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Lutein and Lutein Esters in Whole Grain Flours Made from 75 Genotypes of 5 *Triticum* Species Grown at Multiple Sites

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Supporting Information

ABSTRACT: Concentrations of lutein and lutein esters were determined in an ample collection of 75 wheat genotypes comprising bread wheat (*Triticum aestivum* L.), durum (*Triticum durum* Desf.), spelt (*Triticum spelta* L.), emmer (*Triticum dicoccum* Schrank), and einkorn (*Triticum monococcum* L.) grown in five different environments. Einkorn genotypes showed the highest total amounts of lutein (4.5−7.8 μg/g dry matter), followed by durum (2.0−4.6 μg/g), spelt (0.9−2.0 μg/g), emmer (0.8−1.9 μg/g), and bread wheat (0.7−2.0 μg/g). Due to the observed highly significant genetic variance and high heritability, lutein contents of wheat genotypes may be increased by future plant breeding. Detailed HPLC-DAD-APCI(±)-MS^n data allowing the identification of six lutein monoesters and nine diesters are presented. Linoleic, palmitic, and oleic acids were the most abundant fatty acids in both the lutein esters and total free lipid fractions. Lutein esters were virtually absent in the tetraploid durum and emmer species, whereas their concentrations considerably differed among the genotypes belonging to the other species.

KEYWORDS: *carotenoid ester, heritability, xanthophylls, mass spectrometry, fatty acids*

INTRODUCTION

Wheat (*Triticum* spp.) is one of the most important cereal crops in Europe and worldwide.[1] Although this staple food is mainly consumed as an energy supplier due to its high starch and protein contents, it also contains nutritionally relevant compounds, such as vitamin E and carotenoids.[2] Among the present carotenoids, lutein is predominant in wheat (Figure 1A), accounting for 90−100% of the total carotenoids.[3] Lutein also occurs at high concentrations in the human macula, and its increased dietary intake has been associated with a reduced incidence of eye diseases, such as age-related macular degeneration (AMD), cataract, and retinitis pigmentosa.[4,5] The prevention of mild cognitive impairment and Alzheimer's disease in elderly people by lutein has also been discussed.[6−8] A yet unknown function of lutein in the infant brain has recently been hypothesized, as approximately 60% of the total carotenoids in their brain have been assigned to lutein, and its levels were found to be depleted in preterm infants.[9]

Although lutein concentrations in most wheat products are rather low (0.1−0.5 mg/100 g fresh weight (FW))[10] compared to common lutein-rich foods, such as green leafy vegetables (>2 mg/100 g FW)[11] and egg yolk (approximately 1 mg/100 g FW),[12] their high per capita consumption makes them an important dietary source. In 2007, annual per capita consumption of wheat products in Germany has been estimated to be 87 kg (women) and 114 kg (men), respectively.[13] In addition to its nutritional relevance, the carotenoid content in wheat is an important prerequisite for the product's color, substantially differing when processing flours from different species and genotypes.[3,14] In particular, durum wheat (*Triticum durum* Desf.) was bred for a high carotenoid content, due to its use for the production of semolina and pasta, where the resulting yellowish color hue is highly desirable.[15] Nevertheless, the carotenoid content in einkorn (*Triticum monococcum* L.), an ancient diploid wheat species, was previously reported to be up to 4-fold higher than in other wheat species.[3,14] However, investigations into carotenoid levels of flours from different wheat species are so far limited to a low number of included species and genotypes, additionally lacking multiyear and multisite studies.

According to the semisystematic carotenoid nomenclature,[16] lutein is characterized by hydroxylated β- and ε-rings (Figure 1A). Due to their asymmetric esterification with fatty acids, regioisomers of lutein esters are frequently observed. Recent studies of different nonwheat sources provided detailed information about lutein esters, including their regioisomers.[17−19] Although an earlier study demonstrated that lutein in wheat may occur in both the nonesterified and esterified forms,[20] comprehensive reports about the occurrence and identification of lutein acyl esters in wheat and cereals in general are scarce.[19,20] Particularly, a comprehensive study on carotenoid esters in old wheat species, such as spelt, emmer, and einkorn, has not been carried out to date.

In the present study, the determination of lutein and its esters in an ample set of 75 genotypes from 5 wheat species

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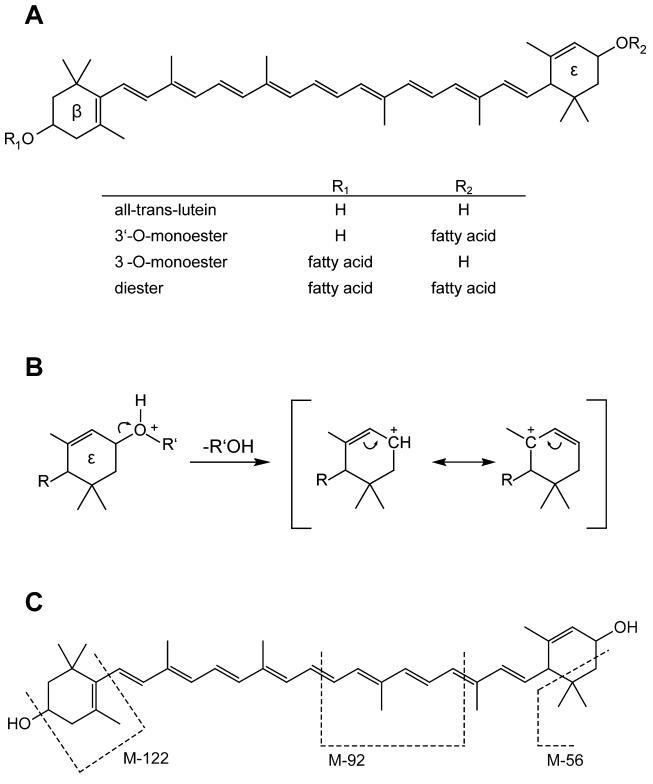

	R_1	R_2
all-trans-lutein	H	H
3'-O-monoester	H	fatty acid
3 -O-monoester	fatty acid	H
diester	fatty acid	fatty acid

Figure 1. (A) Chemical structure of free and esterified lutein. (B) Fragmentation scheme of lutein at the ε-ring applying positive atmospheric pressure chemical ionization (APCI). (C) Characteristic lutein fragments produced by positive APCI.

grown in 5 different environments should illustrate the genetic variation and modificatory variability, that is, diversity of xanthophyll contents among different and identical species as affected by both genotype and environment. Considering the applied HPLC-DAD-MS^n method, the advantages of combining positive and negative ionization techniques for the identification of lutein ester regioisomers are additionally discussed.

MATERIALS AND METHODS

Plant Material and Sample Preparation. Fifteen genotypes each of bread wheat (*Triticum aestivum* L.), durum (*Triticum durum* Desf.), spelt (*Triticum spelta* L.), einkorn (*Triticum monococcum* L.), and emmer (*Triticum dicoccum* Schrank) were grown at four sites in Germany (Hohenheim, Eckartsweier, Oberer Lindenhof, Seligenstadt). All samples originated from harvest 2013, except for those from Hohenheim, which were from 2012 and 2013. A detailed sample list and further information about the different sites are provided in Supplemental Tables 1−6. Cereal samples used in this study were produced by the State Plant Breeding Institute (University of Hohenheim, Stuttgart, Germany) in separate but adjacent trials with two replicates for each species. After harvest, all spelt, einkorn, and emmer samples were dehulled and cleaned with a laboratory seed cleaner (Samatec-Roeber, Bad Oeynhausen, Germany) to remove hulls, straw, and damaged kernels. For chemical analyses, the kernels of the two replicates of one site were combined. The samples were stored under identical conditions (≤18 °C, ≤40% relative humidity) prior to milling to a particle size ≤0.5 mm by a laboratory mill ZM1 (Retsch, Haan, Germany). Subsequently, whole grain flour was stored at −20 °C until further analysis. Dry matter content was determined using an infrared moisture analyzer MA 40 (Sartorius, Göttingen, Germany).

Chemicals. Methanol, methyl *tert*-butyl ether (MTBE), potassium hydroxide (KOH), sodium chloride (NaCl), and ammonium acetate were purchased from VWR International (Darmstadt, Germany). Acetone, diethyl ether, and *n*-hexane were from Merck (Darmstadt, Germany). Lutein (purity ≥ 95%) originated from CaroteNature (Lupsingen, Switzerland). Fatty acids (FAs), their respective methyl esters (FAMEs) of GC purity, and a methanolic boron trifluoride solution (10% BF_3 in methanol) were obtained from Sigma-Aldrich (Steinheim, Germany). A marigold (*Tagetes erecta* L.) extract (10% lutein esters) was kindly provided by BioActives Europe (Frankfurt, Germany). Purified water was prepared using a Sartorius arium 611 Ultrapure Water System.

Extraction of Carotenoids. Carotenoids were extracted for their quantification according to a previously described method.[21] Briefly, an

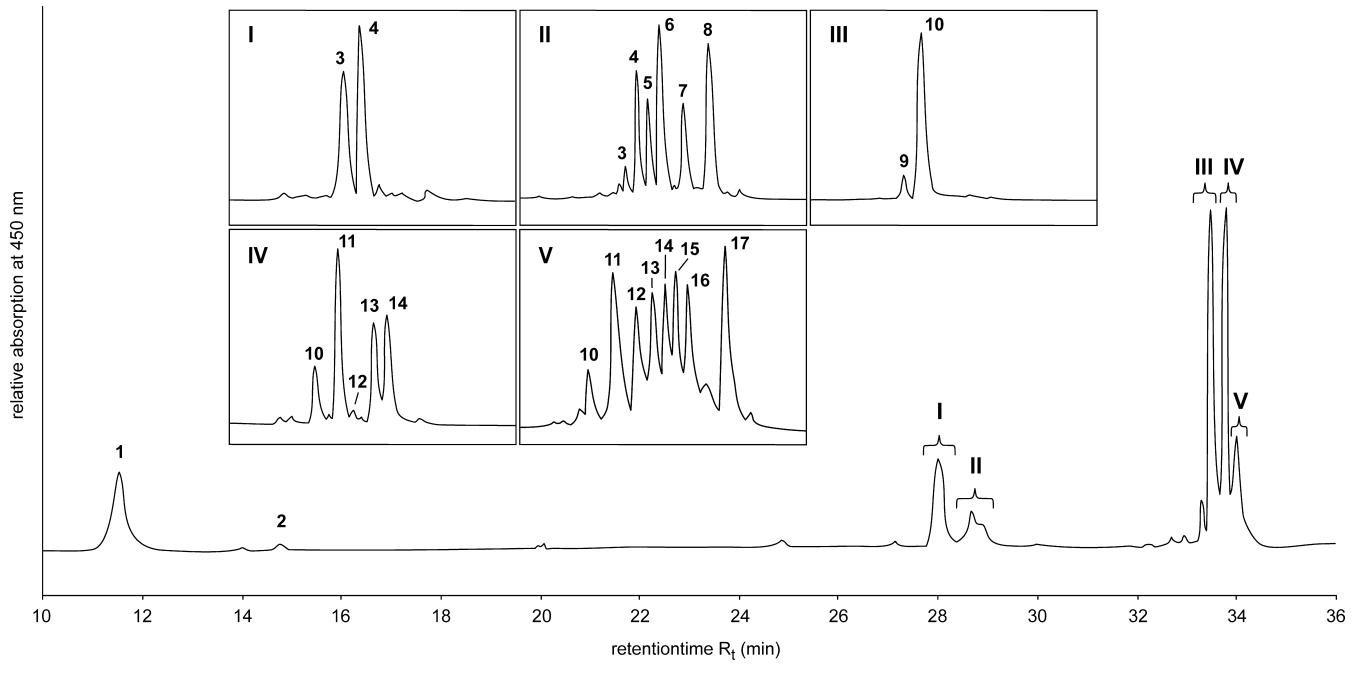

Figure 2. C_{18} HPLC chromatogram of a carotenoid extract from einkorn and C_{30} HPLC chromatograms of the isolated lutein ester fractions (I−V).

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aliquot of 0.70 ± 0.05 g of whole grain flour was extracted three times with acetone (2, 1.5, and 1.5 mL, respectively) using an Ultra Turrax T-25 homogenizer (IKA-Werke, Staufen, Germany). Separation of the acetone extracts from the solid extraction residue was enhanced by centrifugation at 1750*g* for 2 min (Heraeus Labofuge 400R, Thermo Fisher Scientific, Osterode, Germany). The combined acetone extracts were dried under a gentle nitrogen stream and stored at −80 °C until HPLC analyses. As described by Burkhard and Böhm,[22] a soaking step was applied prior to the exhaustive extraction of carotenoids from the tetraploid wheat species durum and emmer. For this purpose, 0.7 mL of water was added to the wheat flour and soaked for 5 min prior to extraction. After the application of this soaking step, average carotenoid yields were increased by 44.5 and 39.1% for durum and emmer when all samples from genotypes of these species were compared, respectively (data not shown). In contrast, total carotenoid contents were decreased by 2.4−17.9% in the case of bread wheat, spelt, and einkorn. Therefore, the data reported for the latter species refer to an extraction omitting the soaking step.

For semipreparative isolation and subsequent identification of lutein esters, the extraction procedure described was scaled up to 40 g of extracted flour. The dried extract was redissolved in 5 mL of MeOH/MTBE (1:1, v/v) and, subsequently, lutein esters were fractionated by semipreparative HPLC using a 250 × 21.2 mm i.d. Phenomenex Aqua 5 μm C_{18} 125 Å column (Phenomenex, Torrance, CA, USA). For this purpose, a linear gradient from MeOH/water (90:10, v/v) to MTBE/MEOH/water (60:36:4, v/v/v) within 40 min was applied. As shown in Figure 2, the lipophilic wheat extracts were separated into five fractions (I−V), evaporated in vacuo (T = 25 °C), and analyzed by HPLC-DAD-MS^n as described below.

Saponification of Carotenoid Extracts. Confirming the occurrence of lutein esters, saponification of carotenoid extracts was conducted as follows. After carotenoid extraction as described above, the dried extracts were saponified following a method described by Mellado-Ortega and Hornero-Méndez.[19] Briefly, the dried extracts were redissolved in 2 mL of diethyl ether. Subsequently, 0.5 mL of methanolic KOH (10%, w/v) was added, and the mixture was stirred (ca. 500 rpm) for 1 h under a nitrogen atmosphere. After the addition of 1 mL of $NaCl_{aq}$ (30%, w/v), carotenoids were extracted with diethyl ether. The combined ether phases were evaporated in vacuo (T = 25 °C), and the dried extracts were stored at −80 °C until HPLC analyses.

HPLC-DAD-MS^n Analyses of Lutein and Lutein Esters. Prior to HPLC-DAD-MS^n analyses, the dried carotenoid extracts were redissolved in MeOH/MTBE (1:1, v/v) and membrane-filtered (0.45 μm, PTFE) into amber glass vials. Separation of carotenoids was performed on a series 1100 HPLC (Agilent, Waldbronn, Germany) equipped with a G1315B diode array detector. The carotenoid C_{30} column (150 × 3.0 mm i.d., 3 μm particle size, YMC, Dinslaken, Germany) with a YMC C_{30} guard column (10 × 3.0 mm i.d.) of the same material was operated at 25 °C. The mobile phase consisted of two different mixtures of MeOH/MTBE/10 g/L ammonium $acetate_{aq}$ (80:18:2 (v/v/v), solvent A; 6:92:2 (v/v/v), solvent B). The gradient program was as follows: 0−60% B (45 min), 60−100% B (5 min), 100% B isocratic (2 min), 100−0% B (3 min). Re-equilibration of the column was achieved at 0% B within 5 min. Total run time was 60 min at a flow rate of 0.42 mL/min. Injection volume was 20 μL. All carotenoids and carotenoid esters were monitored at 450 nm. Additionally, UV−vis spectra were recorded in the range of 220−700 nm. The HPLC system was coupled online to a Bruker (Bremen, Germany) model Esquire 3000+ ion trap mass spectrometer. MS^n analyses of carotenoids were performed using an atmospheric pressure chemical ionization (APCI) source in positive and negative mode in the range of m/z 200−1200 at a scan speed of 13000 Th/s. Nitrogen was used both as drying gas at a flow rate of 5 L/min and as nebulizer gas at a pressure of 55 psi. Vaporizer and dry temperature were set at 350 °C with a capillary voltage of ±2800 V, and the corona was set at 3000 nA in both positive and negative modes. Helium was used as the collision gas for collision-induced dissociation (CID) experiments at a pressure of 4.9×10^{-6} mbar. The fragmentation amplitude was set at 1.0 V.

Identification of carotenoids was carried out by comparing their retention times, UV−vis absorption, and mass spectra with those of authentic standards. Because commercial standards of lutein esters were unavailable, they were identified by their UV−vis absorption matching free lutein and by their characteristic mass spectral behavior as discussed below.[17−19,23] The identification of lutein esters was confirmed by cochromatographic analyses of a marigold (*Tagetes erecta* L.) extract containing known lutein esters (lutein dimyristate, lutein palmitate-myristate, lutein dipalmitate, lutein stearate-palmitate, and lutein distearate).[17] The respective chromatogram and corresponding identification parameters are provided in the Supporting Information.

The quantification of free, mono-, and diesterifed lutein was conducted by RP-C_{18}-HPLC-DAD using an authentic lutein standard recently described.[21] Due to their coelution on RP-C_{18} columns, zeaxanthin was quantitated together with free lutein. However, zeaxanthin contributes only 0−10% to the total carotenoids in *Triticum* according to previous studies[3] and our observations using a C_{30} column. Therefore, peak 1 was assigned to free lutein. Because the chromophore of xanthophylls remains identical after esterification,[24] the concentrations of lutein esters were determined by using the linear calibration for free lutein and molecular weight correction factors when data were converted from micromoles to micrograms. Limits of detection (LOD) and quantitation (LOQ) were determined[25] to be 0.01 and 0.03 μmol lutein/g DM, respectively.

Fatty Acid Extraction, Derivatization, and Analyses by GC-FID. Fatty acids (FAs) were determined according to a previously published protocol[26] with some modifications. Briefly, total free lipids of wheat flours were extracted in a Soxhlet apparatus for 2 h with boiling *n*-hexane. The organic solvent was evaporated in vacuo at 30 °C, and the total free lipid content was gravimetrically determined. Subsequently, an aliquot of 20−30 mg of the obtained total free lipids was combined with 1 mL of methanolic KOH (0.5 M) and heated at 80 °C for 10 min. After the mixture had cooled to room temperature, 1 mL of methanolic BF_3 reagent was added and heated for another 5 min at 80 °C. The samples were subsequently cooled to room temperature, and then 2 mL of $NaCl_{aq}$ (30%, w/v) was added. The fatty acid methyl esters (FAMEs) obtained were then extracted with *n*-hexane prior to GC-FID analyses. Heptadecanoic acid was added as internal standard prior to saponification.

For determination of FAMEs, a Chrompack CP 9001 gas chromatograph (Chromopack, Middleburg, The Netherlands) equipped with a CP 9010 autosampler and a FID detector was used. Separation was performed on a Supelcowax-10 fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm; Supelco, Bellafonte, PA, USA). Helium (purity 5.0) was used as carrier gas with a constant flow of 0.92 mL/min, and the injection volume was 1 μL at a split ratio of approximately 1:55. The oven temperature was ramped from 180 to 240 °C at a heating rate of 3 K/min. The final temperature was held for 3 min. The temperature of both the injector and detector was set at 250 °C. FAMEs were identified by comparison of their retention times with those of authentic standards. Quantitation was performed using linear calibrations of authentic reference compounds, additionally corrected by the internal standard signal (heptadecanoic acid).

Statistical Analysis. Variance components were determined by the restricted maximum likelihood (REML) method, assuming a random model using classical one-stage analyses.[27] Significance of variance component estimates was tested by model comparison with likelihood ratio tests in which halved P values were used as approximation.[28] Heritability h^2 was calculated according to eq 1

$$h^2 = 1 - \frac{\vartheta}{2\sigma_G^2} \quad (1)$$

where ϑ is the mean variance of a difference of two best linear unbiased predictors (BLUP) and σ_G^2 the genetic variance.[29,30] All analyses were performed with the statistical software R (R Development Core Team 2011) and the software ASReml 3.0.[31]

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Table 1. Chromatographic and Spectroscopic Properties of the Lutein and Lutein Esters Isolated from Einkorn

peak[a]	t_R[b] (min)	identity	HPLC-DAD UV−vis spectrum λ_{max} (nm)	APCI(−) $[M]^-$ m/z	APCI(+) $[M + H]^+$ m/z (relative intensity)	HPLC-APCI(+)-MS^2 m/z (relative intensity)[c]
std	9.0	(*all-E*)-lutein standard	445, 473	568	551	[551]: 533 (100), 495 (79), 429 (24), 411 (22), 459 (12), 477 (11), 345 (10)
1	9.0	(*all-E*)-lutein	445, 473	568	551	[551]: 533 (100), 495 (28), 429 (24), 411 (17), 459 (12), 477 (11), 345 (8)
2	10.3	lutein (*Z*)-isomer	329, 441, 468	568	551	
3	21.5	lutein 3′-*O*-linoleate	445, 473	830	551 (100), 533 (13), 831 (4)	[551]: 533 (100), 495 (26) 429 (24), 411 (16), 477 (10) 459 (9), 345 (8)
4	22.4	lutein 3-*O*-linoleate	445, 473	830	813 (100), 533 (51), 831 (8)	[813]: 533 (100), 411 (11), 477 (8)
5	23.3	lutein 3′-*O*-oleate	445, 473	832	551 (100), 533 (21), 833 (9)	[551]: 533 (100), 495 (92), 411 (27), 429 (23), 477 (12), 345 (10), 459 (9)
6	24.1	lutein 3-*O*-oleate	445, 473	832	815 (100), 533 (53), 833 (10)	[815]: 533 (100), 411 (12), 477 (6), 441 (6)
7	25.8	lutein 3′-*O*-palmitate	445, 473	806	551 (100), 533 (36)	[551]: 533 (100), 495 (35), 429 (19), 441 (18), 477 (8), 345 (6), 459 (4)
8	27.7	lutein 3-*O*-palmitate	445, 473	806	789 (100), 533 (43), 808 (5)	[789]: 533 (100), 411 (10), 477 (8), 441 (4)
9	31.6	lutein linoleate-linolenate[d]	445, 473	1090	813 (100), 811 (96), 533 (63)	[813]: 533 (100), 411 (15)
10	32.4	lutein dilinoleate	445, 473	1092	813 (100), 533 (17)	[813]: 533 (100), 411 (12), 477 (7)
11	33.5	lutein 3′-*O*-linoleate- 3-*O*-oleate	445, 473	1094	815 (100), 813 (47), 533 (41)	[815]: 533 (100), 411 (8), 477 (4), 441 (3)
12	34.6	lutein dioleate	445, 473	1096	815 (100), 533 (46)	[815]: 533 (100), 411 (10), 441 (4)
13	35.3	lutein 3′-*O*-palmitate- 3-*O*-linoleate	445, 473	1068	813 (100), 533 (33), 789 (4)	[813]: 533 (100), 411 (13)
14	36.0	lutein 3′-*O*-linoleate- 3-*O*-palmitate	445, 473	1068	789 (100), 533 (42), 813 (8)	[789]: 533 (100), 411 (14), 477 (7)
15	36.5	lutein 3′-*O*-palmitate- 3-*O*-oleate	445, 473	1070	815 (100), 533 (62), 789 (45)	[815]: 533 (100), 411 (9), 477 (6), 441 (4)
16	37.0	lutein 3′-*O*-oleate-3-*O*-palmitate	445, 473	1070	789 (100), 533 (69), 815 (29)	[789]: 533 (100), 411 (9), 477 (6), 441 (5)
17	38.7	lutein dipalmitate	445, 473	1044	789 (100), 533 (52)	[789]: 533 (100), 411 (14), 477 (5), 441 (3)

[a]Peak assignment according to Figure 2 and Supplemental Figure 1. [b]Retention times (t_R) refer to the analytical runs on the C_{30} column. [c]Carotenoid-specific fragments. [d]Identification of regioisomer was impossible.

RESULTS AND DISCUSSION

Identification of Lutein and Its Fatty Acid Esters. *General Considerations.* All compounds monitored at 450 nm revealed identical UV−vis absorption spectra with maxima at 445 and 473 nm, also being identical to that of an authentic lutein standard (Table 1). In contrast to compounds **1** and **2**, compounds **3**−**17** were absent in the saponified carotenoid extracts (Supplemental Figure 1B,C). The highest concentrations of these compounds (**3**−**17**), presumably lutein mono- and diesters as described below (Table 2), were found in the einkorn breeding line '8.103/04', the bread wheat genotype 'Tabasco', and the spelt genotype 'Oberkulmer Rotkorn'. Thus, these genotypes were used for the identification of lutein esters.

For identification by RP-C_{30}-HPLC-DAD-MS^n as described above, the lutein ester-rich extracts were first divided into five fractions (I−V) by RP-C_{18} semipreparative HPLC (Figure 2). For the identification of lutein esters, mass spectral data obtained from an authentic lutein standard were helpful. Because the hydroxyl group of the ε-ring is in an allylic position (Figure 1A), the *in source*-formation of the dehydrated fragment at m/z 551 $[M + H - H_2O]^+$ is highly favored in the positive APCI experiments due to mesomeric effects (Figure 1B). The abundance of this ion at m/z 551 was drastically higher than that of the protonated pseudomolecular ion at m/z 569 $[M + H]^+$ (Table 1), being in agreement with previous studies.[32−34] CID experiments of the *in source*-fragment $[M + H - H_2O]^+$ at m/z 551 revealed several characteristic fragments (Figure 1C),[33,34] such as the predominant daughter ion $[M + H - 2H_2O]^+$ at m/z 533 (base peak), resulting from an additional loss of water from the hydroxyl group of the β-ring. The carotenoid-specific elimination of toluene from the central polyene chain of the carotenoid skeleton resulted in a characteristic loss of 92 amu,[32] leading to product ions at m/z 459 $[M + H - H_2O - 92]^+$ and 441 $[M + H - 2H_2O - 92]^+$. As shown in Figure 1C, the neutral loss of 56 amu may be due to an ε-ring-specific fragmentation following a retro-Diels−Alder reaction as described previously.[32,33] Consequently, the product ions detected at m/z 495 and 477 may correspond to $[M + H - H_2O - 56]^+$ and $[M + H - 2H_2O - 56]^+$, respectively (Table 1). The elimination of the cyclic β- or ε-end groups from the carotenoid skeleton resulted in the fragment ions $[M + H - H_2O - 122]^+$ at m/z 429 and $[M + H - 2H_2O - 122]^+$ at m/z 411 (Figure 1C),[33] whereas the ion at m/z 345 was previously described to be a fragment of $[M + H - H_2O]^+$ at m/z 551.[35]

The described fragmentation pattern of lutein was instrumental for the identification of lutein esters, particularly their regioisomers. By analogy to the *in source* water loss from C3′ of the ε-ring, acyl residues were separated from the ε-ring of lutein esters during APCI(+) experiments. As a result, the protonated molecular ion $[M + H]^+$ of 3′-*O*-monoesters and diesters is commonly observed at poor intensity. However, the differing fragmentation patterns of the β- and ε-ring hydroxyl and acyl moieties allow the differentiation of lutein ester

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Table 2. Total Lutein Content and Relative Composition of Free, Mono-esterified, and Di-esterified Lutein in Different Wheat Species and Genotypes

			total lutein content[a] (μg/g DM)		lutein composition[b] (%)			
species	genotype	n[c]	mean[d]	range	free	monoesterified	diesterified	esterified
bread wheat	Altigo	5	1.35 ± 0.27	0.92−1.57	100.0	0.0	0.0	0.0
	Colonia	5	1.48 ± 0.24	1.18−1.77	74.3	20.2	5.5	25.7
	Cubus	5	1.30 ± 0.29	0.87−1.54	100.0	0.0	0.0	0.0
	Event	5	0.87 ± 0.28	0.56−1.32	71.8	17.8	10.5	28.2
	Genius	5	1.07 ± 0.21	0.74−1.23	74.4	18.1	7.5	25.6
	JBAsano	5	1.35 ± 0.28	0.91−1.61	69.2	19.8	11.0	30.8
	Julius	5	0.69 ± 0.15	0.44−0.81	100.0	0.0	0.0	0.0
	Lear	4	2.01 ± 0.36	1.51−2.36	100.0	0.0	0.0	0.0
	Meister	5	1.34 ± 0.31	0.88−1.71	100.0	0.0	0.0	0.0
	Mulan	5	1.57 ± 0.40	0.98−2.11	100.0	0.0	0.0	0.0
	Orcas	5	1.28 ± 0.31	0.82−1.57	77.3	17.1	5.7	22.7
	Skalmeje	5	1.39 ± 0.37	0.84−1.83	70.4	20.6	9.0	29.6
	Tabasco	5	1.50 ± 0.20	1.20−1.70	61.7	22.3	16.1	38.3
	Tobak	5	1.26 ± 0.28	0.90−1.64	100.0	0.0	0.0	0.0
	Tommi	5	1.52 ± 0.26	1.16−1.84	71.9	19.3	8.8	28.1
	median		1.35		77.3	17.1	5.5	22.7
durum	Auradur	5	3.36 ± 0.70	2.42−4.39	100.0	0.0	0.0	0.0
	Elsadur	4	3.58 ± 0.84	2.65−4.69	100.0	0.0	0.0	0.0
	Karur	5	2.95 ± 0.51	2.16−3.37	100.0	0.0	0.0	0.0
	Logidur	5	2.22 ± 0.38	1.69−2.73	100.0	0.0	0.0	0.0
	Lunadur	5	2.01 ± 0.45	1.41−2.64	100.0	0.0	0.0	0.0
	Lupidur	5	2.35 ± 0.57	1.63−3.20	100.0	0.0	0.0	0.0
	W-05005/02	5	3.46 ± 0.84	2.68−4.88	100.0	0.0	0.0	0.0
	W-05006/01	5	3.00 ± 0.42	2.48−3.62	100.0	0.0	0.0	0.0
	W-05018/04	5	3.56 ± 0.50	3.07−4.38	100.0	0.0	0.0	0.0
	W-05020/01	5	3.02 ± 0.52	2.49−3.79	100.0	0.0	0.0	0.0
	W-05024/01	5	4.56 ± 1.06	3.62−6.33	100.0	0.0	0.0	0.0
	W-05025/01	5	3.37 ± 0.60	2.69−4.33	100.0	0.0	0.0	0.0
	W-05026/03	5	3.25 ± 0.65	2.44−4.17	100.0	0.0	0.0	0.0
	W-05029/02	5	4.28 ± 0.77	3.36−5.49	100.0	0.0	0.0	0.0
	Wintergold	5	3.26 ± 0.56	2.53−4.03	100.0	0.0	0.0	0.0
	median		3.26		100.0	0.0	0.0	0.0
spelt	Badengold	5	1.51 ± 0.21	1.26−1.76	70.9	19.7	9.5	29.1
	Badenkrone	5	1.96 ± 0.43	1.32−2.42	70.0	21.5	8.5	30.0
	Badenstern	5	1.53 ± 0.26	1.15−1.82	70.3	19.9	9.8	29.7
	D-04004/03	5	1.56 ± 0.42	1.07−2.13	71.6	19.3	9.1	28.4
	D-04004/04	5	1.64 ± 0.43	1.11−2.20	69.9	19.0	11.1	30.1
	D-04004/08	5	1.77 ± 0.47	1.18−2.37	68.6	20.5	10.9	31.4
	D-04004/09	5	1.50 ± 0.39	1.00−2.02	71.6	18.6	9.8	28.4
	D-05015/03	5	0.87 ± 0.18	0.62−1.05	74.3	17.1	8.6	25.7
	Divimar	5	1.57 ± 0.36	1.10−1.97	68.6	21.1	10.3	31.4
	Filderstolz	5	1.23 ± 0.28	0.94−1.58	71.0	19.0	10.0	29.0
	Franckenkorn	5	1.28 ± 0.21	1.11−1.63	76.3	17.5	6.3	23.7
	Oberkulmer Rotkorn	5	1.54 ± 0.23	1.30−1.80	64.2	21.9	13.9	35.8
	Samir	5	1.65 ± 0.41	1.20−2.16	72.2	19.0	8.8	27.8
	Schwabenkorn	5	1.51 ± 0.34	1.07−1.89	67.3	21.6	11.1	32.7
	Zollernspelz	5	1.43 ± 0.16	1.29−1.65	68.2	22.0	9.8	31.8
	median		1.53		70.3	19.7	9.8	29.7
einkorn	8.103/04	4	5.56 ± 0.37	5.14−6.05	41.7	18.1	40.2	58.3
	8.108/04	5	5.56 ± 1.01	4.28−7.07	92.7	7.0	0.3	7.3
	8.116/04	5	7.33 ± 0.59	6.92−8.33	88.5	10.1	1.3	11.5
	M-03021/03	5	5.92 ± 0.50	5.44−6.69	89.7	9.3	1.0	10.3
	M-04003/01	5	6.24 ± 0.53	5.73−7.12	94.1	5.6	0.4	5.9
	M-04018/01	5	4.69 ± 0.49	4.03−5.35	93.3	6.4	0.3	6.7
	M-04018/02	5	4.47 ± 0.47	4.03−5.19	94.0	5.7	0.3	6.0

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Table 2. continued

species	genotype	n^c	total lutein content[a] (μg/g DM) mean[d]	range	lutein composition[b] (%) free	monoesterified	diesterified	esterified
	M-04018/03	5	4.69 ± 0.63	4.09−5.62	93.8	6.0	0.2	6.2
	M-04033/01	5	7.66 ± 0.80	6.75−8.83	89.7	9.1	1.1	10.3
	M-04033/03	5	7.83 ± 0.85	6.95−8.95	89.3	9.4	1.2	10.7
	M-07006/01	4	6.06 ± 0.41	5.71−6.64	95.4	4.6	0.0	4.6
	Monlis	3	5.65 ± 0.20	5.42−5.77	95.6	4.4	0.0	4.4
	MVMenket	4	5.72 ± 0.28	5.35−5.96	92.4	6.9	0.7	7.6
	Terzino	5	5.49 ± 0.38	4.89−5.92	90.6	8.2	1.2	9.4
	Tifi	5	5.51 ± 0.48	4.82−5.87	90.5	8.6	0.9	9.5
	median		5.65		92.4	7.0	0.7	7.6
emmer	9.105/06/01	5	1.31 ± 0.18	1.04−1.52	100.0	0.0	0.0	0.0
	9.118/05/01	5	1.13 ± 0.22	0.91−1.44	100.0	0.0	0.0	0.0
	9.121/05	5	1.15 ± 0.17	0.92−1.35	100.0	0.0	0.0	0.0
	9.131/05	5	0.90 ± 0.09	0.76−0.99	100.0	0.0	0.0	0.0
	9.199/06/01	5	1.62 ± 0.15	1.40−1.81	100.0	0.0	0.0	0.0
	CC1E-04058/01	5	1.90 ± 0.19	1.63−2.13	100.0	0.0	0.0	0.0
	CC1E-04059/02	5	1.86 ± 0.32	1.30−2.13	100.0	0.0	0.0	0.0
	CC1E-04059/04	5	1.61 ± 0.21	1.27−1.83	100.0	0.0	0.0	0.0
	CC1E-04059/06	5	1.85 ± 0.22	1.47−2.05	100.0	0.0	0.0	0.0
	CC1E-04059/07	5	1.57 ± 0.26	1.25−1.82	100.0	0.0	0.0	0.0
	E-07086/01	4	1.93 ± 0.31	1.55−2.27	100.0	0.0	0.0	0.0
	E-07087/01	4	1.19 ± 0.14	1.08−1−39	100.0	0.0	0.0	0.0
	E-07087/02	4	0.79 ± 0.10	0.64−0.88	100.0	0.0	0.0	0.0
	Osiris	5	1.53 ± 0.07	1.41−1.58	100.0	0.0	0.0	0.0
	Ramses	5	1.27 ± 0.14	1.13−1.44	100.0	0.0	0.0	0.0
	median		1.52		100.0	0.0	0.0	0.0

[a]Up to 10% of the total lutein content may be zeaxanthin. [b]Mean. [c]Number of environments where the respective genotype was grown. [d]Mean ± standard deviation.

regioisomers, as the predominant *in source*-fragment ion indicates the position of the fatty acid moiety.[17−19] Briefly, lutein 3′-*O*-monoesters showed *in source*-fragments $[M + H - \text{fatty acid}]^+$ at m/z 551 as the most abundant ion, whereas $[M + H - H_2O]^+$ at higher m/z represented the predominant positive fragment ion of 3-*O*-monoesters, for example, at m/z 789 for lutein 3-*O*-palmitate. Similarly, the most abundant *in source*-fragment ion of lutein diesters indicated the loss of the fatty acid located at position 3′ (ε-ring). Consequently, the identity of the fatty acid attached to position 3 (β-ring) can be deduced by exclusion principle. However, this identification strategy requires the unambiguous assignment of the m/z of the (pseudo)molecular ion, which may be intricate when only APCI(+) is used. Therefore, APCI(−) experiments were conducted as described previously.[36,37] Under the chosen MS conditions, the authentic lutein standard showed a predominant ion in the negative mode at m/z 568, corresponding to its molecular ion $[M]^-$. Among others, Breithaupt[36] reported that APCI(−) produced a higher abundance of the negatively charged molecular ions $[M]^-$ of astaxanthin esters than the corresponding $[M - H]^-$ ions.

Identification of Lutein Monoesters. Following the identification strategy described above, six compounds (**3**−**8**) contained in fractions I and II were assigned to lutein monoesters by RP-C_{30}-HPLC-MS^n (Figure 2). Lutein being the core carotenoid of diverse esters was identified on the basis of its characteristic UV−vis absorption spectrum identical to that of free lutein (Table 1) as well as the disappearance of lutein esters after saponification and the concomitant increase of the signal of free lutein. Both 3′-*O*- and 3-*O*-regioisomers of lutein linoleate (**3**, **4**), oleate (**5**, **6**), and palmitate (**7**, **8**) were identified, because characteristic molecular ions $[M]^-$ at m/z 830, 832, and 806 were observed during APCI(−)-MS experiments, respectively. With regard to the 3′-*O*-regioisomers, the *in source*-fragment $[M + H - \text{acyl}]^+$ at m/z 551 was the prevailing ion, whereas the 3-*O*-regioisomers showed predominant dehydrated *in source*-fragment ions corresponding to $[M + H - H_2O]^+$ of lutein linoleate (m/z 813), lutein oleate (m/z 815), and lutein palmitate (m/z 789), respectively. In contrast to APCI(−)-MS, molecular ions $[M + H]^+$ were detected only at very low intensity, if they were detectable at all. However, CID experiments of the above-mentioned predominant *in source*-fragment ions of 3′-*O*-regioisomers provided characteristic daughter ions at m/z 495, 459, and 429, thus indicating the occurrence of a free hydroxyl group at the β-ring as described above. After fragmentation of 3-*O*-regioisomers, the lutein backbone $[M + H - H_2O - \text{fatty acid}]^+$ at m/z 533 was the most abundant ion, followed by product ions at m/z 411 and 477, corresponding to a further lutein-specific fragmentation. In addition, identification of 3′-*O*-regioisomers of lutein monoesters was confirmed by their elution before the respective 3-*O*-isomers as has been previously reported for the RP-C_{30} conditions applied.[17−19]

Identification of Lutein Diesters. Eight compounds (**9**−**17**) present in the isolated fractions III−V (Figure 2) were identified to be lutein diesters due to their characteristic UV−vis absorption spectra identical to that of free lutein (Table 1), their disappearance after saponification, and their characteristic mass spectral behavior.

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The first eluting diester (compound **9**) showed a negative ion $[M]^-$ at m/z 1090. The occurrence of the positive *in source*-fragment ions $[M + H - 278$ (linolenic acid)$]^+$ at m/z 813 and $[M + H - 280$ (linoleic acid)$]^+$ at m/z 811 indicated a lutein linolenate-linoleate. Unexpectedly, elucidation of its regioconfiguration was impossible, due to the almost identical intensities of the above-mentioned *in source*-fragments $[M + H - \text{acyl}]^+$. Possibly, this finding may be explained by the coelution of both regioisomers.

Compound **10** exhibited a predominant molecular ion $[M]^-$ at m/z 1092 in APCI(−), whereas APCI(+) produced corresponding *in source*-fragments $[M + H - 280]^+$ at m/z 813 (100%) and $[M + H - 280 - 280]^+$ at m/z 533 (17%). After CID of the ion at m/z 813, a prevailing fragment at m/z 533 confirmed the relationship of both ions. Due to the absence of a second *in source*-fragment $[M + H -$ fatty acid$]^+$ indicating the loss of a fatty acid other than linoleic acid, compound **10** was identified as a homogeneous diester, namely, lutein dilinoleate.

Due to its $[M]^-$ at m/z 1094 and characteristic *in source*-fragments $[M + H - 282$ (oleic acid)$]^+$ at m/z 815, $[M + H - 280]^+$ at m/z 813, and $[M + H - 282 - 280]^+$ at m/z 533 (Table 1), compound **11** was assigned to lutein 3′-*O*-linoleate-3-*O*-oleate.

Compound **12** revealed a molecular ion $[M]^-$ at m/z 1096. The *in source*-fragments $[M + H - 282]^+$ at m/z 815 allowed its identification as lutein dioleate (**12**), particularly because additional losses of fatty acids other than oleic acid were missing.

A negative molecular ion $[M]^-$ at m/z 1068 was observed for both compounds **13** and **14**, indicating related regioisomers. As described above, the relative ion abundances of the two $[M + H - \text{acyl}]^+$ *in source*-fragments were used for the identification of the regioconfiguration. The occurrence of product ions $[M + H - 256$ (palmitic acid)$]$ at m/z 813 (100%) and $[M + H - 280]^+$ at m/z 789 (4%) identified compound **13** as lutein 3′-*O*-palmitate-3-*O*-linoleate, whereas compound **14** producing ions at m/z 789 (100%) and m/z 813 (8%) occurring at reversed intensity was assigned to lutein 3′-*O*-linoleate-3-*O*-palmitate. By analogy, peaks **15** and **16** were identified as lutein 3′-*O*-palmitate-3-*O*-oleate (**15**) and lutein 3′-*O*-oleate-3-*O*-palmitate (**16**). Compound **17** revealed a molecular ion $[M]^-$ at m/z 1044 as well as a characteristic *in source*-fragment $[M + H - 256]^+$ at m/z 789. Because neutral losses of other fatty acids were not observed, compound **17** was identified as lutein dipalmitate.

In addition to these observations, the CID spectra presented in Table 1 confirmed our proposed structures of compounds **9**−**17**. The identity of the carotenoid core molecule lutein was supported by CID spectra of the respective *in source*-fragments, consisting of a characteristic product ion at m/z 411, which corresponds to the lutein backbone after elimination of one cyclic end group (β- or ε-ring).[33] Furthermore, compounds **10**, **11**, and **14**−**17** revealed a product ion at m/z 477 corresponding to a retro-Diels−Alder fragment of the ε-ring of the lutein backbone. Notably, further fragment ions less important for structure elucidation were observed (data not shown).

Lutein Content and Degree of Esterification in *Triticum* spp. Lutein contents of whole grain flours from 15 genotypes of wheat species comprising bread wheat, durum, spelt, einkorn, and emmer were determined after their cultivation in five different environments, that is, different sites and harvest years. Concentrations of free lutein (compound **1**), total lutein monoesters (fractions I and II, Figure 2), total lutein diesters (fractions III−V), and total lutein (free + esterified) were determined using the whole sample set, whereas concentrations of single lutein esters were determined only in a representative subset of samples (see below).

Table 2 shows the lutein contents and their degree of esterification of the investigated 75 genotypes belonging to 5 species of bread wheat, durum, spelt, einkorn, and emmer. The einkorn genotypes clearly showed the highest total lutein content (total median = 5.65 μg/g DM), followed by the 1.7-fold lower content of durum (3.26 μg/g DM). Spelt (1.53 μg/g DM) and emmer (1.52 μg/g DM) contained comparable amounts, being slightly higher than in bread wheat (1.35 μg/g DM). The highest total lutein contents were found for the einkorn breeding line M-04033/03, consistently over all environments investigated (6.95−8.95 μg/g DM), although not being significantly different from the also lutein-rich einkorn genotypes M-04033/01 (6.75−8.83 μg/g DM) and 8.116/04 (6.92−8.33 μg/g DM). The lutein contents determined in *Triticum* spp. are comparable to previously reported values.[3,14] Similarly, einkorn was previously reported to be the most lutein-rich *Triticum* species.[3,38] Due to its high carotenoid content, the production of pasta may be a promising application of einkorn, because the resulting yellow color is highly desirable. Hence, lutein concentrations up to 0.6 mg/100 g could be reached in vegan pasta, being even higher than in egg pasta (0.5 mg/100 g).[39,40]

The degree of lutein esterification varied strongly among and within species. Interestingly, lutein esters were completely absent in all durum and emmer genotypes. Because durum and emmer are both tetraploid wheat species, the inability to form carotenoid esters may be congenital. However, further investigations may be intricate, because, to the best of our knowledge, the genes and enzymes responsible for carotenoid esterification have not yet been reported. Although the absence of lutein esters in emmer has been reported for the first time, sporadic reports about a few durum genotypes lacking lutein esters are available.[15,23]

With regard to bread wheat, lutein esters were absent in seven genotypes, whereas 22.7−38.3% of total lutein was esterified in the remaining eight genotypes (Table 2). In contrast, all spelt and einkorn genotypes consistently contained lutein esters. However, the degree of esterified lutein in spelt (median = 29.7%) was significantly higher than in einkorn (7.6%). Furthermore, the ratio of mono- to diesters was about 2:1 in spelt. In einkorn, this ratio was even higher, ranging from 7:1 to 20:1, with the einkorn breeding line 8.103/04 being the only exception. The latter contained 48.7−72.6% lutein esters, mainly diesters. A correlation between the amount of esterified lutein and the total lutein content could not be revealed (data not shown).

Notably, the simultaneous occurrence of lutein and its esters in significant amounts, as shown for *Triticum* in the present work, is very unusual. In photosynthetically active tissues, lutein commonly occurs only in its free form, whereas it is predominantly esterified in chromoplasts of diverse fruits or petals.[41]

In previous studies, the occurrence of lutein esters in cereals has been hypothesized to be related to storage conditions such as temperature, humidity, and time.[23,42−44] Because all samples investigated in the presented study were stored under the same conditions and originated from five different environments, we

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hypothesize a significant genetic factor to be responsible for the observed differences among genotypes and species.

Genetic Variance and Heritability of Total Lutein Contents. In all five wheat species, a highly significant genetic variance (σ^2_G) was determined for the total lutein content (Table 3). Furthermore, a very high heritability (h^2) exceeding 0.93 was calculated for all wheat species, thus confirming previous studies of tetraploid wheats.[45,46] Accordingly, total lutein contents in einkorn, emmer, spelt, durum, and bread wheat appear to be mainly affected by genetic factors, whereas environmental effects play a minor role. The variability of the total lutein content of individual genotypes is illustrated in Supplemental Tables 2−6, showing comparatively small differences among the different cultivation sites or harvest years. However, over all environments, the same einkorn genotypes revealed the highest total lutein contents (M-04033/03, M-04033/01, and 8.116/04), whereas the bread wheat genotypes Julius and Event consistently showed the lowest lutein contents. The wheat species durum and einkorn both exhibiting the highest average lutein contents also showed the largest genetic variance. Thus, especially in the latter species, a future increase of total lutein content by plant selection efforts seems to be promising.

Table 3. Genetic Variance (σ^2_G), Heritability (h^2), and Least Significant Difference (LSD) of Total Lutein Content across Five Locations, Which Have Been Separately Determined for Each Species

variable	bread wheat	durum wheat	spelt	einkorn	emmer
σ^2_G	0.09*[a]	0.46*	0.06*	0.94*	0.12*
h^2	0.98	0.97	0.93	0.97	0.94
LSD	0.13	0.32	0.19	0.51	0.26

[a]*, significant at the 0.001 probability level.

Quantification of Individual Lutein Esters in Selected Genotypes. Concentrations of individual lutein esters were determined in bread wheat (var. Tabasco), spelt (var. Oberkulmer Rotkorn), and einkorn (var. 8.103/04). As shown in Table 4, all esters so far identified were found in einkorn, which exhibited the highest total ester content of the investigated genotypes (7.7 μg/g DM). The monoesters lutein 3-*O*-oleate (**6**) and lutein 3-*O*-palmitate (**8**), as well as the diesters lutein dioleate (**12**) and lutein 3′-*O*-oleate-3-*O*-palmitate (**16**), could not be detected in bread wheat and spelt. In the latter species, lutein linoleate-linolenate (**9**) and lutein 3′-*O*-palmitate-3-*O*-oleate (**15**) were found only in traces (<LOQ). Lutein dilinoleate was the prevailing ester in all three species, with particularly high concentrations in einkorn (2.637 μg/g DM). This lutein diester seems to be characteristic of cereals, because it has previously been described in bread wheat and tritordeum (×*Tritordeum* Ascherson et Graebener).[19,42] To the best of our knowledge, its occurrence in other plants has not been reported so far.

In agreement with a previous study on lutein regioisomers from tritordeum grains,[19] 3-*O*-regioisomers of lutein monoesters occurred at higher concentrations than their related 3′-*O*-regioisomers, displaying a ratio of about 2:1 in einkorn (Table 4). However, because bread wheat and spelt showed the opposite with lower or even undetectable amounts of 3-*O*-regioisomers, conclusions about preferred regiospecific esterification cannot be drawn. By analogy, this specific einkorn genotype contained 3.8-fold higher diester levels than monoesters, whereas bread wheat and spelt contained almost equal amounts of both forms.

Fatty Acid Composition in Lutein Esters and Total Free Lipids. To reveal putative similarities, the fatty acid pattern of the carotenoid esters was compared to that of total free lipids in wheat after saponification of a limited subset of samples (Table 5). Linoleic acid was the predominant fatty acid

Table 4. Contents of Individual Lutein Esters in Einkorn, Bread Wheat, and Spelt

		content[a] (μg/g DM)		
peak	identity	bread wheat (Tabasco)	spelt (Oberkulmer Rotkorn)	einkorn (8.103/04)
1	(*all-E*)-lutein	0.666 ± 0.018	0.616 ± 0.005	1.551 ± 0.187
2	lutein *Z*-isomer	0.113 ± 0.007	0.057 ± 0.005	0.187 ± 0.013
3	lutein 3′-*O*-linoleate	0.151 ± 0.004	0.132 ± 0.008	0.441 ± 0.010
4	lutein 3-*O*-linoleate	0.103 ± 0.008	0.123 ± 0.017	0.732 ± 0.021
5	lutein 3′-*O*-oleate	0.040 ± 0.002	0.038 ± 0.007	0.062 ± 0.003
6	lutein 3-*O*-oleate	nd[b]	nd	0.122 ± 0.014
7	lutein 3′-*O*-palmitate	0.152 ± 0.009	0.148 ± 0.003	0.086 ± 0.006
8	lutein 3-*O*-palmitate	nd	nd	0.160 ± 0.009
9	lutein linoleate-linolenate	tr[c]	tr	0.365 ± 0.009
10	lutein dilinoleate	0.195 ± 0.002	0.165 ± 0.003	2.637 ± 0.063
11	lutein 3′-*O*-linoleate- 3-*O*-oleate	0.067 ± 0.001	0.068 ± 0.001	1.015 ± 0.025
12	lutein dioleate	nd	nd	0.178 ± 0.014
13	lutein 3′-*O*-palmitate-3-*O*-linoleate	0.174 ± 0.005	0.164 ± 0.005	0.681 ± 0.019
14	lutein 3′-*O*-linoleate-3-*O*-palmitate	0.059 ± 0.004	0.057 ± 0.003	0.772 ± 0.21
15	lutein 3′-*O*-palmitate-3-*O*-oleate	tr	tr	0.162 ± 0.015
16	lutein 3′-*O*-oleate-3-*O*-palmitate	nd	nd	0.112 ± 0.015
17	lutein dipalmitate	0.053 ± 0.003	0.051 ± 0.005	0.202 ± 0.003
	total monoesters	0.446	0.440	1.604
	total diesters	0.550	0.504	6.126
	total lutein esters	0.996	0.945	7.729

[a]Mean ± standard deviation (n = 3). [b]nd, not detectable, ≤LOD. [c]tr, traces, ≥LOD, ≤LOQ.

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Table 5. Fatty Acid Composition in the Total Free Lipids of Different Wheat Species and Genotypes

species (genotype)	fatty acid composition[a] (%) palmitic (C 16:0)	stearic (C 18:0)	oleic (C 18:1)	linoleic (C 18:2)	linolenic (C 18:3)	arachidic (C 20:0)	eicosenoic (C 20:1)
bread wheat (Tabasco)	17.68 ± 0.11	0.92 ± 0.01	14.36 ± 0.02	61.57 ± 0.04	4.67 ± 0.05	0.15 ± 0.01	0.65 ± 0.01
durum (Auradur)	13.98 ± 0.12	1.34 ± 0.02	18.72 ± 0.03	58.94 ± 0.12	5.88 ± 0.13	0.21 ± 0.04	0.93 ± 0.02
spelt (Oberkulmer Rotkorn)	15.33 ± 0.32	1.10 ± 0.02	22.98 ± 0.07	55.99 ± 0.20	3.52 ± 0.19	0.17 ± 0.01	0.90 ± 0.05
einkorn (8.103/04)	13.39 ± 0.08	0.85 ± 0.01	29.22 ± 0.08	50.73 ± 0.04	4.15 ± 0.04	0.19 ± 0.01	1.47 ± 0.03
emmer (Ramses)	15.84 ± 0.14	1.19 ± 0.02	21.96 ± 0.08	55.51 ± 0.19	4.44 ± 0.40	0.26 ± 0.02	0.81 ± 0.07

[a]Mean ± standard deviation (n = 2).

(50.7−61.6%) of the total free lipids in all five genotypes investigated. As previously reported,[47,48] palmitic acid was the second most common fatty acid in bread wheat (17.7%), followed by oleic acid (14.4%), whereas it was vice versa in the other four species investigated (13.4−15.8% palmitic and 18.7−29.2% oleic acid). Linolenic acid accounted for 3.5−5.9% of the total fatty acids. Stearic acid, eicosenoic acid, and arachidic acid occurred only in minor amounts. Major fatty acids of the total free lipids were found to be identical with those being esterified with lutein. Figure 3 illustrates the strong correlation (Pearson

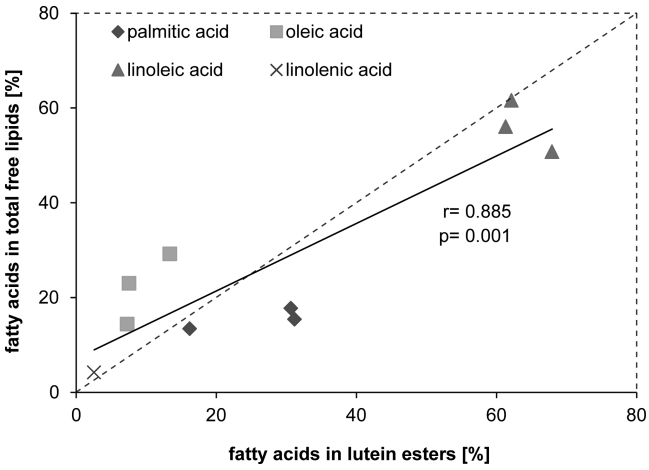

Figure 3. Correlation between the abundance of fatty acids in the total free lipids and of lutein esters.

correlation coefficient r = 0.885, p = 0.001) between the fatty acids present in carotenoid esters and those of total free lipids. As demonstrated by the dashed reference line (slope = 1), the abundance of linoleic and palmitic acid in the lutein esters was significantly higher (61−68 and 16−31%, respectively), that of oleic and linolenic acid being relatively underrepresented (7−13 and 2%, respectively). However, these findings do not yet allow the hypothesis of a highly fatty acid-specific xanthophyll acyltransferase in wheat, as has previously been suggested for tritordeum.[19,23] Briefly, Mellado-Ortega and Hornero-Méndez[19] found palmitic acid to be the most common fatty acid in lutein esters, whereas its relative abundance among the fatty acids of the total free lipids was lower. In addition, oleic acid was found to be present at high concentrations in the total lipids of tritordeum, being absent in its lutein esters. Therefore, they suggested a highly specified enzyme involved in the esterification of lutein.

In our study, the relative amounts of fatty acids being esterified with carotenoids were determined, showing that only up to 0.06% w/w of the total fatty acids in all wheat species was esterified with lutein.

In conclusion, einkorn genotypes showed the highest lutein contents among all genotypes belonging to five *Triticum* species investigated in the present study. The presence of lutein esters was demonstrated to be a characteristic trait of specific genotypes and species, being independent of their total carotenoid content. The absence of lutein esters in tetraploid wheat species (i.e., durum, emmer) was found to be a general trait, which merits further investigation. Genotypes devoid of and being rich in lutein esters may be useful tools for the identification of the yet unknown genes and enzymes responsible for carotenoid esterification. Whether the bioavailabilities of lutein and its esters from wheat differ is yet unknown, although their esterification should impart a highly distinct solubility in dietary lipids and, thus, also different liberation and absorption behaviors from wheat products. In addition, lutein and lutein esters may be deposited in different plastidal substructures, which may further affect their bioavailability.[49,50] Therefore, a nutritional preference of lutein ester-containing sources to those mainly containing free lutein should be based on in vitro and in vivo experiments. Although the lutein contents in vegetables and fruits are by far higher than in wheat (i.e., up to 20-fold higher in spinach or kale than in einkorn),[51] wheat products are generally consumed more frequently and in higher quantities in many countries worldwide. Therefore, wheat and, in particular, the yet underutilized species einkorn and durum should be considered as valuable sources of lutein in the human diet. In addition, manufacture of yellow-tinted vegan wheat products may become feasible without the addition of egg yolk or other colorants by the sole use of einkorn flours.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental Tables 1−7 and Supplemental Figure 1. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b01477.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

amu, atomic mass unit; APCI, atmospheric pressure chemical ionization; CID, collision-induced dissociation; DM, dry matter; FA, fatty acid; FAME, fatty acid methyl ester; FW, fresh weight; LOD, limit of detection; LOQ, limit of quantitation

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