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Highlights

- Lutein esters were solely present in tritordeum fractions
- Lutein esters profile was similar in germ and endosperm of tritordeum
- Endosperm was the preferential place for the biosynthesis of lutein esters
- Esterified lutein content was 3-fold higher in endosperm than germ
- Esterification facilitates carotenoids accumulation and their even deposition throughout the grain

**Effect of lutein esterification on the differential distribution of carotenoids in germ
and endosperm fractions from tritordeum grains.**

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Abstract

The effect of lutein esterification on the carotenoid distribution profile in the different fractions of tritordeum grains was studied. Durum wheat, a cereal lacking of lutein esters, was included for comparison in the study. Although carotenoid contents in endosperm and germ were significantly different for both cereals, the pigment distribution showed a marked dependence on the cereal's genetic background. Thus pigment content in durum wheat was 3 times higher in germ (2.52 $\mu\text{g/g}$ fresh weight (fw)) than the endosperm (0.74 $\mu\text{g/g}$ fw). In contrast, carotenoids in tritordeum were distributed more homogeneously (4.16 and 4.59 $\mu\text{g/g}$ fw for germ and endosperm, respectively). Lutein esters were exclusively present in tritordeum fractions, with a 3-fold higher content in the endosperm, which suggests a preferential esterifying activity in this tissue. The fatty acid profile indicated that the presence of lutein esters could be limited by the existence of specific XAT (xanthophyll acyltransferase) enzymes and not by substrate availability. A positive impact of esterification on the even deposition pattern of pigments throughout the tritordeum grain was observed. These data could be useful for optimizing the retention of lutein through the food chain as well as to direct the breeding of crops enriched in lutein esters.

Keywords: Carotenoids, lutein esters, endosperm, germ.

Chemical compounds studied in this article:

β -Carotene (PubChem CID: 5280489)

Zeaxanthin (PubChem CID: 5280899)

Lutein (PubChem CID: 5368396)

1. Introduction

A diet rich in whole grains and derived products has been associated with a reduced risk of developing chronic diseases (cardiovascular diseases, type 2 diabetes, some cancers, etc.). This is attributed in part to their antioxidant content; however, more information is required about the composition and distribution of antioxidant phytochemicals (including carotenoids) in cereal grains (Fardet, 2010). Carotenoids are a class of natural pigments that are ubiquitous components of all photosynthetic organisms because they are required for the assembly and function of the photosynthetic apparatus. In wheat grains, the antioxidant activities of carotenoids protect the seed from deterioration and contribute to the successful progress of the germination process (Howitt and Pogson, 2006). Carotenoid functions in humans are no less important: β -carotene, among others, is a precursor of vitamin A, and lutein and zeaxanthin have an important role in the prevention of ocular diseases (Landrum and Bone, 2001). Humans and animals are unable to synthesize carotenoids *de novo* and rely upon dietary sources of these compounds. Fruits and vegetables are generally considered to be the main dietary source of carotenoids. In addition, staple cereals often contain minor amounts of carotenoids in their grains. Consequently, cereals are being bred to boost their levels of these phytochemicals by both conventional and transgenic breeding methods. Certain cereals, such as yellow corn, durum wheat, and specialty primitive wheat (e.g., Einkorn and Khorasan), contain relatively high amounts of carotenoids, mainly lutein (reviewed by Shewry and Hey, 2015). Some synthetic cereals, such as tritordeum ($\times$ *Tritordeum* Ascherson et Graebner), offer even better properties than these ancient cereal species. Hexaploid tritordeum is the amphiploid derived from the cross between a wild barley (*Hordeum chilense* Roem. et Schultz.) and durum wheat (*Triticum turgidum* conv. *durum*). Special attention has been focused on this new cereal due to its increased

carotenoid content (about 5–8 times higher than durum wheat) and high proportion of lutein esterified with fatty acids (including both lutein monoesters and diesters) (Atienza et al., 2007; Mellado-Ortega and Hornero-Mendez, 2012).

Current research has shown that the total phytochemical content and antioxidant activity of whole grains have been commonly underestimated in the literature, so that whole grains contain more phytochemicals than previously reported (Hentschel et al., 2002). The carotenoid profile of cereals is mainly composed of xanthophylls, with lutein the most abundant, followed by zeaxanthin and β -cryptoxanthin, as well as small amounts of carotenes such as α - and β -carotene. In general, the bioactive compounds are concentrated in the outer layers (bran) of the cereal grain (Fardet, 2010). The highest concentration of carotenoids can be found in the embryo, although this part of the seed only represents 3–5% of the grain's total weight. However, the endosperm, representing around 80–85% of the grain has the most influential contribution in terms of the total carotenoid content. Moreover, the distribution of the carotenoid content and its profile seems to vary between and within cereal varieties (Ziegler et al., 2016). Most studies of carotenoid pigments in cereals attribute their location to the endosperm, while only a few considered the distribution of these pigments in the different fractions of the grain kernels (Borrelli et al., 2008). One study developed a new chromatographic method to verify the qualitative and quantitative distribution of carotenoids in the kernel fractions and in cereal by-products (Panfili et al., 2004). Later, Adom et al. (2005) published the detailed carotenoid composition of the milled fractions (endosperm and bran/germ) of three different wheat varieties. Significant differences in the carotenoid content (lutein and zeaxanthin) between different millstreams, including bran, of purple wheat, blue wheat, and black barley were reported by Siebenhandl et al. (2007). More recently, Ndolo and Beta (2013) and Masisi et al. (2015) provided data about the carotenoid

composition of the aleurone layer of different cereals, a grain fraction with high levels of potentially health-promoting compounds including zeaxanthin and lutein. Although the esterification of xanthophylls has been found to increase the accumulation and stability of carotenoid pigments during grain storage and processing, there is a lack of information regarding their tissue distribution in wheat grains (Ahmad et al., 2015). The aim of this study was to investigate the effect of esterification on the distribution profile of carotenoids in the two major grain fractions, endosperm/bran and germ/embryo. For this purpose, durum wheat and tritordeum grains were selected as none- and high-carotenoid ester containing cereals, respectively. A better understanding of lutein ester formation and distribution within the grain will provide critical information to help optimize the retention of lutein through the food chain.

2. Materials and methods

2.1. Plant material

Grains from a tritordeum line (high-carotenoid advanced line HT621) and a commercial durum wheat variety (Simeto) were used in the present study. Tritordeum HT621 is an advanced line developed by the Cereal Breeding Program of the Institute for Sustainable Agriculture (IAS-CSIC) in Córdoba, Spain. The Simeto variety is one of the most widely cultivated durum wheat varieties in Europe due to its excellent quality parameters: high semolina yield, extraordinary gluten content, and high protein value. Both grain samples are good representatives of the two cereal genotypes and their carotenoid profiles have previously been characterized (Atienza et al., 2007; Mellado-Ortega and Hornero-Méndez, 2012; Mellado-Ortega et al., 2015; Mellado-Ortega and Hornero-Méndez, 2017).

2.2. Sample preparation and isolation of the germ-endosperm grain fractions.

For each cereal type, 1000 grains were manually dissected under a magnifying glass to separate the germ from the endosperm using a scalpel. Strictly speaking, the endosperm fraction also contains the bran, which consists of the aleurone and pericarp layers, although in this study, this fraction will simply be referred to as endosperm. Isolated germs and endosperms were individually weighed and subsequently collected into the corresponding fractions. **Table 1** shows the average weight (mg) of whole grain and the percentage proportions for the germ and endosperm fractions of both cereals.

2.3. Chemicals and reagents

HPLC-grade deionised water was produced with a Milli-Q Advantage A10 system (Merck Chemicals and Life Science, Madrid, Spain) and HPLC-grade acetone, methanol, toluene, and heptane were supplied by BDH Prolabo (VWR International Eurolab, Barcelona, Spain). Heptadecanoic acid (C17:0), butylated hydroxytoluene (BHT), 2,2-dimethoxypropane, and fatty acid methyl ester (FAME) standard mixtures were purchased from Sigma–Aldrich Química, (Madrid, Spain). Other reagents were all of analytical grade.

2.4. Extraction of carotenoids

Carotenoid pigments were extracted from grain fractions (germ and endosperm) according to the method of Atienza et al. (2007) with some modifications (Mellado-Ortega and Hornero-Mendez, 2012). Briefly, 0.1 g and 1 g of milled grain sample from germ and endosperm, respectively, was placed in a 15 mL round-capped polypropylene tube, and then extracted with 4 mL of acetone (containing 0.1% BHT) for 2 min by vortexing, following sonication for 1 min. The mixture was centrifuged at $4,500 \times g$ for 5 min at 4° C. The extraction process was repeated three times and the acetone fractions

were pooled. The solvent was gently evaporated under a nitrogen stream, and the pigments were dissolved in 0.5 mL of acetone for both grain fractions. Prior to chromatographic analysis, samples were centrifuged at $13,000 \times g$ for 5 min at 4° C. The analyses were carried out in quadruplicate for each sample. All operations were performed under dimmed light to prevent the isomerization and photo-degradation of carotenoids.

2.5. HPLC analysis of carotenoids

The identification of carotenoid pigments in tritordeum grains and durum wheat has been described in previous works (Atienza et al., 2007; Mellado-Ortega and Hornero-Méndez, 2012). 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 Mellado-Ortega and Hornero-Méndez (2017). The HPLC system consisted of a Waters e2695 Alliance chromatograph fitted with a Waters 2998 photodiode array detector, and controlled with Empower2 software (Waters Cromatografía, Barcelona, Spain). A reversed-phase column (Mediterranea SEA18, 3 μm , 20 $\times$ 0.46 cm; Teknokroma, Barcelona, Spain) was used. Separation was achieved by a binary-gradient elution using acetone (solvent A) and deionized water (solvent B). An initial composition consisted of 75% A, which was increased linearly to 95% A in 10 min, then raised to 100% A 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 UV-Visible spectra were acquired online (350–700 nm wavelength range). Quantification was carried out using calibration curves prepared with pigment standards purified from natural sources (Mínguez-Mosquera and Hornero-Méndez, 1993). Eight-point calibration curves were

prepared in the pigment concentration range of 0.5–50 µg/mL. Taking into consideration that the esterification of xanthophylls with fatty acids does not modify the chromophore properties, lutein ester content were estimated by using the calibration curve for free lutein. Therefore, the concentration of lutein esters was expressed as free lutein equivalents. The concentration of (Z)-isomers of lutein was determined by using the calibration curve for (all-*E*)-lutein. Data were expressed as µg/g fresh weight (µg/g fw). Analyses were carried out in quadruplicate (n=4).

2.6. Analysis of fatty acids by gas chromatography

The fatty acid composition of the total lipid pool was analysed by gas chromatography (GC) as methyl esters (FAMES), which were prepared as described by Mellado-Ortega and Hornero-Méndez (2012). Milled grain fractions (0.01 g) were placed in 18-mL test tubes with Teflon® caps together with 50 µL of heptadecanoic acid (C17:0) as internal standard (10 mg/mL stock solution). After the addition of 1.65 mL of methanol:toluene:2,2-dimethoxypropane:H₂SO₄ (39:20:5:2 v/v/v/v) and 0.85 mL of heptane (containing 0.05% BHT), the mixture was flushed with nitrogen and heated at 80° C for 2 h. After cooling to room temperature, 0.5 mL of the upper organic phase, corresponding to heptane, was collected for GC analysis. FAMES were separated on a Supelcowax 10 fused silica capillary column (30 m length; 0.32 mm i.d.; 0.25 µm film thickness) (Sigma–Aldrich Química, Madrid, Spain) by using an Agilent Technologies 7890A gas chromatograph (Agilent Technologies, Madrid, Spain) fitted with a flame ionisation detection (FID), a split/splitless injector and a 7683B autosampler. Helium was used as carrier gas with a constant linear flow of 1.75 mL/min. The injector and detector temperature were 250° and 260° C, respectively. The oven temperature program started at 145° C, increasing with a ramp of 15° C/min to 230° C with a 10 min hold.

Injection volume was 1 μ L at a split ratio of 1:50. Fatty acids were identified by comparison with known standards. Quantification was performed using the area of the internal standard. Analyses were carried out in quadruplicate (n=4).

2.7. Statistical analysis

Pigment contents were expressed as mean and standard deviation (SD). 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 content distribution in durum wheat and tritordeum kernels.

As summarized in **Table 1**, the contribution of germs was higher in tritordeum grains (7.1%) when compared with durum wheat (2.91%), although durum wheat grains are double the weight of tritordeum grains. The carotenoid composition for both cereal types, as determined by HPLC analysis (**Table 2** and **Figure 1**), was consistent with previous studies (Atienza et al., 2007; Mellado-Ortega & Hornero-Méndez, 2012; Mellado-Ortega et al., 2015; Mellado-Ortega and Hornero-Méndez, 2017). As shown in **Table 2**, the carotenoid content was significantly higher in the germ and endosperm fractions of tritordeum (4.16 and 4.59 μ g/g fw, respectively) compared to durum wheat (2.52 and 0.74 μ g/g fw, respectively). The endosperm of tritordeum presented a carotenoid content about 6.2 times higher than that of durum wheat, and the germ a 1.65-fold higher content. The carotenoid contents were significantly different ($p < 0.05$) for the germ and endosperm fractions, although a clear genetic background effect was evident. Thus, the pigment content in the durum wheat germ (2.52 μ g/g fw) was 3 times

higher than that of the endosperm (0.74 $\mu\text{g/g}$ fw). Similarly, Adom et al. (2005) found that the bran/germ fractions of three bread wheat varieties had 4-fold more lutein, 12-fold more zeaxanthin, and 2-fold more β -cryptoxanthin than the endosperm fractions. A significantly higher total carotenoid content for the germs in comparisons to the other grain fractions was also reported by Okarter (2010). This data supports the well-established nutritional recommendation for the consumption of whole grains (Liu, 2007).

The carotenoid content was distributed more homogeneously among both the grain fractions of tritordeum (4.16 and 4.59 $\mu\text{g/g}$ fw for germ and endosperm, respectively). These data underline the genetic dependence of the carotenoid distribution within the cereal grain. Similarly, Fratianni et al. (2005) reported 15% more lutein, zeaxanthin, and β -carotene in whole meal than in semolina for low-carotenoid (low-YP) durum wheat cultivars, probably due to the contribution of the germ content. Moreover, in agreement with our finding for tritordeum, Abdel-Aal et al. (2007) reported a homogenous distribution pattern of pigments in 19 high-carotenoid (high-YP) wheat cultivars (such as Einkorn and Khorasan). These studies suggest that the distribution of carotenoids in the kernel strongly depends on whether the genotype is a high-YP or low-YP variety. We hypothesized that the esterification of lutein would enhance its accumulation in the germ and endosperm tissues.

The germ fraction of both cereals presented the typical chloroplastic pigment composition (i.e., lutein, neoxanthin, violaxanthin and β -carotene), revealing the operability of the carotenogenic pathway, which is characteristic of photosynthetic tissues (Howitt and Pogson, 2006). Lutein was the main pigment in both cereals (47 and 80% of the total carotenoid content for durum wheat and tritordeum, respectively), followed by zeaxanthin (31 and 11%, respectively), violaxanthin (8 and 2%, respectively), neoxanthin (7 and 5%, respectively) and β -carotene (5 and 2%,

respectively). β -Cryptoxanthin, antheraxanthin and α -carotene, the last one only present in durum wheat endosperm, were also detected but at very low levels. The highest β -carotene content was observed in durum wheat germ (0.13 $\mu\text{g/g}$ fw), with levels almost 20 times higher than for the endosperm; as such, the germ's nutritional contribution to provitamin A content is very relevant. Again, this difference among grain fractions was not so marked in tritordeum, with a content of β -carotene (0.07 $\mu\text{g/g}$ fw) in the germ (50% lower than in durum wheat), compared to the content (0.02 $\mu\text{g/g}$ fw) in the endosperm of tritordeum. Similarly, for zeaxanthin, a higher content in durum wheat germ (0.79 $\mu\text{g/g}$ fw) was observed, exceeding the content found in the endosperm by about 11 fold. This would indicate that the zeaxanthin contents found in durum wheat grains, and which characterize this cereal, are significantly contributed by the germ of the grain. In fact, zeaxanthin has previously been reported to be concentrated in the germ fraction of wheat and barley (Masisi et al., 2015; Ndolo and Beta, 2013; Panfili et al., 2004). In agreement with previous studies in tritordeum (Atienza et al., 2007; Mellado-Ortega and Hornero-Méndez, 2012), no zeaxanthin was detected in endosperm tissue, although it was found in the germ. However, the low contribution of the germ in relation to the whole grain (**Table 1**) could explain the overall low amount of zeaxanthin and the subsequent inability to detect it during the analysis of whole-grain of tritordeum (Atienza et al., 2007; Mellado-Ortega et al., 2015). Similarly, the absence of zeaxanthin in the endosperm of barley, oat, and soft wheat varieties has been reported (Ndolo and Beta, 2013; Masisi et al., 2015). Zeaxanthin is a major precursor of the carotenoids involved in the synthesis of abscisic acid (ABA), namely neoxanthin and violaxanthin. The marked accumulation of ABA in the embryo of cereal grains is well described (King, 1979), and this could be the reason for the occurrence of zeaxanthin in this tissue. In contrast, lutein showed a more even distribution in the fractions of durum

wheat and tritordeum grains, which is in accordance with previous studies (Adom et al., 2005; Panfili et al., 2004).

3.2. Lutein esters distribution in tritordeum grains.

The detailed analysis showed clear qualitative differences in tritordeum grains, highlighting the presence of lutein esters in both fractions; in contrast, lutein esters were not detected in durum wheat (**Table 2**). Lutein esters were present in the germ and endosperm of tritordeum, and no qualitative difference in their esterification profile were noted. The carotenoid composition was in accordance with previous studies (Mellado-Ortega and Hornero-Méndez, 2012). Total lutein ester content was 3 times higher in the endosperm (1.92 $\mu\text{g/g}$ fw) of tritordeum grains than the germ (0.64 $\mu\text{g/g}$ fw). This fact indicated that endosperm is the preferential place for the biosynthesis and accumulation of lutein esters. Moreover, up to 42.1% of the lutein present in the endosperm was esterified, with a 3:1 monoester to diester ratio, whereas in the germ only 19.1% of the lutein was esterified (with a 4:1 monoester to diester ratio). A similar analysis was carried out by Ahmad et al. (2015) in three synthetic hexaploid wheat lines characterized by a high-lutein content, who concluded that the occurrence of lutein esters was negligible in the germ, despite the presence of high lutein concentrations, but very relevant in both the seed coat (up to 45%) and the endosperm (up to 75%).

The higher ester content in the endosperm fraction, together with the lower values for the monoester to diester ratio (3:1), compared with that found in the germ (4:1), revealed a greater esterifying activity in the endosperm. The presence of esters in the germ could be derived from a contamination of endospermic tissue during the dissection and isolation of the grain fractions; however, this possibility can be ruled out

since a similar relationship between the mono- and diesterified lutein should be maintained in such a case.

Lutein monopalmitate content was higher than lutein monolinoleate in both fractions, reaching a ratio of about 1.5. Accordingly, the most abundant diesters were those with palmitic acid in their composition (lutein dipalmitate and lutein palmitate linoleate). Moreover, analysis of lutein ester regioisomers confirmed that the hydroxyl group at the position 3 (β -end ring) of the lutein molecule was preferentially esterified regardless of the grain fraction or the fatty acid involved. As shown in **Figure 2**, the ratio lutein-3-*O*-linoleate to lutein-3'-*O*-linoleate regioisomers in the tritordeum germ reached values of 3.5 whereas the ratio lutein-3-*O*-palmitate to lutein-3'-*O*-palmitate was 1.6. In the case of the endosperm, the observed values were higher (4.6 and 2.1, respectively) but followed the same trend, in accordance with a greater esterifying activity in endosperm. As proposed in a previous work (Mellado-Ortega and Hornero-Méndez, 2012), these results suggest that the responsible esterifying enzyme (xanthophyll acyltransferase, XAT) has a preferential acylating action for the β -end ring of lutein compared to the ϵ -end ring, as well as a higher selectivity for palmitic acid.

3.3. Fatty acid composition, lutein esters occurrence, and grain fractions.

The relative fatty acid composition was analysed in order to determine if the available fatty acid pool in each cereal species and tissue type was a determinant for the presence of lutein esters. As shown in **Figure 3**, the fatty acid profile did not change with respect to tissue type or cereal species. In all cases the most abundant fatty acid was linoleic acid, reaching slightly higher values in the endosperm (up to 59%) than the germ tissue (55–58%). The main differences observed were a higher proportion of palmitic acid (26%) and a smaller content of oleic acid (9%) in the endosperm

compared to the germ (16–21%). Linolenic acid accounted for 3.3 and 4.1% in the endosperm and 4.7 and 7.0% in the germ of tritordeum and durum wheat, respectively. Stearic and palmitoleic acids were only present in minor amounts (less than 0.4 and 1.5%, respectively). These data are in agreement with those from previous studies that have reported the fatty acid composition of wheat grains (Ziegler et al., 2015), wheat germ oil (Panfili et al., 2003), and wheat flour (Arunga and Morrison, 1971).

As monogalactosyl-diacylglycerol (MGDG) and digalactosyl-diacylglycerol (DGDG) are the major lipids in wheat endosperm, we hypothesized that these molecules could be involved in the donation/transfer of acyl groups during the esterification of xanthophylls. Moreover, linoleic, palmitic, and oleic acids have been found to be the main fatty acids present in MGDG and DGDG from wheat flour (Arunga and Morrison, 1971). The sn-2 position of the diacylglycerols was almost exclusively esterified with C18 fatty acids (mainly linoleic acid, 83%), while the participation of palmitic acid at the sn-1 position was very marked (11 and 26% for MGDG and DGDG, respectively). The presence of oleic acid was similar (4–9%) for the two positions (sn-1 and sn-2) in MGDG and DGDG. Strikingly, this fatty acid profile is similar to the fatty acid composition of the lutein esters in tritordeum grains (Mellado-Ortega and Hornero-Méndez, 2012). Galactolipids (MGDG and DGDG) has been demonstrated to act as acyl donors during the biosynthesis of fatty acid phytyl esters in chloroplasts of *Arabidopsis* (Lippold et al., 2012), a process with some similarities with the esterification of xanthophylls. Other authors proposed the membrane phospholipids as potential acyl donors involved in the esterification of lutein in wheat endosperm (Konopka et al., 2004; Ahmad et al., 2015).

Since there were no marked differences in the fatty acid pools associated with both cereal genotypes, we hypothesized that the key factor necessary for the

biosynthesis of lutein esters could be determined by the presence of specific XAT enzymes. In line with this, the influence of the genetic background on the ability to produce and accumulate lutein esters has been recently suggested by Ziegler et al. (2015). Our data are in accordance with previously published results in relation to the genetic basis of the esterification in tritordeum, in which lutein is preferentially esterified with palmitic acid, although linoleic acid is more abundant in the lipid pool containing the acyl-donors, indicating the existence of substrate specificity (Mattera et al., 2015; Mellado-Ortega and Hornero-Mendez, 2012). In fact, for both cereals, the linoleic acid content was 2-fold higher in the endosperm and 3-fold in the germ compared to palmitic acid (**Figure 3**). Moreover, the esterification of lutein in wheat showed a preferential acylating action over the β -end ring compared to the ε -end ring, suggesting that the involved enzymes have a marked regioselectivity (Mellado-Ortega and Hornero-Mendez, 2012). In the case of tritordeum, this trait has been mapped to chromosome 7H^{ch} from *H. chilense* (Mattera et al., 2017).

The fact that tritordeum showed a markedly increase of the lutein content in the endosperm (similar to the content in germ), in contrast to durum wheat, corroborates the hypothesis that carotenoid accumulation in grain tissues is facilitated by the esterification of xanthophylls (lutein in this case) with fatty acids.

4. Conclusion

This study reports the distribution of lutein esters (including monoesters, diesters, and their regioisomers) in tritordeum and durum wheat grains for the first time. The esterification of lutein in tritordeum seems to have a positive effect on the even deposition pattern of pigments throughout the grain. The information reported in this study could help to direct the breeding of cultivars enriched in lutein esters.

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Conflict of interest statement

The authors have declared no conflict of interests.

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Table 1. Average weight and germ-endosperm distribution (%) in *Triticum turgidum* sp. *durum* (Simeto) and *tritordeum* (HT621 line) grains.

	Durum wheat (Simeto variety)	Tritordeum (HT621 advanced line)
Average weight whole grain (mg)*	68.1 ± 3.2	34.5 ± 2.6
Germ (%)	2.9 ± 0.4	7.1 ± 0.6
Endosperm (%)	97.1 ± 5.1	92.9 ± 4.7

* Data are the mean ± standard deviation (n=1000).

Table 2. Carotenoid composition ($\mu\text{g/g}$ fresh weight) in the germ and endosperm fractions of durum wheat (Simeto) and tritordeum (HT621 line) grains.

HPLC Peak [§]	Pigment	Concentration ($\mu\text{g/g}$ fw) [*]			
		Durum wheat (Simeto variety)		Tritordeum (HT621 line)	
		Germ	Endosperm	Germ	Endosperm
1	(9Z)-Neoxanthin	0.17 ± 0.01	-	0.20 ± 0.01	-
2	(all-E)-Violaxanthin	0.20 ± 0.00	-	0.07 ± 0.00	-
3	(all-E)-Antheraxanthin	0.02 ± 0.00	-	0.04 ± 0.01	-
4	α + β -Cryptoxanthin	0.04 ± 0.00	-	-	-
5	(all-E)-Zeaxanthin	0.79 ± 0.03^a	0.07 ± 0.00^b	0.45 ± 0.00	-
6	(all-E)-Lutein	0.87 ± 0.04^a	0.51 ± 0.02^b	2.23 ± 0.04^a	2.30 ± 0.04^a
7	(9Z)-Lutein	0.12 ± 0.01^a	0.07 ± 0.00^b	0.22 ± 0.00^a	0.20 ± 0.00^b
8	(13Z)-Lutein	0.19 ± 0.09^a	0.08 ± 0.00^b	0.25 ± 0.01^a	0.14 ± 0.00^b
9	Lutein-3'-O-linoleate	-	-	0.04 ± 0.00^a	0.10 ± 0.00^b
10	Lutein-3-O-linoleate	-	-	0.15 ± 0.00^a	0.46 ± 0.00^b
9+10	Lutein monolinoleate	-	-	0.20 ± 0.00^a	0.56 ± 0.01^b
11	Lutein-3'-O-palmitate	-	-	0.12 ± 0.00^a	0.28 ± 0.01^b
12	Lutein-3-O-palmitate	-	-	0.20 ± 0.00^a	0.59 ± 0.01^b
11+12	Lutein monopalmitate	-	-	0.32 ± 0.00^a	0.87 ± 0.02^b
13	(all-E)- α -Carotene	-	traces	-	-
14	(all-E)- β -Carotene	0.13 ± 0.00^a	0.01 ± 0.00^b	0.07 ± 0.00^a	0.02 ± 0.00^b
15	Lutein-3,3'-dilinoate	-	-	0.02 ± 0.00^a	0.06 ± 0.00^b
16	Lutein-3'-O-linoleate-3-O-palmitate plus Lutein-3'-O-palmitate-3-O-linoleate	-	-	0.05 ± 0.00^a	0.23 ± 0.00^b
17	Lutein-3,3'-dipalmitate	-	-	0.05 ± 0.00^a	0.21 ± 0.00^b
	Total lutein	1.18 ± 0.06^a	0.66 ± 0.02^b	3.34 ± 0.05^a	4.57 ± 0.07^b
	Total free lutein	1.18 ± 0.06^a	0.66 ± 0.02^b	2.70 ± 0.05^a	2.64 ± 0.05^b
	Lutein monoesters	-	-	$(80.92 \pm 1.41)^{**}$	(57.90 ± 1.02)
	Lutein diesters	-	-	(15.36 ± 0.11)	(31.11 ± 0.51)
	Total lutein esters	-	-	(3.71 ± 0.04)	(10.99 ± 0.15)
	Total carotenoids	2.52 ± 0.09^a	0.74 ± 0.02^b	4.16 ± 0.04^a	4.59 ± 0.07^b

[§] Peak numbers in accordance with Figure 1.

^{*} Data are the mean $\pm$ standard deviation (n=4). Different letters for each pigment, within the same cereal, indicate significant differences ($p < 0.05$) as determined by the Duncan test.

^{**} The relative composition (%) is shown within parentheses for the free and esterified lutein fractions in tritordeum.

FIGURE CAPTIONS

Figure 1. HPLC chromatograms obtained of the germ and endosperm fractions of durum wheat (Simeto) and tritordeum (HT621) grains. Peak identities: 1, (9Z)-Neoxanthin; 2, (all-*E*)-Violaxanthin; 3, (all-*E*)-Antheraxanthin; 4, β -Cryptoxanthin (included also α -Cryptoxanthin); 5, (all-*E*)-Zeaxanthin; 6, (all-*E*)-Lutein; 7, (9Z)-Lutein; 8, (13Z)-Lutein; 9, Lutein 3'-*O*-linoleate; 10, Lutein 3-*O*-linoleate; 11, Lutein 3'-*O*-palmitate; 12, Lutein 3-*O*-palmitate; 13, (all-*E*)- α -Carotene; 14, (all-*E*)- β -Carotene; 15, Lutein dilinoleate; 16, Lutein 3'-*O*-linoleate-3-*O*-palmitate and Lutein 3'-*O*-palmitate-3-*O*-linoleate; 17, Lutein dipalmitate. UV-visible spectrophotometric detection was carried out at 450 nm.

Figure 2. The content of lutein monoesters ($\mu\text{g/g}$ fresh weight) and the 3/3' regioisomers ratio (represented by a black triangle) present in the germ and endosperm fractions of tritordeum (HT621 advanced line) grains. The values shown are the mean and standard deviation ($n=4$).

Figure 3. Fatty acid composition (%) in germ and endosperm fractions of durum wheat (Simeto variety) and tritordeum (HT621 advanced line) grains. The values shown are the mean ($n=4$). Fatty acid identities: palmitic acid (C16:0); palmitoleic acid (C16:1); stearic acid (C18:0); oleic acid (C18:1); linoleic acid (C18:2); linolenic acid (C18:3).

Figure 1

Figure 2

Figure 3