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Genetic Variability of Carotenoid Concentration and Degree of Esterification among Tritordeum (×*Tritordeum* Ascherson et Graebner) and Durum Wheat Accessions

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The higher carotenoid content (commonly referred as "yellow pigment content") of tritordeum seeds as compared to wheat and the potential of this species as a donor of useful traits to wheat led us to investigate the detailed carotenoid composition of 53 accessions of hexaploid tritordeums originating from different stages of the tritordeum breeding program developed at IAS-CSIC. In addition, seven durum wheat accessions were also studied for comparison. Lutein was the unique carotenoid detected, either free or esterified with fatty acids. On average, tritordeum had 5.2 times more carotenoids than durum wheat, which suggests a high potential of this species to become a functional food. In addition, the most outstanding result of this work is the high esterification degree of lutein found in tritordeums as compared to durum wheat. This difference may indicate the differential esterification ability between tritordeum and durum wheat species. The implications of this high level of lutein esterification on both carotenoid accumulation and stability are discussed.

KEYWORDS: Carotenoid; esterification; HPLC; lutein; tritordeum (×*Tritordeum* Ascherson et Graebner)

INTRODUCTION

Plant carotenoids are C_{40} isoprenoids with polyene chains that may contain up to 15 conjugated double bounds (*1*). More than 650 different carotenoids have been identified to date (*2*). Carotenoids can only be synthesized *de novo* by plants, certain bacteria, and fungi. In contrast, animals are unable to synthesize carotenoids, so they need to take them from the diet. Carotenoids are essential components of the photosynthetic apparatus and are involved in the assembly of the photosystems, playing a role in light harvesting (*3*). Besides, they act as photoprotectors preventing photo-oxidative damage, thus limiting membrane damage (*1*, *3*). An additional, and many times underestimated, important role of carotenoids in plants is to furnish flowers and fruits with distinct colors to attract animals and to ensure pollen and seed dispersion.

Carotenoids can be classified into two distinct classes: carotenes, which are hydrocarbons either linear or cyclized at one or both ends of the molecule, and xanthophylls, which are oxygenated derivatives of carotenes (*4*). The consumption of carotenoid-rich foods has been associated with a decrease in the risk of developing certain types of cancer (*5*−*8*) and other degenerative and chronic diseases (*9*−*11*). In particular, lutein and zeaxanthin have been implicated in preventing age-related macular degeneration (*12*). In addition, other carotenoids (mainly β-carotene, α-carotene, and β-cryptoxanthin) have provitamin A activity (*7*, *13*).

Carotenoids are also related with end-use quality. Indeed, carotenoids are added to many foods to make then more appealing, since colors play an important role in consumer choice (*14*, *15*).

In grasses, carotenoids contribute to the yellow pigment content of seeds. The yellow pigment content is being considered as a selection criterion for durum wheat breeding worldwide. Yellow color is the result of the carotenoid pigments present in the seeds, and it is an essential requirement for pasta products, particularly to make good-quality pasta products where a bright yellow color is desired (*16*). In addition to this, a bright yellow color is required for yellow alkaline noodles (*17*). Furthermore, some consumers seem to desire a yellow color in bread (*18*).

Wild Triticeae species constitute an interesting gene pool to improve yellow pigment contents in durum wheat. For example, the yellow pigment gene *Y* (*19*) from *Lophopyrum ponticum* (Podp.) Löve has been transferred to durum wheat to improve the yellow pigment content (*20*). The species *Hordeum chilense* Roem. et Schulz. constitutes a source of additional variability for yellow pigment content. This variability may be used in both durum and in bread wheat breeding through the amphiploids called tritordeums (×*Tritordeum* Ascherson et Graebner) (*21*). The hexaploid tritordeum was developed from the hybrid

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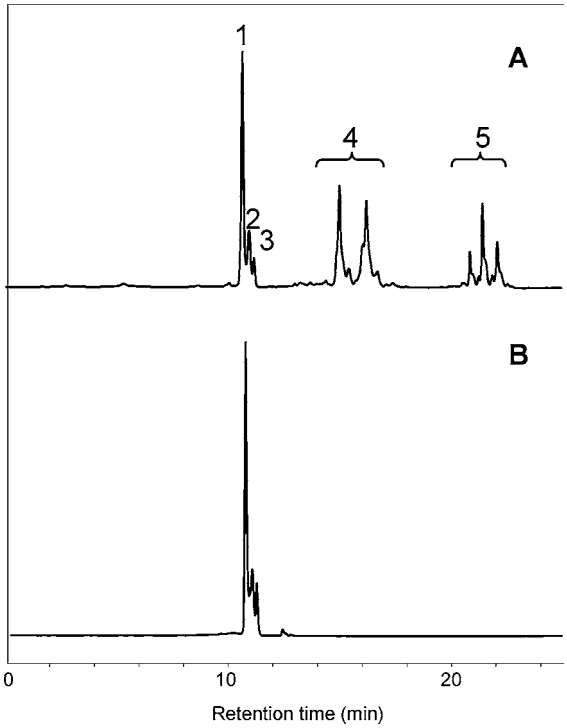

Figure 1. HPLC chromatograms obtained for a carotenoid extract prepared from tritordeum seeds (accession HT28) before (**A**) and after (**B**) submitting the sample to saponification with 10% (w/v) KOH–MeOH. Peak identities: 1, all-*trans*-lutein; 2, *cis* isomer of lutein; 3, *cis* isomer of lutein; 4, peaks corresponding to ME lutein; and 5, peaks corresponding to DE lutein.

derived from the cross between *H. chilense* and *Triticum turgidum* Desf (*22*). Tritordeums have a high yellow pigment content (*23*), which is much higher than that found in their wheat progenitors (*24*).

Lutein is the main carotenoid found in seeds of *Triticum* spp. (*25*−*29*). Although tritordeum seeds have been reported to have a higher yellow pigment content than their durum wheat parents, to date, their carotenoid composition, not the variability within the species, has been never quantified. The development of biofortified staple foods, such as cereal grains, is a powerful tool to combat malnutrition (*30*), which is especially important for developing countries where populations frequently rely on a single crop.

The main objective of the present work was to characterize a collection of tritordeum genotypes for carotenoid content, to compare these values with durum wheat accessions, and eventually to evaluate the potential of tritordeum to become a functional food.

MATERIALS AND METHODS

Plant Material. Fifty-three accessions of tritordeum and seven accessions of durum wheat were grown in greenhouse conditions at the Institute for Sustainable Agriculture (Córdoba, Spain). Tritordeums are the amphiploids derived from the chromosome doubling of the hybrid between *H. chilense* and durum wheat. Tritordeums were divided into two different groups: primary and secondary tritordeums. Primary tritordeums (HT1°) (24 accessions) are those directly obtained from the chromosome doubling of the hybrid between *H. chilense* and durum wheat as described by ref *22*. Thus, these accessions have not been subjected to selection in the breeding program.

Secondary tritordeums (HT2°) include 29 accessions derived from the tritordeum breeding program. Therefore, they have been subjected to several cycles of crossing and selection for agronomic performance; therefore, they are characterized by a higher yield and bigger seeds than primary tritordeums. The distinction of both groups is useful to investigate whether breeding for bigger seeds affects carotenoid contents.

Simeto, Vitron, Don Pedro, and Cocorit are durum wheat varieties; DH2652 is a double haploid durum wheat developed at IAS-CSIC; T155 and T60 correspond to the entries 20 and 221 from the CIMMYT crossing block 1986 and 1987, respectively.

Sample Preparation. Grains were milled by using a spice hand mill, and subsequently, 2.0−2.5 g of the resulting flour was stored at −30 °C until carotenoid extraction and analysis.

Extraction of Carotenoids. One gram of milled grain sample was placed in a capped plastic centrifuge tube (15-mL) and extracted with 5 mL of acetone (containing 0.1% BHT) by sonication for 1 min and vortexing the tube for about 2 min. Extracted carotenoids contained in the acetone phase were collected with a Pasteur pipet after the tube was centrifuged at 5000 rpm for 5 min at 4 °C. The extraction operation was repeated three times, and all of the acetone fractions were pooled together in a test tube. The solvent was evaporated under nitrogen stream, and the pigment extract was dissolved in 1 mL of acetone. The sample was stored at −30 °C until high-performance liquid chromatography (HPLC) analysis within a period of a week. Samples were centrifuged at 12000 rpm prior to the chromatographic analysis. All operations were carried out under dimmed light to prevent isomerization and photodegradation of carotenoids. The analyses were carried out in duplicate.

Pigment Identification. Routine procedures for the isolation and identification of carotenoid pigments, already described in detail in previous publications (*31*), were used. Briefly, the identification procedure consisted of the following: separation of pigment by thin-layer chromatography (TLC) and cochromatography with purified pigments; observation of the pigment color on TLC plates under white, UV254 nm, and UV360 nm lights; acquisition of UV−vis spectra in different solvents and comparison with the values reported in the literature; and chemical derivatization microscale tests for the examination of 5,6-epoxide, hydroxyl, and carbonyl groups (*2*, *32*−*34*). Carbonyl and hydroxyl groups were also investigated by Fourier transform infrared spectroscopy. Lutein and zeaxanthin standards were obtained in the laboratory by means of TLC from a de-esterified carotenoid extract from mint (*Menta arvensis* L.) and red pepper (*Capsicum annuum* L.), respectively (*31*, *35*).

Chemicals. Solvents (of analytical or HPLC grade as required) and reagent grade chemicals were purchased from Teknokroma (Barcelona, Spain), Microdur (Sevilla, Spain), and Sigma Aldrich Quimica (Madrid, Spain).

HPLC Analysis of Carotenoids. The analysis of carotenoids by HPLC was carried out according to the method of Mínguez-Mosquera and Hornero-Méndez (*31*). The HPLC system consisted of a Waters 2690 Alliance together with a Waters 996 photodiode array detector. The system was controlled with Millennium32 software (Waters Cromatografía, S.A., Barcelona, Spain). A 20 cm × 0.46 cm i.d., 3 μm Spherisorb ODS2 (Teknokroma) column was used. Separation was achieved by a binary-gradient elution using an initial composition of 75% acetone and 25% deionized water. That proportion was maintained constant for 5 min, and subsequently, the acetone proportion was increased linearly to 95% in 5 min and maintained for 20 min. After that period, the acetone percentage was raised to 100% in 2 min and maintained for 5 min. Initial conditions were reached in 5 min. An injection volume of 20 μL and a flow rate of 1 mL/min were used. Detection was performed at 450 nm, and online spectra were acquired in the 350−600 nm wavelength range. Quantification was carried out using a calibration curve obtained with lutein standard. This calibration curve was used to quantify both free and esterified lutein, since the esterification of xanthophylls with fatty acids does not modify the chromophore properties. According to ref *36*, the present chromatographic conditions allowed us to distinguish three different lutein fractions with respect to the degree of esterification, that is, free (F), monoesterified (ME), and diesterified (DE) lutein, respectively. For the quantification of these three fractions, peaks belonging to each one were integrated together before applying the calibration curve.

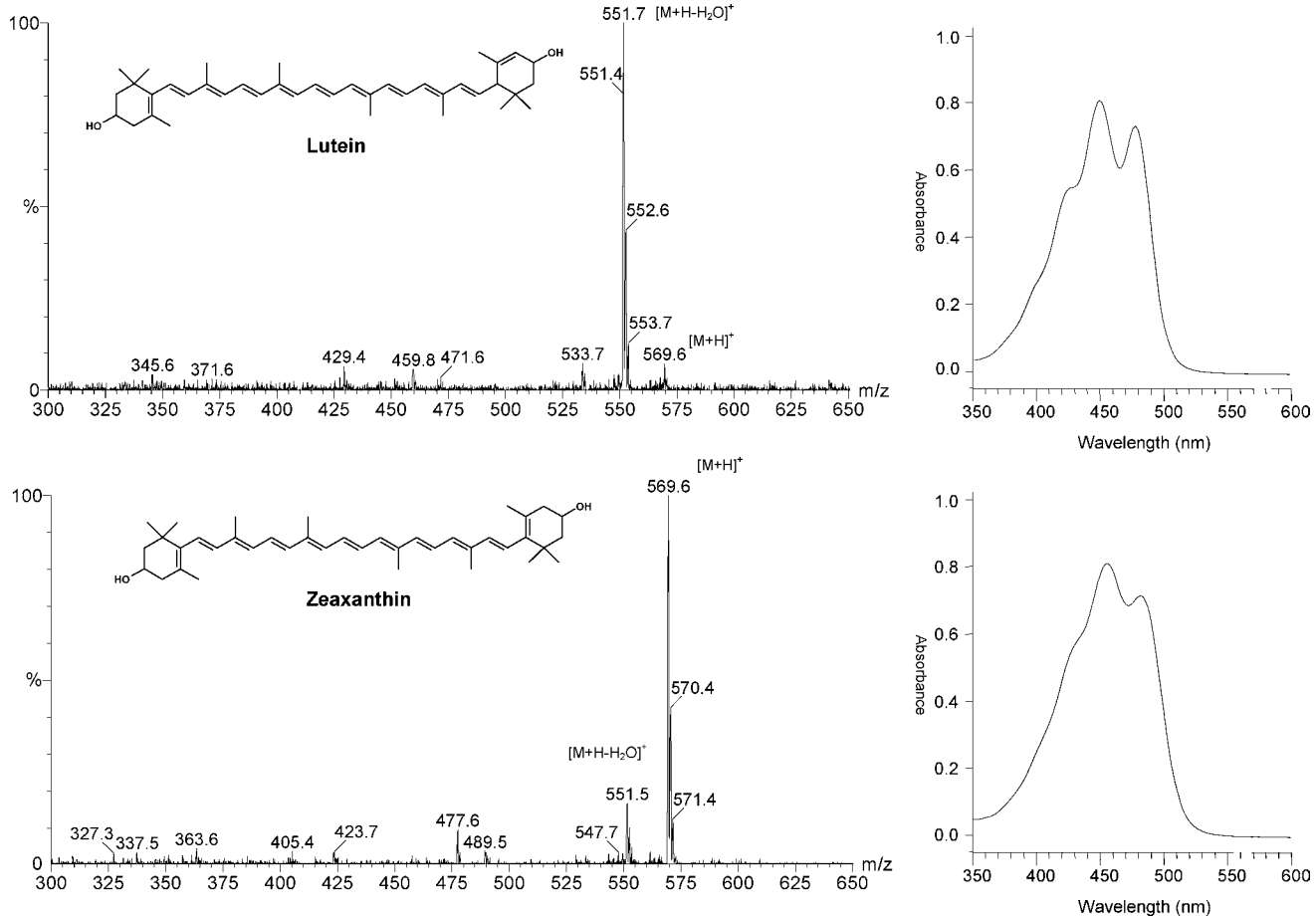

Figure 2. Mass (APCI, positive mode) and UV–visible spectra for the lutein peak present in a saponified extract of tritordeum seeds (accession HT28) and for the corresponding obtained with a standard zeaxanthin solution.

Liquid Chromatography−Mass Spectrometry (LC-MS). LC-MS was performed on the same chromatographic system described before, coupled to a Micromass ZMD4000 (Manchester, United Kingdom) mass spectrometer. The atmospheric pressure chemical ionization (APCI) source was heated at 150 °C, and the APCI probe was kept at 400 °C. The corona voltage was fitted at 3.7 kV, the HV lens to 0.5 kV, and the cone voltage to 30 V. Nitrogen was used as the desolvation and cone gas at 300 and 75 L/h, respectively. Mass spectra of carotenoids were acquired within an *m*/*z* 200−1000 scan range. The UV−visible absorbance was recorded at 450, together with the spectra in the range of 350−650 nm, by using Waters 996 photodiode array detector connected in-line after the pump and before the mass spectrometer. Data were acquired and processed with MassLynx 3.2 (Micromass LTD, Manchester, U. K.) software. Chromatographic conditions were identical to those used for routine HPLC analysis.

Statistical Analyses. Analysis of variance was carried out on all of the traits considered as main factor species (primary tritordeum vs secondary tritordeum vs durum wheat). Because durum wheat samples had no detectable lutein diesters, we used the nonparametric Kruskal−Wallis test for evaluating the existence of dissimilarities among groups. When significant differences ($p \leq 0.01$) were detected, the least significant difference method was computed. All of the statistical analyses were performed with Statistix v. 8.0.

RESULTS AND DISCUSSION

Carotenoid Composition. To assess the genetic variability of the carotenoid concentration and profile of wholemeal flour between tritordeum and durum wheat species, a detailed analysis (qualitative and quantitative) of the carotenoids composition was carried out by HPLC for tritordeum and durum wheat accessions. Prior to quantification, a screening and identification of the carotenes and xanthophylls occurring in the tritordeum and durum wheat accessions under study were performed in a randomly selected set of three samples, respectively. Lutein was the major carotenoid identified in all cases, accounting for up to 99% of the composition, and these data were later confirmed for all accessions considered in the present work. Lutein was present in the free stage but also esterified with fatty acids (mono- and diesters). This fact can be easily demonstrated by comparing the HPLC chromatogram before and after submitting the carotenoid extract to alkaline saponification with KOH−methanol (**Figure 1**), with the presence in the latter case of a unique peak corresponding to free lutein, having the area under the peak increased as a result of the hydrolysis of lutein esters. Lutein was unambiguously identified by LC-MS analysis of the saponified extract. **Figure 2** shows the mass spectrum for lutein together with the UV−visible online spectra. For comparison purposes, the same data were obtained for a sample containing zeaxanthin standard. Beside that, the UV−visible is different for both pigments: Lutein shows the characteristic APCI-MS spectra, showing a strong loss of water at *m*/*z* 551.7 $[M + H - H_2O]^+$, which is due to the presence of an allylic hydroxyl group. On the contrary, zeaxanthin shows a strong quasimolecular ion at *m*/*z* 569.6. An identical MS fragmentation pattern was found for the two small peaks accompanying the main lutein peaks (data not shown). This fact together with the presence of a characteristic UV−visible absorption maximum at 320−340 nm suggested that these two peaks correspond to the *cis* isomer of lutein (probably 9- and 13-*cis*-lutein, respectively). Taking into consideration the precaution taken during sample extraction and analysis, it is feasible to assume that these isomers were already naturally present in the grains.

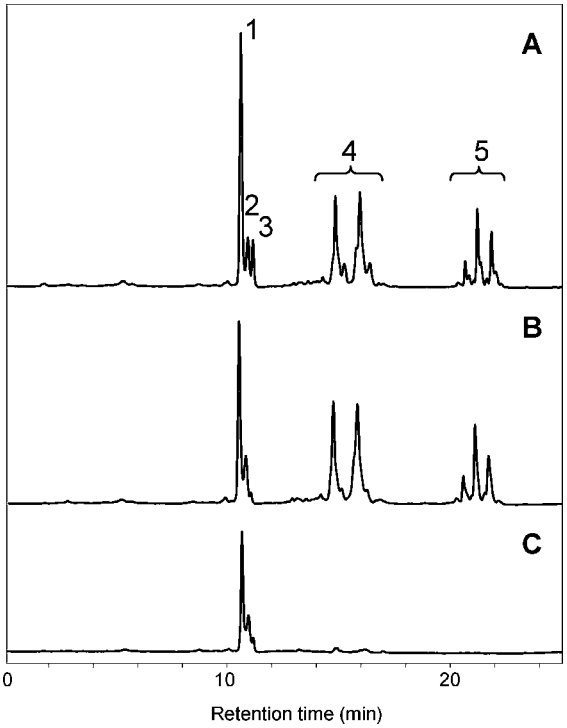

Figure 3. HPLC chromatograms obtained for a carotenoid extract prepared from HT1° (HT131; **A**) and HT2° (HT609; **B**) tritordeum accessions and from a durum wheat accession (Don Pedro; **C**). Peak identities are as detailed in **Figure 1A**.

Lutein has been reported as the main carotenoid found in seeds of *Triticum* spp. For instance, lutein is the main carotenoid in einkorn (*27*, *28*, *37*), durum wheat (*25*, *26*, *28*), and common wheat (*25*, *28*, *38*). However, differences are reported regarding the composition of minoritary carotenoids. First, while we only detected lutein and its esters in tritordeum and durum wheat accessions, zeaxanthin has been detected at trace levels by several authors in several Triticeae species (*25*, *26*, *28*, *39*). Besides, both α- and β-carotenes have been found in several einkorn accessions (*27*).

Second, carotenoids are reported to account for between 30 and 100% of the yellow pigment content. On one hand, cumulative data from the last century suggest that the yellow pigment content of durum wheat consists of carotenoids. On the other hand, carotenoids amount to only 30−50% of the yellow pigment content in durum wheat according to ref *26*. In consequence, they proposed that other compounds not yet identified contribute to yellow color in durum wheat seeds. In the present work, we compared the carotenoid content determined by HPLC with the yellow pigment content determined by absorbance reading at 448 nm for tritordeum accessions. No significant differences were found between both methods (data not shown); therefore, only HPLC data were considered due to the higher sensibility of this detection method. Besides, to our knowledge, only carotenoids have been reported to be related to seed yellow pigment contents in cereals to date.

The third general difference found among works is related to the detection or not of lutein esters. **Figure 3** shows the chromatographic separation of lutein and its esters in HT1°, HT2°, and durum accessions. In general, yellow pigments of durum wheat are assumed to consist of ∼90% lutein and its esters. For instance, lutein accounts for 84.8% of the yellow pigments, lutein monoesters for 9.8%, and lutein diesters for 5.3% (*29*). More recently, the analysis of 138 wheat cultivars for seed carotenoid contents detected lutein esterification in 129 of them (*40*). However, recent works have not normally detect lutein esters in wheat species (*26*, *28*). In the present work, we detected lutein esterification in both durum wheat and tritordeum species.

Carotenoid Content. Before performing any quantitative determination and taking into consideration the poor recoveries reported in some studies for carotenoids from cereal grains, we performed a preliminary work for the optimization of the extraction procedure. Previous works proposed different solvents for extraction (methanol, tetrahydrofurane, water-saturated *n*-butanol, etc.); however, our previous experience in other plant material suggested acetone as a first choice. Acetone was shown to be an excellent extraction solvent when the sample was previously milled, and up to 84% of the color was extracted in a first step. In view of this result, we assayed successive extractions, and we found that a second treatment with acetone obtained an extra 12% of the extractable color, and the rest (4%) was obtained in the third consecutive extraction. As a routine procedure, and just to ensure that all of the color was exhausted, four successive extractions were carried out. With this simple and fast procedure, we found very reproducible results, with relative standard deviations for intraday and interday lower than 4.1 and 7.2%, respectively.

Using the proposed analytical protocol detailed in the Materials and Methods, the carotenoid content of tritordeum and durum wheat accessions was determined (**Table 1**). Tritordeums were divided into two different groups (primary [HT1°] and secondary [HT2°]) as described in the Materials and Methods.

Ranges and mean values are also shown in **Table 1** for the three groups considered in the work (HT1°, HT2°, and durum). The total carotenoid content ranged between 2.6 and 8.8 μg/g, with an average of 5.8 μg/g for primary tritordeums. Secondary tritordeums ranked between 3.7 and 9.4 μg/g with an average of 6.6 μg/g for total carotenoid content. On average, the carotenoid concentration of tritordeum accessions analyzed in the current study was 5.2 times higher than in durum wheat accessions.

These results are not surprising at all, since tritordeums are characterized by high yellow pigment content (*23*, *24*, *41*), which is much higher than the content found in durum wheat varieties. In spite of this previous knowledge, until now, the nature of the compounds responsible of the yellow color in tritordeum seeds had never been investigated. The higher carotenoid content of tritordeums as compared to durum wheat may hold potential as high-lutein functional cereal. Tritordeum shares this potential with einkorn wheat. On average, einkorns have between two and three times more lutein than durum wheat accessions (*27*, *28*, *37*). Therefore, tritordeums may hold a higher potential to produce carotenoids than einkorn wheat since on average they have more than five times a higher carotenoid content than durum wheat.

Tritordeums showed significantly higher means than durum wheat ($p \leq 0.01$) for total carotenoid content, free lutein, lutein monoesters, and lutein diesters (**Table 2**). However, the most outstanding finding was the much higher degree of esterification of carotenoids in tritordeums as compared to durum wheat. On average, 49.2 and 62.1% esterification were found in primary and secondary tritordeums, respectively. Besides, lutein diesters were only detected in tritordeum samples, while these compounds were almost absent in durum wheat genotypes.

There appears to be little or no esterification in just harvest-ripe seeds of wheat (*42*); the presence of lutein esters is related to wheat genotype (*29*). In addition to this, lutein esterification

Table 1. Carotenoid Content (μg/g) in Tritordeums and Durum Wheat Accessions

			lutein						
species[a]	accession	carotenoid content	free	monoester	diester	% free	% monoester	% diester	% esterified
HT1°	HT1	8.8	3.0	4.2	1.7	34.2	47.1	18.7	65.8
HT1°	HT7	3.9	2.3	1.3	0.3	59.8	33.4	6.8	40.2
HT1°	HT27	7.1	2.7	3.3	1.1	37.6	46.9	15.5	62.4
HT1°	HT51	5.0	2.5	2.1	0.4	50.0	42.3	7.7	50.0
HT1°	HT55	2.9	1.8	0.9	0.1	63.3	32.0	4.7	36.7
HT1°	HT71	7.4	3.6	3.1	0.7	49.0	41.9	9.1	51.0
HT1°	HT75	7.8	3.7	3.1	0.9	47.9	40.0	12.0	52.1
HT1°	HT79	7.5	3.6	3.1	0.8	48.2	41.4	10.5	51.8
HT1°	HT80	6.5	3.4	2.5	0.6	52.3	37.8	10.0	47.7
HT1°	HT84	5.9	2.6	2.4	0.8	45.0	41.0	14.0	55.0
HT1°	HT86	6.9	3.4	2.7	0.8	49.3	39.3	11.4	50.7
HT1°	HT89	7.2	2.5	3.4	1.2	35.6	47.6	16.8	64.4
HT1°	HT91	5.0	3.0	1.7	0.3	59.6	33.5	6.9	40.4
HT1°	HT96	8.2	4.0	3.2	0.9	49.3	39.7	11.0	50.7
HT1°	HT114	3.7	2.8	0.9	0.1	74.7	22.7	2.6	25.3
HT1°	HT127	4.7	2.3	2.0	0.4	48.9	42.0	9.1	51.1
HT1°	HT131	5.3	2.4	2.3	0.7	44.3	43.1	12.7	55.7
HT1°	HT138	2.6	1.3	1.0	0.3	49.4	40.3	10.3	50.6
HT1°	HT164	5.2	2.7	2.0	0.5	51.6	38.7	9.7	48.4
HT1°	HT176	3.5	1.4	1.7	0.4	41.2	47.9	11.0	58.8
HT1°	HT195	3.6	2.1	1.3	0.3	58.0	35.2	6.9	42.0
HT1°	HT198	7.1	4.0	2.7	0.4	55.9	38.4	5.6	44.1
HT1°	HT223	5.5	2.5	2.3	0.7	45.4	41.4	13.2	54.6
HT1°	HT224	8.4	5.9	2.3	0.3	69.5	26.8	3.7	30.5
mean		5.8	2.9	2.3	0.6	50.8	39.2	10.0	49.2
max		8.8	5.9	4.2	1.7	74.7	47.9	18.7	65.8
min		2.6	1.3	0.9	0.1	34.2	22.7	2.6	25.3
HT2°	HT2	4.5	1.7	2.1	0.8	36.9	46.1	17.1	63.1
HT2°	HT9	8.0	2.0	3.8	2.1	25.2	48.3	26.5	74.8
HT2°	HT10	7.7	3.5	3.5	0.8	45.1	45.0	9.9	54.9
HT2°	HT13	4.9	1.8	2.3	0.8	37.6	46.1	16.4	62.5
HT2°	HT28	7.2	2.3	3.3	1.7	31.7	45.2	23.1	68.3
HT2°	HT31	6.6	1.7	3.0	1.9	25.4	45.9	28.7	74.6
HT2°	HT64	8.0	2.7	3.7	1.5	34.4	46.5	19.1	65.6
HT2°	HT110	6.3	1.7	2.8	1.9	26.4	43.7	29.9	73.6
HT2°	HT143	6.7	3.0	2.9	0.8	44.9	42.8	12.3	55.1
HT2°	HT148	9.4	4.8	3.6	1.0	51.1	38.1	10.8	48.9
HT2°	HT150	7.6	3.1	3.2	1.3	40.9	42.1	17.1	59.1
HT2°	HT152	7.2	3.6	2.7	0.8	49.9	38.4	11.7	50.1
HT2°	HT157	8.4	4.8	3.1	0.5	56.8	36.9	6.4	43.2
HT2°	HT221	6.4	2.9	2.9	0.7	44.8	44.7	10.4	55.2
HT2°	HT240	5.8	2.2	2.5	1.0	39.9	42.8	17.2	60.1
HT2°	HT263	9.0	4.0	3.8	1.1	45.0	42.1	12.8	54.9
HT2°	HT265	7.0	2.5	3.3	1.2	36.1	46.7	17.2	63.8
HT2°	HT290	5.5	1.4	2.5	1.6	25.3	46.0	28.7	74.7
HT2°	HT292	5.8	1.8	2.6	1.5	30.9	43.8	25.2	69.1
HT2°	HT320	5.6	1.9	2.7	1.0	33.8	48.3	17.9	66.2
HT2°	HT323	6.1	2.1	2.8	1.2	33.7	46.0	20.3	66.3
HT2°	HT325	4.4	0.9	1.9	1.6	19.4	43.3	37.3	80.6
HT2°	HT327	3.7	1.4	1.6	0.7	38.9	42.9	18.2	61.1
HT2°	HT332	4.6	2.2	1.9	0.4	48.4	42.3	9.4	51.6
HT2°	HT333	5.8	1.9	2.8	1.1	32.7	47.7	19.6	67.3
HT2°	HT335	6.6	2.3	3.0	1.3	35.8	45.1	19.1	64.2
HT2°	HT609	8.1	2.8	3.6	1.7	34.6	44.1	21.3	65.4
HT2°	HT630	8.6	3.9	3.8	1.0	44.8	43.7	11.5	55.2
HT2°	HT632	6.1	3.0	2.4	0.7	48.8	39.3	12.0	51.2
mean		6.6	2.5	2.9	1.2	37.9	43.9	18.2	62.1
max		9.4	4.8	3.8	2.1	56.8	48.3	37.3	80.6
min		3.7	0.9	1.6	0.4	19.4	36.9	6.4	43.2
durum	DH2652	1.2	1.0	0.2	ND	84.6	15.4	ND	15.4
durum	Don Pedro	1.5	1.2	0.3	ND	80.5	19.5	ND	19.5
durum	Simeto	0.9	0.7	0.2	ND	77.7	22.3	ND	22.3
durum	T155	1.8	1.4	0.4	ND	79.6	20.4	ND	20.4
durum	T22	0.7	0.6	0.1	ND	83.7	16.3	ND	16.3
durum	T60	1.0	0.8	0.2	ND	76.3	23.7	ND	23.7
durum	Vitrón	1.1	0.9	0.2	ND	81.7	18.3	ND	18.3
mean		1.2	0.9	0.2	0.0	80.6	19.4	ND	19.4
max		1.8	1.4	0.4	0.0	84.6	23.7	ND	23.7
min		0.7	0.6	0.1	0.0	76.3	15.4	ND	15.4

[a] HT1°, primary tritordeums; HT2°, secondary tritordeums; durum, durum wheat; ND, nondetectable, that is, lower than the detection limit.

Table 2. Carotenoids Average Content (μg/g) of Tritordeum and Durum Wheat Accessions

			free lutein		lutein monoester		lutein diester		
species[a]	seed weight	carotenoid content	total	%	total	%	total	%	percentage esterified
HT1°	35.9 c	5.8 a	2.9 a	50.8 b	2.3 b	39.2 b	0.6 b	10.0 b	49.2 b
HT2°	40.5 b	6.6 a	2.6 a	37.9 c	2.9 a	43.9 a	1.2 a	18.2 a	62.1 a
durum	56.4 a	1.2 b	0.9 b	80.6 a	0.2 c	19.4 c	0.0 c	0.00 c	19.4 c

[a] HT1°, primary tritordeums; HT2°, secondary tritordeums; and durum, durum wheat. Means within a column followed by the same letter are not significantly different ($p \leq 0.01$).

increases with the progress of the storage time in most wheat cultivars analyzed (*40*). However, other studies have found decay in lutein concentration with the age of the grain, suggesting that lutein may play an important role as an antioxidant within the grain. On the contrary, there appears to be little esterification in durum wheat cultivars (*43*). The high level of esterification in tritordeum seeds may be influenced, at least partly, by storage time since seeds were not immediately processed after harvesting.

Significant differences ($p \leq 0.01$) for kernel weight were detected among the three groups considered in this work (**Table 2**). The yellow pigment content of seeds is not correlated with kernel weight in durum wheat (*44*). In the current study, kernel weight and carotenoid contents (all the traits) were not correlated ($p \leq 0.05$) in agreement with previous findings on durum wheat (*44*).

Is Differential Esterification Ability Related to Differences in Total Carotenoid Content? The most important finding of this work is the high degree of lutein esterification found in tritordeum seeds. This characteristic may be very important to increase the potential of tritordeums as a functional food for two reasons. First, esterification increases the stability of carotenoids (*45*−*47*). Second, esterification of carotenoid pigments may help to improve their bioavailability as the presence of fatty matter is a determinant factor for increasing their absorption and transport (*48*).

In addition, differential esterification ability between tritordeums and durum wheat may influence the higher yellow pigment content of tritordeum seeds. In plants, carotenoids are produced in specialized organelles, chloroplasts, and chromoplasts, where they are accumulated in association with membranes and lipids. Chromoplasts accumulate carotenoids in specialized lipoprotein-sequestering structures (*49*, *50*). The biosynthesis and accumulation of carotenoids in chromoplast are thought to be regulated by two different mechanisms: (i) increases in carotenoid accumulation due to increased transcript abundance of regulatory biosynthetic genes and (ii) increased and novel carotenoid accumulation caused by the presence of sequestering structures capable of storing carotenoids within the plastid (*3*). These lipoprotein structures can be classified as globular, crystalline, membranous, fibrilar, and tubular (*49*, *50*). Some of them, for instance fibrils, consist of equal parts of proteins and lipids, and within the apolar components are the esterified xanthophylls. Thus, differential esterification ability may play an important role in carotenoid accumulation. Indeed, esterification is a common means to sequester carotenoids in plants and flowers, and it is postulated to increase xanthophylls accumulation by increased lipophilic properties and integration into the lipid-rich plastoglobules (*51*).

Therefore, carotenoid accumulation is influenced by both the level of activity of carotenoid biosynthetic genes and the presence of structures capable of storing and accumulating carotenoids. Within a species, where cellular components are conserved, enzyme levels are expected to play a more important role than sequestering structures in regulating carotenoid content (*3*). However, between species, both mechanisms may be equally important. In our case, tritordeums have a higher yellow pigment content than durum wheat. Until now, it has been assumed that this difference relied on genes located on chromosome $7H^{ch}$ (*52*) and in the chromosome $2H^{ch}$ (*53*). However, differential esterification ability between tritordeums and durum wheat may also play an important role in explaining the differences in carotenoid content between both species.

In conclusion, tritordeums contain much higher carotenoid contents than durum wheat; lutein is the only carotenoid responsible for the yellow color of the grain. The high levels of lutein esterification found in tritordeum seeds may underline a differential esterification ability that together with the activity of biosynthetic genes may explain the high seed carotenoid content of this species.

The biochemical, molecular, and genetic mechanisms governing the esterification of xanthophylls are still to be discovered; therefore, further multidisciplinary research is needed. In the case of wheat, an increase on the lutein ester formation during the grain storage period has been reported. However, other studies have found decay in lutein concentration with the age of the grain, suggesting that lutein may play an important antioxidant role within the grain.

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