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Wheat Lipoxygenase Activity Induces Greater Loss of Carotenoids than Vitamin E during Breadmaking

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The current study was undertaken to provide solutions to optimize the unsaponifiable antioxidants content of bread. We report a complete description of changes in wheat carotenoids and vitamin E content from grain to bread and highlight the most important processing steps affecting their level in wheat bread. Major carotenoids losses occurred during kneading. A close correlation ($r^2 = 0.97$; $P = 0.05$) was found between carotenoid pigment losses and lipoxygenase (LOX) activity, both parameters depending on wheat genotype. The use of wheat species exhibiting high carotenoid contents and low LOX activity was shown to preserve significant carotenoid level in the bread. No relation was found between vitamin E losses during doughmaking and LOX activity. In addition, moderate kneading resulted in higher vitamin E retention in comparison with carotenoids (12% and 66% losses, respectively). It is concluded that carotenoids are more susceptible to oxidation by endogenous lipoxygenase than vitamin E during breadmaking. This study showed that bread nutritional quality, in terms of antioxidant content, could be improved by selecting suitable cereal genotypes, if this potential is preserved by milling and baking processes.

KEYWORDS: Wheat; einkorn; milling fractions; breadmaking; carotenoids; lipoxygenase; vitamin E

INTRODUCTION

Increasing evidences indicate that dietary intakes of antioxidants may protect against long-term health risks associated with oxidative stress (*1*). Several studies have emphasized the antioxidant properties of wheat (*2*, *3*), a major crop and component of the human diet across the world. Among the antioxidant phytochemicals identified in wheat, polar ones, such as phenolic acids, have been well characterized and appeared to be correlated with the antioxidant capacity of the water-soluble extract of wheat grain (*4*). Carotenoids and tocols are another group of well-recognized apolar antioxidants. Owing to their lipid-soluble properties, these compounds inhibit lipid peroxidation processes of polyunsaturated fatty acids and other compounds in cell membranes and consequently are considered to play a key role in delaying the pathogenesis of a variety of degenerative diseases (*5*). Lutein is the main carotenoid of yellow-colored kernels such as wheat. Known mostly for its importance for eye health, lutein consumption and serum levels of lutein have also been shown to reduce the risk of developing cardiovascular disease (*6*, *7*). Moreover, carotenoids pigments contribute to grain color and are screened in durum wheat breeding programs as indicators of the quality of yellow-colored pasta products (*8*). Cereal grains are the second most important dietary source of vitamin E, after vegetal oils. The term vitamin E describes a family of eight isomers. Wheat grains mainly contain alpha (α)- and beta (*β*)-tocopherol and α- and *β*-tocotrienol. Tocol derivatives are responsible for the vitamin E activity of plant tissues, but α-tocopherol is the only form of vitamin E that is actively maintained in the human body (*9*). This form accounts for most of the lipid-soluble chain-breaking antioxidant activity in mammalian tissues and plasma (*10*). The antioxidant effect of α-tocopherol has been associated with lower risk for heart disease (*11*), diabetes, and cancer (*12*). It has been suggested that the protective effect of vitamin E may occur primarily from consumption of vitamin E obtained from foods, with no additional benefits from supplementation (*13*).

Most of the wheat produced in the world is processed with roller flour mills into white flour or semolina for use in the production of bread, biscuits, or pasta. A recent American survey found bread to be the fourth highest contributor to vitamin E intake for both men and women (*14*). Clearly, the frequency of consumption of bread, despite its relatively limited vitamin E content, positions it as a major contributor. To date, some studies

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have been performed to determine the impact of the breadmaking process on the lipid-soluble antioxidant content of wheat and to understand the mechanisms of loss of these compounds (*15*−*18*). However, little effort has been made to define strategies to preserve these antioxidants in bread.

As a result of their antioxidant activity, carotenoids and tocols are themselves subject to destruction by oxygen. This process is accelerated by light, heat, and exposure to hydroperoxides formed during lipid oxidation, all factors occurring during breadmaking The oxidation of polyunsaturated fatty acids (mostly linoleic and linolenic) by lipoxygenases present in wheat gives rise to hydroperoxides which react with flour pigments. LOX−linoleate system are activated during doughmaking resulting in carotenoid bleaching (*15*, *17*, *18*). Previous studies have shown that breadmaking also affects vitamin E content of wheat (*16*), but it remains to check the hypothesis of an enzyme-catalyzed oxidation.

The breadmaking process of French bread made in the traditional way was examined in the present study, with emphasis on carotenoid and vitamin E recovery. The susceptibility of these nonpolar antioxidants during the breadmaking process was compared. The role of endogenous oxidases was investigated by comparing different wheat genotypes. Bread quality could be improved by breeding wheat species for a high level of antioxidants, if this potential is preserved after the milling and baking processes.

MATERIALS AND METHODS

Plant Material. Seeds of einkorn (*Triticum monococcum*), durum wheat (*Triticum durum*), bread wheat (*Triticum aestivum*), and their corresponding white flour (type 65) were purchased as mixes of varieties from the organic market (Markal, ZA Les Plaines, 26320 St Marcel Lès Valence). Grains were ground into whole-meal flour with an electric millstone (Rondella, Les moulins à céréales du Tyrol de l'Est, Chalon sur Saône, France).

Seeds of bread wheat (cv. Apache) were provided by ULICE (ZAC des portes de Riom 63200 Riom) to study carotenoids distribution within the milling fractions. Grains were roller-milled (by Mezonnier, Nesher, France) into flour at an extraction rate of ≈72%, and the other flour mill streams (coarse and thin brans, brown and white midds) were recovered separately.

Breadmaking. Five different batches of bread were prepared simultaneously using freshly milled flours. The recipe was based on wheat flour (whole-wheat flour or white flour "type 65"), water (60% of the flour weight), baker's yeast (fresh compressed yeast purchased at the local bakery), and salt, according to the "French tradition" designation. Ingredients were mixed with a mechanical oblique axis kneader (VMI Mahot labo 25, Montaigu, France) at room temperature (21 °C) for 8 min at low speed and then for 8 min at high speed. The dough was left to proof at 30 °C at a relative humidity of 80% for 120 min. The dough (3 kg) was divided manually into 350 g pieces. The loaves were allowed to rest for about 5 min before being shaped. The formed dough was then fermented at 28 °C at a relative humidity of 80% for 60 min and baked at 250 °C for 25 min. Triplicate samples of dough were taken immediately after mixing the ingredients with water (starting point), after kneading, after each proofing, and after the baking stage. They were immediately stored at −80 °C for subsequent analytical procedures. Samples were freeze-dried before analysis.

High-Performance Liquid Chromatography Analysis for Carotenoid and Tocol Determination. *Chemicals and Reagents*. Echinenone, lutein, zeaxanthin, α-tocoacetate, α-tocopherol, and β-tocopherol were from Sigma Chemical Company; α-tocotrienol and β-tocotrienol were purchased from Calbiochem (an affiliate of Merck Kga, Darmstadt, Germany). Ammonium acetate (7.5 M), HPLC-grade acetonitrile, methanol, ethanol, dichloromethane, and hexane were obtained from Sigma (l'Isle d'Abeau Chesnes, France). HPLC water was obtained by a MilliQ Plus water purification system (Millipore).

Preparation of Standards. Stock solutions of carotenoid and tocol isomers standards were stored at −20 °C in tetrahydrofuran and ethanol, respectively. With the use of their respective extinction coefficient, concentrations of standard solutions were determined spectrophotometrically after dilution in ethanol. Final dilutions were made in mobile phase (2.5 μg/mL) (working solution). Echinenone and α-tocoacetate were used as internal standards for carotenoid and tocol quantification, respectively.

Chromatographic System. The HPLC apparatus consisted in a Waters system equipped with a pump (Waters 610 fluid unit), a regulator (Waters 600 controller), a cooled autosampler (Waters 717 plus), and a UV−vis photodiode array detector (Waters 996); 32 software (version 3.05.01) from Waters was used for instrument control, data acquisition, and data processing. Analyses were performed on a 150 mm × 4.6 mm, RP C_{18}, 3 μm Nucleosil column (Interchim, Montluçon, France) coupled with a 250 mm × 4.6 mm RP C_{18}, 5 μm, Vydac TP54 (Hesperia, CA) and a 20 mm × 4.6 mm C_{18}, 5 μm, Hypersil guard column. The mobile phase consisted of acetonitrile/methanol containing 50 mM ammonium acetate/water/dichloromethane (70/15/5/10; v/v/v/v). The methanol fraction was first prepared by dilution of acetate ammonium (7.5 M) in methanol. Then this mixture was added to the other solvents of the mobile phase. The flow rate was 2 mL/min. The run time was set at 45 min allowing both carotenoid and tocol detection. Wavelength detection was set up at 450 and 290 nm for the determination of carotenoids and tocols, respectively.

Sample Extraction. Individual carotenoid and tocol derivatives were extracted as in a method previously described (*19*) with a preliminary step of saponification. All extractions were performed at room temperature under yellow light, to minimize light-induced isomerization. Freeze-dried milled grain (200 mg) was saponified with 1 mL of potassium hydroxide (10% in ethanol) in a 37 °C water bath for 1 h and then deproteinized by addition of 1 mL of ethanol containing the internal standards. Carotenoids and tocols were extracted twice by the addition of 2 mL of hexane containing pyrogallol. The mixture was mixed 30 s and then centrifuged for 5 min at 500*g*. Both upper hexane phases were collected and then evaporated to dryness under nitrogen. The residue was dissolved in 200 μL of acetonitrile/dichloromethane mixture (50/50; v/v), and 80 μL was injected for HPLC analysis.

Quantification. Six-point external standard curves (ranging from 10 to 200 ng) were constructed from dilutions of working solutions of standards in the mobile phase. Concentrations of echinenone, lutein, zeaxanthin, α-tocoacetate, α-tocopherol, β-tocopherol, α-tocotrienol, and β-tocotrienol were calculated using a linear regression $ax + b$ (concentration vs area) of the six-point external standards curves and were adjusted by percent recovery of the added internal standard. The recovery was greater than 90%.

Vitamin E activity was defined in terms of α-tocopherol equivalents (α-TE). The calculated values were the sum of the weight (expressed in milligrams) of each tocol derivative multiplied by the appropriate biological activity factor. The factor was 1 for α-tocopherol, 0.5 for β-tocopherol, 0.3 for α-tocotrienol, and 0.05 for β-tocotrienol (adapted from (*20*)).

Measurement of Oxidases Activity. *Enzyme Extraction.* Flour (4 g) was homogenized with 10 mL of 0.1 M sodium phosphate buffer (pH 7.5) in an ice bath using an Ultra Turrax homogenizer for 15 s followed by a 30 s rest period and another 15 s treatment. Homogenates were immediately centrifuged at 37 800*g* for 20 min at 4 °C. The supernatant was directly used for the measurement of lipoxygenase (LOX) activity and after dilution (1/8) in the same buffer for peroxidase (POD) activity.

LOX activity was determined polarographically according to the method previously described by (*21*): the substrate (1 mL), a mixture of linoleic acid (1 mm) and Tween 20 in a molar ratio of 4:1, dispersed in a phosphate buffer (pH 6.5, 100 mm), was saturated by air at 30 °C. The reaction started by addition of 50−100 μL of enzyme extract. LOX activity is expressed in nkat, i.e., in nmol of oxygen consumed·s^{-1} per g of dried matter in the assay conditions.

POD activity was measured by a spectrophotometric method (*22*) based on the decrease of absorbance at 310 nm for ferulic acid (FA) using a diode array spectrophotometer. The reaction mixture contained FA (100 μM), $CaCl_2$ (20 mM), and H_2O_2 (500 μM) in sodium acetate

	MILLING YIELD (%)
Coarse brans	13,7
Thin brans	3,8
Brown midds	5,4
White midds	2,8
Flour	74,3

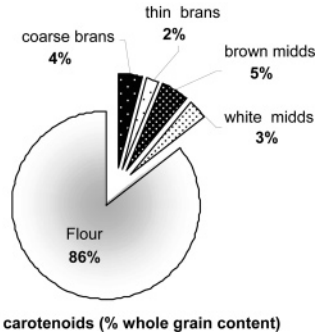

Figure 1. Distribution of total bread wheat carotenoids (calculated as the sum of lutein and zeaxanthin concentrations) in the milling streams, expressed in percent of the whole-grain content according to the milling yield (relative amount of each milling stream in percent).

buffer (0.1 M, pH 5.6). The reaction was performed at 25 °C and started by addition of 25 μL of the diluted enzyme extract. The activity was determined by the initial slope from the linear decrease in absorbance at 310 nm, using the extinction coefficient of FA $\epsilon = 8400$ M·cm^{-1}. It is expressed in μkat, i.e., in μmol of FA consumed·s^{-1} per g of dried matter in the assay conditions. All activity values were obtained from two extractions and two assays per extract.

Statistical Analysis. Data from this study are presented as the mean ± SD. Differences between means were determined using Student−Newman−Keuls' pairwise multiple-comparison test, run on Instat software (San Diego, CA). Differences with $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Carotenoids and vitamin E extracted from all doughs and breads studied in the present study originated from flour, since traditional French breads are only made from flour, water, salt, and a leavening agent (baker's yeast or sourdough).

Distribution of Wheat Carotenoids in Milling Streams and Evolution of Whole-Wheat Carotenoids Content during Breadmaking. Bread production starts with the milling of wheat grains that results in different types of fractions containing endosperm, bran, and germ in varying proportions. The amounts of carotenoid pigments vary in the different milling fractions according to their repartition in the kernel. **Figure 1** shows the distribution of carotenoids in the different flour mill streams obtained with an industrial roller mill. It was calculated according to the milling yield, which indicates the relative quantity of each mill streams produced from a given grain weight. Carotenoids were present in all milling fractions, but the high concentration observed in the flour points to a higher concentration in the wheat endosperm than in other parts of the grain. Taking into account the proportion of carotenoids in white flour together with the fact that white flour represents about 75% of the wheat kernel, 86% of total grain carotenoids were recovered in white flour. According to Panfili et al., lutein is the main carotenoid found, equally distributed throughout the kernel, whereas zeaxanthin is principally localized in the germ (*23*). However, it must be noted that the germ does not appear as a separated milling fraction since it is recovered in the different byproduct fractions and in flour depending on the extraction rate. It was concluded that most grain carotenoids are recovered in white flour after the milling process.

Carotenoid pigments have been determined after each step of breadmaking using either whole-wheat flour obtained by grinding a mix of bread wheat varieties or white flour produced industrially from the same grains. Measurements were performed

Table 1. Total Carotenoids Contents, Tocol Isomers Contents, and Vitamin E Activities before (Whole-Wheat Flours) and after Breadmaking (Whole-Wheat Breads), Expressed in μg/g of Dry Weight[a]

	carotenoids (μg/g dw)	tocopherol (μg/g dw)		tocotrienol (μg/g dw)		vitamin E activity (μg α-TE/g dw)
		a	b	a	b	
			T. monococcum			
whole flour	5.75	11.03	7.78	4.87	40.94	15.8
whole bread	3.34	9.17	6.16	3.02	14.83	12.5
			T. durum			
whole flour	3.32	11.52	6.93	6.81	31.41	17
whole bread	1.61	9.46	5.43	3.75	7.57	13
			T. aestivum			
whole flour	1.24	14.93	6.24	9.7	44.97	21.9
whole bread	0.10	12.24	5.49	5.11	13.7	16.4

[a] Values are means of triplicates ± SD.

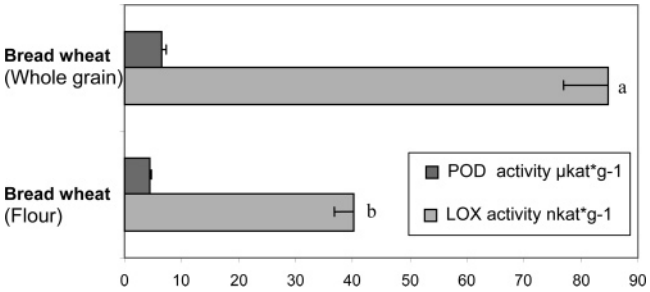

Figure 2. Lipoxygenase activity (nkat·g^{-1}) and peroxidase activity (μkat·g^{-1}) of whole-grain flour and white flour from bread wheat. Values are means of triplicates ± SD. Bars with different letters differ significantly ($p < 0.001$).

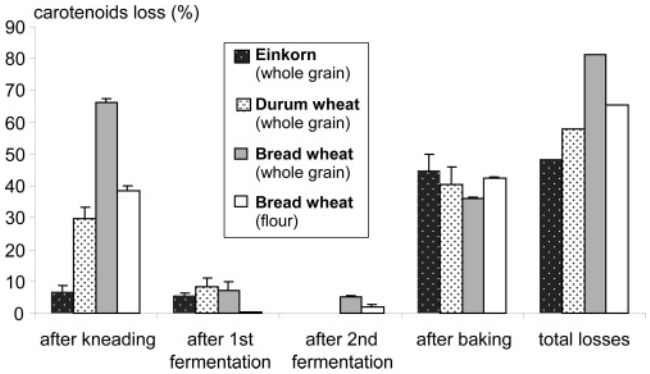

Figure 3. Carotenoid losses after each step of the breadmaking process, expressed in percent of losses from the previous step. Values are means of triplicates ± SD.

on the initial flour, on the dough after kneading, and on freshly baked bread. Total carotenoid content, calculated by summing lutein and zeaxanthin concentrations, decreased after breadmaking (**Table 1**). The carotenoid content of bread wheat decreased by about 66% after kneading **(Figure 3**), probably because of the presence in wheat flour of enzymes such as lipoxygenase (LOX) and peroxidase (POD), which become active when water is added. It has been previously shown that doughmaking results in a substantial incorporation of oxygen in the dough, facilitating the lipoxygenase (LOX)-catalyzed oxidation of polyunsaturated fatty acids that can lead to oxidation of carotenoid pigments by coupled reaction (*24*). Very little difference in carotenoids concentration (about 10%) was observed after the two fermentation periods, which were not favorable to oxidation since dough was left to proof at 30 °C

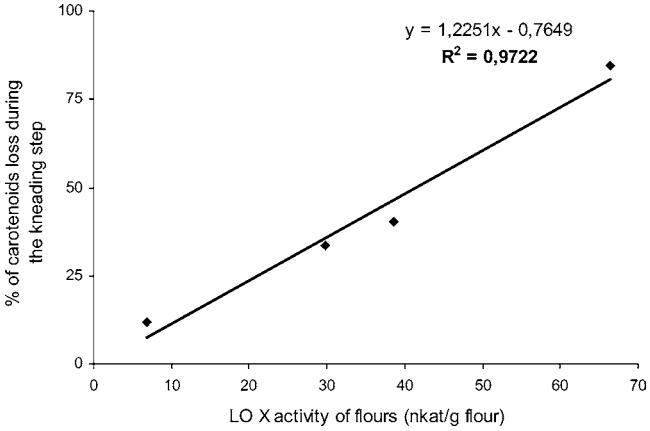

Figure 4. Relationship and correlation between the percentage of carotenoids lost during the kneading step and LOX activity of whole flour and white flour from bread wheat, whole flour from durum wheat, and whole flour from einkorn.

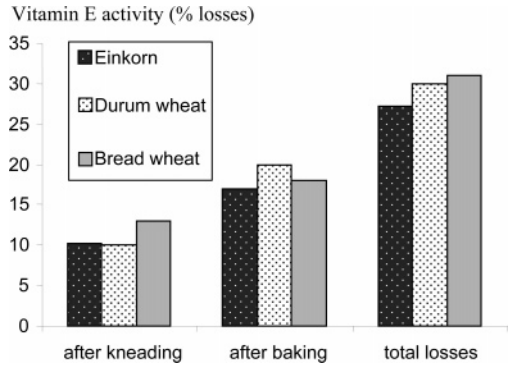

Figure 5. Losses in vitamin E activity during the breadmaking process, expressed in percent of losses after each step and total losses for three different wheat species.

in a closed and dark environment. Moreover, oxygen is consumed by the baker's yeast. The 10% losses could have occurred between the two periods of fermentation, when the initial dough (3 kg) was divided and remolded into smaller pieces. The baking stage (250 °C for 25 min) resulted in carotenoids losses ranging from 36% to 45% depending on wheat species, which are easily explained by the known susceptibility of carotenoids to heat. Doughmaking was therefore the stage of the breadmaking process which resulted in major losses of carotenoid pigments. It must be noted that kneading conditions were different and lighter (8 min at mixing speed and 8 min at high speed) than common practices. Dough is traditionally kneaded for at least 20 min (5 min at low speed and 15 min at high speed) in order to produce loaves with high volume and white crumb. To evaluate the impact of endogenous LOX activity on carotenoid destruction during kneading, three *Triticum* species, which are phylogenetically close to bread wheat and have been shown to exhibit highly different levels of carotenoids and LOX activity (*25*), were simultaneously subjected to the breadmaking procedure. LOX in einkorn grains (11.9 nkat g^{-1}) was about 3 times less active than in durum wheat grains which was 2.5 times less active than in bread wheat varieties. It is interesting to note that the percent carotenoid loss was inversely related to the grain carotenoid content thereby validating the report of Trono et al. (*26*).

LOX activity was also analyzed in white flour from bread wheat and was 50% lower than in the whole flour (40.2 ± 3.4 nkat g^{-1} vs 84.7 ± 7.7 nkat g^{-1}, respectively) (**Figure 2**). This is in agreement with a previous study reporting that germ and bran exhibited the greatest LOX activity within wheat grain (*27*). These data were also used to highlight a possible correlation between LOX activity and carotenoid destruction, and the evolution of carotenoid content of white flour (produced from bread wheat) was studied and compared with that of whole-wheat flour. The proportion of carotenoids lost at each step of breadmaking are reported in **Figure 3**. There was very little loss after einkorn doughmaking (<7%) and about 50% less losses for durum wheat (30%) and for white flour of common wheat (38%) than for whole-meal flour (66%) during kneading. These percentage of pigments losses were highly correlated ($r^2 = 0.97$) to LOX activity of the respective flours (**Figure 4**). As a result, carotenoid oxidation during kneading was assumed to depend mainly on LOX activity. POD was far less active than LOX and should have a minor impact on dough bleaching in comparison with that of LOX. Wheat LOX has been shown to have an optimal bleaching activity at pH 5.2, the pH in a dough when yeast is used as leavening agent (*28*).

Total losses for a given dough closely reflected those observed during kneading, because practically no changes were observed during fermentation and losses occurring during baking were not significantly different between species. The carotenoid content of the different wheat species and the corresponding breads are shown on **Table 1**. The remaining pigment concentration in common wheat bread was negligible. As a result of the high carotenoid/lipoxygenase activity ratio of einkorn, its bread contained significantly higher amounts of carotenoids, about twice that of durum wheat bread. Einkorn bread contained almost 3 times as much carotenoid pigments as were initially present in common wheat grains. Bread produced from durum wheat contained as much pigments as common wheat grains. Einkorn bread and durum wheat bread would thus contribute more significantly to carotenoid intake than common wheat bread.

Changes in Wheat Tocol Content and Vitamin E Activity during Breadmaking. In the wheat grain, tocopherols are most abundant in the germ and least in the endosperm (*29*). However, commercial milling of wheat flour aims at maximum extraction of the endosperm with minimum possible contamination by bran and germ which are considered as byproducts for the industrial bakery. Since α-tocopherol is the most biologically active of all homologues, it would not be appropriate for study of the changes in vitamin E content from a commercialized white flour. **Table 1** shows the concentration of the different tocol isomers present in whole-grain flours of einkorn, durum wheat, and bread wheat, which express low, middle, and high LOX activity, respectively. In contrast to carotenoids, there were no significant differences for vitamin E activity between the three wheat species, which ranged from 15.8 to 21.9 $\mu g \cdot g^{-1}$ α-TE. The remaining concentration in the corresponding whole-wheat breads is presented in the same table. Vitamin E losses after breadmaking were close to 30% without significant differences between the three wheat species. This is in agreement with a previous study reporting between 20% and 40% tocopherols losses, depending on the breadmaking method (*16*). The same authors associated tocopherols losses both to direct oxidation and to the one dependent on lipoxygenase. **Figure 5** presents vitamin E losses after each critical step of breadmaking. There were no varietal differences for vitamin E activity during kneading, despite large varietal differences for LOX activity. Therefore, tocols losses during breadmaking could be attributed to direct oxygenation during doughmaking and heat destruction during baking. On the basis of another finding described in the literature, most carotenoids are better electron donors than

α-tocopherol (*30*). It may be suggested that carotenoid oxidation protects vitamin E from LOX activity.

Conclusions. Wheat grains are source of many phytonutrients with potential health benefit, but the nutritional properties of wheat will only be fully exploited if whole-wheat products are available. Bread is a staple food for humans since it satisfies many of the energy and proteins needs Nevertheless, bread nutritional quality should be reconsidered taking into account the factors associated with grain carotenoids and vitamin E losses during wheat processing. Breeding could be one approach for improving nutritional value of wheats. Abdel-Aal et al. first demonstrated that diploid and tetraploid wheats contain higher level of lutein than bread wheat and proposed to develop bakery products from high-lutein wheats such as einkorn or durum wheat to raise carotenoids dietary intake significantly (*31*). Our previous study also reported variations in carotenoids content among high-yield hexaploid wheats (*25*). Second, the generalized use of white flour in the bakery production means that other parts of the grain structure, where most bioactive compounds are concentrated (such as germ and bran), are discarded after the grain milling process. Since wheat germ is the richest source of tocopherols, its recovery in flour would be an easy strategy to improve vitamin E content of bread. Wheat contains 2−3% of germ which can be extracted from byproduct milling fractions and could be directly added to produce germ-rich bread, independently of the milling yield. However, germ should be first stabilized to avoid oxidative rancidity. Third, some parameters of the breadmaking process could be adapted to optimize antioxidant retention in bread. Concerning carotenoid stability, a major role appeared to be played by LOX during doughmaking as strongly suggested by the positive correlation found between carotenoid losses and LOX activity. The addition of other sources of LOX (soy or broad bean flours), as done in some industrial white breads recipes, could increase carotenoids and vitamin E oxidation. Since varietal differences have been shown for LOX activity, selection programs should consider carotenoids content together with LOX activity for bread production. The present study emphasized that high-carotenoid genotypes also express very low LOX activities. In particular, einkorn appears to be a potential candidate for developing high-carotenoid breads.

A reduction in kneading time and intensity associated with a longer period of dough fermentation may spare carotenoids and vitamin E by limiting oxygen incorporation.

Until now, carotenoid pigments have had little attention in a breadmaking perspective, since they contribute to color of the flour, whereas white breads are traditionally popular. However, an important aspect of the nutritional quality of breads would be improved by increasing wheat carotenoids and vitamin E retention.

ABBREVIATIONS USED

α-TE, α-tocopherol equivalent; LOX, lipoxygenase; POD, peroxidase.

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