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Research Article

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Esterification enhances carotenoid retention during bread-making

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Abstract

BACKGROUND: Carotenoids are plant-derived antioxidants that contribute to human health and represent key quality traits in wheat-based foods. However, they are highly unstable and prone to degradation during processing. Xanthophyll esterification has been identified as a natural mechanism that enhances carotenoid stability during grain storage. This study investigated carotenoid retention throughout the bread-making process, with particular focus on the potential protective effect of esterification.

RESULTS: Six genotypes with contrasting carotenoid content and esterification capacity, including bread wheat, tritordeum, and *Hordeum chilense*–wheat chromosome substitution lines, were analyzed. Carotenoids were quantified by high-performance liquid chromatography (HPLC) at sequential processing stages: grain, flour, dough, freshly baked bread, and bread stored for 48 h. Lutein, present in both free and esterified forms, was the predominant carotenoid in all the genotypes evaluated, with slight genotype-dependent differences. Esterified carotenoids consistently showed higher retention compared to their free forms. Baking was identified as the most detrimental step for carotenoid degradation in our study, whereas storage after baking resulted in only minor additional changes. Our results indicated that carotenoid esterification may continue during the early stages of bread-making.

CONCLUSIONS: Carotenoid esterification represents a promising strategy to enhance carotenoid preservation during bread-making. This approach could complement both technological optimizations in processing and the genetic selection and improvement of wheat varieties with elevated carotenoid content. These optimizations should focus primarily on baking, as this was the step that had the most detrimental effect on carotenoid retention.
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Keywords: bread wheat; carotenoids; food processing; lutein esters; retention; tritordeum

INTRODUCTION

The Triticeae tribe comprises a group of cereal species that are economically important as staple food sources and for their contribution to human health through their content of essential dietary micronutrients, including carotenoids.[1,2] Carotenoids are naturally occurring pigments that play key roles in human health by acting as potent antioxidants.[3-5] Their intake has been associated with a reduced risk of developing chronic conditions, such as cardiovascular disease, cancer, neurodegenerative disorders, obesity, and age-related macular degeneration.[5,6] Since humans are unable to synthesize carotenoids *de novo*, adequate levels of these nutrients must be maintained through dietary intake. In plant-derived foods, carotenoids are the primary source of provitamin A, contributing to both the nutritional value and the visual appeal of food products.

In cereal grains, particularly wheat, carotenoids are responsible for the yellow pigment content (YPC), which is an important quality trait for wheat-based products like bread, pasta, and pastries.[1,7] Numerous efforts have been made to characterize YPC, and or carotenoid content, in durum wheat (*Triticum turgidum* (L.) ssp. *durum* (Desf.) Husn.),[8-11] einkorn (*Triticum monococcum* L. subsp. *monococcum*),[12] emmer wheat (*Triticum dicoccum* L.),[13] and bread wheat (*Triticum aestivum* L.).[7] In addition, emerging cereals such as tritordeum (× *Tritordeum martinii* A. Pujadas), an amphiploid resulting from the cross between durum wheat and *Hordeum chilense*, have gained considerable commercial success due to its high carotenoid content, which provides a characteristic golden colour to their derived-products (https://www.tritordeum.com/).

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However, wheat grains are rarely consumed in their whole form. They are usually stored for extended periods and subjected to industrial or domestic processing (e.g., milling, pearling, kneading, or baking) to produce food products, which can negatively affect their carotenoid content and, consequently, reduce their nutritional quality and visual appeal.[14,15] The degradation of carotenoids during food processing is influenced by both external factors (e.g., heat, light, oxygen exposure) and internal factors (e.g., enzymatic activity such as lipoxygenase activity).[16] Several studies have demonstrated the impact of grain processing on carotenoid content and composition showing that milling,[16,17] extrusion or puffing,[18] boiling[19] and pasta production[20,21] contribute to carotenoid losses. A better understanding of these degradation mechanisms is essential for developing strategies to preserve carotenoid levels in wheat-derived foods. In this context, carotenoid esterification has emerged as a promising and pivotal process in biofortification programmes. This natural mechanism facilitates the sequestration, accumulation, and storage of carotenoids in plants.[22] Esterification of xanthophylls with fatty acids enhances carotenoid stability against thermo-oxidative degradation[23] and may increase their bioavailability and bioaccessibility during digestion, particularly in the presence of dietary fats.[24]

In Triticeae species, lutein is the most abundant carotenoid in the endosperm of grains and occurs either in free form or esterified with fatty acids (mostly palmitic and linoleic acids) as monoesters and diesters.[22] The biosynthesis of xanthophyll esters is mediated by xanthophyll acyltransferase (XAT) enzymes. In wheat, lutein fatty acid esters are specifically formed after grain maturation, a process that is promoted by low relative humidity and temperatures ranging from 30 to 60 °C.[25-29] Several studies have shown that esterification enhances the stability and accumulation of carotenoids compared to their free forms during the storage of cereal grains[26,30,31] and flours.[32,33] Furthermore, lutein diesters appear to be more resistant to lipoxygenase-mediated degradation than free lutein in noodle sheets.[34]

Understanding how the different stages of bread production including milling, dough preparation, fermentation and baking influence carotenoid levels is essential for optimizing wheat-based foods to enhance their nutritional value. The objectives of this study were to (i) quantify carotenoid losses at each step of bread-making, (ii) identify the stage with the greatest impact on carotenoid degradation, and (iii) evaluate whether carotenoid esterification contributes to enhanced carotenoid retention. To achieve this, we analysed variations in carotenoid content and profile across the sequential stages of the bread-making process using six genotypes with contrasting carotenoid levels and esterification capacities, including bread wheat, tritordeum and a set of *H. chilense*–bread wheat chromosome substitution lines.

MATERIALS AND METHODS

Plant material and field testing

Six genotypes with carotenoid esterification capacity were used: the high-lutein bread wheat line DM5685*B12, developed at the University of Adelaide (Australia)[26]; the high-carotenoid tritordeum line HT621 (AABBH^{ch}H^{ch})[35]; the bread wheat 'Chinese Spring' (CS) cultivar; and three *H. chilense*–bread wheat (CS) substitution lines: CS(7A)/7H^{ch}, CS(7B)/7H^{ch}, and CS(7D)/7H^{ch}, in which chromosome pairs 7A, 7B and 7D were replaced by chromosome pair 7H^{ch}, respectively.[36] The analysis of *H. chilense*–bread wheat (CS) substitution lines revealed the important role of chromosomes 7H^{ch} and 7D on lutein esterification and showed that the simultaneous occurrence of both chromosomes resulted in high levels of lutein esters.[36] All genotypes were cultivated outdoors at Finca Alameda del Obispo (Córdoba, Spain), under a protective structure equipped with an anti-bird netting structure and with an anti-weed mesh. A randomized block design with two replications was implemented, using two-row plots of 3 m in length and spaced 0.2 m between rows. Samples were harvested at physiological maturity, and the grains were stored at 4 °C until sample preparation.

Preparation of samples: grain, flour, dough, bread and bread stored for 48 h after baking

The carotenoid content and profile were analysed at different stages of wheat processing for bread-making: whole grain, flour, dough, freshly baked bread and bread stored for 48 h after baking.

A representative 5 g portion of the harvested grains was stored at −30 °C for carotenoid extraction of whole grain. The remaining grain was milled using a hammer mill (Laboratory Mill 3100, Falling number, Huddinge, Sweden), producing wholemeal flour. A 5 g subsample of this flour was stored at −30 °C for subsequent carotenoid extraction, while the rest was used for bread dough preparation.

To prepare the dough, 300 g of wholemeal flour, 4.8 g of salt, 6.0 g of fresh commercial baker's yeast (Lesaffre Iberica S.A., Valladolid, Spain) and 180 mL of warm water (approximately 30 °C) were mixed in a food processor (Thermomix tm31) set to kneading mode for 1 min. The dough preparation process included 5 min of kneading, followed by dividing the dough into three bread buns (approximately 150 g each). The buns were left to rise for 1 h in an incubator (Memmert ICP-500; Germany) set at 37 °C to promote fermentation, after which each dough was kneaded again for an additional 5 min. After the rising process, one of the three buns was immediately frozen for carotenoid analysis, while the remaining two were baked at 200 °C for 30 min in a forced-air oven (Memmert UFE-500) equipped with a water tray to maintain humidity during baking. After baking, the two buns were allowed to cool. One was subsequently frozen for carotenoid analysis, while the other was covered with a cloth and stored at room temperature (approximately 25 °C) for 48 h. This bun was then also frozen until analysis. All frozen samples (dough and bread) were lyophilized in the dark for 72 h using a VirTis Benchtop 2 K lyophilizer (SP Scientific, Stone Ridge, NY, USA). The resulting dry samples were ground in a food processor and stored at −30 °C until carotenoid extraction and analysis.

Extraction of carotenoids and HPLC analysis

Carotenoids were extracted and analysed in accordance with the protocol described by Rodríguez-Suárez *et al.*[37] A one-step grinding-extraction procedure was used for grain samples, while the standard protocol was applied to all other sample types. In all cases, approximately 1.25 g of sample, weighted to the nearest 0.0001 g, was used for the extraction. All operations were performed under dimmed light to prevent carotenoid isomerization and photodegradation. Each sample was extracted and analysed in duplicate.

Carotenoid analysis was conducted using high-performance liquid chromatography (HPLC), following the methodologies described in the literature.[37,38] The HPLC system comprised a Waters e2695 Alliance chromatograph, which was equipped with a Waters 2998 photodiode array detector, and was operated using Empower2 software (Waters Cromatografía, S.A., Barcelona,

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Spain). Chromatographic separation was carried out on a reversed-phase C18 analytical column (Mediterranea SEA18, 3 μm, 20 × 0.46 cm; Teknokroma, Barcelona, Spain). A binary gradient elution was employed, using acetone (solvent A) and deionized water (solvent B). The gradient programme started at 75% A and 25% B, increased linearly to 95% A over 10 min, held for 7 min, then ramped to 100% A over 3 min and maintained for 13 min. The system was returned to its initial conditions within 5 min. The injection volume was 20 μL, and the flow rate was set to 1 mL/min. Carotenoids were detected at 450 nm, and ultraviolet (UV)-visible spectra were recorded online within a wavelength range of 350–650 nm. Quantification was based on calibration curves constructed by plotting peak areas against pigment concentrations of authentic carotenoid standards (lutein, zeaxanthin and *β*-carotene) in concentrations ranging from 0.5 to 45.0 μg/mL. Lutein esters were quantified as free lutein equivalents, and the concentration of (*Z*)-isomers of lutein was estimated using the calibration curve for (all-*E*)-lutein. All analyses were performed in duplicate and performed on the same day that the extracts were prepared. Results were expressed in micrograms per gram of dry weight (μg/g dw).

Data analysis

Differences in carotenoid contents during bread-making were assessed using least significant difference (LSD) test after analysis of variance (ANOVA) using Statistix version 9.0 (Analytical Software, Tallahassee, FL, USA). A repeated measured analysis was applied to compare the means of retention of free and esterified carotenoids during bread-making separately.

In accordance with the recommendations of Wasserstein *et al.*,[39] *P*-values were reported as continuous quantities.

RESULTS AND DISCUSSION

Carotenoid esterification profile and its relationship with the genetic constitution of the genotypes

The carotenoid content and profile of the grain samples were determined at harvest (Table 1). Among the genotypes, the tritordeum line HT621 exhibited the highest total carotenoid (TC) content (8.83 μg/g dw), followed by the high lutein bread wheat DM5685*B12 (4.416 μg/g dw). The elevated TC content in HT621 is attributed to carotenogenic genes from the *H. chilense* genome, particularly the *Psy_H^{ch}1* (*Phytoene synthase 1*) gene.[40,41]

Similarly, the high-lutein bread wheat DM5685*B12 carries a gene likely involved in enhanced lutein biosynthesis, probably the *Psy-E1*, associated with a *Thynopirum distichum* translocation on chromosome 7DL,[42] where the *Psy1* gene is located in the Triticeae tribe.[43] Likewise, the chromosome substitution lines of CS, in which each homoeologous chromosome 7A, 7B or 7D was individually replaced by its orthologous chromosomes 7H^{ch} chromosome from *H. chilense*, also showed higher TC content compared to the unsubstituted CS line due to the presence of *Psy1_H^{ch}1* (Table 1). Specifically, while CS showed 1.095 μg/g dw TC, the substitution lines exhibited 4.348 μg/g dw (CS(7A)/7H^{ch}), 3.041 μg/g dw (CS(7B)/7H^{ch}) and 3.913 μg/g dw (CS(7D)/7H^{ch}).

The carotenoid profile observed in the mature grains was consistent with previous studies.[7,44-46] Lutein, including its (all-*E*) and (*Z*) isomers and esterified forms, was the predominant carotenoid, accounting for 84–96% of the TC content, followed by zeaxanthin and *β*-carotene in lower amounts. Carotenoid esterification is a common phenomenon occurring in bread wheat grains,[36,46-48] although genotypes lacking carotenoid esters have also been reported.[46,47,49] In bread wheat and tritordeum, carotenoid esterification is controlled by *XAT-D1* and *XAT-H^{ch}1* genes, respectively.[28,29] All the genotypes evaluated in this study carry one or both of these genes. Specifically, the chromosome substitution lines CS(7A)/7H^{ch} and CS(7B)/7H^{ch} carry both *XAT-D1* and *XAT-H^{ch}1* since they harbour chromosomes 7D and 7H^{ch}, while HT621 and CS(7D)/7H^{ch} carry only *XAT-H^{ch}1*, and CS and DM5685*B12 carry only *XAT-D1*. All the genotypes produced lutein esters with linoleic and palmitic acids, including monoesters (lutein monolinoleate and lutein monopalmitate) and diesters (lutein dipalmitate, lutein dilinoleate and lutein linoleate-palmitate) (Table 1). Esters with palmitic acid predominated in genotypes carrying only *XAT-H^{ch}1* (HT621 and CS(7D)/7H^{ch}), whereas the other genotypes showed balanced levels of lutein esters with both fatty acids (Table 1). In addition to this, the genotypes with both genes resulted in higher percentage of carotenoid esterification (34.1% and 46.1% in CS(7A)/7H^{ch} and CS(7B)/7H^{ch}, respectively). The balanced esterification pattern and the higher percentage of carotenoid esterification when both *XAT-H^{ch}1* and *XAT-D1* are present, support the hypothesis that *XAT-D1* and *XAT-H^{ch}1* encode enzymes with complementary substrate specificities.[36]

Carotenoid degradation during bread-making

The aims of the present study were to investigate the extend of TC degradation during bread-making, to identify the most detrimental step for carotenoid retention, and to evaluate whether carotenoid esterification enhances carotenoid retention during the bread-making process. To address these objectives, carotenoid content and profile were analysed at key stages of the bread-making process, including whole grain flour, dough, freshly baked bread and bread stored for 48 h after baking (Table 1), and compared to the values in intact grain (Fig. 1). Overall, TC content decreased at all stages for all genotypes, with a few exceptions (Fig. 1). For instance, although the TC content in the flour of HT621, CS(7B)/7H^{ch} and CS(7D)/7H^{ch} was lower than in grains (Fig. 1), these differences were minor (Table 1).

The choice of milling equipment used for flour preparation significantly affects nutrient retention due to temperature differences during grinding. Stone, plate, hammer and roller mills generated temperatures of 90, 85, 55 and 35 °C.[50] In this study, a hammer mill was used, that is intermediate in terms of temperature reached (55 °C). Therefore, TC losses during milling are likely due to cellular disruption and oxygen exposure rather than temperature alone. Stone and plate mills, which reach higher temperatures, may cause greater TC degradation. In fact, damage of endosperm storage proteins, essential for bread quality, has been reported for temperatures above 85 °C generated during milling, as revealed by sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE).[50]

In this study, baking was identified as the most critical step for carotenoid degradation, leading to the highest carotenoid losses in all accessions analysed (Fig. 1). Similarly, findings have been reported for bread crust and water biscuits made from einkorn (*Triticum monococcum*)[20] and for biofortified wheat products enriched with *β*-carotene[51] or with pumpkin (*Cucurbita maxima* Dutch).[52] However, other studies have found dough preparation to be the most detrimental step for carotenoid stability and retention,[49] likely due to lipoxygenase activity.[16,53] Indeed, lipoxygenases are commonly used in the baking industry as bleaching agents to produce whiter bread and flour.[54]

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Table 1. Carotenoid composition (micrograms per gram of dry weight, μg/g dw) in grain, flour, dough, freshly bread and bread stored for 48 h

Genotype	Stage	Zeax	Lut	cisLut	βCar	LutMLin	LutMPal	LutDLin	LutDLinPal	LutDPal	TC
HT621	Grain	0.202 ± 0.006c	6.429 ± 0.107a	0.640 ± 0.028d	0.080 ± 0.004d	0.395 ± 0.006de	0.917 ± 0.017b	0.015 ± 0.001hijk	0.06 ± 0.001h	0.091 ± 0.002d	8.830 ± 0.133a
	Flour	0.160 ± 0.008e	6.370 ± 0.107a	0.620 ± 0.002d	0.069 ± 0.005e	0.415 ± 0.013cd	0.967 ± 0.005a	0.006 ± 0.001Lm	0.060 ± 0.002h	0.096 ± 0.002d	8.764 ± 0.124a
	Dough	0.177 ± 0.007de	5.483 ± 0.204b	0.681 ± 0.029c	0.074 ± 0.006de	0.437 ± 0.003bc	0.979 ± 0.024a	0.008 ± 0.001kLm	0.062 ± 0.001h	0.099 ± 0.003d	7.999 ± 0.156b
	Bread	0.076 ± 0.002j	2.091 ± 0.020fg	0.836 ± 0.009a	0.048 ± 0.001hi	0.218 ± 0.003i	0.495 ± 0.006c	0.007 ± 0.001lm	0.030 ± 0.002ijk	0.045 ± 0.001hi	3.846 ± 0.033de
	Bread 48 h	0.097 ± 0.016hi	2.086 ± 0.118g	0.796 ± 0.047b	0.044 ± 0.004i	0.206 ± 0.006ij	0.457 ± 0.021de	0.002 ± 0.001m	0.028 ± 0.001ijk	0.042 ± 0.001i	3.760 ± 0.208de
DM5685*B12	Grain	0.232 ± 0.003b	2.820 ± 0.078cd	0.230 ± 0.003gh	0.118 ± 0.003b	0.389 ± 0.021e	0.407 ± 0.022fg	0.054 ± 0.006fg	0.111 ± 0.014fg	0.055 ± 0.007fg	4.416 ± 0.145c
	Flour	0.138 ± 0.005f	2.412 ± 0.038e	0.192 ± 0.007i	0.114 ± 0.001b	0.355 ± 0.0017f	0.366 ± 0.014hi	0.047 ± 0.004g	0.100 ± 0.008fg	0.047 ± 0.003ghi	3.771 ± 0.071de
	Dough	0.115 ± 0.004gh	1.926 ± 0.054gh	0.232 ± 0.004g	0.114 ± 0.002b	0.389 ± 0.014e	0.390 ± 0.009gh	0.052 ± 0.005fg	0.113 ± 0.010ef	0.053 ± 0.003fgh	3.385 ± 0.103f
	Bread	0.062 ± 0.004jkl	0.585 ± 0.034kl	0.216 ± 0.011ghi	0.053 ± 0.002gh	0.144 ± 0.009 k	0.148 ± 0.008kl	0.019 ± 0.002h	0.041 ± 0.004i	0.02 ± 0.001j	1.289 ± 0.076kl
	Bread 48 h	0.051 ± 0.005l	0.604 ± 0.011kl	0.202 ± 0.004ghi	0.059 ± 0.001g	0.139 ± 0.006kl	0.135 ± 0.006l	0.016 ± 0.002hij	0.034 ± 0.003i	0.016 ± 0.002jk	1.257 ± 0.020klm
CS	Grain	0.159 ± 0.002e	0.605 ± 0.021kl	0.080 ± 0.003klm	0.014 ± 0.0003Lm	0.081 ± 0.001o	0.092 ± 0.002m	0.016 ± 0.0003hi	0.032 ± 0.001ij	0.016 ± 0.001jk	1.095 ± 0.021lmno
	Flour	0.103 ± 0.004h	0.565 ± 0.010kl	0.069 ± 0.002m	0.013 ± 0.0003lm	0.081 ± 0.001o	0.092 ± 0.002m	0.022 ± 0.0004h	0.031 ± 0.001ik	0.015 ± 0.001jk	0.991 ± 0.019no
	Dough	0.099 ± 0.001h	0.437 ± 0.005lm	0.076 ± 0.004lm	0.016 ± 0.0003l	0.084 ± 0.002no	0.096 ± 0.003m	0.022 ± 0.0004h	0.034 ± 0.001i	0.016 ± 0.001jk	0.879 ± 0.008o
	Bread	0.056 ± 0.002kl	0.193 ± 0.006n	0.084 ± 0.002klm	0.009 ± 0.001m	0.040 ± 0.002p	0.040 ± 0.0005n	0.009 ± 0.001jklm	0.016 ± 0.001kl	0.008 ± 0.001kl	0.454 ± 0.015p
	Bread 48 h	0.055 ± 0.005kl	0.178 ± 0.005n	0.084 ± 0.001klm	0.010 ± 0.0005lm	0.041 ± 0.0005p	0.044 ± 0.0004n	0.010 ± 0.0004ijkl	0.015 ± 0.0004jkl	0.008 ± 0.001kl	0.445 ± 0.010p
CS(7A)/7H^{ch}	Grain	0.236 ± 0.006b	2.278 ± 0.048ef	0.221 ± 0.001ghi	0.132 ± 0.003a	0.485 ± 0.007a	0.488 ± 0.007c	0.135 ± 0.004cd	0.257 ± 0.008c	0.117 ± 0.004c	4.348 ± 0.039c
	Flour	0.170 ± 0.020de	2.042 ± 0.031g	0.196 ± 0.002hi	0.131 ± 0.002a	0.474 ± 0.013a	0.476 ± 0.013cd	0.126 ± 0.005e	0.235 ± 0.009d	0.111 ± 0.004c	3.961 ± 0.035d
	Dough	0.130 ± 0.014fg	1.336 ± 0.050i	0.227 ± 0.01ghi	0.120 ± 0.005b	0.445 ± 0.005b	0.435 ± 0.005ef	0.131 ± 0.003de	0.254 ± 0.007c	0.114 ± 0.004c	3.192 ± 0.044fg
	Bread	0.079 ± 0.002ij	0.600 ± 0.002kl	0.231 ± 0.001gh	0.067 ± 0.001ef	0.201 ± 0.003ij	0.199 ± 0.004j	0.052 ± 0.002g	0.103 ± 0.003fg	0.048 ± 0.002ghi	1.58 ± 0.015j
	Bread 48 h	0.071 ± 0.003jk	0.572 ± 0.011kl	0.215 ± 0.007ghi	0.061 ± 0.001fg	0.192 ± 0.001j	0.184 ± 0.001j	0.050 ± 0.001g	0.097 ± 0.002g	0.047 ± 0.001hi	1.488 ± 0.01jk8
CS(7B)/7H^{ch}	Grain	0.180 ± 0.008d	1.270 ± 0.076ij	0.127 ± 0.007j	0.060 ± 0.002fg	0.334 ± 0.016fg	0.388 ± 0.021gh	0.152 ± 0.008a	0.343 ± 0.019a	0.185 ± 0.010a	3.041 ± 0.133g
	Flour	0.131 ± 0.005fg	1.124 ± 0.076j	0.109 ± 0.005jkl	0.059 ± 0.004g	0.322 ± 0.012gh	0.368 ± 0.014hi	0.141 ± 0.001bc	0.317 ± 0.003b	0.170 ± 0.002b	2.741 ± 0.112h
	Dough	0.107 ± 0.009h	0.745 ± 0.067k	0.140 ± 0.012j	0.057 ± 0.004g	0.307 ± 0.018h	0.346 ± 0.017i	0.143 ± 0.004b	0.325 ± 0.002b	0.177 ± 0.003ab	2.348 ± 0.132i
	Bread	0.052 ± 0.001l	0.308 ± 0.014mn	0.129 ± 0.005j	0.030 ± 0.001jk	0.131 ± 0.001kl	0.147 ± 0.002kl	0.060 ± 0.002f	0.128 ± 0.003e	0.071 ± 0.002e	1.055 ± 0.019lmno
	Bread 48 h	0.046 ± 0.002l	0.283 ± 0.023mn	0.115 ± 0.010jk	0.028 ± 0.001k	0.122 ± 0.005klm	0.133 ± 0.006l	0.051 ± 0.001g	0.110 ± 0.004fg	0.061 ± 0.002f	0.949 ± 0.054o
CS(7D)/7H^{ch}	Grain	0.259 ± 0.004a	2.854 ± 0.063c	0.414 ± 0.007e	0.090 ± 0.003c	0.105 ± 0.009mn	0.170 ± 0.005jk	0.004 ± 0.001lm	0.010 ± 0.003l	0.009 ± 0.001kl	3.913 ± 0.087d
	Flour	0.187 ± 0.016cd	2.657 ± 0.031d	0.392 ± 0.007e	0.092 ± 0.002c	0.118 ± 0.0002lm	0.169 ± 0.002jk	0.004 ± 0.001lm	0.011 ± 0.0004l	0.010 ± 0.0004kl	3.640 ± 0.033e
	Dough	0.131 ± 0.003fg	1.781 ± 0.108h	0.380 ± 0.015e	0.089 ± 0.003c	0.121 ± 0.006lm	0.171 ± 0.010jk	0.006 ± 0.001lm	0.010 ± 0.0001l	0.008 ± 0.0002kl	2.697 ± 0.144h
	Bread	0.078 ± 0.004j	0.692 ± 0.038k	0.277 ± 0.018f	0.044 ± 0.003i	0.051 ± 0.003p	0.068 ± 0.004mn	0.003 ± 0.0002lm	0.004 ± 0.0003l	0.004 ± 0.001l	1.222 ± 0.071lmn
	Bread 48 h	0.055 ± 0.004kl	0.577 ± 0.028kl	0.226 ± 0.009ghi	0.037 ± 0.002j	0.044 ± 0.002p	0.057 ± 0.003n	0.003 ± 0.0002lm	0.004 ± 0.0005l	0.005 ± 0.001l	1.009 ± 0.046mno

Note: Values represent mean ± standard error. For each carotenoid compound, values with the same letter are not significantly different at $P < 0.05$, determined at least significant difference (LSD) test. Abbreviations: Zeax, (all-*E*)-zeaxanthin; Lut, lutein ((all-*E*)-lutein + (*Z*)-lutein isomers); cisLut, (*Z*)-lutein isomers; βCar, (all-*E*)-β-carotene; LutMLin, lutein monolinoleate; LutMPal, lutein monopalmitate; LutDLin, lutein dilinoleate; LutDLinPal, lutein linoleate–palmitate; LutDPal, lutein dipalmitate; TC, total carotenoids.

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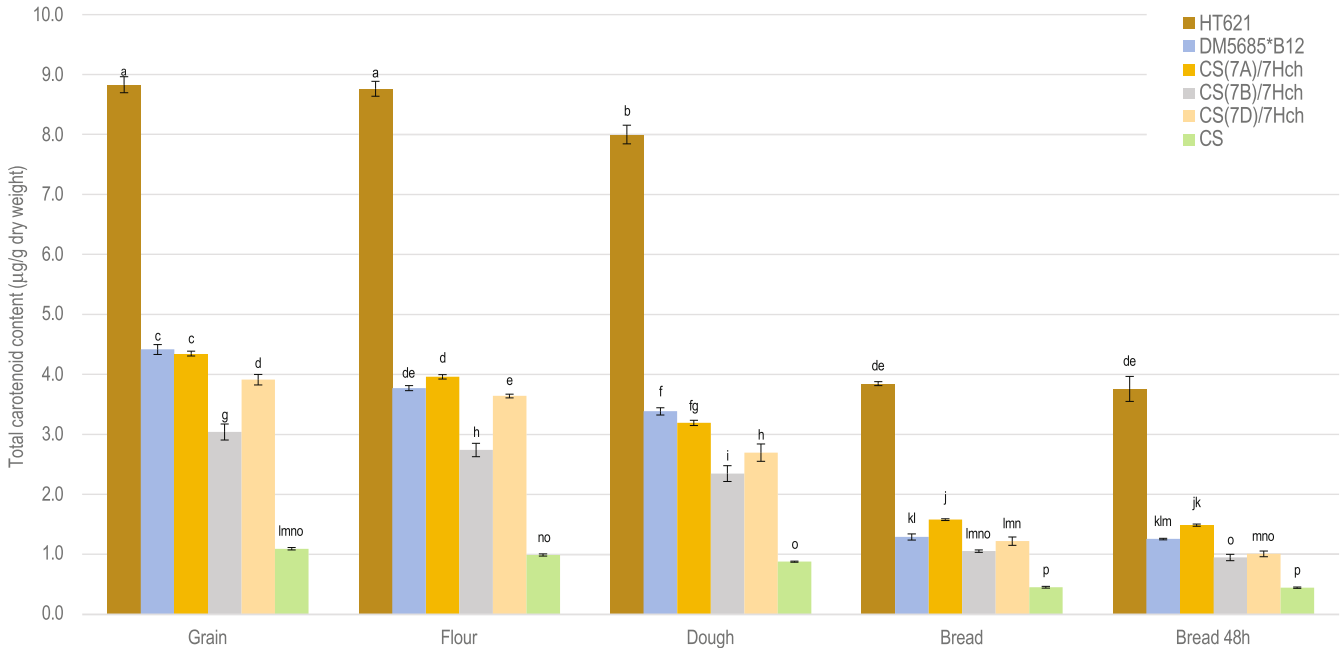

Figure 1. Evolution of total carotenoid content during different stages of bread processing (grain, whole-meal flour, dough, freshly baked bread and bread stored for 48 h after baking). In parentheses: carotenoid content retention (%) relative to intact grains. Total carotenoid content expressed in micrograms per gram (µg/g) dry weight (mean ± standard error). Values with the same letter are not significantly different at $p < 0.05$ after least significant difference (LSD) test.

At the final stage (bread stored for 48 h), the carotenoid retention relative to intact grains ranged from 25.8% in CS(7D)/7H^{ch} to 42.6% in HT621 (Table 1). Similar retention values have been reported for the yellow endosperm wheats 'Citrus' and 'Bona Vita', although these values were relative to flour instead of intact grains.[49]

Both thermal and non-thermal processes contribute to carotenoid degradation during food processing and storage.[55] Oxidative enzymes such as lipoxygenases and polyphenol oxidases play a significant role in this degradation.[16,53] Given that the dough preparation method used here was similar to that used in previous studies, the observed differences in carotenoid losses suggest variability in these enzymatic systems among genotypes.

Once baked, TC content experienced only minimal losses during storage (Fig. 1). These findings are consistent with previous studies that reported limited carotenoid degradation in baked wheat products over short-term storage periods.[49,51,56] The stability of carotenoids in baked bread suggests that the baking process itself, although responsible for the most significant losses, may also contribute to the stabilization of these compounds by inactivating oxidative enzymes such as lipoxygenases and polyphenol oxidases.

Collectively, these results indicate that once the bread is baked, it does not undergo substantial carotenoid degradation during subsequent storage. This reinforces the notion that post-baking conditions, such as reduced enzymatic activity, limited oxygen exposure, and lower moisture content, play a protective role in preserving carotenoids. Therefore, optimizing carotenoid retention should focus primarily on the earlier stages of bread-making, where the majority of losses occur.

Does esterification protect carotenoids during bread-making?

An additional objective of the present study was to determine whether carotenoid esterification provides a protective effect during bread-making. To investigate this, carotenoid retention was assessed separately for free and esterified carotenoids at each stage of the bread-making process (Fig. 2). At each stage considered, esterified carotenoids consistently exhibited higher retention rates than free carotenoids, relative to the initial carotenoid content in grain suggesting a potential stabilizing role. Notably, our results indicate that carotenoid esterification may continue during the early stages of bread-making. This is particularly evident during dough preparation, where the content of esterified carotenoids was higher than in intact grains, resulting in a retention value above 100% (Fig. 2). As *de novo* carotenoids cannot be synthetized during bread-making, these findings may indicate that XAT enzymes remain active at least until dough preparation stage. In wheat grain, carotenoid esterification only occurs post-maturation, when the breakdown of the cellular compartment enables interaction between XAT and carotenoids.[28] This process has also been observed during grain storage in both wheat and tritordeum, where *de novo* esterification continues to take place.[26,30] These findings support the hypothesis that enzymatic activity involved in esterification may persist beyond harvest and into the initial phases of food processing. Together, these observations support the persistence of esterification activity beyond harvest and into the initial phases of food processing. Importantly, this behaviour was observed only in genotypes exhibiting carotenoid esterification, indicating that its occurrence depends on the presence of functional esterification capacity, although its magnitude may vary among genotypes.

Although esterified carotenoids consistently exhibited higher retention values than free carotenoids throughout the bread-making process, they appeared to undergo greater degradation than free carotenoids as a result of baking (Fig. 2). This may indicate hydrolysis (de-esterification) during baking or that esterified carotenoids are more susceptible to thermal degradation. In addition to this, since carotenoid esters continue to be produced

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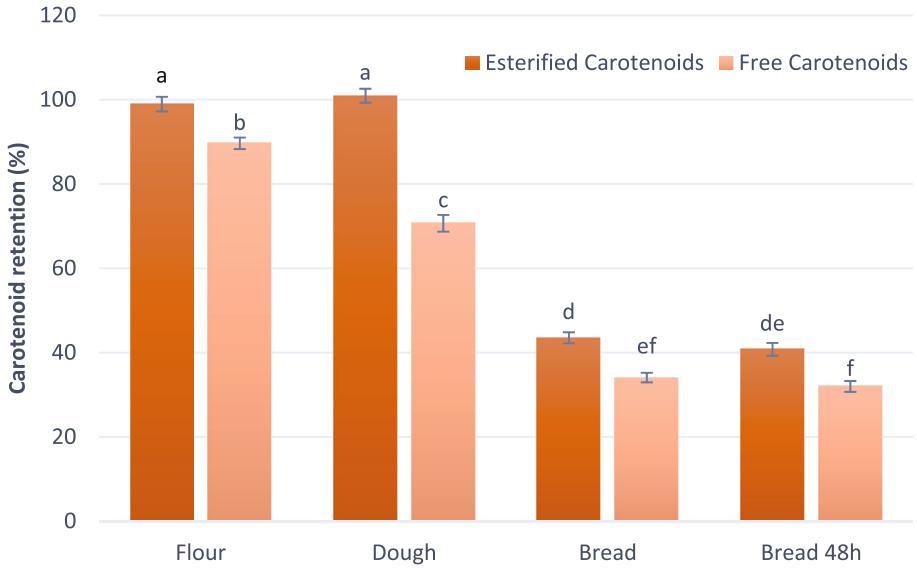

Figure 2. Retention (%) of free and esterified carotenoids in flour, dough, freshly baked bread and bread stored for 48 h after baking compared to the initial grain stage considering all genotypes. Values with the same letter are not significantly different at $p < 0.05$ after least significant difference (LSD) test.

during the early stages – particularly up to dough preparation – it is not possible to definitively conclude whether their higher retention is due to greater chemical stability or to ongoing esterification. Similarly, the disappearance of free lutein during the early stages of bread-making cannot be attributed solely to its degradation, but may also be partly due to its transformation into the esterified form. This distinction is critical, as it affects the interpretation of esterified carotenoids as protective compounds. Moreover, the genotypes analysed in this study are expected to differ in lipoxygenase activity, which introduces an additional source of variation in carotenoid degradation. Lipoxygenases are known to catalyse oxidative reactions that degrade carotenoids, and their activity may vary significantly among wheat lines. Therefore, although our results demonstrate that esterified carotenoids exhibit higher retention than free (non-esterified) carotenoids during bread-making, further research is needed to fully elucidate the interaction between carotenoid esterification and lipoxygenase-mediated degradation. To address this, we are currently developing plant materials differing in carotenoid content, esterification capacity and lipoxygenase activity. These resources will enable controlled experiments to isolate the effects of each factor and better understand their roles in carotenoid stability during food processing.

Improving carotenoid retention during bread-making will likely require a combination of technological innovations in processing methods and genetic improvements in wheat germplasm. Wheat lines with high TC content and low lipoxygenase activity are particularly promising for the development of wheat-based products with high carotenoid content and enhanced nutritional value. Carotenoid esterification represents an additional strategy to improve carotenoid retention during bread-making; however, it remains unclear whether its protective effect arises from reduced degradation or from continued *de novo* ester synthesis during the initial stages of bread-making. Clarifying this mechanism will be essential for optimizing both breeding and processing approaches aimed at preserving carotenoids in cereal-based foods.

CONCLUSIONS

Carotenoid losses were observed throughout all stages of the bread-making process, with baking identified as the most critical step contributing to carotenoid degradation in our study. Notably, esterified carotenoids exhibited greater stability and retention compared to their free counterparts. These findings suggest that carotenoid esterification represents a promising strategy to enhance carotenoid preservation during bread-making. This approach could complement both technological optimizations in processing and the genetic selection and improvement of wheat varieties with elevated carotenoid content.

AUTHOR CONTRIBUTIONS

María Dolores Requena-Ramírez: investigation, validation, visualization, formal analysis, writing – review and editing. Cristina Rodríguez-Suárez: investigation, validation, writing – review and editing. Dámaso Hornero-Méndez: conceptualization, methodology, writing –review and editing, funding acquisition. Sergio G. Atienza: conceptualization, methodology, writing – review and editing, funding acquisition.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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