Rosser, 2000). For example, the biofortification of staple crops, due to their widespread consumption, is a useful tool to combat and alleviate the malnutrition problems in micronutrients such as vitamin A, which is a prevalent problem in developing countries (Shahriari, Heidari, Cheraghi, & Shahriari, 2013).

In order to assist in the development of carotenoid-enhanced cereals, it is necessary to investigate the capacity of the cereal endosperm tissue to store of these pigments, since storage seems to be an important control mechanism for carotenoid biosynthesis (Cuttriss, Cazzonelli, Wurtzel, & Pogson, 2011; Li & Eck, 2007). Although significant progress has been made in investigations with maize (*Zea mays*), the cereal with the highest carotenoid accumulation, up to 33 µg/g dry weight (Kurilich & Juvik, 1999), there is still very little knowledge about the post-carotenogenic

metabolism in these staple foods during the storage period (Wurtzel, Cuttriss, & Vallabhaneni, 2012) detailed understanding of the biochemistry of this process is needed in order to carry out predictable genetic engineering and/or breeding practices (Shumskaya & Wurtzel, 2013).

Lutein (3R,3'R,6'R-β,ε-carotene-3,3'-diol) is the main carotenoid found in cereal seeds (Adom, Sorrells, & Liu, 2003). Tritordeum (×*Tritordeum* Ascherson et Graebner) is a novel functional cereal obtained as an amphiploid (2n=6x=42, $AABBH^{ch}H^{ch}$), resulting from the cross between a wild barley (*Hordeum chilense* Roem. & Schult.) and durum wheat (Martín & Sanchez–Monge Laguna, 1982). Lutein content in tritordeum is about 5–8 times higher than in durum wheat being further characterized by a high esterification profile, in which two fatty acids – linoleic (C18:2), and palmitic (C16:0) acids – are involved (Atienza, Ballesteros, Martín, & Hornero–Méndez, 2007; Mellado–Ortega & Hornero–Méndez, 2012). The latter property is derived from the parental genetic background of *Hordeum chilense* (Mellado–Ortega & Hornero–Méndez, 2015). The analysis of the characteristic mass spectrometry fragmentation pattern of lutein has recently allowed the unambiguous structural assignment of the lutein esters regioisomers present in tritordeum (Mellado–Ortega & Hornero–Méndez, 2012), allowing the investigation of the structural effect of esterification (nature of the fatty acid and position) on the formation, accumulation, and stability of xanthophyll esters.

The storage conditions of raw materials and processing techniques for food production involve changes in food components. In the case of cereals, grains are stored in silos for long periods and may undergo important physical, chemical, and physiological modifications that, in general, result in a reduction in the phytochemical content (including carotenoids). These reductions are directly proportional to the duration of storage and the increase and intensity of other variables such as temperature,

moisture, or other degradative processes. Carotenoid degradation processes are usually promoted by enzymatic and non-enzymatic oxidation reactions (Doblado–Maldonado, Pike, Sweley, & Rose, 2012). The rate at which these changes take place has been an active research topic in food technology. The literature about the kinetics of carotenoid degradation in food is numerous and the derived results are highly variable. The proliferation of such studies reflects the importance of carotenoid pigments not only from a nutritional point of view, but also for the food industry in order to identify the optimal conditions needed to ensure a greater stability and retention of the pigments. Color is considered one of the main criteria for the acceptance of food products by consumers. This criterion is also important in the baking industry and the pasta/semolina production sector, especially for products derived from durum wheat. The investigations in this regard try to reproduce the conditions of the processing and storage of foods, including cereals, in order to estimate their shelf life and the effect on bioactive components. Most studies carried out with plant foods conclude that the kinetics of carotenoid degradation follow a zero- and first-order reaction; however, the literature is particularly limited for cereals even though they are known to experience long storage periods as part of their industrial technological treatment (Hidalgo & Brandolini, 2008).

Temperature has been frequently considered as the variable that has the greatest influence on the stability of carotenoid pigments during the storage of cereals (Quackenbush, 1963). A previous study evaluating the effect of storage temperatures on the carotenoid content of durum wheat and tritordeum grains during postharvest storage (for up to 90 days) showed the ability of this variable to significantly stimulate the *de novo* esterification of xanthophylls, which has been directly related with a higher carotenoid retention in tritordeum. In addition, the maturity stage of the cereal grains

affected the evolution of the carotenoid content during storage (Mellado–Ortega, Atienza, & Hornero–Méndez, 2015).The present study aims to assess the thermooxidative degradation of carotenoid pigments and the influence of pigment esterification during the long-term storage of grains under temperature-controlled conditions.

2. Materials and methods

2.1. Plant material, storage conditions and sample preparation

An advanced tritordeum line characterized by a high carotenoid content, registered as HT621 germplasm line, and developed in the Cereal Breeding Program of the Institute for Sustainable Agriculture (IAS-CSIC, Córdoba, Spain), (Ballesteros, Ramírez, Martínez, Atienza, & Martín, 2005) and a commercial durum wheat variety (Don Pedro) were used in the present study. Both samples are representative of the two cereal genotypes (Atienza et al., 2007; Mellado–Ortega et al., 2015; Mellado–Ortega & Hornero–Méndez, 2012). Plants were grown under greenhouse conditions with supplementary lights providing a day/night regime of 12/12 h at 22/16 °C. Plants were grown in 1-L pots until maturity, and the harvested grains were stored at 4 °C. Seeds corresponding to previous seasons (2006-2008) were selected in order to ensure a high proportion of xanthophyll esters in the carotenoid profile of tritordeum. The durum wheat grains were also selected from the same harvesting season of tritordeum. For both cereals, grains were distributed in lots of approximately four grams in round-capped polypropylene 15-mL centrifuge tubes, which were stored under controlled temperature conditions (-32, 6, 20, 37 and 50 °C) and low relative humidity for a period of 12 months, conditions that are representative of the ones that cereal grains may be subjected to on an industrial scale. A control sample (t=0 days) consisting of five

subsamples was taken for each cereal type and stored at -30 ºC until analysis. Triplicate samples (three tubes for each temperature and time) were taken at monthly intervals and analyzed in duplicate. During the course of the experience, a continuous monitoring of the storage temperature was performed.

2.2. Chemicals and reagents

HPLC-grade acetone was supplied by BDH Prolabo (VWR International Eurolab, S.L., Barcelona, Spain), and HPLC-grade deionized water was produced with a Milli-Q 50 system (Millipore Iberica S.A., Madrid, Spain). The rest of reagents were of analytical grade.

2.3. Extraction of carotenoids

Pigments from the mature grains were extracted by using a one-step grinding-extraction procedure. Briefly, 1 gram of grains was placed into 25 mL stainless steel grinding jar together with two stainless steel balls (15 mm), 6 mL of acetone containing 0.1 % (w/v) BHT and a known amount of internal standard (β-apo-8′-carotenal; 1.75 and 3.50 μg for durum wheat and tritordeum samples, respectively). The samples were crushed in an oscillating ball mill Retsch Model MM400 (Retsch, Haan, Germany) at 25 Hz for 1 min. Only a one-step solvent treatment was necessary for the complete extraction of pigments (data not shown). The resulting mixture was centrifuged at 4,500×*g* for 5 min at 4 °C and the supernatant collected in a clean tube. The solvent was gently evaporated under a nitrogen stream, and the pigments were dissolved in 0.5 mL of acetone. Prior to the chromatographic analysis, samples were centrifuged at 13,000×*g* for 5 min at 4 ºC. The analyses were carried out in duplicate for each sample. All

operations were performed under dimmed light to prevent isomerization and photo-degradation of carotenoids.

2.4. HPLC analysis of carotenoids

The procedures for the isolation and identification of carotenoid pigments and its esters have already been described in detail in previous works (Atienza et al., 2007; Mellado–Ortega & Hornero–Méndez, 2012). Briefly, the identification of carotenoid pigments was mainly based on the chromatographic (retention time) and spectroscopic (UV-visible and MS) properties obtained by HPLC-DAD and HPLC-DAD-MS(APCI+), as well as some micro-scale chemical tests for the determination of the presence 5,6-epoxide, hydroxyl and carbonyl groups. As described by Mellado-Ortega and Hornero-Méndez (2012) the structural assignment of the lutein esters, including the regioisomers, was mainly based on the fragmentation pattern obtained under the liquid chromatography mass spectrometry (LC-MS (APCI+)) conditions described below. Moreover, the tentative identification of *cis* isomers of lutein was based on the presence and relative intensity (%A_B/A_{II}) of the *cis* peak at about 330-340 nm in UV-visible spectrum, a reduction in the fine structure and a small hypsochromic shift in λmax with respect to the all-*trans* lutein, and the chromatographic behavior in the C18 HPLC column (the *cis* isomers are slightly more retained than the all-*trans* isomer).

Quantitative analysis of carotenoids was carried out by HPLC according to the method of Mínguez-Mosquera and Hornero-Méndez (1993) with the modifications detailed by Atienza et al. (2007). The HPLC system consisted of a Waters 2695 Alliance chromatograph fitted with a Waters 2998 photodiode array detector, and controlled with Empower2 software (Waters Cromatografía, S.A., Barcelona, Spain). A

reversed-phase column (Mediterranea SEA18, 3 μm, 20×0.46 cm; Teknokroma, Barcelona, Spain) was used. Separation was achieved by a binary-gradient elution using an initial composition of 75% acetone and 25% deionized water, which was increased linearly to 95% acetone in 10 min, then raised to 100% in 2 min, and maintained constant for 10 min. Initial conditions were reached in 5 min. An injection volume of 10 μL and a flow rate of 1 mL/min were used. Detection was performed at 450 nm, and the online spectra were acquired (350-700 nm wavelength range). Quantification was carried out using calibration curves prepared with lutein, zeaxanthin, α-carotene and β-carotene standards isolated and purified from natural sources (purity higher than 98% as checked by HPLC and UV-visible spectroscopy) (Mínguez-Mosquera & Hornero-Méndez, 1993). Eight-point calibration curves were prepared in the pigment concentration range of 0.5-45 μg/ml. Lutein ester content were estimated by using the calibration curve for free lutein, since the esterification of xanthophylls with fatty acids does not modify the chromophore properties (Britton, 1995). The calibration curve of free lutein was also used to determine the concentration of the (*Z*)-isomers of lutein. Data were expressed as μg/g fresh weight (μg/g fw).

2.5. Degradation kinetics modeling

To determine the degradation reaction order of the carotenoid content, zero- and first-order kinetics were hypothesized by applying the general reaction rate expression - $dC/dt = kC^n$, where C is the concentration of the compound (μg/g fw), k is the reaction rate constant ($months^{-1}$), t is the reaction time (months), and n is the order of the reaction (Upadhyay, 1996). Table 1 summarized the reaction rate expression and the kinetic parameters for zero-order and first-order models. The selected order of the reaction was that showing the best correlation (R^2) and the best correspondence among

the experimental values and the half-life of the compound ($t_{1/2}$) and D ($t_{1/10}$) [time needed for the concentration of a reactant to fall to half and one tenth its initial value respectively, where $t_{1/2} = C_0/2k$ and $t_{1/10} = 0.9C_0/k$ for zero-order and $t_{1/2} = \ln(2/k)$ and $t_{1/10} = \ln(10/k)$ for first-order].

2.6. Statistical analysis

Total and individual pigments are expressed as mean and standard error of the mean (SEM). The significant differences between means were determined by one-way ANOVA, followed by a post-hoc test of mean comparison using the Duncan test for a confidence level of 95% ($p < 0.05$) utilizing the STATISTICA 6.0 software (StatSoft Inc.).

3. Results and discussion

3.1. Carotenoid composition

For both types of cereals, the initial carotenoid composition of the grains used for the experiments (Table 2, at t=0 months) was consistent with previous studies (Atienza et al., 2007; Mellado–Ortega et al., 2015; Mellado–Ortega & Hornero–Méndez, 2012). The average content of total carotenoids was about five times higher in tritordeum (line HT621) with respect to durum wheat (Don Pedro).

As is common in most cereals, (all-*E*)-lutein was the major pigment in durum wheat, accounting for 92% of total carotenoids (1.11±0.01 µg/g fresh weight), followed by lower amounts of (all-*E*)-zeaxanthin (6%; 0.08±0.00 µg/g fresh weight) and (all-*E*)-β-carotene (2%; 0.02±0.00 µg/g fresh weight). It is interesting to note that in the present study the carotenoid profile of durum wheat grains was characterized by a small, but detectable, presence of lutein monoesters (0.04±0.00 µg/g fresh weight). However, this

esterified fraction represented only 3.2% of the total lutein content in durum wheat, in contrast to the 40% in tritordeum (Table 2). Lutein diesters were absent in durum wheat. The percentage composition (Table 2) revealed a higher abundance of the lutein monopalmitate (52.37±0.84% of the total monoester fraction) than lutein monolinoleate (47.60±1.72%). In addition, the content of lutein-3-*O*-palmitate regioisomer was about three times higher with respect to the other regioisomer, lutein-3'-*O*-palmitate. As indicated by Lepage and Sims (1968), the lutein ester content among cereal species may vary widely. According to previous studies, the presence of lutein esters in durum wheat is probably derived from storage conditions (Atienza et al., 2007; Mellado–Ortega et al., 2015; Ziegler, Wahl, Würschum, Longin, Carle, & Schweiggert, 2015). In addition, the carotenoid profile is not only determined by the grain's genetic component but also by environmental factors, including the storage conditions (Ahmad, Asenstorfer, Soriano, & Mares, 2013; Kaneko, Nagamine, & Yamada, 1995; Kaneko & Oyanagi, 1995). On the other hand, unlike other vegetables and staple crops in which development processes are associated with an increase in the xanthophyll esterification (Mínguez–Mosquera & Hornero–Méndez, 1994; Subagio & Morita, 1997), this factor does not affect the lutein ester content (Rodríguez–Suárez, Mellado–Ortega, Hornero–Méndez, & Atienza, 2014) of cereal grain, although, as noted above, such development processes may alter the total pigment content (Mellado–Ortega et al., 2015; Ramachandran, Pozniak, Clarke, & Singh, 2010). In any case, in the present study, the maturity of the grains was fully guaranteed so that this factor would not be influence the obtained results.

Lutein accounted for 99% of the total carotenoid content in tritordeum grains. (all-*E*)-Lutein was the major carotenoid (4.18±0.09 µg/g fresh weight), accompanied by lower amounts of 9(*Z*)- and 13(*Z*)-lutein isomers (0.19±0.00 and 0.30±0.01 µg/g fresh weight, respectively), and a significant occurrence of lutein fatty acid esters (3.05±0.01

µg/g fresh weight). Additionally, a very low but detectable amount of β-carotene (1%; 0.07±0.00 µg/g fresh weight) was present. The esterified lutein fraction accounted for 39.48±0.31% of the total lutein, with the monoester fraction present at higher proportions than the diesters (28.16±0.14% vs 11.32±0.17%, respectively). Regioisomers of lutein monoesters at position 3 of the β-end ring (lutein-3-*O*-linoleate and lutein-3-*O*-palmitate) were found at higher concentration levels than the regioisomers at the position 3' of the ε-end ring (lutein-3'-*O*-linoleate and lutein-3'-*O*-palmitate). These data were consistent with the regioisomer profile described for lutein monoesters in other advanced tritordeum lines (Mellado–Ortega & Hornero–Méndez, 2012). The lutein diester fraction, which was only present in tritordeum, was observed at a higher content (44.05±0.63%) for the lutein linoleate-palmitate heterodiester (comprised of two regioisomers, lutein-3'-*O*-linoleate-3-*O*-palmitate and lutein-3'-*O*-palmitate-3-*O*-linoleate) and for the homodiester with palmitic acid (C16:0), lutein dipalmitate (42.22±0.35%), with a lower occurrence of the homodiester with linoleic acid (C18:2), lutein dilinoleate (13.73±0.52%). Therefore, the lutein esters presenting palmitic acid in their structure, both mono- and diesterified, were the most abundant. As has been proposed in previous work (Mellado–Ortega & Hornero–Méndez, 2012), these results suggest the preferential acylating action of the responsible esterifying enzyme (XAT: xanthophyll acyltranferase) during the synthetic process for the β-end ring of lutein compared to the ε-end ring, as well as a higher selectivity for palmitic acid. These arguments are also applicable to durum wheat.

3.2. Changes in the carotenoid content during long-term storage of durum wheat and tritordeum grains

As can be deducted from Figures 1 and 2 (see also Tables S1 and S2) the variables imposed during storage – temperature and time – clearly affected the average content of pigments, resulting in a progressive reduction that became increasingly pronounced according to the magnitude of both variables. However, the comparison between the two cereal types indicated that carotenoid pigments were more stable in tritordeum (Figure 2). The pigment content reduction at the end of the storage period at -32, 6 and 20 °C were similar for both cereals. In contrast, significant differences were observed at the higher assayed temperatures (37 and 50 °C), with pigment losses of 77.3 and 99 % for durum wheat and 57.9% and 90% for tritordeum grains at 37 and 50 °C, respectively. It is important to note that in durum wheat grains, most pigments (99%) were destroyed after 10 months of storage at 50 °C, whereas under the same conditions, tritordeum grains retained 19% of their initial pigment content.

In general, the evolution of individual free carotenoids in durum wheat (Figure 3) followed the same pattern described for total carotenoid content. The free pigments completely disappeared after the tenth month of storage at 50 °C. The effect of the lowest assayed thermal conditions (-32 and 6 °C) derived in similar losses (17–28% range) for all pigments. A similar effect was described by Craft et al. (1993) who found no significant differences in the lutein concentration when creamed corn samples were stored at -80, -20 and 4 °C during 12 months. An exception to this was β-carotene, presenting a higher loss at 6 °C (27.2%) than at -32 °C (10.4%) after the storage. In any case, these data appear to be somewhat contradictory since the losses at -32 °C were expected to be negligible in all cases (Hidalgo & Brandolini, 2008). As for most phytochemicals, the stability of carotenoids, either in concentrated extracts or as part of the food, increases with a decrease in the storage temperature, with lower temperatures being the most appropriate for their preservation (Britton & Khachik, 2009; Craft &

Wise, 1993). The experimental temperatures recorded throughout our experiment (data not shown) indicated that there were no significant fluctuations that could have affected our results. In accordance with the trends observed for the total carotenoid content, the individual pigment degradation was more pronounced at 37 and 50 °C, with losses of 79.4% for (all-*E*)-lutein and 61.7% for (all-*E*)-zeaxanthin at the end of the storage period. The total disappearance of pigments was only observed at 50 °C. The (all-*E*)-lutein isomers experienced greater declines than lutein (*Z*)-isomers, which could be explained by a partial compensation of the degradation by the generation of new (*Z*)-isomers, since the *E* to *Z* isomerization is favored by the temperature. Geometric isomerization, together with the enzymatic and non-enzymatic oxidation, is one of the main processes that affects carotenoid pigments when they are exposed to heat treatment and/or during storage and food processing (reviewed by Schieber and Carle, 2005).

A clearly differentiated behavior of the monoesterified forms of lutein with respect to the free pigments was observed (Figure 3), which could be attributed to the esterified nature of the molecules. The physical state in which carotenoids exist in the food matrix, the degree of processing (or the cellular integrity) (Britton, Gambelli, Dunphy, Pudney, & Gidley, 2002) and their chemical structure (Pérez–Gálvez & Mínguez–Mosquera, 2002), are determinants of the reactivity and stability of the pigments during storage or food processing. Lutein esters experienced less marked losses and a similar behavior at -32, 6, 20 and 37 ºC. The concentration decline at 50ºC accounted for 76% in contrast to the total disappearance of the free pigments. These data confirm the protective role played by the esterification on the pigments. It merits mentioning that lutein monoesters were the only remaining carotenoids in durum wheat grains stored at the more severe conditions. The profile observed for the esterified

pigments at 37 °C was very interesting, suggesting the participation of two opposed processes, biosynthesis and degradation, leading to a more attenuated degradation process with a 28% concentration reduction. In fact, a previous study revealed an intense esterifying activity in tritordeum grains during their postharvest storage at 37 ºC, so that the relative contents of lutein monoesters and diesters increased 2- and 9-fold, respectively, coinciding with a concomitant decrease in the free (all-*E*)-lutein levels (Mellado–Ortega et al., 2015). Other authors have recently obtained similar results indicating that the storage of bread wheat grains at 37 ºC for two months significantly promoted the esterification of lutein (Ahmad et al., 2013). It is well-known that temperature is an important factor that significantly influences the biosynthesis and metabolism of carotenoids (Gross, 1987). Our data corroborate that the esterification process is modulated by the temperature, and 37 °C (among the assayed temperatures) was shown to be the temperature at which the esterification reaction of lutein was most favored (Figure 4).

The evolution of free and esterified pigments in tritordeum grains followed the same temperature-dependent degradative pattern (Figure 5) as that described for durum wheat. However, it should be noted that the degradative effects at 50 °C for the free carotenoids during the last stages of the storage period (10–12 months) were not so severe compared to those observed for durum wheat, showing 6 and 20% retention of the initial pigment content for (all-*E*)-lutein and lutein (*Z*)-isomers, respectively. As an exception, (all-*E*)-β-carotene was only observed until the seventh month, which indicates that this pigment was less thermostable than (all-*E*)-lutein under the imposed conditions. Similar results were obtained by Quackenbush (1963) in his pioneer studies. Within the free-pigment pool, lutein (*Z*)-isomers again reflected smoother decreases at most temperatures (from -32 to 37 °C). For example, lutein (*Z*)-isomers showed a 46%

decrease at 37 ºC, in contrast to 77 and 82% for (all-*E*)-lutein and (all-*E*)-β-carotene, respectively, under the same conditions. On the other hand, some increases were observed after the first month at 37 °C (12%) and 50 °C (29%). Such increases are consistent with a *cis-trans* isomerization process promoted by the heating treatment that compensates for the degradation processes. Updike and Schwartz (2003) confirmed that the thermal processing of corn increased the amount of lutein (*Z*)-isomers by 12%. Similarly, Aman et al. (2005) reported a rise in the presence of lutein and zeaxanthin (*Z*)-isomers from 12% to 30% and 7% to 25%, respectively. As commented before, the monoesterified lutein fraction is an intermediate stage between the free and the fully esterified lutein (diester). Concentration declines for the monoesterified lutein fraction at 37 and 50 °C were higher (41 and 86%, respectively) than those observed for durum wheat, which can be derived from the effect of the degradative conditions but also from their transformation into diesters, especially at 37 ºC (Figure 5). From analysis of the diester fraction, it is possible to conclude that lutein diesters have a higher stability than the monoesterified forms, as deducted from the losses recorded at the end of storage at 37 and 50 °C (15 and 60%, respectively). The progress of the relative content (%) of free, monoesterified, and diesterified lutein fractions during long-term storage of tritordeum grains (Figure 4) reflects a modulation of the esterification process with temperature, also indicating that this process is still active in the grains, at least during the early months of the storage. In conclusion, the stability of hydroxylated xanthophylls increases with the degree of esterification (Ahmad et al., 2013; Mellado–Ortega et al., 2015; Subagio, Wakaki, & Morita, 1999).

3.4. Evolution and stability of esterified lutein fractions during long-term storage of durum wheat and tritordeum grains

The analysis of the monoesterified lutein fraction in durum wheat showed differences in stability over the storage period according to the type of fatty acid and its position in the lutein molecule (regioisomers at the positions 3 in the β-end ring and 3' in the ε-end ring). The evolution profile of lutein monolinoleate (Figure 6) was approximately constant (losses of 1 to 10 %) for the temperature range of -32 to 37 °C, whereas at 50 °C, the corresponding losses accounted for 56% at the end of the storage period. As deducted from the graphic profile, a net synthesis of this monoester took place at the beginning of the storage period for all tested temperatures, a phenomenon that explains the low losses observed. In contrast, lutein monopalmitate experienced greater content reductions, which progressed following a temperature-dependent pattern, reaching 94.5% loss at 50 °C. These data indicate a clear difference in stability between both monoesters, and lutein monolinoleate was the most stable under the assayed conditions. The observed decrease of the ratio between lutein monopalmitate and lutein monolinoleate with increased temperature and time also supports this. The changes in the composition of monoesters, including the regioisomers, during the storage are summarized in Tables S1 and S2 (supporting information). The differences in stability could be due to structural dissimilarities between the two fatty acids. In fact, linoleic acid (C18:2) is characterized by a longer chain than palmitic acid (C16:0) with two unsaturations in the *cis* configuration that result in a different space-filling conformation. These structural features are also different from those of carotenoids, which have a polyene chain with a long conjugated double bond system (mostly in *trans* configuration) that gives the molecules a rigid structure without the possibility of torsion (Skibsted, 2012). In plant cells, the carotenoids are associated with proteins (for instance in the membranes of chloroplasts) or stored within specific lipoprotein structures (i.e. the plastoglobule present in the chromoplasts) due to their hydrophobic

character (Vishnevetsky, 1999). This change in the spatial conformation adopted by the binding of a polyunsaturated fatty acid may favor greater interactions between the carotenoid and its molecular environment, providing a more efficient integration in subcellular structures. Consequently, the degree of unsaturation of the fatty acid could be an important factor for the *in vivo* stability of xanthophyll esters. However, these results are somewhat contradictory since unsaturated fatty acids are known to be more susceptible to oxidation. Carnevale et al. (1979) reported in an earlier study a higher stability for β-carotene in the presence of linoleic acid compared to lauric (C12:0) and oleic (C18:1) acids.

The changes in the regioisomer composition for lutein monoesters in durum wheat grains were consistent with the behavior described for the total monoester fractions (Figure 6). For both monoesters, the regioisomeric forms with the fatty acid at position 3 (lutein 3-*O*-linoleate and lutein-3-*O*-palmitate) were more abundant, and the ratios between lutein 3-*O*-linoleate/lutein 3'-*O*-linoleate and lutein 3-*O*-palmitate/lutein 3'-*O*-palmitate remained mostly constant over the storage period (see Table S1 in supporting information). The lutein monolinoleate regioisomers showed differences in their losses during storage, suggesting that the esterifiying positions (3 and 3') are not equivalent in terms of stability. Lutein-3'-*O*-linoleate underwent higher concentration reductions than lutein-3-*O*-linoleate, with the latter even experiencing increases at the lower temperatures (-32, 6 and 37 ºC). The data recorded at 37 ºC is particularly interesting; whereas lutein 3'-*O*-linoleate presented losses up to 12%, lutein 3-*O*-linoleate showed a 9% increase at the end of the storage period. However, at 50 °C, position 3' (with 50% loss) appeared to be the more favorable, presenting lower losses than the regioisomer at position 3 (60% reduction). Therefore, overall, the regioisomers of monoesters at position 3’ appeared to be more thermostable. This observation could

indicate that the regioisomeric selectivity of the XAT enzymes is modified with higher temperatures with respect to the preferential esterification position in the lutein molecule under normal conditions (position 3 at the β-end ring) (Mellado–Ortega & Hornero–Méndez, 2012). In the case of lutein monopalmitate, both regioisomers showed a similar behavior, suggesting that in this case, both positions 3 and 3' in the lutein structure were equivalent.

In tritordeum, the stability differences attributed to the fatty acid involved in each monoester were not very relevant, although at the highest temperatures (37 and 50 °C), these differences were intensified (Figure 7). At 37 °C, the degradation of lutein monolinoleate was 27% compared to 49% for lutein monopalmitate at the end of the tested period. Stability differences between the regioisomers of lutein monolinoleate were observed at 37 and 50 °C, resulting in reductions in the lutein-3-*O*-linoleate content of up to 30 and 86%, and reductions in the lutein-3'-*O*-linoleate content of 10 and 62%, respectively. These results were also reflected in the evolution of the lutein-3-*O*-linoleate to lutein-3'-*O*-linoleate ratio during storage (Table S2 in supporting information). The data reported at 37 °C could suggest that the synthesis of diesters presenting linoleic acid in their structure occurs with a higher rate and/or selectivity for the regioisomer at position 3 (lutein-3-*O*-linoleate) rather than a change in the XAT enzyme specificity for position 3. The degradation of the lutein monopalmitate regioisomers at the end of the storage period was similar for both monoester forms across the range of tested temperatures, indicating that both positions (3 and 3') in the lutein structure were equivalent, as previously observed for durum wheat. Thus, the ratio between lutein-3-*O*-palmitate/lutein-3'-*O*-palmitate was constant during storage (Table S2 in supporting information). The evolution of lutein diesters during the course of storage (Figure 8) significantly reflected the competition phenomena between

synthesis and degradation. At 50 ºC, losses of 15% were recorded for lutein dilinoleate, in contrast to 58% for lutein linoleatepalmitate and 78% for lutein dipalmitate. This confirms the greater stability that the linoleic acid confers to the lutein molecule, when compared to palmitic acid, in the cereal grains.

These data, together with those derived from the kinetic analysis (to be described below), allow the pigment stability to be analyzed, not only in terms of the total esterified fraction but also at the level of lutein monoester regioisomers. Such an analysis provides useful information from a structural point of view that could contribute to a better understanding of the biochemical pathway governing the esterification of carotenoids, which is still to be deciphered.

3.5. Degradation kinetics of carotenoids during long-term storage of durum wheat and tritordeum grains

Tables 3 and 4 summarize the data characterizing the evolution of both free and total pigments (including esterified pigments) during long-term storage, which assumes a zero- and first-order kinetic model, respectively. The first-order kinetic model showed the best adjustment to the data, indicating that the degradation reaction rate is directly proportional to the concentration of pigment ($-\mathrm{d}C/\mathrm{d}t = kC$; see *Materials and methods* and Table 1). Previous studies on foods with low water content, such as those subjected to the dehydration processes, including the cereal grains, have also explained the rate of carotenoid degradation during food processing and/or storage with first-order kinetic models (Ahmad et al., 2013; Da–Jing, Jiang–Feng, & Chun–Quan, 2014; Hidalgo & Brandolini, 2008).

It is important to note the difference in the evolution of the esterified fractions, (Figures 3 and 5) with respect to free carotenoids, where a comprehensive kinetic study

was not possible due to the high retention of these pigments during storage (Table 5). However, the rate constant values (*k*), especially at 37 and 50 °C, and the contribution of the esterified fractions to the total carotenoids provided useful data on the influence of the chemical structure of the pigments to their stability.

The kinetic study for both cereal species indicated a progressive increase of the rate constants (*k*) with increasing temperature. Table 4 shows that the degradation rate of free pigments (both individual and total fractions) was faster in tritordeum than in durum wheat at the lower assayed temperatures (-32, 6 and 20 ºC). For the major pigment, (all-*E*)-lutein, the *k* values were higher in tritordeum at -32, 6 and 20 ºC than for durum wheat, and similarly for the total free lutein fraction. However, at 37 ºC, the values were similar for both cereals, and at 50 ºC, tritordeum presented lower *k* values than for durum wheat for all pigments (on average 1.2 fold lower), indicating a greater thermostability of the carotenoids in tritordeum. An exception was (all-*E*)-β-carotene, with *k* values higher in tritordeum that durum wheat for all the temperatures tested. The kinetic data obtained did not show marked differences between the rate at which the carotenes (β-carotene) and the xanthophylls are degraded, which is consistent with results from other similar studies carried out with cereals (Hidalgo & Brandolini, 2008).

In tritordeum, the total pigments fractions (total lutein and total carotenoids; Table 4), showed a decrease in *k* values at 20, 37 and 50 ºC compared to the corresponding *k* values of total free pigment fractions. These results could suggest that the degradation reaction of carotenoids is delayed due to the presence and/or contribution of the esterified pigments in tritordeum. This decrease was more significant at 37 ºC (1.6 fold) and 50 °C (1.5 fold). In durum wheat, this effect was not clearly observed for the total pigment fraction because the contribution of the esterified fraction was very low; however, a detailed analysis of the data did provide some relevant

information. The pigment thermostability differences between both cereals were intensified at 50 ºC. This was particularly significant for the total carotenoids fractions where the *k* values for durum wheat were double those for tritordeum.

Quantitative analysis of the esterified fractions and their respective regioisomers (Table 5) identified differences for the free pigments that might be due to the pigment's structure and nature of esterification, and were independent of the cereal genotype. The degradation rates at 37 and 50 °C were always lower than the corresponding rates for total free lutein, reinforcing the conclusion that the esterified pigments have higher stability than the free pigments in the two cereals studied. In durum wheat, the reaction rate constants (*k*) for the total monoesters fraction at 37 and 50 °C were about 5 and 4 fold lower, respectively, when compared to those obtained for the total free lutein fraction under the same conditions. Unfortunately, the *k* values were only obtained for the lutein monopalmitate regioisomers (lutein-3-*O*-palmitate and lutein-3'-*O*-palmitate), with no apparent differences among them. For the lutein monolinoleate regioisomers at 50 °C, the *k* value could only be determined for lutein-3'-*O*-linoleate. The *k* value was lower than the corresponding value obtained for the palmitic acid counterpart, which is consistent with the results discussed above.

In tritordeum, *k* values of the different regioisomers of lutein monolinoleate at 50 ºC revealed a difference in the reaction rate depending on the position of the fatty acid in the xanthophyll molecule (Table 5). Lutein-3-*O*-linoleate showed a reaction rate about 2.4 fold higher than lutein-3'-*O*-linoleate, as indicated above. In contrast, the regioisomers of lutein monopalmitate presented closer *k* values. In general, the *k* values were lower for the diester fraction than for the monoesters. The data obtained suggested that lutein dipalmitate was the diester with the lowest stability, with *k* values at 50 ºC higher than those for the total diester fraction. Lutein dilinoleate and lutein

linoleatepalmitate showed a profile over the storage period that did not allow for an adequate kinetic analysis (Figure 8). It is remarkable that the *k* values at 50 °C for the total monoester and diester fractions were about two and five times lower compared to the free lutein. The data reported for the rate constants (*k*) affirms the greater stability of diesterified xanthophylls followed by the monoesterified forms and the free carotenoid.

As summarized in Table 4, half-life values ($t_{1/2}$; months) and D-values ($t_{1/10}$; months) can be used as good indicators of the lifetime of a pigment, and provide a useful tool to estimate chronologically the pigment concentration that will be present in grains stored under controlled temperature conditions. Both parameters are inversely related to *k* values, such that increases to the grain storage temperature result in a reduction of the $t_{1/2}$ and $t_{1/10}$ values. The half-life and D-values determined in this study for tritordeum and durum wheat were consistent with the above stated *k* values. At 37 ºC, the $t_{1/2}$ and D-values were 9 and 30 months for tritordeum, and 6 and 19 months for durum wheat, respectively. Similarly, at 50 ºC, a half and tenth of the total carotenoid content was retained in tritordeum after 4 and 13 months, respectively, and was double the corresponding values for durum wheat. Therefore, tritordeum is not only a cereal with a higher carotenoid content than others cereals such as wheat, but it also has a greater capacity to retain the carotenoids when the grains are stored, which represents a direct benefit from the nutritional point of view.

The differences described in the present kinetic study for the retention of carotenoids in tritordeum and durum wheat grains should be attributed to the proportion of xanthophylls that are esterified with fatty acids. The results are consistent with a degradation process that is modulated by the chemical structure of the carotenoid and in which the behavior and stability of carotenoids are influenced by variables such as temperature and oxygen. The esterification process of xanthophylls is highly specific

and has a significant effect on the stability of the carotenoids according to the fatty acids involved in the esterification their position on the lutein.

4. Conclusion

These results indicate that beside the genetic and biochemical control of lutein esterification (Ahmad, Mather, Law, Li, Yousif, Chalmers, Asenstorfer, & Mares, 2015; Ziegler et al., 2015) this process is induced by environmental conditions (especially the temperature), such that the control of such conditions can be a powerful tool to modulate the carotenoid profile in cereals. However, there is some controversy about the contribution of the genetic background and the environmental conditions. In this work, we have shown how cereal varieties classified as "*no ester*", such as durum wheat, are able to synthesize xanthophyll esters (lutein esters) after storage under appropriate conditions, consequently improving the stability and retention of the total carotenoid content. It is important to note that these two factors are clearly enhanced in tritordeum, making it a more promising cereal in the field of functional foods. The results from this study advance our understanding of the *in vivo* characterization of post-carotenogenesis in these staple foods. However, more research is needed in order to address this characterization for the different processing steps of cereals in order to fully decipher the metabolic pathways involved and as well as the mechanism controlling the accumulation and deposition of this pigments.

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Figure captions

Figure 1. HPLC chromatograms obtained during long-term storage (12 months) at five different temperatures (-32, 6, 20, 37 and 50 °C) of durum wheat (Don Pedro) and tritordeum (HT621) grains. Peak identities: 1, (all-*E*)-Zeaxanthin; 2, (all-*E*)-Lutein; 3, (9*Z*)-Lutein; 4, (13*Z*)-Lutein; 5, Lutein 3'-*O*-linoleate; 6, Lutein 3-*O*-linoleate; 7, Lutein 3'-*O*-palmitate; 8, Lutein 3-*O*-palmitate; 9, (all-*E*)-β-Carotene; 10, Lutein dilinoleate; 11, Lutein 3'-*O*-linoleate-3-*O*-palmitate and Lutein 3'-*O*-palmitate-3-*O*-linoleate; 12, Lutein dipalmitate. Internal Standard (IS): β-apo-8'-carotenal.

Figure 2. Evolution of the total carotenoid content (μg/g fresh weight) in durum wheat (Don Pedro variety) and tritordeum (HT621 advanced line) grains during long-term storage under temperature-controlled conditions (-32, 6, 20, 37 and 50 °C). The values shown are the mean and standard error (n=5 for the starting sample, n=3 for the rest of the samples). Pigment losses (%) are indicated at the end of storage.

Figure 3. Evolution of the individual carotenoid content and esterified fractions (μg/g fresh weight) in durum wheat (Don Pedro variety) grains during long-term storage under temperature-controlled conditions (-32, 6, 20, 37 and 50 °C). The values shown are the mean and standard error (n=5 for the starting sample, n=3 for the rest of the samples). Pigment losses (%) are indicated at the end of storage.

Figure 4. Effect of temperature on the esterification of lutein during the long-term storage of durum wheat (Don Pedro variety) and tritordeum (HT621 advanced line) grains. Data represents the relative contribution of each fraction (free, monoesterified,

and diesterified) in relation to temperature and storage time. The values shown are the mean and standard error (n=5 for the starting sample, n=3 for the rest of the samples).

Figure 5. Evolution of the individual carotenoid content and esterified fractions (μg/g fresh weight) in tritordeum (HT621 advanced line) grains during long-term storage under temperature-controlled conditions (-32, 6, 20, 37 and 50 °C). The values shown are the mean and standard error (n=5 for the starting sample, n=3 for the rest of the samples). Pigment losses (%) are indicated at the end of storage.

Figure 6. Quantitative changes in the xanthophyll ester fractions (μg/g fresh weight) in durum wheat (Don Pedro variety) grains during long-term storage under temperature-controlled conditions (-32, 6, 20, 37 and 50 °C). The values shown are the mean and standard error (n=5 for the starting sample, n=3 for the rest of the samples). Pigment losses (%) are indicated at the end of storage.

Figure 7. Evolution of the monoesterified lutein (μg/g fresh weight), including the regioisomers, in tritordeum (HT621 advanced line) grains during long-term storage under temperature-controlled conditions (-32, 6, 20, 37 and 50 °C). The values shown are the mean and standard error (n=5 for the starting sample, n=3 for the rest of the samples). Pigment losses (%) are indicated at the end of storage.

Figure 8. Evolution of the diesterified lutein (μg/g fresh weight) in tritordeum (HT621 advanced line) grains during long-term storage under temperature-controlled conditions (-32, 6, 20, 37 and 50 °C). The values shown are the mean and standard error (n=5 for

the starting sample, n=3 for the rest of the samples). Pigment losses (%) are indicated at the end of storage.

Table 1. Expression of the reaction rate depending on the reaction order (*n*) and kinetic parameters derived.

	Reaction rate expression	**Integrated expression y= mx**	**Graphical representation**	**Half-life[a] ($t_{1/2}$)**	**D[b] ($t_{1/10}$)**
Order 0			C-C_0 *vs* t		
n=0	$-dC/dt = k\,C^0 = k$	C-C_0=-$k\,t$	Slope = -k	$t_{1/2} = C_0/2k$	$t_{1/10}$=0.9 C_0/k
Order 1			Ln(C/C_0) *vs* t		
n=1	$-dC/dt = k\,C^1 = k\,C$	Ln(C/C_0)=-$k\,t$	Slope = -k	$t_{1/2}$= Ln2/k	$t_{1/10}$= Ln10/k

[a] Time needed for the concentration of a reactant to fall to half of its initial value.
[b] Time needed for the concentration of a reactant to fall one tenth of its initial value.

Table 2. Initial carotenoid composition in *Triticum turgidum* cv. *durum* (Don Pedro) and *tritordeum* (HT621 line) grains subjected to long-term storage (12 months) under temperature controlled conditions.

		Concentration (μg/g fresh weight)[b]			
HPLC Peak [a]	**Pigment**	**Durum wheat (Don Pedro variety)**		**Tritordeum (HT621 advanced line)**	
1	(all-*E*)-Zeaxanthin	0.08 ± 0.00		-	
2	(all-*E*)-Lutein	1.11 ± 0.01		4.18 ± 0.09	
3	(9*Z*)-Lutein	0.06 ± 0.00		0.19 ± 0.00	
4	(13*Z*)-Lutein	0.11 ± 0.00		0.30 ± 0.01	
5	Lutein-3´-*O*-linoleate	0.01 ± 0.00	(23.80 ± 0.48)	0.14 ± 0.00	(6.35 ± 0.03)
6	Lutein-3-*O*-linoleate	0.01 ± 0.00	(23.80 ± 2.38)	0.68 ± 0.00	(31.09 ± 0.21)
5+6	Lutein monolinoleate	0.02 ± 0.00	(47.60 ± 1.72)[d]	0.82 ± 0.00	(37.44 ± 0.15)
7	Lutein-3´-*O*-palmitate	0.01 ± 0.00	(14.28 ± 0.00)	0.43 ± 0.00	(19.64 ± 0.09)
8	Lutein-3-*O*-palmitate	0.01 ± 0.00	(38.09 ± 1.20)	0.93 ± 0.01	(42.92 ± 0.28)
7+8	Lutein monopalmitate	0.02 ± 0.00	(52.37 ± 0.84)	1.36 ± 0.01	(62.56 ± 0.21)
9	(all-*E*)-β-Carotene	0.02 ± 0.00		0.07 ± 0.00	
10	Luteína-3,3´-dilinoleate	n.d. [c]	-	0.12 ± 0.00	(13.73 ± 0.52)
11	Lutein-3´-*O*-linoleate-3-*O*-palmitate and Lutein-3´-*O*-palmitate-3-*O*-linoleate	n.d.	-	0.39 ± 0.01	(44.05 ± 0.63)
12	Lutein-3,3´-dipalmitate	n.d.	-	0.37 ± 0.00	(42.22 ± 0.35)
	Lutein monoesters	0.04 ± 0.00	(3.19 ± 0.01)[e]	2.18 ± 0.01	(28.16 ± 0.14)
	Lutein diesters	-	-	0.88 ± 0.01	(11.32 ± 0.17)
	Total lutein esters	0.04 ± 0.00	(3.19 ± 0.01)	3.05 ± 0.01	(39.48 ± 0.31)
	Total free lutein	1.28 ± 0.03	(96.80 ± 1.08)	4.67 ± 0.19	(60.51 ± 1.24)
	Total lutein	1.31 ± 0.01		7.73 ± 0.07	
	Total carotenoids	1.42 ± 0.02		7.79 ± 0.07	

Regioisomers ratios		
Lutein-3-*O*-linoleate/ Lutein-3´-*O*-linoleate	1	5
Lutein-3-*O*-palmitate/ Lutein-3´-*O*-palmitate	3	2

[a] Peak numbers are according to Figure 1.
[b] Data are the mean ± standard error (n=5)
[c] *n.d.* not detected
[d] The relative composition (%) is shown within parentheses for the monoesterified lutein fraction, including the corresponding regioisomers
[e] The relative composition (%) is shown within parentheses for the free and esterified lutein fractions.

Table 3. Reaction rate constant (*k*; month $^{-1}$) for the total carotenoid content in durum wheat (Don Pedro variety) and tritordeum (HT621 line) grains during a long-term storage period (12 months) at -32, 6, 20, 37 and 50 ºC following the zero-order kinetic model ($C\text{-}C_{0=}\text{-}kt$).

Pigment	T (°C)	Durum wheat (Don Pedro variety)		Tritordeum (HT621 advanced line)	
		k (x10^{-3} month^{-1})	R^2	*k* (x10^{-3} month^{-1})	R^2
(all-*E*)-Zeaxanthin	-32	15	0.94	-	-
	6	20	0.88	-	-
	20	30	0.94	-	-
	37	45	0.95	-	-
	50	84	0.80	-	-
(all-*E*)-Lutein	-32	23	0.97	106	0.67
	6	28	0.90	124	0.86
	20	45	0.92	201	0.87
	37	85	0.78	332	0.54
	50	123	0.07	446	0.03
Total free (*Z*)-Lutein	-32	13	0.33	11	0.58
	6	25	0.58	14	0.78
	20	44	0.92	13	0.80
	37	90	0.93	18	0.83
	50	167	0.82	39	0.89
(all-*E*)-β-Carotene	-32	0.3	0.56	2	0.56
	6	0.5	0.76	2	0.63
	20	0.9	0.90	4	0.81
	37	2	0.83	6	0.57
	50	3	0.33	8	0.19
Total free Lutein	-32	24	0.96	117	0.67
	6	31	0.91	138	0.86

	20	50	0.93	214	0.87
	37	94	0.82	349	0.63
	50	139	0.10	485	0.24
Total free	-32	35	0.80	119	0.67
Carotenoids	6	41	0.70	140	0.86
	20	61	0.84	218	0.87
	37	106	0.73	356	0.63
	50	152	0.00	493	0.24
Total Lutein	-32	25	0.96	24	0.89
	6	32	0.91	31	0.79
	20	51	0.93	50	0.87
	37	95	0.83	94	0.81
	50	142	0.14	140	0.54
Total Carotenoids	-32	35	0.83	203	0.89
	6	42	0.71	215	0.79
	20	61	0.85	283	0.87
	37	107	0.74	432	0.81
	50	154	0.04	698	0.53

Table 4. Reaction rate constant (*k*; month^{-1}), half-life time ($t_{1/2}$; months) and D ($t_{1/10}$; months) for the total carotenoid content in durum wheat (Don Pedro variety) and tritordeum (HT621 line) grains during a long-term storage period (12 months) at -32, 6, 20, 37 and 50 ºC following the first-order kinetic model ($Ln(C/C_0) = -kt$).

Pigment	T[a] (°C)	Durum wheat (Don Pedro variety)				Tritordeum (HT621 advanced line)			
		k (x10^{-3} month^{-1})	r^2	$t_{1/2}$ (months)	$t_{1/10}$ (D) (months)	*k* (x10^{-3} month^{-1})	r^2	$t_{1/2}$ (half-life) (months)	$t_{1/10}$ (D) (months)
(all-*E*)-	-32	19	0.94	36	121	-	-	-	-
Zeaxanthin	6	28	0.89	25	83	-	-	-	-
	20	44	0.96	16	52	-	-	-	-
	37	75	0.98	9	31	-	-	-	-
	50	220	0.99	3	10	-	-	-	-
(all-*E*)-Lutein	-32	23	0.97	30	100	29	0.71	24	80
	6	29	0.93	24	79	35	0.89	20	67
	20	51	0.95	14	45	63	0.93	11	37
	37	130	0.97	5	18	135	0.99	5	17
	50	356	0.93	2	6	301	0.93	2	8
Total free (*Z*)-	-32	5	0.35	43	274	24	0.61	29	96
Lutein	6	16	0.58	43	142	33	0.81	21	71
	20	31	0.91	23	75	31	0.79	22	74
	37	76	0.92	9	30	45	0.84	15	51
	50	213	0.97	3	11	158	0.88	4	15
(all-*E*)-β-	-32	13	0.57	54	180	32	0.54	22	72
Carotene	6	27	0.76	26	87	40	0.62	17	57
	20	48	0.91	14	48	83	0.90	8	28
	37	131	0.97	5	18	157	0.92	4	15
	50	298	0.93	2	8	361	0.96	2	6
Total free	-32	21	0.96	33	101	28	0.71	25	81
Lutein	6	27	0.93	25	84	34	0.89	20	67
	20	48	0.96	14	48	59	0.93	12	39
	37	121	0.98	6	19	122	0.92	6	19
	50	328	0.94	2	7	275	0.94	3	8
Total free	-32	28	0.86	24	81	28	0.71	24	81
Carotenoids	6	35	0.79	20	66	34	0.89	20	67
	20	56	0.92	12	41	59	0.93	12	39
	37	129	0.96	5	18	122	0.92	6	19

	50	337	0.92	2	7	276	0.94	3	8
Total Lutein	-32	21	0.96	33	111	30	0.91	23	78
	6	27	0.94	26	85	32	0.82	22	73
	20	47	0.95	15	49	44	0.92	16	52
	37	116	0.97	6	20	76	0.94	9	30
	50	361	0.93	2	6	177	0.94	4	13
Total	-32	28	0.88	25	83	30	0.91	23	78
Carotenoids	6	34	0.80	20	67	32	0.82	22	72
	20	55	0.92	13	42	44	0.90	16	52
	37	124	0.96	6	19	77	0.94	9	30
	50	371	0.93	2	6	178	0.94	4	13

Table 5. Reaction rate constant (k; month^{-1}) for the esterified carotenoid content in durum wheat (Don Pedro variety) and tritordeum (HT621 line) grains during a long-term storage period (12 months) at 20, 37 and 50 ºC following the first-order kinetic model ($Ln(C/C_0) = -kt$).

		Durum wheat (Don Pedro variety)		**Tritordeum (HT621 advanced line)**	
Pigment	**T (°C)**	***k*** **(x10^{-3} month^{-1})**	**R^2**	***k*** **(x10^{-3} month^{-1})**	**R^2**
Lutein monolinoleate	20	-	-	-	-
	37	-	-	-	-
	50	-	-	109	0.92
Lutein-3´-*O*-linoleate	20	-	-	-	-
	37	-	-	-	-
	50	52	0.79	52	0.70
Lutein-3-*O*-linoleate	20	-	-	-	-
	37	-	-	-	-
	50	-	-	126	0.92
Lutein monopalmitate	20	50	0.95	35	0.75
	37	72	0.87	56	0.97
	50	169	0.83	152	0.93
Lutein-3´-*O*-palmitate	20	53	0.72	38	0.74
	37	78	0.86	67	0.97
	50	175	0.81	144	0.93
Lutein-3-*O*-palmitate	20	49	0.94	34	0.74
	37	70	0.85	51	0.95
	50	166	0.84	156	0.93
Total monoesters	20	24	0.79	28	0.77
	37	26	0.79	40	0.94
	50	88	0.86	134	0.94
Lutein dipalmitate	20	-	-	-	-
	37	-	-	31	0.89
	50	-	-	100	0.76
Total diesters	20	-	-	-	-
	37	-	-	-	-
	50	-	-	59	0.84
Total esters	20	24	0.79	24	0.73
	37	26	0.79	29	0.90
	50	88	0.86	106	0.94

Figure 1

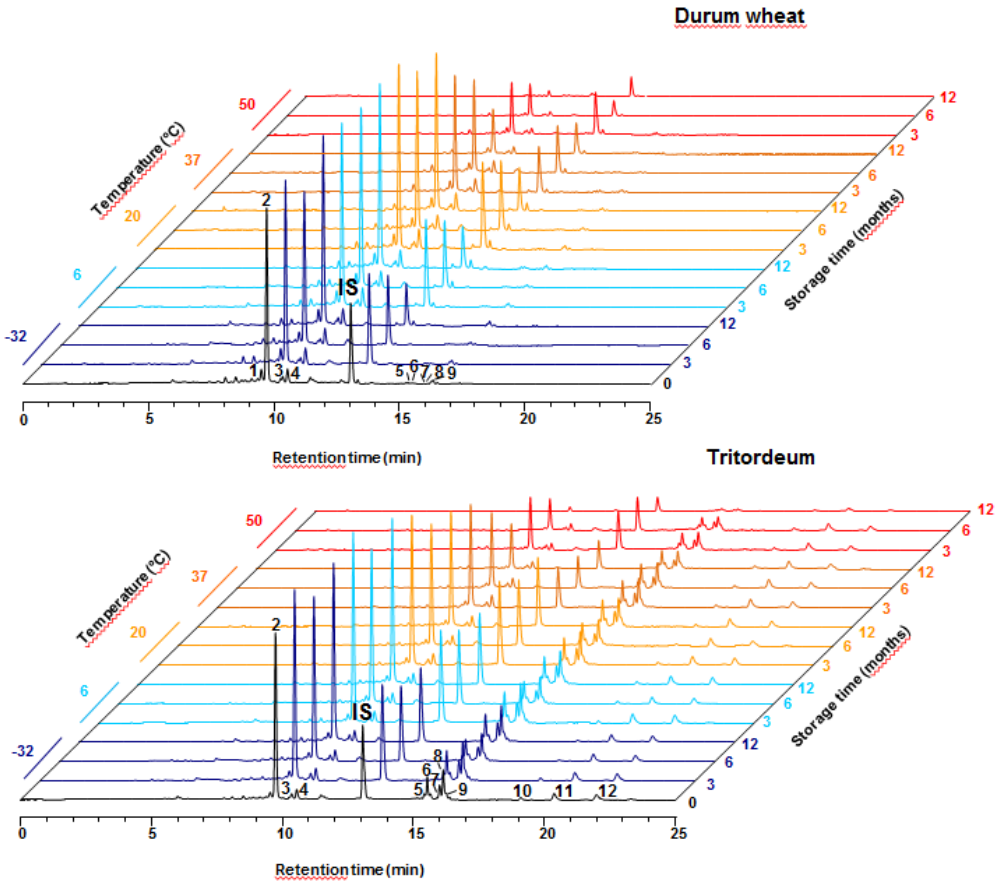


Figure 2

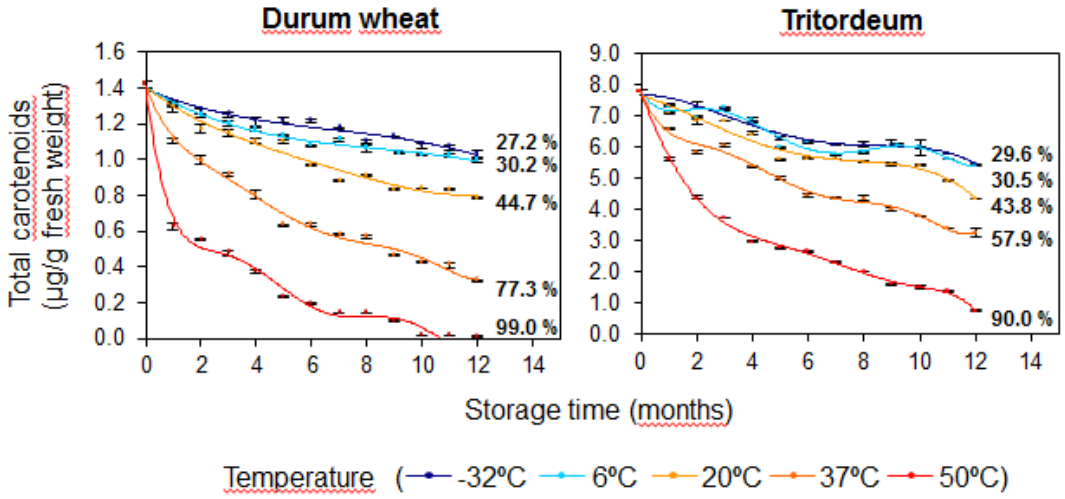
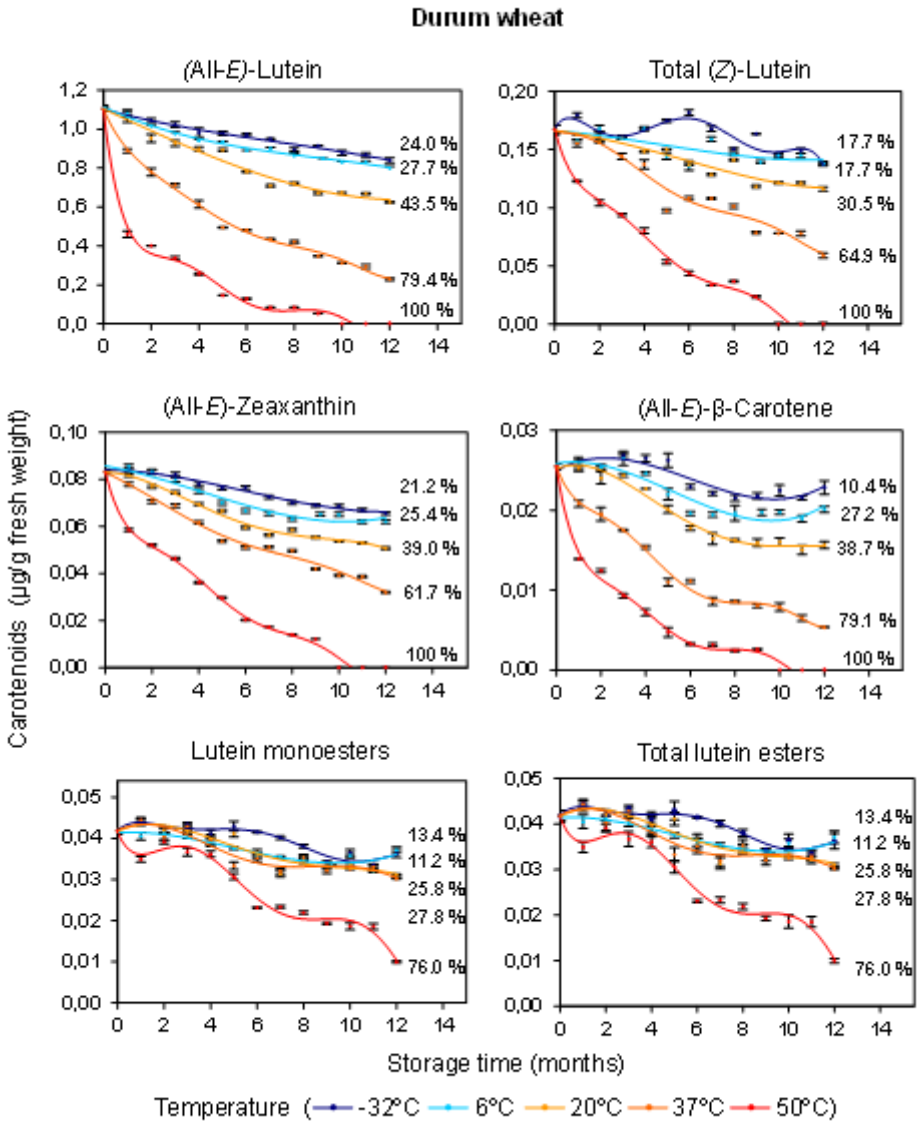


Figure 3



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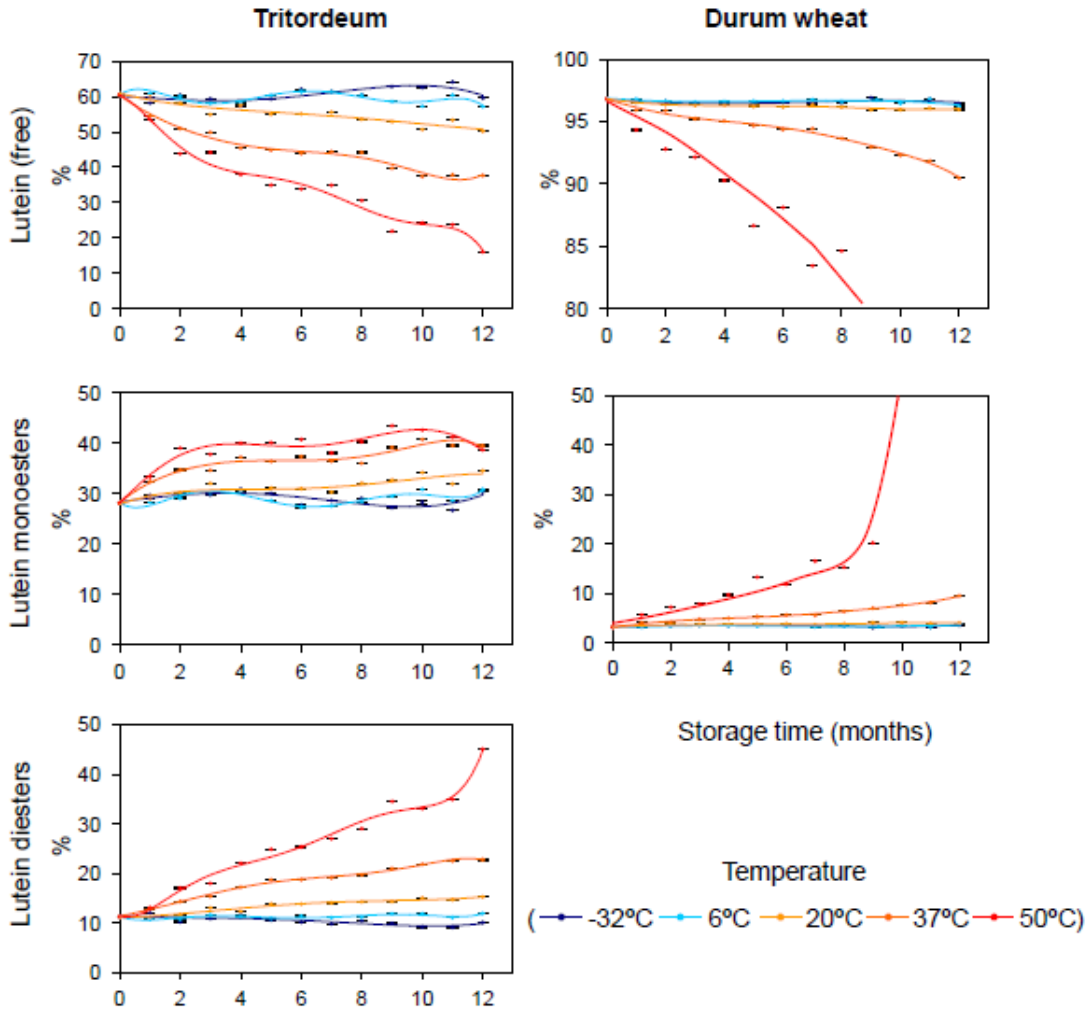
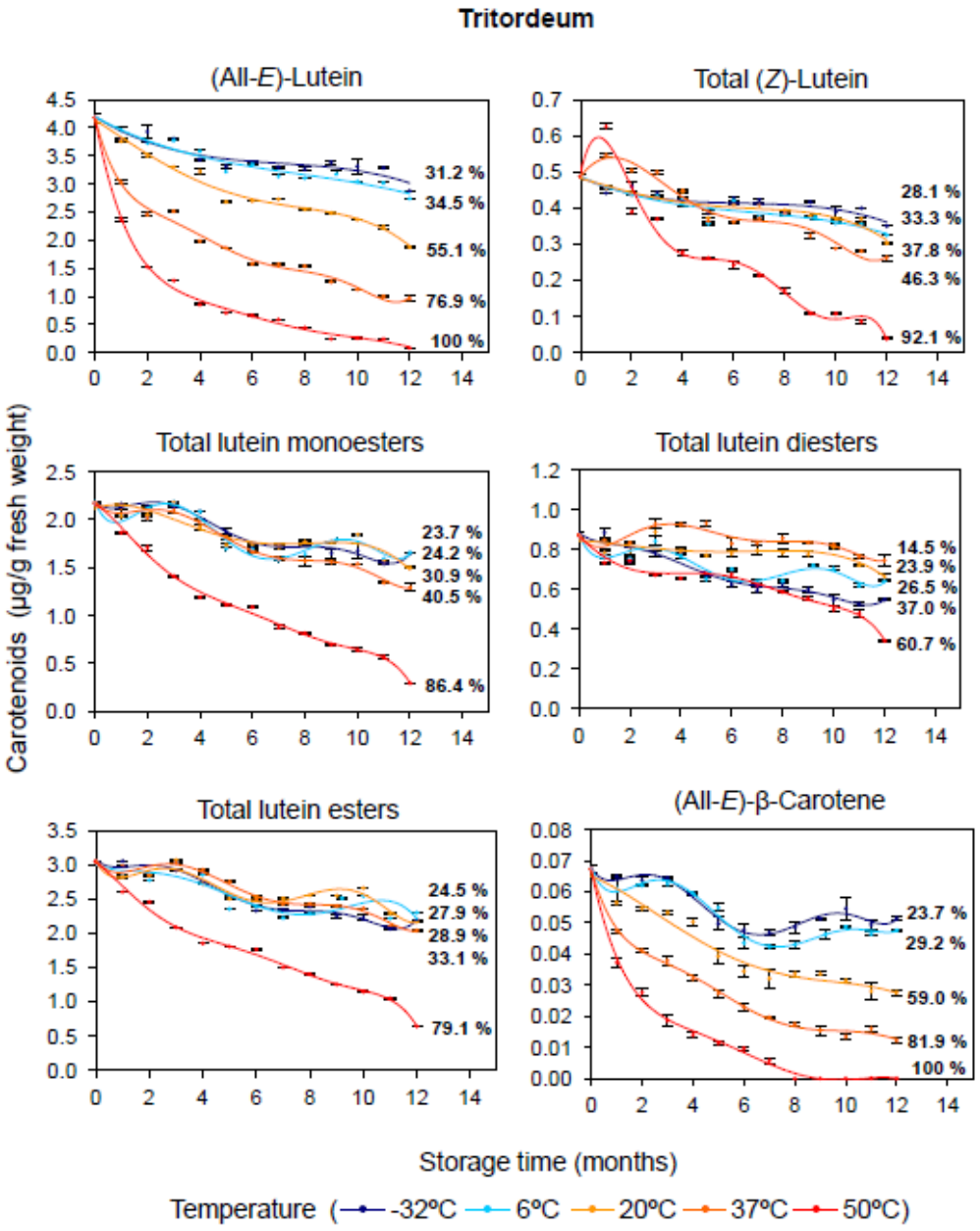


Figure 5



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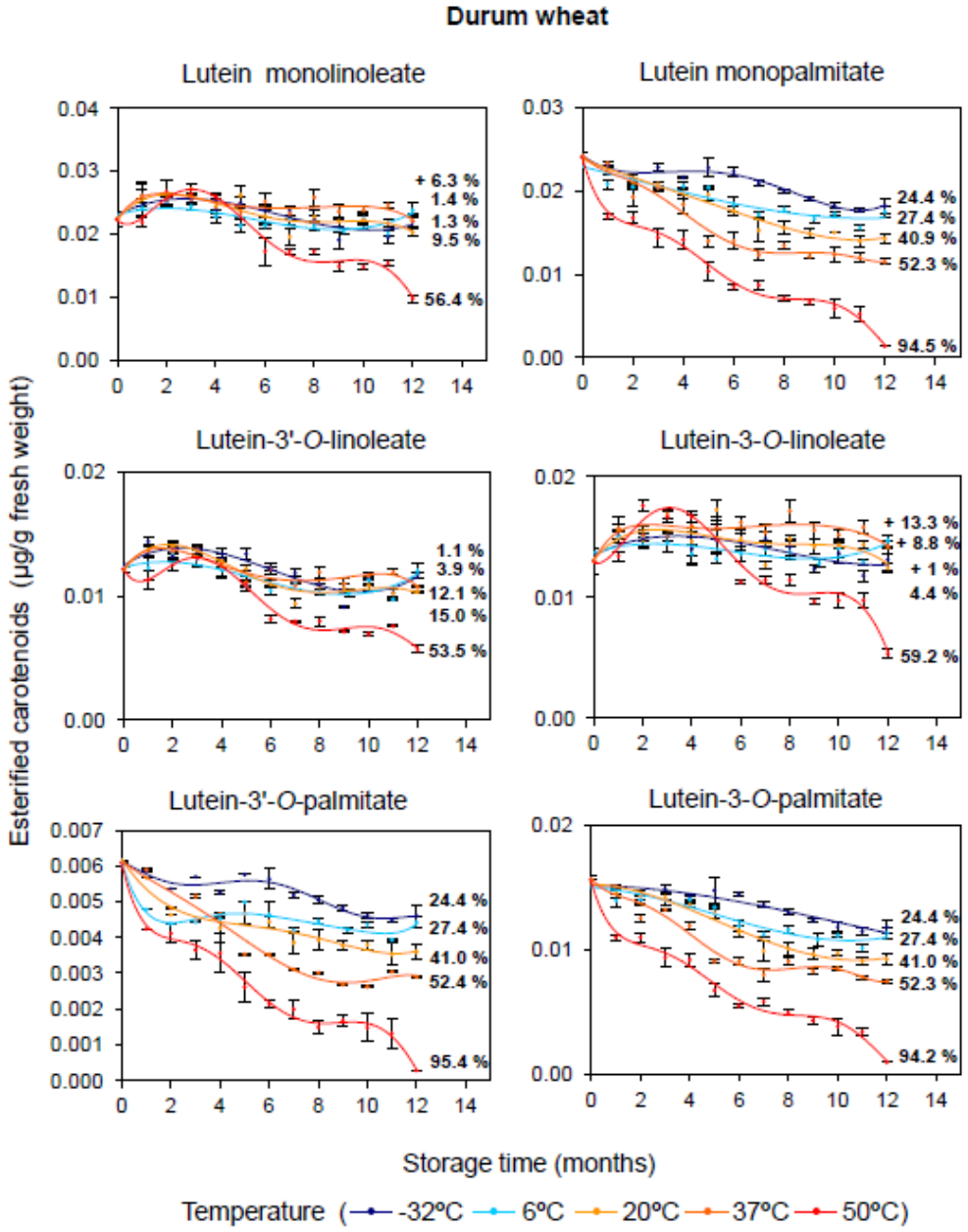


Figure 7

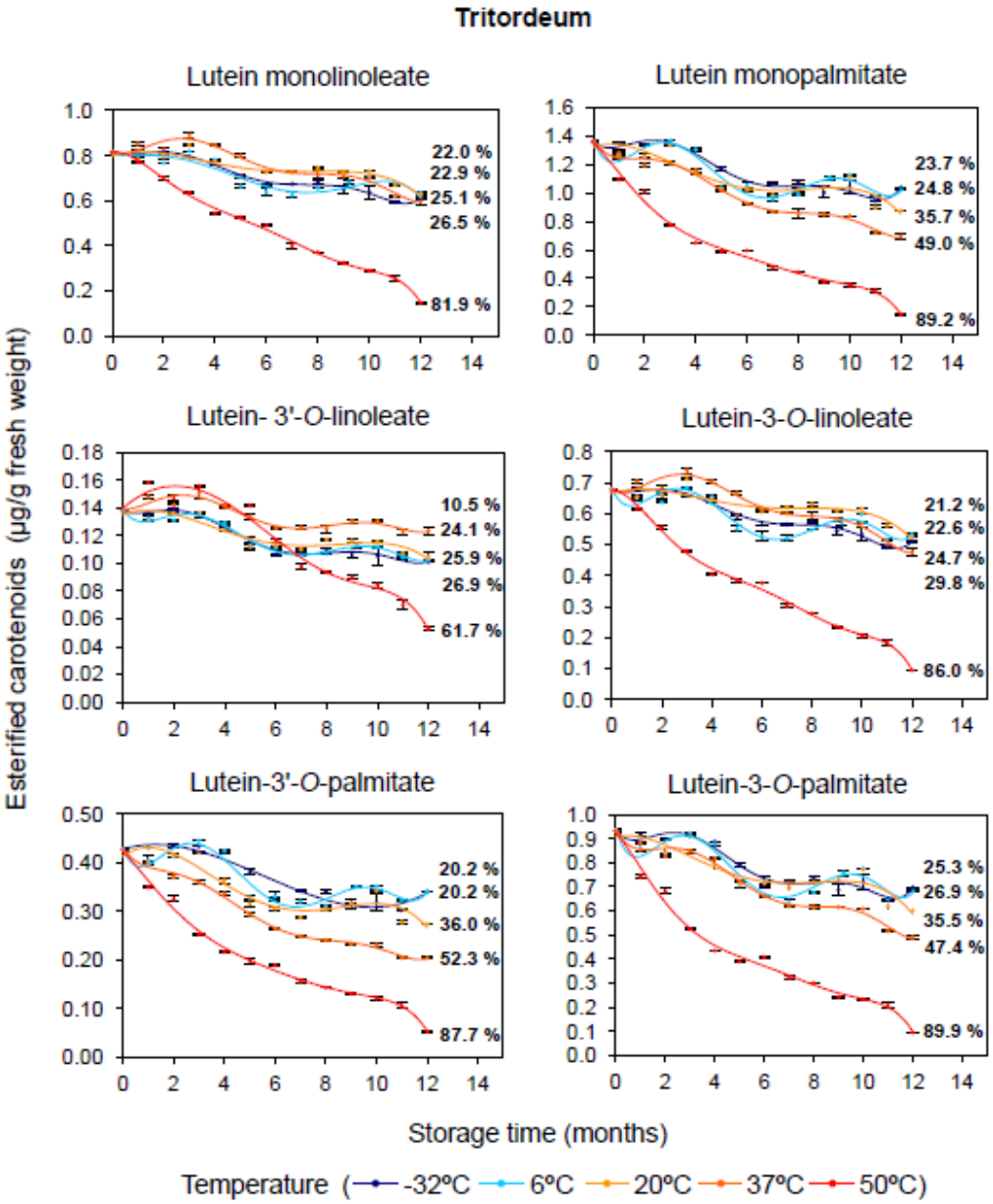
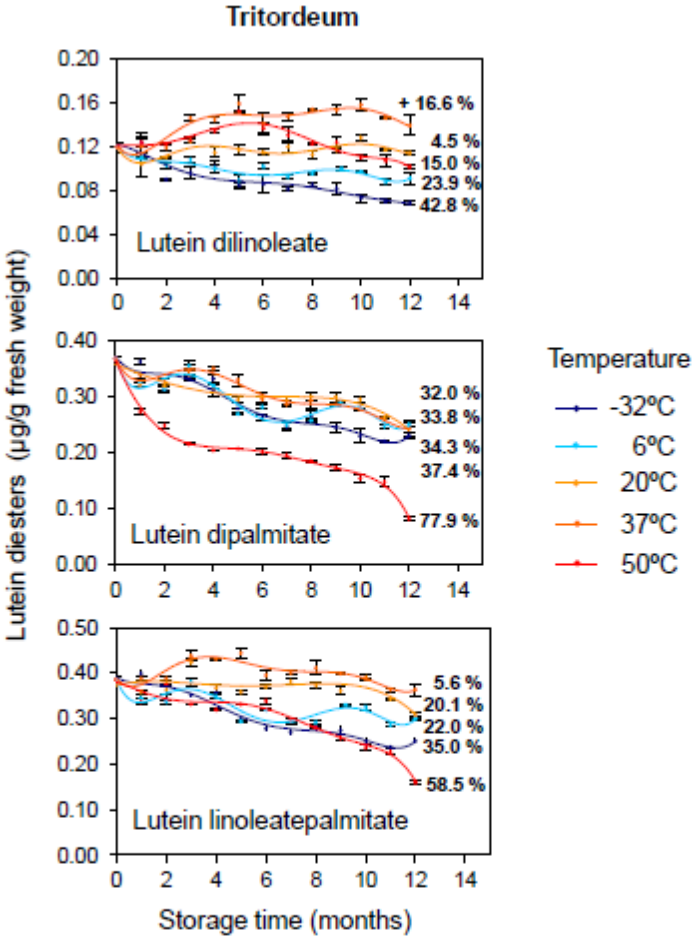
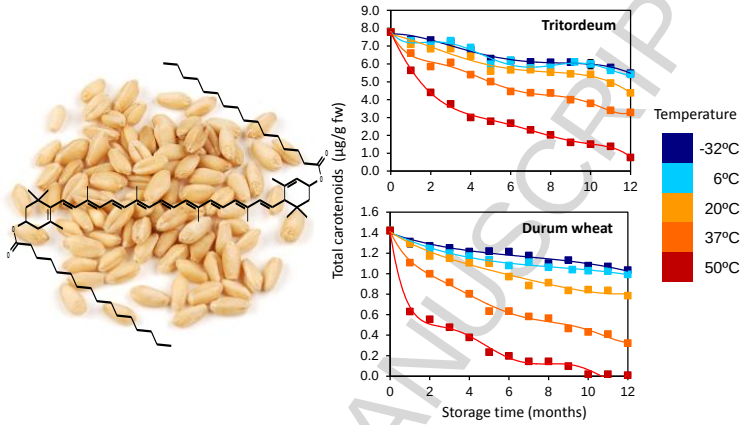


Figure 8



Graphical abstract



Highlights

- Long-term storage affects carotenoids in durum wheat and tritordeum grains
- Total carotenoid content decreased according first-order degradative kinetic model
- Carotenoid retention was higher in tritordeum than durum wheat
- Esterified xanthophylls were more stable than the free ones
- Xanthophyll esterification can be promoted by environmental conditions