Incorporation of Lutein into Wholegrain Bread as a Functional Ingredient and Antioxidant

By

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ABSTRACT

INCORPORATION OF LUTEIN INTO WHOLEGRAIN BREAD AS A FUNCTIONAL INGREDIENT AND ANTIOXIDANT

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The lutein carotenoid plays significant roles in human health but its consumption is low worldwide. The current study was aimed to investigate the effect of using different baking formulas and lutein forms on lutein distribution and stability in wholegrain bread to improve its lutein content and antioxidant properties. One bound wholegrain breads were made using three lutein forms (lutein powder, lutein in oil emulsion, and lutein in ethanol suspension) with two baking formulas (basic and enriched). Lutein and other carotenoids were measured in bread loaf, crust, top crumb and center crumb. Lutein-enriched breads had significantly higher lutein compared with the non-enriched respective ones. The lutein powder with basic formula was more effective in preserving lutein during baking process. The lutein content remained fairly stable during bread storage at room temperature up to 7 days. Enrichment of wholegrain bread with lutein resulted in significant increases in antioxidant properties as measured by three assays DPPH, ABTS and ORAC, particularly for breads made from the enriched formula. The study provides insights into the production of wholegrain bread enriched with lutein to boost lutein consumption and its anticipated positive health effects. More research is needed to investigate lutein bioavailability and health benefits of the developed products.
DEDICATION

I would like to dedicate this thesis to my wonderful family; my beloved parents—my mother Eman, my father Abdulhafidh, my uncle Amir, my sisters and my brothers for their unconditional, selfless love, support and understanding.
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CHAPTER 1:
INTRODUCTION

Lutein is a yellow pigment from the carotenoid family found in fruits, dark green vegetables and grains. It plays significant roles in human health particularly the health of eyes and skin and is linked with reduced risk of age-related macular degeneration (AMD), cataracts, cancer and cardiovascular disease. Lutein along with zeaxanthin constitute the main pigments found in the yellow spot of the human retina providing several protective functions such as protection of the macula from damage by blue light and scavenging harmful reactive oxygen species. They also reduce or inhibit the oxidation of vulnerable molecules in food products or human body. Since carotenoids can’t be produced in humans, they must be provided in the diet.

Wholegrain bread is considered a healthy food because it is a good source of many bioactive components such as dietary fiber, antioxidants, polyphenols, carotenoids and tocols. Lutein is the main carotenoid found in wheat but its concentration is not enough to meet the suggested physiological dose (e.g. 5-6 mg per day). Several approaches can be used to boost lutein content in wheat such as bio-fortification (e.g. developing high-lutein wheat) or fortification with food-grade lutein products. Fortification of wheat with lutein supplement was found to be a practical and economic way to boost lutein content in wheat products particularly for high-fat baked products such as cookie and muffin (Read et al. 2015, Abdel-Aal et al. 2010). However, incorporation of lutein in a low-fat food system such as bread is a big challenge. Lutein is a lipophilic compound and is difficult to uniformly distribute in an aqueous food system. Thus a special care should be taken when incorporating lutein into a food system that is low in its fat content.

Lutein stability in wholegrain bakery products is significant consideration. Lutein is
sensitive to oxidization and isomerization due to heat, storage, temperature and baking conditions which could affect its content and antioxidant properties. A high fat content in baked products causes a greater loss of lutein because carotenoids are soluble in fat and can be exposed to isomerization and oxidation during baking process. In a low-fat bakery product lutein is less prone to oxidization and isomerization due to its lipophilic nature. On the other hand, incorporation of lutein in an aqueous or low-fat food system could pose a technological challenge for the same reason. This could result in uneven distribution of lutein in bread products (Read et al. 2015). To this end, the bread formula needs to be modified when fortified with lutein supplement to improve lutein content and its distribution in the end product.

There has been interest in bakery products due to their popularity and widespread consumption. To this end, previous work has been done showing that bakery products (bread, muffin and cookie) can be enriched with lutein to produce high-lutein foods (Abdel-Aal et al. 2010). The baked products were assessed for stability of lutein and in terms of antioxidant properties (Abdel-Aal and Rabalski 2013). More research is needed to improve uniformity and stability of lutein in baked products especially the low-fat ones. The overall goal of the current study was to improve incorporation and stability of lutein in wholegrain bread using different forms of lutein. It was hypothesized that the form of lutein (e.g. powder versus liquid) and the method of addition of lutein (e.g. dry mix, aqueous ethanol suspension and oil emulsion) could affect incorporation and stability of lutein in wholegrain bread. Additionally, baking ingredients (e.g. enriched formula versus lean formula) could also affect incorporation and stability of lutein in wholegrain bread. Therefore, the following objectives were investigated: 1) to investigate impact of lutein enrichment in various forms on lutein distribution in wholegrain bread; 2) to study effect of lutein incorporation in various forms on compositional and structural changes of lutein; 3) to investigate impact of lutein
incorporation in various forms on antioxidant properties of wholegrain bread; 4) to determine differences between basic (lean) and enriched baking formula on incorporation of lutein; 5) to investigate stability of lutein in wholegrain bread during storage.

Enhancing stability of lutein in wholegrain bread is of interest to researchers and food industry because bakery products are the most appropriate vehicles to transfer the recommended dietary intake of essential nutrients. Fortified wholegrain bread with lutein would hold a promise for the development of high-lutein functional foods. The current study is directed at achieving a better understanding of the factors influencing lutein stability and distribution in bread as they are linked with health effects. The outcome of this research would help boost the daily intake of lutein and increase consumption of wholegrain products through the development of wholegrain bread enriched with lutein supplement.
CHAPTER 2:
LITERATURE REVIEW

2.1. Wholegrain and wholegrain foods

The American Association of Cereal Chemists International (AACCI) defines wholegrain as “wholegrains shall consist of the intact, ground, cracked, or flaked caryopsis (kernel or seed), whose principal anatomical components—the starchy endosperm, germ, and bran—are present in the same relative proportion as they exist in the intact caryopsis” (AACCI 2010). The grain or kernel contains three main parts: endosperm (80 - 85% of the grain), germ (2-3%) and outer layers or bran (13-17%). The endosperm is composed of cells having starch granules embedded in a protein matrix. The germ is rich in oil and lipid-soluble vitamins, and the bran contains high concentrations of minerals, cellulose and hemicelluloses. In addition, the outer layers are rich in bioactive components such as phenolic compounds, anthocyanins, β-glucan and dietary fiber (Ragaee et al. 2013; Abdel-Aal et al. 2006).

Phenolic acids and flavonoids are the most common phenolic compounds in wholegrain (Abdel-Aal and Rabalski 2013; Abdel-Aal et al. 2012; Liu 2007). Ferulic acid is the dominant phenolic acid found profusely in wheat bran (Abdel-Aal et al. 2001; 2011). Carotenoids are very wide spread pigments in plants, and are found in wheat particularly einkorn, durum and Kumat at relatively high levels (Abdel-Aal et al. 2002; 2007). They are present at exceptional high concentration in corn (Abdel-Aal et al. 2007). Lutein is the main carotenoid found in wheat, followed by zeaxanthin and β-cryptoxanthin (Abdel-Aal et al. 2007).

AACCI defined wholegrain food as “A whole grain food must contain 8 grams or more of wholegrain per 30 grams of product” (AACCI 2013). Wholegrain foods such as wholegrain bread and pasta are a unique source of dietary fiber (Liu 2003). They are
also rich in vitamins E and B, and minerals such as selenium, copper, zinc, proteins, iron and magnesium. They are also containing phytochemicals such as antioxidants that play significant roles in human health and disease prevention. They deliver several nutrients to protect human body from cardiovascular disease (CAD), type II diabetes, cancer and other chronic diseases (Slavin et al. 2001). Wholegrain wheat products contain a diverse array of bioactive compounds such as dietary fiber, β-glucan, tocopherols, tocotrienols, phenolic acids, anthocyanins, carotenoids and phytosterols. Many of these compounds have demonstrated human health benefits.

2.2. Bioactive compounds in wholegrain

Cereal grains are rich sources of many health-enhancing and/or disease-preventing components known as bioactive compounds. These components make wholegrain products healthier than their corresponding refined ones. Many of the bioactive compounds are phytochemicals produced by plants primarily for protection against predators and diseases. Phytochemicals have also been found to protect humans against certain chronic diseases. In general, phytochemicals are natural and non-nutritive bioactive compounds produced by plants that act as protective agents against external stress and pathogenic attack (Chew et al. 2009). They are secondary metabolites that are crucial for plant defense which enable plants to overcome temporary or continuous threats integral to their environment. Phytochemicals could exhibit several bio-activities such as antimutagenic, anticarcinogenic, antioxidant, antimicrobial, and anti-inflammatory properties (Okarter and Liu 2010). The type and concentration of phytochemicals vary among grains, and grain species and genotypes (Adom et al. 2003). For example, total phenolic content significantly varied between wheat species and cultivars ranging from 881 µg/g to 2382 µg/g (Abdel-Aal and Rabalski 2008). Ferulic acid concentration (220-574 µg/g) ranged broadly in the wheat species due to their environmental and genetic diversity used in the study. Four tocols including α-tocopherol,
β-tocopherol, α-tocotrienol and β-tocotrienol were the predominant compounds in all the wheat species examined. All-trans lutein is the main carotenoid found in wheat species (Abdel-Aal et al. 2002; 2007). This polar dihydroxylated carotenoid constituted about 77-83% of the total carotenoids in high lutein wheat species (Abdel-Aal et al. 2007). Since the study goal is to improve incorporation of lutein in wholegrain bread, in the following section emphases will be put on carotenoids in cereal grains and their role in human health and nutrition.

2.3. Carotenoids

2.3.1. Structure

Carotenoids, in general, are a collection of tetraterpenoid compounds, with the basic carotenoid structural backbone consisting of isoprenoid units formed either by head-to-tail or by tail-to-tail biosynthesis. The primary groups of carotenoids are carotenes and xanthophylls. Carotenes are carotenoids that contain only hydrocarbon made up from isoprene units, such as α-, β-, and γ-carotene and lycopene. Xanthophylls are carotenoids that contain oxygen as hydroxyl, keto, carboxyl, methoxyl, and epoxy group, such as lutein and zeaxanthin (Figure 2.1) (Abdel-Aal and Akhtar 2006).

Lutein is defined structurally as a long carbon chain with conjugated double bonds, and at the ends of the carbon backbone, the molecule includes a construction of cyclic hexenyl with an attached hydroxyl group. The zeaxanthin has a similar structure as lutein, the only structural differences between them is in the location of the double bonds in the hexenyl ring or the ionone ring. Zeaxanthin has 2 β-ionone rings (symmetric molecule), while lutein contain β-ionone ring and ε-ionone ring (asymmetric molecule). Lutein and zeaxanthin are distinguished from other carotenoids because of the presence of a hydroxyl group at the ends of the molecule. The absorbance of a specific light wavelength and the release of other wavelengths create characteristic color properties in the molecules as a result of the structure
of the nine double bonds. The yellow or orange color of lutein can be identified based on its concentration, which affects the amount of blue light absorption.

Lutein is also present in several isomers and forms such as trans, cis, and epoxy lutein. However, it is still unclear whether bioavailability or other physiological functions are affected by lutein isomers. The trans form of lutein is the primary isomer in plants and vegetables, and processing may cause changes in this significant isomer. In nature, lutein exists as a fatty ester in which one or two of the hydroxyl groups are bound to a fatty acid (Kijlstra et al. 2012) but is also found in free form (Abdel-Aal et al. 2007). There are 17 various esters detected in commercial lutein supplements, the level of monoesters was very low (1.5%), mixed diesters were present at about the same level as the homogeneous diesters (50.2 vs 48.0%, respectively), and palmitic acid was the predominant fatty acid (57.2%) followed by myristic acid (30.2%), stearic acid (10.2%), and lauric acid (2.4%) (Piccaglia et al. 1998). Abdel-Aal and Young (2009) observed low levels of two pairs of regioisomeric monoesters and nearly equal levels of three homogeneous diesters and five pairs of mixed diesters in a commercial lutein supplement. Identifying lutein esters would help in improving quality of lutein-based products by fortifying the wheat species to deliver the physiological dose (Young et al. 2007). Lutein is readily absorbed from foods and dietary supplements, whereas to enter the bloodstream, the esters require prior de-esterification by intestinal enzymes (Alves-Rodrigues and Shao 2004).
Figure 2.1: Structure of all-trans-lutein, all-trans-zeaxanthin, all-trans-β-cryptoxanthin, all-trans-β-carotene, and 13-cis-lutein found in wheat (source: Abdel-Aal et al. 2007).
2.3.2. Occurrence of lutein in cereal grains

Carotenoids, specifically lutein, are the main yellow pigment in wheat (Abdel-Aal et al. 2007). They are effective antioxidants due to the long series of alternating double and single bonds (Okarter and Liu 2010). The concentration of carotenoids in cereal grains exhibited a wide range from very low in white and red wheat to relatively high in einkorn and durum wheat (Abdel-Aal et al. 2002; 2007). The lutein in wheat is the main carotenoid existing in a high concentration ranging from 26.4 to 143.5 µg/100 g grain, followed by zeaxanthin ranging from 8.7 to 27.1 µg/100 g grain, and then β-cryptoxanthin ranging from 1.1 to 13.3 µg/100 g grain (Adom et al. 2003). In corn flour, there is 11.5 µg/g of lutein and 17.5 µg/g of zeaxanthin content and 3.7 µg/g of β-cryptoxanthin, (Brenna and Berardo 2004). In a study by Abdel-Aal et al. (2007) showed that corn is exceptionally high in lutein at a concentration of 21.9 µg/g showing a good potential as blending flour in the development of high-lutein wheat-based functional foods. It also has a high concentration of all-trans-zeaxanthin 10.3 µg/g and small concentrations of all trans β-cryptoxanthin and all-trans-β carotene 0.95 and 0.31 µg/g, respectively.

Several studies have shown that einkorn is a rich source of carotenoids (Abdel-Aal et al. 2002; 2007; 2010; Hidalgo et al. 2006). High levels of all-trans-lutein (7.41 µg/g) were identified in einkorn wheat with small amounts of all-trans-zeaxanthin, cis-lutein isomers, and α-carotene (Abdel-Aal et al. 2007). The lutein content is distributed in the endosperm of einkorn and bread wheat (74.6 % and 69.4 %, respectively) (Hidalgo and Brandolini 2008). Adom et al. (2003) reported that the lutein content of the bran plus germ-milled fraction of wheat was about 4-fold higher than that of the flour fraction. In the United States, the average consumption of lutein is below the recommended daily intake which is 1.5 mg to 2 mg per day (Fullmer and Shao 2001), 2.2 mg per day in Europe (O’Neill et al. 2001) and 1 mg to 2 mg per day in Canada (Lyle et al. 1999; Wright et al. 2003). In a human diet, in addition to
cereals, fruits and vegetables, such as dark green vegetables (e.g., green lettuce and Brussels sprouts) are also considered a major source of carotenoids, and their consumption is recommended as a way to increase dietary intake of lutein and zeaxanthin (Sommerburg et al. 1998). Egg yolk is also a good source of highly bioavailable lutein and zeaxanthin (Handelman et al. 1999)

2.3.3. Functionality

Plant carotenoids provide the pigments for photosynthesis as an important role in the protection against photo-oxidation. They play a fundamental role in plastid pigments and in provision of substrate for biosynthesis of plant growth regulator (Tanaka et al. 2008). The function of carotenoids’ pigments is determined by whether the tissue is photosynthesis or non-photosynthesis. In photosynthesis tissue, the most significant function is to be photoprotective in plant life against harmful oxygen species while the pigments in non-photosynthesis tissue provide the color for fruits and flowers (Bartley and Scolnik 1995; Tanaka et al. 2008).

Carotenoids have the ability to absorb the blue light of the spectrum in which absorbed energy can be transformed to chlorophylls. Then, they function as light harvesting of radiant light in a region of the spectrum that not covered by the chlorophylls. Carotenoids are needed for photo-protection. Plants would suffer photo-oxidative damage, in the absence of carotenoids which can lead to the death of the organism. Chromoplasts, which are plastids that responsible for pigment synthesis, contain carotenoids that impart the orange, red and yellow pigments to roots, flower and fruits. Carotenoids are stored at very high level in chromoplasts to provide the severe color of plants (Bartley and Scolnik 1995).

Carotenoids as phytochemicals can also function biologically. They are effective antioxidants due to their ability of reducing free electrons and scavenging harmful free radicals from biological system resulting in inhabitation of oxidative reactions.
Phytochemicals can modulate cellular physiology at the molecular, biochemical and physiological levels. They also manage infectious diseases as antibacterial, antiulcer, antiviral and antifungal (Vattem and Shetty 2005). Carotenoids impart foods their yellow and orange colors and provide several beneficial effects as dietary antioxidants, health-enhancing and disease-prevention components.

2.4. Role of lutein in human health

2.4.1. Health of eye

The structure of the eyes is relatively more complex than that of any other part of the human body, and vision loss is considered a widespread problem, especially among the elderly (Stringham and Hammond 2005). Mares-Perlman et al. (2002) have suggested that lutein and zeaxanthin help to decrease the danger of developing eye diseases, such as cataracts and age-related macular degeneration (AMD), which are the most common eye diseases in elderly people. More than 20% of these populations might have this disorder (Lim et al. 2012). Abdel-Aal et al. (2013) also reported the prevalence of major eye diseases and the role of lutein and zeaxanthin in reducing AMD and cataracts. AMD leads to legal blindness and numerous visual losses, and involves a complex interaction of metabolic and functional factors (Nowak 2006; Klein et al. 2004; Kuehn 2005). Thus, to protect against AMD, a diet with low glycemic index such as high intake of wholegrain products and low intake of saturated and polyunsaturated fats might be needed to help moderate the risk (Mares and Moeller 2006). Epidemiological studies have proven that lutein plays a protective role with respect to the development of macular degeneration, the primary cause of blindness in the elderly in Western countries, and the consumption of meal containing significant amounts of carotenoids is associated with a lower risk of macular degeneration. Stringham and Hammond (2005) have added that giving patients who suffered from cataracts 15 mg of lutein three times per week for two years resulted in improved visual acuity. In addition, the
macular pigments, lutein and zeaxanthin, can decrease both longitudinal and lateral chromatic aberrations that damage the retinal image due to the wavelength that is concentrated in the retina. This macular pigment can also enhance visual acuity (Stringham and Hammond 2005). High macular pigment density (MPD), macular pigment optical density (MPOD) and macular pigment (MP) have been associated with reduced AMD (Abdel-Aal et al. 2013).

2.4.2. Health of skin

The skin is an organ of human body that requires protection from environmental damage by several mechanisms. First, the presence of natural antioxidant system can protect skin cells by neutralizing the free radicals produced by sunlight exposure. Second, some cells in the skin called melanocytes produce melanin that results in the tanning of the skin. This tan provides protection to the skin by filtering sunlight because melanin has the ability to absorb the wavelengths of sunlight. Lastly, hyperplasia process reduces the damage that the light may cause in the viable cells of the skin. The antioxidants present in the skin at the time of light exposure provide a protection against the damage caused by sunlight in the three mechanisms. However, antioxidant capacity can be significantly reduced by sunlight exposure. Lutein may have the ability to absorb visible blue-light wavelengths in the skin (Roberts et al. 2009). Lutein and zeaxanthin, as a result of dietary intake, are found in human skin. Both components help to protect the skin against damage caused by UV light, against skin swelling and against hyperplasia (González et al. 2003). Roberts (2013) has proved that lutein and zeaxanthin supplementation helps to reduce the effect of UV radiation exposure and help to provide skin protection against cutaneous effects by UV. The accumulation of lutein and zeaxanthin may also help to decrease the formation of reactive oxygen species (ROS).

2.4.3. Lutein and cancer

The absorption of total provitamin A carotenoids such as α-carotene, β-carotene and
β-cryptoxanthin have helped to reduce the risk of lung cancer (Rock 2009). There was an inverse association between serum concentrations of α-carotene, β-carotene, lutein, lycopene and β-crypoxanthin, and the risk of lung cancer exist (Woggon and Kundu 2004). Carotenoids, single or in combination form, have the ability to remove free radicals and enhance the immune response against tumor development. A study by Freudenheim et al. (1996) proved that the intake of carotenoids especially lutein and zeaxanthin is correlated with reduced risk of premenopausal breast cancer. According to Mares-Perlman et al. (2002) among women who have a history of breast cancer, the defensive effect of zeaxanthin and lutein is very strong.

2.5. Effects of processing and storage on lutein

2.5.1. Milling

The milling process entails transforming raw materials into finer and primary products for subsequent processing. With respect to cereal grains, milling separates the bran and germ from the starchy endosperm to produce white flours for use in making bakery products. Because of the removal of the kernel outer layers (pericarp, testa, and aleurone), milling has a significant influence on the health-promoting components found in grains, such as phytochemicals, so the concentration of grain bioactive compounds is significantly reduced through this process (Ragaee et al. 2012). Wheat milling consists of controlled breaking, reduction, and separation to produce a variety of milled products for various end uses. The objective of wheat milling is to separate the pericarp and germ of the wheat kernel from the endosperm to produce white flours. On the other hand durum wheat is milled into a granular product called semolina for pasta production. Significant differences in the composition and concentration of phenolic acids in eight durum wheat samples were observed between kernel parts (starchy endosperm, aleurone layer and pericarp) (Peyron et
al. 2002). The starchy endosperm was characterized by a low content of ferulic acid, the aleurone layer was rich in trans-sinapic acid, while the pericarp exhibited a high content of ferulic acid dehydrodimer. In a study on two Canadian wheats, durum and bread wheat, bioactive constituents were mainly concentrated in the outer layers of grains with the bran fraction having the highest antioxidant capacity compared with shorts and flours (Liyana-Pathirana and Shahidi 2007).

Corn can also be fractionated into its components either through a dry milling process, in which the kernel is separated into endosperm, bran, and germ, or through a wet milling process, in which the corn is separated into starch, protein, fiber, and oil. Qualitatively, the phenolic composition of corn kernels is similar to that of other cereal grains (McDonough et al. 1983). The carotenoids in milled corn have been extracted and analyzed using high-performance liquid chromatography (HPLC). The results indicated that the level of lutein and zeaxanthin in milled corn were 1.60 and 7.83 µg/g respectively (Mamatha et al. 2012). Reducing the particle size of corn grains through a processing operation such as milling or grinding could enhance the extractability of the carotenoids. For example, the levels of lutein and zeaxanthin were 1.60 and 7.83 µg/g in milled corn respectively) and they reduced by 5.2% milled corn after over drying of (Mamatha et al. 2012). Therefore, to reduce the loss of bioactive compounds and possibly increase the health benefits of end products, removal of the outer layers of cereal grains during the milling process should be considered (Ragaee et al. 2012).

2.5.2. Baking

Baking is a traditional process for making assorted types of products such as breads, cakes, pastries, pies, tarts, quiches, cookies, and crackers. Abdel-Aal et al. (2010) studied the stability of lutein and zeaxanthin in unfortified or fortified baked products (pan bread, flat
bread, cookies, and muffins) using different baking recipes and baking conditions. Fortified flat bread contained about 0.9-1.1mg of lutein/serving (serving=30g), whereas the unfortified einkorn had <0.2 mg/serving. The unfortified pan bread had relatively small amounts of lutein, about 0.1-0.2 mg/serving. In addition to lutein, einkorn/corn pan bread contained zeaxanthin at about 0.1mg/serving. Baking of flat bread resulted in a significant reduction in all-trans-lutein, being about 37-41% for the unfortified breads and 29-33% for the fortified breads. Hidalgo et al. (2010) also showed carotenoids loss during processing. Bread crumbs lost 21% of the carotenoid content, while 47% of the carotenoids were lost in bread crusts due to manufacturing. A minimal amount of carotenoids were lost in water biscuits (19%) due to leavening. During baking, all carotenoids were reduced except β-cryptoxanthin, which is concentrated in bread crusts and water biscuits (Hidalgo et al. 2010). Only a small effect on carotenoids was evident as a result of the bread leavening, whereas significant losses occurred during baking (Abdel-Aal et al. 2010). In bread leavening, insignificant carotenoid losses of only 3% were observed (Hidalgo et al. 2010). Moreover, variations in the dough fermentation time from 0 h to 48 h resulted in no significant effect on antioxidant properties. Longer dough fermentation times and increased baking times or temperatures could be considered a potential approach for enhancing the antioxidant properties of whole wheat pizza crust (Moore et al. 2009). The long kneading time involved in pasta production also decreased the amount of carotenoids significantly, but the degradation was not significant during the drying step (Hidalgo et al. 2010). Kneading caused 9% degradation in bread and 6% in water biscuits. Kneading step mixes oxygen and water in the dough, increasing lipoxygenase (Hidalgo et al. 2010) and peroxidase (Leenhardt et al. 2006), and thus oxidizing carotenoids (Hidalgo et al. 2010).

Abdel-Aal et al. (2010) also investigated stability of lutein and zeaxanthin in cookies.
produced from einkorn flour alone or in blend with corn flour, either fortified with lutein or unfortified. Lutein content in fortified cookies was lower than that in flat bread, ranging from 0.5 to 0.6 mg/serving. The results indicated that the fortified einkorn and control cookies had significant losses of lutein concentrations, but that only a moderate decline was evident in the unfortified einkorn cookies. The decline in lutein was about 62% in unfortified einkorn, 65% in fortified einkorn, and 63% in the control cookies. These losses were considered to be due to the baking recipe and the concentration of lutein. A high fat content caused a greater loss of lutein in the cookies than in the bread because carotenoids are soluble in fat and can be exposed to isomerization and oxidation during baking. The degradation rate of zeaxanthin during baking was lower than that of lutein because of its low concentration in the baking formula. The muffin recipe is made of einkorn and corn flour, either fortified with lutein or unfortified and high fat, so the fat could help cause a degradation of the lutein during the baking process because of its solubility in fat, which destroys the lutein through oxidation and isomerization (Hidalgo et al. 2010). Muffins had a reasonable amount of lutein at 0.8 mg/serving. The unfortified cookies and muffins contained small amounts of lutein, about 0.1 mg/serving. The decline in their lutein content was 64% in the unfortified muffins and 55% in the fortified muffins (Abdel-Aal et al. 2010).

Abdel-Aal et al. (2010) has assessed the formation of cis-lutein and cis-zeaxanthin isomers in bread, cookie, and muffin products during baking and subsequent storage. When lutein-containing products undergo thermal processing or long-term storage (up to a few years), lutein may partially convert into cis-isomers. In cookie, the formation of 13- and 13'-cis-isomers was dominant and higher compared to the other products. In the unfortified flat bread, small amounts of cis-isomers were formed whereas cis-isomer concentrations in fortified flat breads were lower than that of their respective flours. On baking of fortified cookies, significant amounts (~57%) of cis isomers were formed. However, the unfortified
cookies exhibited only slight reduction (~21%) in cis-isomer concentrations. As in cookies, fortified muffins had significant amounts of cis-isomers formed during baking, in particular 13- and 13'-cis-lutein, whereas the concentration of cis-isomers in the unfortified cookies dropped slightly during baking.

2.5.3. Steaming

Hidalgo et al. (2008) studied the effect of a variety of steaming treatment conditions on carotenoids in hulled einkorn and bread wheat. Their experiment revealed that steaming has considerable influences on the concentration of carotenoids. Extreme steaming conditions led to significant degradation in antioxidants. For example, lutein that exhibited the least stability of the carotenoids lost more than 25% of its content. The chemical and technological characteristics of wholegrain flours, which made up with germ, endosperm and bran, were strongly affected by the steaming treatments (Hidalgo et al. 2008). Updike and Schwartz (2003) also reported that thermal processing using a microwave affects the isomerization of lutein and zeaxanthin in vegetables.

A recent study by Junpatiw et al. (2013) examined the effect of steaming of sweet corn cultivars on lutein, zeaxanthin, β-carotene, and β-cryptoxanthin, demonstrating increases in all carotenoids concentrations, with the highest for lutein, zeaxanthin and β-cryptoxanthin at 232%, 457% and 405%, respectively, while the increase in β-carotene was only 88%. This is might be because of the difference in tissue structure of both samples and cooking time for the steaming process. Steaming is also not a very effective technique for transferring heat comparing with boiling, but the results of both domestic methods indicated that thermal treatment enhanced availability of the carotenoid. The carotenoid content in sweet corn cultivars could thus be preserved and enhanced through appropriate cooking methods and conditions that associated with an increasing in tissue breakdown during heating (Junpatiw et al. 2013). Bengtsson et al. (2008) found that steaming of orange-fleshed potato resulted in a
decrease in all trans-ß-carotene and the retention was between 69% and 81% of all trans-ß-carotene.

2.5.4. Extrusion

Since 1930 extrusion has been one of the most significant cooking technologies employed in food processing. It is used extensively for processing ready-to-eat food and breakfast cereal products (Brennan et al. 2011; Riaz et al. 2009; Cheftel 1986). It is preferred over other processing technologies because of the short cooking time involved and the variety of products made by extrusion (Brennan et al. 2011). Several studies have demonstrated the significant reduction in bioactive compounds in food products after extrusion processing (Korus et al. 2007; Delgado-Licon et al. 2009; Shih et al. 2009). The conditions associated with this process would cause a reduction in bioactive compounds in general, and in particular, a significant decrease in ß-carotene has been shown after the extrusion of sweet potato and orange (Delgado-Licon et al. 2009; Shih et al. 2009). The reduction in phenolic compounds occurs during extrusion because of the high temperature of the barrel and the high moisture content, which encourage the polymerization of phenols and finally lead to decarboxylation (Dlamini et al. 2007; Repo-Carrasco-Valencia et al. 2009).

Fonseca et al. (2008) compared the amount of carotenoids lost in dehydrated and extruded products. During dehydration, the total amount of carotenoids in sweet potato flour cultivars did not significantly decreased, but extrusion cooking caused a significant degradation. The total carotenoid reductions in sweet potato flour, orange sweet potato flour and cream sweet potato flour were 24.3% and 50.5%, respectively. When sweet potato flour is mixed with rice flour, the reduction in carotenoids in the mixed flours (orange and cream sweet potato flours) is smaller than for the unmixed sweet potato flours, the losses were 2.6%, 3.8%, and 16.2% for sweet potato, orange, and cream flour, respectively. The mixed
flour had small losses of total carotenoids because of the composition of rice flour. Borrelli et al. (2003) reported that composition of rice flour (9% of protein, 0.75% of crude fiber and 1% of lipid) could create carotene-lipid-protein net to protect carotenoids from thermic denaturation. The appropriate processing conditions are important to reduce or prevent the loss of constituents during extrusion process (Camire et al. 1990). Altan et al. (2009) found a significant reduction in both antioxidant capacity (60%-68%) and total phenolics (46%-60%) in barley extradites compared with those reported for unprocessed barley flour.

2.5.5. Storage

Hidalgo and Brandolni (2008) studied the effect of storage temperature on degradation of carotenoids in white and whole meal flour of einkorn (cv. Monlis) and bread wheat (cv. Serio) at a range of temperature (-20, 5, 20, 30, and 38 °C) for more than 239 days. The temperature degree had a substantial effect on the amount of the carotenoids decreased in stored einkorn wheat flour, which can also be affected by temperature and time according to the first-order kinetics. The reduction of lutein and total carotenoids in both white and whole meal flour was similar. However, losses of lutein were faster in bread wheat than in einkorn. The temperature 20°C or less has been considered to be an appropriate for preserving carotenoids for long-term storage. Storing flat bread at the ambient temperature for more than 5 days also had little effect on the all-trans-lutein in unfortified products, and linear losses that adhered to first-order kinetics occurred in lutein-fortified products (Abdel-Aal et al. 2010).

Carotenoid compounds were destroyed at higher storage temperatures. At 38 °C, the carotenoids content in einkorn flour had greater stability than in bread wheat (Hidalgo and Brandolini 2008). The difference could be due to the significant initial concentration of these compounds in both the whole meal and white flour (Trono et al. 1999), which subsequently decrease the antioxidant activity of carotenoids (El-Agamey et al. 2004). It can
also be due to the lower lipoxygenase activity in einkorn (Leenhardt et al. 2006). In einkorn, because of the higher concentration of lipoxygenase in the bran portion, with the exception of β-cryptoxanthin, carotenoids are more stable in white flour than in whole meal flour (Rani et al. 2001). In a study on white and golden corn, lutein and zeaxanthin did not really change in canned corn that was kept in a sugar/salt brine solution for 12 min at 126.7 °C, but, α-carotene decreased to 62 % (Scott and Eldridge 2005). The study did not measure cis-isomers of lutein and zeaxanthin, which were found to increase in canned vegetables (Updike and Schwartz 2003). Junpatiw et al. (2013) have studied the carotenoid content in sweet corn after freezing in a domestic freezer for one month. They found that the carotenoid content in frozen corn was higher than fresh corn, which would make it a greater dietary source of carotenoids than fresh corn. This improvement in carotenoid content after freezing is the result of the release of the bound carotenoids from the structural matrix (Dewanto et al. 2002).

2.6. Effect of storage and processing on antioxidant properties

Wholegrain cereals, fruits and vegetables are the most important dietary sources of antioxidants. Storage of breakfast cereals at room temperature slightly changed antioxidant capacity, but storing breakfast cereals at 100°F for two months, which equivalent to eight months at room temperature, resulted in about 10% loss of antioxidant (Miller et al. 2000). Nicoli et al. (1999) have reported that the antioxidant properties of polyphenol products can be increased or decreased because of the oxidative reactions.

Moore et al. (2009) have studied the effect of processing conditions (fermentation, baking time and temperature) and bran size on antioxidant properties in whole wheat pizza crust using 2,2′ azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Oxygen radical absorbance capacity (ORAC) methods. The
results of ABTS, ORAC and DPPH ranged from 16.5 to 18.5, 16.1 to 21.2 and 1.6 to 1.9 μmol trolox equivalents/g, respectively. The study indicates that decreasing wheat bran particle size did not significantly affect antioxidant capacities of pizza crust during processing. In addition, micronization of wheat bran improved the extraction of antioxidant components, but it’s not clear, in the study, if the improvement due to an increase in the extract surface area or the thermal processing (Zhou et al. 2004). In terms of fermentation for 0, 18 and 48 h at 4°C, the three assays ABTS, DPPH and ORAC indicate that there is no significant effect on antioxidant properties of pizza dough. However, baking time and temperature showed significant changes in antioxidant properties. Two baking conditions of the pizza crust (from 7 to 14 min at 204°C) and (for 7 min at 204°C and 288°C) increased ABTS scavenging capacity from 42% to 47% with no significant different in samples baked at 204°C for 7 min. The DPPH scavenging capacity also increased from 50% to 82%. The ORAC value significantly increased from 47% to 51% when baking conditions changed from 204°C to 288°C for 7 min (Moore et al. 2009). Millard reactions, that contain high molecular weight brown compounds from thermal treatments help to increase the antioxidant capacity of the crust (Lindenmeier and Hofmann 2004). Thermal processing at 115°C decreased total antioxidant capacity of bound phytochemicals from sweet corn samples by 21.9, 27.9 and 49.8% (after 10, 25 and 50 min, respectively) when compared with the raw samples. However, the antioxidant capacity of free phytochemicals from sweet corn samples at 115°C for 10, 25 and 50 min significantly increased by 35.4, 44.0 and 94.0%, respectively, compared with the raw samples (Dewanto et al. 2002). Releasing bound phenolic from food matrix with thermal processes could explain the improved in antioxidant capacity (Eberhardt et al. 2000).

In general, there are several possible effects on antioxidant properties of grain products during storage and processing. First, in some cases insignificant or slight changes
could occur during storage and processing such as changes in carotenoids like lycopene and β-carotene, which are stable even in intense heat treatments. Second, naturally occurring antioxidants can be lost because many compounds are relatively not stable or they can be increased due to storage and/or processing conditions and type of antioxidant compounds. Third, new compounds such as Millard reaction products can be formed. Finally, interactions among different food components (e.g. between lipid and natural antioxidants, and between lipids and Millard reaction products) have uncertain effects on antioxidant properties and stability of food (Nicoli et al. 1999).

2.7. High-lutein functional foods

The Food and Nutrition Board of the National Academy of Sciences in USA defines functional food as “any modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains (Hasler 2002). The International Life Sciences Institute in the United State suggests slightly different definition: “foods that, by virtue of the presence of physiologically active components, provide a health benefit beyond basic nutrition” (Hasler 2002). Heath Canada defines functional foods as “functional food is similar in appearance to, or may be, conventional food, is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic natural functions.” Functional foods are attractive to consumers and may be preferred to pure bioactive compounds, dietary supplements or drugs as evidenced by the incredible growth of the functional foods market in North America in recent years (Lewis 2008). Sheeshka and Lacroix (2008) conducted an official survey indicating that Canadian dietitians encourage the improvement and intake of functional foods as well as foods with labels stating health claims on products. Because of the increase in the availability of functional foods in the market, appropriate legislation should be enacted with respect to some
products in order to protect consumers. An additional consideration is that functional baked products are a very attractive way to deliver bioactive compounds such as lutein because they can be produced easily (Read 2011). Functionalizing baked products also increase the profitability and marketability of grains and cereals (Sheeshka and Lacroix 2008).

In terms of grains, wheat species such as einkorn (ancient wheat) and durum (pasta wheat) and corn were identified as promising ingredients for the development of high-lutein functional foods based on their relatively higher levels of lutein compared with other wheat species such as spelt, soft and hard wheat (Abdel-Aal and Akhthar 2006; Fregeau-Reid and Abdel-Aal 2005; Abdel-Aal et al. 2002; 2013; Abdel-Aal and Hucl 2014). The use of grain with high endogenous lutein content has been shown to improve carotenoid content in food products (Read 2011). Approximately 70% of the total carotenoids are accumulated in corn-milled fractions which makes corn a promising blending flour ingredient in the development of high-lutein functional foods (Abdel-Aal et al. 2007). The high-lutein wholegrain bread, cookie and muffin were found to be able to scavenge peroxyl, ABTS and DPPH radicals (Abdel-Aal and Rabalski 2013). Unbound and bound phenolic extracts were found to contribute to the antioxidant properties of the products with a higher share given by the bound phenolic compounds (Abdel-Aal and Rabalski 2013).

Three wholegrain foods with high level of lutein (about 1 mg per 30 g serving) were developed and evaluated in terms of lutein stability during baking process (Abdel-Aal et al. 2010) and antioxidant properties (Abdel-Aal and Rabalski 2013). The wholegrain bakery products include high-lutein flat bread, high-lutein cookie and high-lutein muffin. Lutein was found to drop significantly during baking process (28% to 64% loss) due to oxidation and isomerization. The isomers 13- and 13′-cis-lutein were found as dominant cis-isomers. Different approaches have been used to protect the lutein during processing and storage and to compensate for the losses of lutein. For example, functional food products have been
fortified with lutein. In addition, wheat and corn verities, with higher lutein content than the existing ones, have been developed or under development. The fortified baked products were found to contain reasonable concentrations (up to 1 mg/serving) of lutein despite of its significant losses. The wholegrain bakery products are also considered good sources of phenolic antioxidants (Abdel-Aal et al. 2013).
CHAPTER 3:

Effect of Lutein Enrichment on its Stability and Distribution in Wholegrain Bread

3.1. Abstract

The carotenoid lutein plays significant roles in human health but its daily intake is low. Bread is a staple food which could be a good vehicle for lutein delivery. This study was designed to investigate the effect of two different bread formulas (basic and enriched) and three forms of lutein (e.g. lutein powder, lutein in oil emulsion and lutein in ethanol suspension) on stability and distribution of lutein in wholegrain bread. Total carotenoid content and lutein concentration were measured in three different parts of bread loaf (crust, top crumb, and center crumb) using spectrophotometry and liquid chromatography. The stability of lutein during storage of bread at room temperature up to 7 days was also investigated. There were no significant differences in total carotenoid content between the basic and enriched formula breads. While the concentration of lutein in the bread parts (crust, top crumb and center crumb) varied by the baking formula and form of lutein used. The crust had significantly lower carotenoids than the crumb, with no significant differences between the top crumb and center crumb. The addition of lutein in a powder form was more stable in both formulas than the other two forms with lutein in oil emulsion had the lowest stability in both formulas. The presence of fat in the formula would make lutein more accessible during baking process causing more degradation. Higher levels of lutein were found in center and top crumb for breads made from basic formula than their counterparts made from the enriched formula. Slight reductions in lutein content were observed with storage up to 7 days, but in general, the content remained fairly stable. The results suggest that enrichment of bread, especially bread made from basic formula, with lutein powder would be promising food to boost the consumption of this important carotenoid.
3.2. Introduction

Carotenoids are a group of pigments that impart the red, yellow, and orange colors in fruits, vegetables, and cereal grains (Borneo and León 2012). The concentration of carotenoids in cereal grains ranges from very low in white and red wheat to relatively high in einkorn and durum wheats (Abdel-Aal et al. 2007). The primary carotenoid in wheat is lutein present in high concentrations ranging from 26.4 µg/100 g to 143.5 µg/100 g of grain, followed by zeaxanthin, which ranges from 8.7 µg/100 g to 27.1 µg/100 g of grain, and then β-cryptoxanthin, at 1.1 µg/100 g to 13.3 µg/100 g of grain (Adom et al. 2003). In a human diet, in addition to cereals, dark green vegetables such as spinach, green lettuce and brussels sprouts are considered a major source of lutein (Sommerburg at al. 1998). Lutein and zeaxanthin are oxygenated carotenoids containing two hydroxyl groups attached to the two ionone rings. They have similar chemical structures with a slight difference in their ionone ring whereas lutein has β-ionone ring and ε-ionone ring, while zeaxanthin has two β-ionone rings (Abdel-Aal et al. 2010). They are the primary carotenoids in wheat, durum and corn (Abdel-Aal et al. 2002; 2007; Kean et al. 2008).

Lutein and zeaxanthin play significant roles in promoting the health of eyes and skin (Abdel-Aal et al. 2013) as well as reducing the risk of age-related macular degeneration (AMD) and cataracts (Mares-Perlman et al. 2002) and cardiovascular disease (Slavin et al. 2001). They accumulate in the macular region of human retina that gives them a curative function (Handelman et al. 1999). β-carotene (another carotenoid pigment) also reduces proxy radicals and exhibit antioxidant properties that defend against oxidative harm (Mares-Perlman et al. 2002). Because of the significant roles in human health, daily intake of lutein has been boosted although it is low worldwide. For example, In the United States, the average consumption of lutein is 1.5-2.0 mg per day (Fullmer and Shao 2001), 2.2 mg per day in Europe (O’Neill et al. 2001) and 1 mg to 2 mg per day in Canada (Lyle et al. 1999;
Wright et al. 2003). These amounts are below the suggested daily intake of lutein (5-6 mg). Dark green vegetables are considered as a good source of carotenoids and their consumption is recommended as a way to increase dietary intake of lutein and zeaxanthin (Sommerburg et al. 1998).

Bread is a staple food that has been used as a suitable vehicle to deliver vitamins and minerals. The incorporation of lutein into bread products could be a big challenge due to the nature of lutein as a lipophilic compound. Lutein is also a sensitive molecule to heat, light and oxygen which can lead to isomerization and oxidation during baking process. In a previous study (Abdel-Aal et al. 2010), the stability of lutein in breads, cookies and muffin baked by naturally high-lutein and lutein-enriched whole wheat was found to substantially declined. The loss of lutein in cookies and muffin was greater at 64 and 55% compared to flat bread at 31%. The use of einkorn as a high lutein wheat with or without enrichment of flours with lutein showed the potential to boost lutein content in baked products (Read 2011). The current study is intended to find a way to improve incorporation of lutein into bread recipes. The study aimed to investigate the effect of two different bread formulas (basic and enriched) and three forms of lutein (lutein powder, lutein in oil emulsion and lutein in ethanol suspension) on stability and distribution of lutein in wholegrain bread. Distribution of lutein in three parts of loaf (e.g. crust, top crumb and center crumb) was evaluated. The stability of lutein during storage of bread at room temperature up to 7 days was also investigated.

3.3. Material and Methods

3.3.1. Materials

Wholegrain flour was obtained from P&H milling group (Cambridge, ON, Canada). The wholegrain flour contains protein, ash and total dietary fiber at average of 13.7, 1.4 and 11.5%, respectively. Crisco shortening and oil, commercial dry yeast, sugar, salt and whey protein were purchased from the retail market in Guelph, ON, Canada. Standards all-trans-
lutein (90% purity) and Methyl tert-butyl ether and butanol were obtained from Sigma (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), all-trans zeaxanthin (95% purity), and all-trans β-cryptoxanthin (95% purity) were purchased from ChromaDex (Santa Ana, CA). Ethanol 95% (food grade) was purchased from LCBO (Guelph, ON, Canada).

3.3.2. Methods

3.3.2.1. Preparation of lutein formulas

Lutein was obtained in two forms, lutein powder (85%) and lutein paste (20%) from (Lyc-O-Lutein, LycoRed Corp., Orange, NJ). The concentration of lutein in each product was confirmed using HPLC as outlined later. Lutein powder was used “as is”, and was added to the wholegrain flour in a dry mix. Lutein paste was used to prepare lutein oil emulsion and lutein ethanol suspension. Lutein oil emulsion was prepared by solubilizing 160 mg of the lutein paste in 5 mL oil in the presence of 160 mg whey protein as a stabilizer. Lutein in ethanol suspension was prepared by solubilizing 160 mg of the lutein paste in 5 mL ethanol in the presence of 160 mg whey protein. Lutein enrichment was performed to achieve a level of about 1.0 mg of free lutein/serving of baked product (30 g).

3.3.2.2. Preparation of wholegrain bread

The approved methods, optimized straight dough (Method 10-10-03) and basic straight dough (Method 10-09.01) (AACC 2011) were used to prepare one bound pan bread loaf from wholegrain flour with and without lutein enrichment. Water absorption of wholegrain flours was previously determined using Farinograph-E (C.W. Brabender Inc., Hackensack, NJ, USA). The average water absorption of wholegrain flour was approximately 70%. Wholegrain flour was enriched with the three lutein forms (powder, oil emulsion, ethanol suspension) before or during mixing with other baking ingredients. The lutein powder were carefully and evenly distributed with flour in a dry mix, lutein in oil emulsion and lutein in ethanol suspension were added into flours with other ingredients (yeast suspension, sugar
solution, water) during mixing step.

3.3.2.3. Quality of breads and bread preparation for analysis

Control and lutein-enrichment breads were taken from oven and left at room temperature for about 2 hr to cool down. Breads were evaluated based on loaf volume, loaf weight and specific volume. Bread Loaves were weighed in grams (g) using an analytical balance and loaf volume was measured according to the rapeseed displacement method of measuring volume in cubic centimeters (cm$^3$) as described by the AACC Intl. approved method 10-05-01 (AACCI 2011). Specific volume was calculated as cm$^3$/g by dividing the volume (cm$^3$) of the loaf by its weight (g). For bread storage experiment, sub-samples of bread (6 loaves per treatment) were stored in a polyethylene Zip-lock bag (21×15 Uline, Ca) at room temperature for 7 days. For bread analysis bread loaves were cut with an electric knife into crust 21.5% (carefully removed without crumb), top crumb 37.6% (the crumb that under crust taken about 1 cm layer) and center crumb 40.8% (the inside center of crumb). Each part was cut into slices and dried in oven at 35°C. Dried bread samples (fresh and stored) were milled using UDY Cyclone mill equipped with a 0.5 mm screen and kept at -20°C for further analysis.

3.3.2.4. Analysis of total carotenoid content and individual carotenoids

Bread crust, top crumb and center crumb were extracted with water-saturated 1-butanol for the determination of total carotenoid content as outlined by Abdel-Aal el al. 2007). Total carotenoid content was determined according to the AACC Intl. approved method 14-60-01 (AACCI 2011). Carotenoid in extracts were separated and quantified by high performance liquid chromatography (HPLC) using an 1100 series chromatograph (Agilent, Mississauga, ON, Canada) as described by Abdel-Aal et al. (2010). The HPLC system was equipped with a model G1311A quaternary pump, a G1329A temperature-controlled injector, a G1316A temperature-controlled column thermostat, a G1322A
degasser, a G1315B photodiode array detector (PDA), and a Chem Station v.8.04 data acquisition system with the capability of conducting isoabsorbance plotting and three-dimensional graphic analyses. The separation was performed on a 4.6 x 100 mm C30 reverse-phase Carotenoid column (Waters, Mississauga, ON, Canada). The column was operated at 35°C and eluted with a gradient mobile system consisting of (A) methanol/methyl tert-butyl ether/nano pure water (81:15:4, v/v/v) and (B) methyl tert-butyl ether/methanol (90:10, v/v) at 1mL/min. The gradient was programmed as follows: 0-9 min, 100-75%A; 9-10 min, 75-0% A; 10-12 min, hold at 0% A; 12-13 min, 0-100% A; and 13-15 min, hold at 100% A for the short column. The separated carotenoids were detected and measured at 450 nm, and the identity of carotenoids was based on the congruence of retention times and UV/vis spectra with those of pure authentic standards. Five concentrations (0.0-5 mg/mL) were prepared for each carotenoid standard in butanol and used to check linearity, to optimize the analytical method, and to generate regression equations for quantification. The regression analysis of response area and injected amount within the above range showed a linear relationship with a coefficient of determination ($R^2$) ranging from 0.9921 to 0.9953. The purity of each compound in extracts was verified on the basis of the spectroscopic properties of each peak using isoabsorbance plot or three-dimensional graphic and peak purity analyses provided with the ChemStation software. Peak purity analysis allows the spectrum of the identified compounds to be identified and confirmed and to determine whether interference occurs.

3.4. Statistical Analysis

All analyses were performed at least in duplicate for (crust, top crumb and center crumb) and the mean values are reported. Analysis of variance was performed using IBM SPSS Statistics 21 software (Armonk, New York, USA). Significant differences (P <0.05) among means were detected using Tukey’s multiple range test at fixed level of α= .05.
3.5. Results and Discussion

3.5.1. Bread quality

Wholegrain baked products are rich sources of nutrients and health-enhancing components such as dietary fiber and phenolic antioxidants (Abdel-Aal and Rabalski 2013). They have also been linked with reducing the risk of chronic diseases such as heart disease (Osganian et al. 2003), cancer (Michaud et al. 2000) and diabetes (Slavin et al. 2001). In the current study wholegrain bread was investigated in terms of lutein enrichment to determine the possibility of incorporating lutein (lipophilic compound) into a bread product and its stability during baking and subsequent storage. On the basis of average loaf volume, loaf weight and loaf specific volume slight differences were observed between control (non-enriched) and enriched breads (Table 3.1). The loaf specific volume was quite similar (2.3-2.4 cm$^3$/g) in breads baked from basic or enriched formula. The addition of lutein in different forms did not have substantial effects on the overall quality based on bread loaf measurements. Pan breads baked from blends of bread wheat flour with einkorn flour (ancient wheat) or with einkorn and corn flours had lower loaf volume than control bread (Abdel-Aal et al. 2010). In the current study high quality flour was used to produce acceptable wholegrain breads since the use of wholegrain flour is anticipated to produce low quality breads compared to that made from refined flours. Further enhancement of the quality of wholegrain bread could be needed for making more appealing products.

3.5.2. Effect of baking on lutein and other carotenoids

Lutein is essential for human health and it has been linked with reduced risk of AMD and cataracts (Abdel-Aal et al. 2013). It can’t be synthetized by humans, and its daily intake is low worldwide (Fullmer and Shao 200; O’Neill et al. 2001; Lyle et al. 1999; Wright et al. 2003). Thus lutein-enriched foods could be a good strategy to promote the consumption of this important carotenoid. Incorporation of lutein into the baking formula is a challenge
because of its lipophilicity and difficulty in evenly distributing it in a dough system (Read et al. 2015). The addition of lutein in oil emulsion was effective in cookies and muffins but not in flatbread. The form of lutein (e.g. free vs ester or powder vs oil-based) could affect distribution and concentration of lutein in the end product. In the current study, free lutein was added in 3 various formulas, i.e. powder form, in oil emulsion and in ethanol suspension. Additionally whey protein was added to the formula to enhance dispersion of lutein in the colloidal system. As expected enrichment of flours with lutein produced enriched breads having total carotenoid content that are significantly higher than that of the control bread made from non-enriched flours (Table 3.2). The enriched breads had total carotenoid content about 5 to 8 times higher than the control bread. The baking recipe or ingredients (basic versus enriched) and how lutein was incorporated in the formula slightly affected the product total carotenoid content. Since total carotenoid content is not specific measurement and other components in the extract could contribute to the measurement, the comparison between products should be taken with caution. The method would provide a rough idea regarding carotenoid content in the bread products, but it is essential to measure individual carotenoid compounds and their concentration in the end products. Previous work on total carotenoid content in comparison with that measured by HPLC method showed that the colorimetric method overestimates the content by 20% (Abdel-Aal et al. 2007).

The total carotenoid content of the loaf parts (crust, top crumb and center crumb) in the enriched formula was slightly lower compared with their corresponding parts of the basic formula (Figure 3.1A). For crumb parts, however, no significant differences were observed among the 3 loaf parts in all enrichment treatments in both formulas (Figure 3.1B, C). These findings indicate that baking ingredients are more pronounced on carotenoids in bread crust during baking process. Variations in total carotenoid content could be due to the addition of lutein and/or changes in natural pigments during baking process which could influence
spectrophotometer readings. Hidalogo et al. (2010) found that bread crumbs lose 21% of the total carotenoid content and 47% in bread crusts. A less reduction occurred during kneading where carotenoids reduced by 9% in bread and 6% in water biscuit. Abdel-Aal et al. (2010) reported that bread leavening has a small effect on carotenoids, whereas significant carotenoid losses occurred during baking. The long kneading time involved in pasta production significantly decreased the amount of carotenoids, but the degradation was not significant during the drying phase (Hidalgo et al. 2010). Lipoxygenase and peroxidase could lead to carotenoids oxidation in the presence of oxygen and water in the dough.

The concentration of lutein in the bread parts (crust, top crumb and center crumb) varied by the baking formula and form of lutein added to the baking formula (Figure 3.2 and Table 3.3). In general, the enriched formula resulted in significant reductions in lutein concentration in all parts of the bread, with crust showing a much greater level of lutein degradation than both crumbs (top and center) in both bread formulas. Within each formula, significant differences were observed among the 3 loaf parts with the center crumb receiving the lowest lutein reduction, followed by the top crumb and finally the crust. This can be anticipated since the center crumb has the lowest temperature during oven baking. The stability of lutein was significantly influenced by baking formula and lutein form, i.e. how lutein was added (powder form, oil emulsion or ethanol suspension). When lutein was added in a powder form, the concentration of lutein in all bread parts was significantly higher than those enriched with other lutein forms (oil emulsion or ethanol suspension) in both baking formulas. The incorporation of lutein in an oil emulsion produced breads with the lowest concentration of lutein (Figure 3.2). This finding was very obvious in the enriched baking formula. The addition of lutein in an oil emulsion to the enriched baking formula that contains oil to makes lutein (lipophilic compound) more accessible and vulnerable to baking conditions resulting in more degradation through oxidation and isomerization. Similar results
were obtained in cookies and muffins (high fat products) compared with bread products (low fat products) (Abdel-Aal et al. 2010). Read et al. (2015) indicated that forms of lutein such as free vs ester or powder vs oil-based could have an effect on the distribution of lutein in end products. Their study has also showed that using whey protein in the baking formula improves carotenoids distribution in cookies and muffin but not in flat bread. The current study is the first to evaluate the content of lutein in wholegrain breads. The crumb seems to be a good preserve of lutein through baking as compared to crust, and the basic formula enriched with lutein powder in a dry mix appears to be a more effective way for making lutein-enriched breads. Since bioavailability of lutein is essential for the delivery of anticipated health benefits of lutein, its content in a food along with bioavailability are needed to determine the product efficacy as a functional food.

The concentration of zeaxanthin in the lutein-enriched and non-enriched or control bread is presented in Table 3.3. Interestingly, the lutein-enriched breads had higher zeaxanthin concentrations that the control ones. Additionally, the lutein-enriched breads baked form the basic formula contained more zeaxanthin compared with that made from the enriched formula. Similar to lutein the presence of fat in the enriched formula and lutein formula (oil emulsion) caused more zeaxanthin degradation during the baking process because of its solubility in fat as a lipophilic compound. Among all treatments, using the basic formula bread enriched with lutein powder had the highest lutein and zeaxanthin concentrations in all loaf parts which underscore the question of lutein forms and baking ingredients. All-trans-lutein is the dominant configuration in wheat flour with trace amount of cis-isomers (Abdel-Aal et al. 2007). When the flours undergo thermal processing or long term storage, potion of all-trans-lutein could be converted into its cis-isomers. In this study, it was noticeable that trace amounts of cis-isomers such as 13-cis-lutein, 13′-cis-lutein, 9-cis-lutein, and 9′-cis-lutein are found in breads enriched with lutein (Figure 3.3). Abdel-Aal et al.
(2010) found that 9, 9’-, 13-, 13’-cis-isomers are, generally, detected in bakery products. Also, they found that the concentration of 13-, 13’-cis-lutein in enriched flat bread is lower than in the respective flours. Besides lutein and zeaxanthin, other carotenoids including α-carotene, β-carotene and β-cryptoxanthin are found in wheat products (Abdel-Aal et al. 2007; 2010).

In this study, the average mg of total carotenoid, lutein and zeaxanthin per serving (30g) in lutein-enriched and control breads is presented in Figure 3.4. The enriched breads had total carotenoid of 1.3-1.10 mg/serving, lutein 1.03-0.4 mg/serving and zeaxanthin about 0.07-0.02 mg/serving compared with approximately 0.1, <0.06 and <0.01 mg/serving for control or non-enriched breads, respectively. In previous study on flat bread using einkorn flour (high carotenoid wheat), lutein-enriched flat bread contained approximately 0.9-1.1 mg lutein/serving, whereas the non-enriched einkorn had < 0.2 mg/serving (Abdel-Aal et al. 2010). In addition, the lutein einkorn/corn pan bread contained zeaxanthin at approximately 0.1 mg/serving. These lutein enriched baked products would boost the daily intake of lutein and other carotenoids on the basis of the recommended 3-8 servings per day.

3.5.3. Effect of bread storage on lutein and other carotenoids

Carotenoids are vulnerable molecules particularly when subjected to unfavorable conditions such as high temperature, oxygen, light, etc. In the present study, storage of lutein-enriched breads at room temperature up to 7 days had no significant effects on total carotenoid content (Figure 3.5). Samples stored for 3 and 7 days showed no significant differences in total carotenoid content in the crust and both crumbs of basic and enriched formulas compared with their corresponding fresh breads. We observed a slight reduction in the concentration of total carotenoid content in the crust and top crumb of the breads made from the enriched formula when lutein incorporating in a suspension form. In general, storage for 7 days did not affect total carotenoid content for lutein-enriched breads. Storing
flat bread at ambient temperature for more than 5 days had little effects on the all-trans-lutein in non-enriched products, but linear losses followed first-order kinetics occurred in lutein-enriched products (Abdel-Aal et al. 2010). It is known that carotenoid compounds can be destroyed at higher storage temperatures. But storage time and temperature are the factor that could affect carotenoids stability of bread wheat (Hidalgo and Brandolini 2008). Lutein and zeaxanthin content of white and golden corn preserved in a sugar/salt brine solution in cans for 12 min at 126.7°C did not change (Scott and Eldridge 2005). Similar to carotenoids, storage for 7 days at ambient temperature had a little effect on lutein content in bread curst, top crumb and center crumb except for bread made from enriched baking formula with lutein added in a suspension form (Figure 3.6). In general the results showed that different forms of lutein in wholegrain breads preserve the stability of lutein and total carotenoid content for 7 days storage at ambient temperature.

3.6. Conclusion

The current study is the first to evaluate the content of lutein in wholegrain bread made from different baking recipes and enriched with lutein in a powder or liquid form. The crumb seems to be a good preserve of lutein through baking as compared to crust, and the basic formula enriched with lutein powder in a dry mix appears to be a more effective way for making lutein-enriched breads. The enriched formula resulted in significant reductions in lutein concentration in all parts of the bread, with crust showing a much greater level of lutein degradation than both crumbs (top and center) for both bread formulas (basic and enriched). The relatively higher temperature of crust compared to that of crumb during baking resulting in more lutein degradation. In general, baking formula and lutein form (powder versus liquid form) affect the distribution and concentration of lutein in lutein enriched breads. Since bioavailability of lutein is essential for the delivery of anticipated health benefits of lutein, its content in a food along with bioavailability are needed to determine the product efficacy as a
functional food. Thus more research is needed to determine bioavailability of lutein. The storage of breads at room temperature for 7 days did not affect the concentration of lutein in most lutein-enriched and non-enriched breads. A low fat-product (basic formula) enriched with lutein in a powder form could hold a promise for the development of high lutein functional bread and boost the daily intake of lutein.
Table 3.1: Loaf volume, weight and specific volume of non-enriched and lutein-enriched wholegrain breads baked from basic and enriched formulas (mean ±SD)

<table>
<thead>
<tr>
<th>Baked type</th>
<th>Loaf Volume (cm$^3$)</th>
<th>Loaf Weight (g)</th>
<th>Loaf specific volume (cm$^3$/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic formula</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (non-enriched)</td>
<td>1760±8.5</td>
<td>714±5.7</td>
<td>2.4±0.03</td>
</tr>
<tr>
<td>Enriched - lutein powder</td>
<td>1740±5.7</td>
<td>713±4.9</td>
<td>2.4±0.01</td>
</tr>
<tr>
<td>Enriched – lutein in oil emulsion</td>
<td>1720±7.1</td>
<td>718±7.1</td>
<td>2.3±0.02</td>
</tr>
<tr>
<td>Enriched -lutein in ethanol suspension</td>
<td>1760±14.1</td>
<td>721±6.36</td>
<td>2.4±0.01</td>
</tr>
<tr>
<td>Enriched formula</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (non-enriched)</td>
<td>1780±8.5</td>
<td>727±9.2</td>
<td>2.4±0.01</td>
</tr>
<tr>
<td>Enriched -powder lutein</td>
<td>1760±11.3</td>
<td>720±7.1</td>
<td>2.4±0.02</td>
</tr>
<tr>
<td>Enriched – lutein in oil emulsion</td>
<td>1780±10.7</td>
<td>720±7.8</td>
<td>2.4±0.02</td>
</tr>
<tr>
<td>Enriched -lutein in ethanol suspension</td>
<td>1720±11.3</td>
<td>721±5.7</td>
<td>2.3±0.02</td>
</tr>
</tbody>
</table>
Table 3.2: Total carotenoid content of non-enriched and lutein-enriched wholegrain bread baked from basic and enriched formulas (µg/g, wb)

<table>
<thead>
<tr>
<th>Lutein form</th>
<th>Crust</th>
<th>Crumb</th>
<th>Full loaf</th>
<th>Crust</th>
<th>Crumb</th>
<th>Full loaf</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Top</td>
<td>Center</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Basic Formula</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (non-enriched)</td>
<td>5.12±0.10</td>
<td>3.95±0.13</td>
<td>4.75±0.06</td>
<td>4.52±0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enriched-powder lutein</td>
<td>40.17±2.34</td>
<td>43.41±3.82</td>
<td>44.01±2.55</td>
<td>42.91±2.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enriched – lutein in oil emulsion</td>
<td>43.67±2.48</td>
<td>45.01±2.69</td>
<td>46.11±0.85</td>
<td>45.12±0.82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enriched – lutein in ethanol suspension</td>
<td>40.60±2.77</td>
<td>42.91±0.28</td>
<td>43.36±0.84</td>
<td>42.55±1.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Enriched formula</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (non-enriched)</td>
<td>7.59±1.50</td>
<td>6.10±0.88</td>
<td>6.39±0.16</td>
<td>6.53±0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enriched-powder lutein</td>
<td>40.74±1.08</td>
<td>39.54±2.96</td>
<td>38.17±3.88</td>
<td>39.19±2.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enriched – lutein in oil emulsion</td>
<td>37.61±1.69</td>
<td>37.23±1.31</td>
<td>39.37±1.58</td>
<td>38.14±1.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fortified-lutein in ethanol suspension</td>
<td>35.55±0.03</td>
<td>36.03±0.99</td>
<td>38.45±3.29</td>
<td>36.88±1.72</td>
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</tr>
</tbody>
</table>
Table 3.3: Concentrations of lutein and zeaxanthin (µg/g, wb) of non-enriched and lutein-enriched wholegrain breads baked from basic and enriched formulas

<table>
<thead>
<tr>
<th>Bread part</th>
<th>Lutein</th>
<th>Zeaxanthin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>LP</td>
</tr>
<tr>
<td>Basic formula</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crust</td>
<td>1.64±0.02</td>
<td>32.50±1.0</td>
</tr>
<tr>
<td>Top crumb</td>
<td>1.73±0.15</td>
<td>34.36±2.4</td>
</tr>
<tr>
<td>Center crumb</td>
<td>1.86±0.04</td>
<td>35.94±2.7</td>
</tr>
<tr>
<td>Enriched formula</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crust</td>
<td>0.70±0.1</td>
<td>19.32 ±1.5</td>
</tr>
<tr>
<td>Top crumb</td>
<td>1.10±0.01</td>
<td>18.29±0.05</td>
</tr>
<tr>
<td>Center crumb</td>
<td>1.12±0.1</td>
<td>17.68±0.1</td>
</tr>
</tbody>
</table>

LP, LOE and LES stand for lutein powder, lutein in oil emulsion, and lutein in ethanol suspension, respectively.
Figure 3.1: Effect of baking on total carotenoid content in crust (A), top crumb (B) and center crumb (C) of breads made from basic and enriched formulas. LP-B, LOE-B, LES-B, LP-E, LOE-E and LES-E stand for lutein powder, lutein in oil emulsion, and lutein in ethanol suspension with basic baking formula; and lutein powder, lutein in oil emulsion and lutein in ethanol suspension with enriched baking formula, respectively. Different letters indicate significant differences between means at p<0.05 (standard deviation values presented by error bars).
Figure 3.2: Effect of baking on lutein content in crust (A), top crumb (B) and center crumb (C) of breads made from basic and enriched formulas. LP-B, LOE-B, LES-B, LP-E, LOE-E and LES-E stand for lutein powder, lutein in oil emulsion, lutein in ethanol suspension with basic baking formula; and lutein powder, lutein in oil emulsion and lutein in ethanol suspension with enriched baking formula, respectively. Different letters indicate significant differences between means at \( p<0.05 \) (standard deviation values presented by error bars).
Figure 3.3: High-performance liquid chromatogram of carotenoids extracted from non-enriched bread (control) (A), enriched bread with lutein (basic formula) (B) and enriched bread with lutein (enriched formula) (C). Peaks: 1, all-trans-lutein; 2, all-trans-zeaxanthin; 3, 13’-cis-lutein; 4, 13 cis lutein; 5, 9-cis-lutein; 6, 9’-cis-lutein.
Figure 3.4: Amount per serving (mg/30g) of total carotenoids (A), lutein (B) and zeaxanthin (C) in full loaf. LP-B, LOE-B, LES-B, LP-E, LOE-E and LES-E stand for lutein powder, lutein in oil emulsion, and lutein in ethanol suspension with basic baking formula; and lutein powder, lutein in oil emulsion and lutein in ethanol suspension with enriched baking formula, respectively. Different letters indicate significant differences between means at p<0.05 (standard deviation values presented by error bars).
Figure 3.5: Effect of storage at ambient temperature on total carotenoids in crust (A), top crumb (B) and center crumb (C) of breads made from basic and enriched formulas. LP-B, LOE-B, LES-B, LP-E, LOE-E and LES-E stand for lutein powder, lutein in oil emulsion, and lutein in ethanol suspension with basic baking formula; and lutein powder, lutein in oil emulsion and lutein in ethanol suspension with enriched baking formula, respectively. Different letters indicate significant differences between means at p<0.05 (standard deviation values presented by error bars).
Figure 3.6: Effect of storage at ambient temperature on lutein in crust (A), top crumb (B) and center crumb (C) of breads made from basic and enriched formulas. LP-B, LOE-B, LES-B, LP-E, LOE-E and LES-E stand for lutein powder, lutein in oil emulsion, and lutein in ethanol suspension with basic baking formula; and lutein powder, lutein in oil emulsion and lutein in ethanol suspension with enriched baking formula, respectively. Different letters indicate significant differences between means at p<0.05 (standard deviation values presented by error bars).
CHAPTER 4:
Antioxidant Properties of Wholegrain Bread Enriched with Lutein

4.1. Abstract

Dietary antioxidants such as carotenoids have a significant role in disease prevention due to their ability to scavenge reactive oxygen species and other free radicals. Thus, improving antioxidant properties of wholegrain products could have significant health benefits in reducing the risk of chronic diseases. With this goal, we have examined the antioxidant properties of two different bread formulas (basic and enriched) and three forms of lutein enrichment (lutein powder, lutein in oil emulsion, and lutein in ethanol suspension) on antioxidant properties of wholegrain bread. Because of the presence of diverse free radicals present in wholegrain bread, multiple types of oxidative assays were used. In the current study antioxidant capacity of bread products was measured using the DPPH, ABTS and ORAC assays. Using the DPPH assay, there was a general trend for higher antioxidant capacity in the enriched formula bread samples. In addition, the bread crust had higher DPPH scavenging capacity than that of bread crumb. When the antioxidant capacity measured using the ABTS assay, there were no significant differences between bread formulas (basic versus enriched), bread portions (crust versus crumb), or lutein enrichment method (powder versus liquid). The ORAC assay revealed significantly higher antioxidant capacity in bread enriched with lutein than non-enriched bread. The results indicate that enrichment of wholegrain products with lutein could provide an important source of dietary antioxidants.
4.2. Introduction

Dietary antioxidants could prevent or mitigate oxidative damage to cellular components through their ability to scavenge reactive oxygen species and other free radicals (Abdel-Aal and Rabalski 2013). Dietary antioxidants include a variety of components such as carotenoids, phenolic acids, flavonoids, tocols and anthocyanins. They are present in wholegrain foods at various extents subject to grain type, product type and processing technology. At present a number of antioxidant assay are available which are either based on hydrogen atom transfer such as inhibition of induced low-density lipoprotein autoxidation and Oxygen radical absorbance capacity (ORAC), or based on electron transfer such as 2, 2-diphenyl-1 picrylhydrazyl (DPPH) and 2, 2 azino-bis (3-ethylbenzthiazoline-6sulfonic acid) (ABTS) (Huang et al. 2005). Due to the variety of antioxidants in a food matrix the use of a single antioxidant assay to measure antioxidant capacity is inadequate and more than one assay should be used to properly determine the antioxidant capacity (Abdel-Aal and Rabalski 2013). In the current study, the antioxidant properties were measured based on three assays, DPPH, ABTS and ORAC using “QUENCHER’’ method which is QUick, Easy, New, CHEap and Reproducible” (Serpen et al. 2008). The method is based on the direct measurement of antioxidant capacity by mixing samples with the reagents followed by a subsequent spectrometric measurement (Serpen et al. 2008).

Wholegrain products are considered a good source of dietary fiber and phytonutrients known as bioactive compounds. The bioactive compounds found in grains and seeds include carotenoids, phenolic acids, tocopherols, tocotrienols, anthocyanins and flavonoids (Ragaee et al. 2012). They are associated with the promotion of human health and reduced risk of chronic diseases such as cardiovascular disease (Osganian et al. 2003), cancer (Michaud et al. 2000) and diabetes (Slavin et al. 2001).

Several high-lutein functional foods using naturally high-lutein grain materials or
Lutein-enrichment grain flours as a source of antioxidants and dietary fiber were developed (Abdel-Aal et al. 2010). Lutein is the primary yellow pigment carotenoid found in wheat. Its concentration was found to be between 77-83% of the total carotenoids in high lutein wheat species such as einkorn, Khorasan and durum (Abdel-Aal et al. 2007). It was found at relatively lower concentrations in white and red wheat as compared to einkorn and durum wheat. Lutein was also found at high concentration in vegetables such as spinach and kale (Perry et al. 2009), eggs yolk (Schaeffer et al. 1988) and pasta (Humphries and Khachik 2003). Lutein plays significant roles in promoting the health of eyes and skin (Abdel-Aal and Akhtar 2006) and in reducing the risk of age-related macular degeneration (AMD) (Bone et al. 2001), cataracts (Olmedilla et al. 2001), cancer (Michaud et al. 2000) and cardiovascular disease (CVD) (Osganian et al. 2003). Abde-Aal et al. (2010) have studied the stability of lutein in bakery products that indicated significant reductions of lutein (28-62% loss) subject to product type and baking ingredients and conditions. But when the products were enriched with lutein, reasonable concentrations of lutein were found in the final baked products.

The use of einkorn as a high lutein wheat with or without enrichment of flours with lutein showed the potential to boost lutein content in baked products (Read 2011). Abdel-Aal and Rabalski (2013) have demonstrated the ability of high-lutein wholegrain bread, cookie and muffin to scavenge peroxyl, ABTS and DPPH radicals. Since lutein is a lipophilic compound, its incorporation into a bread formula could be a big challenge. In a previous study, lutein was successfully added to cookie and muffin as compared with flat bread due to their higher fat content (Read et al. 2015). The current study is aimed to investigate the effect of two different bread formulas (basic and enriched) and three forms of lutein (lutein powder, lutein in oil emulsion and lutein in ethanol suspension) on antioxidant capacity of bread products using three antioxidant assays namely, DPPH, ABTS and ORAC. Stability of lutein and its content in bread was discussed in chapter 3 and also reported in previous studies.
4.3. Materials and Methods

4.3.1. Materials

Wholegrain flour was obtained from P&H milling group (Cambridge, ON, Canada). The wholegrain flour contains protein, ash and total dietary fiber at average of 13.7, 1.4 and 11.5%, respectively. Crisco shortening and oil, commercial dry yeast, sugar, salt and whey protein were purchased from the retail market in Guelph, ON, Canada. Fluorescein, 2,2’-Azobis (2-methylpropion-amidine) dihydrochloride (AAPH), 2, 2 azino-bis (3-ethylbenzthiazoline-6sulfonic acid) (ABTS), 2, 2-diphenyl-1 picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and potassium persulfate were purchased from Sigma (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). Ethanol (95%) was purchased from Commercial Alcohols (Brampton, ON, Canada).

4.3.2. Methods

4.3.2.1. Preparation of lutein formulations

Lutein was obtained in two forms, lutein powder (85%) and lutein paste (20%) from (Lyc-O-Lutein, LycoRed Corp., Orange, NJ, USA). The concentration of lutein in each product was confirmed using HPLC as outlined later. Lutein powder was used “as is”, and was added to the wholegrain flour in a dry mix. Lutein paste was used to prepare lutein oil emulsion and lutein ethanol suspension. Lutein oil emulsion was prepared by solubilizing 160 mg of the lutein paste in 5 mL oil in the presence of 160 mg whey protein as a stabilizer. Lutein in ethanol suspension was prepared by solubilizing 160 mg of the lutein paste in 5 mL ethanol in the presence of 160 mg whey protein. Lutein enrichment was performed to achieve a level of about 1.0 mg of free lutein/serving of baked product (30 g).

4.3.2.2. Preparation of wholegrain bread

The approved methods, optimized straight dough (Method 10-10-03) and basic
straight dough (Method 10-09.01) (AACC 1) were used to prepare one bound pan bread loaf from wholegrain flour with and without lutein enrichment. Water absorption of wholegrain flours was previously determined using Farinograph-E (C.W. Brabender Inc., Hackensack, NJ, USA). The average water absorption of wholegrain flour was approximately 70%. Wholegrain flour was enriched with the three lutein forms (powder, oil emulsion, ethanol suspension) during mixing with other baking ingredients. The lutein powder were carefully and evenly distributed with flour in a dry mix, while lutein in oil emulsion and lutein in ethanol suspension were added into flours with other ingredients (yeast suspension, sugar solution, water) during mixing step.

4.3.2.3. Bread preparation for analysis

Control (non-enriched) and lutein-enrichment breads were taken from the oven and left at room temperature for about 2 hr to cool down. Bread loaves were cut with an electric knife into crust 21.5% (carefully removed without crumb), top crumb 37.6% (under crust crumb that close to crust taken about 1 cm layer) and center crumb 40.8% (the inside center of crumb). Each part was cut into slices and dried in an oven at 35°C. Dried bread samples were milled using UDY Cyclone mill equipped with a 0.5 mm screen and kept at -20°C for further analysis.

4.3.2.4. DPPH radical scavenging capacity assay

A stock solution of 2, 2-diphenyl-1 picrylhydrazyl (DPPH) was prepared daily by dissolving 40 mg of DPPH in 100 mL of ethanol, and then diluted with 100 mL of deionized water. A working DPPH solution was prepared by diluting 200 mL of stock solution with approximately 800 mL of 50% ethanol to obtain a solution having an absorbance value of 0.75–0.80 at 525 nm (Brand-Williams et al. 1995; Serpen et al. 2012). Direct QUENCHER method was used in this study (Serpen et al. 2012). Ten mg ±1.0 mg of ground bread samples was weighed into a centrifuge tube. The antioxidant reaction was initiated by adding 10 mL
of the working DPPH solution. The tube was shaken rigorously for 1 min and placed on an orbital shaker in the dark. The mixture was shaken at 300–400 rpm at room temperature to facilitate the surface reaction between the solid bread particles and the reagent. Centrifugation was performed at 9,200g for 2 min. The clear supernatant (2 mL) was transferred into a cuvette and the absorbance was measured at 525 nm at room temperature. The inflection point (where the curve changes from convex to concave or vice versa) of DPPH was calculated by using regression analysis and approximation Polynomials to determine reading times (Table 4.1). Four different times (5, 31, 48 and 60 min) were chosen for the DPPH depletion measurement. The DPPH scavenge capacity of non-enriched and enriched wholegrain bread extract was measured and calculated as µmole trolox equivalents/g sample. A standard trolox solution was prepared in ethanol at a concentration range between 0 and 600 µg/mL. Exactly 0.1 mL of each trolox concentration was added to 9.9 ml of DPPH radical solution. After 30 min of incubation at room temperature, 2 mL of the reaction solution was transferred into a cuvette and the absorbance was measured at 525 nm. A reagent blank was prepared using deionized water instead of trolox solution. The determination coefficient ($R^2$) of the method is 0.9743 and regression equation is $y=0.0096x + 0.0608$.

4.3.2.5. **ABTS cation radical scavenging capacity assay**

A solution of 7 mmol/L ABTS was prepared by adding 5 mL of deionized water to 38.41 mg of ABTS and 2.45 mmol/L potassium persulfate solution was prepared by adding 5 mL of deionized water to 6.615 mg potassium persulfate. Five mL from each solution were mixed to a stock solution of ABTS which is kept in the dark at room temperature for 12–16 hr before use (Re et al. 1999). A working ABTS solution was prepared daily by diluting the 10 mL of stock solution with approximately 800 mL of 50% ethanol to obtain a solution having an absorbance value of 0.75–0.80 at 734 nm (Serpen et al. 2012). Direct QUENCHER
method was prepared as described above in DPPH assay. The inflection point of ATS was also calculated to determine reading times (Table 4.1). Four different times (5, 26, 45 and 60 min) were chosen for the ABTS depletion measurement. The ABTS scavenge capacity of non-enriched and lutein-enriched wholegrain bread samples was measured and calculated as μmole trolox equivalents/g sample. A standard trolox solution was prepared as explained above in DPPH assay. The determination coefficient of the method is 0.9733 and regression equation is y=0.0112x + 0.0611.

4.3.2.7. ORAC assay

The ORAC method is based on the method of Ou et al. (2001). Ten mg of ground samples was weighed into eppendorf tube with 1mL of 75 mM phosphate buffer pH 7.5. The mixture was vortexed for 1min and centrifuged at 9,000g for 45 sec. The sample extracts were diluted several times until an appropriate curve was obtained over the test period. Twenty-five μL sample extract, trolox standard solution or nano pure water (blank) were mixed with 150 μL of fluorescein in each of the 96 micro-plate well. The mixture was conditioned at 37˚C for 30 min, then 25 μL of 2,2’–Azobis (2-methylpropion-amidine) dihydrochloride (AAPH) as a peroxyl radical generator was added to start the decaying of fluorescein. The degradation of fluorescein progressed for 60 min in the heated chamber of BioTech FLX800TBI with the following settings: the fluorescence excitation 485 nm, the emission wavelength 528 nm, and reading was taken every min for 1 hr. The micro-plate fluorescent reader was operated by Gen 5 software version 1.11.5 (BioTek). In this assay, the scavenge capacity of wholegrain bread extracts was also calculated as μmole trolox equivalents/g sample. The determination coefficient of the method is 0.9906 and regression equation is y=0.2552x + 1.6293.

4.4. Statistical Analysis

All analyses were performed at least in duplicate for (crust, top crumb and center
crumb) and the mean values are reported. Analysis of variance was performed using IBM SPSS Statistics 21 software (Armonk, New York, USA). Significant differences (P <0.05) among means were detected using Tukey’s multiple range test at fixed level of α=.05.

4.5. Results and Discussion

4.5.1. Antioxidant properties measured by DPPH assay

Dietary antioxidants have the ability to scavenge reactive oxygen species and free radicals that could damage cellular components. Wholegrain foods are a good source of antioxidants and phytonutrients which could promote human health and/or reduce the risk of chronic diseases (Abdel-Aal and Rabalski 2013). Lutein-enriched wholegrain bread could play significant roles in human diet as being a good source of lutein and other antioxidant components such as polyphenols. The current study assessed antioxidant properties of lutein-enriched wholegrain bread products made from basic and enriched baking formulas in comparison with non-enriched bread. QUENcher method was used to evaluate the antioxidant capacity of wholegrain bread products to avoid the problems associated with extraction of antioxidants due to their diverse chemical structures as being hydrophilic and lipophilic compounds. In this method food samples directly interact with radical reagents. The QUENcher method was applied for DPPH and ABTS assays. No doubt, it would be preferable to use a single test to evaluate antioxidant or radical scavenging capacity, but currently there is no single test that could characterize antioxidant properties of foods due to the presence of multiple free radicals and oxidants in foods or biological systems. Such complex system would require more than one test for antioxidant measurement and characterization (Abdel-Aal and Rabalski 2013).

In the DPPH assay, the ability of food or food extract to scavenge the DPPH free radicals is measured through the reduction of the color intensity of the radical reduced in the presence of an antioxidant. The color intensity of DPPH radicals with no antioxidants or
grain extracts remains stable over the test time (Ragaee et al. 2012). Scavenging of DPPH radical allows evaluation of the electron-donating potency of food components (Brand-Williams et al. 1995). The DPPH radical scavenging capacity of lutein-enriched and non-enriched breads expressed as micromole trolox equivalents per gram is presented in Figure 4.1. The DPPH scavenging capacity was measured in the bread crust (Figure 4.1A), top crumb (Figure 4.1B) and center crumb (Figure 4.1C) to understand their contribution to antioxidant properties and changes in antioxidant components during baking process. The bread crust showed the highest ability to scavenge DPPH radicals despite its lowest content of lutein as it received the highest degradation in the bread crust (chapter 3). This indicates that other antioxidant components contribute to the DPPH scavenging capacity. Maillard reaction products could contribute to the overall scavenging capacity (Yilmaz and Toledo 2005). Lutein-enriched bread crusts had higher DPPH scavenging capacity than non-enriched bread crust. Previous studies have shown DPPH scavenging capacity of lutein-enriched flat bread, cookie and muffin products was significantly higher than non-enriched ones (Abdel-Aal and Rabalski 2013).

DPPH scavenging capacity of top crumb of breads from lutein-enriched and enriched formula showed higher DPPH scavenging capacity as compared to that made from basic formulas (Figure 4.1B). In addition, top crumbs from lutein-enriched breads had higher ability to scavenge DPPH than those from non-enriched or control breads. Once again this indicates the contribution of lutein to the DPPH scavenging attribute as previously reported (Abdel-Aal and Rabalski 2013). Figure 4.1C presents the DPPH scavenging capacity of the center crumb of lutein-enriched and non-enriched breads. In general, the center crumb had slightly lower DPPH scavenging capacity than that of the top crumb (Figure 4.1B). In other words the DPPH scavenging capacity progressively increased from the bread center to the top. This could reflect the extent of non-browning reactions occurred in the crumb and crust.
during baking. Yu and Nanguet (2013) have reported that the average scavenging ability decreased by 32% for whole wheat bread during making process. This loss might be because of the loss of phytochemicals due to high temperature during baking. The extraction of antioxidants from various food matrices (e.g. wheat flour versus bread) may also affect the level of antioxidant capacity (Yu and Nanguet 2013). The kinetics of scavenging capacity of DPPH in the presence of non-enriched and lutein-enriched wholegrain bread crust, top crumb and center crumb is shown in Figure 4.2A, B and C. The DPPH radical reaction exhibited a non-linear reaction pattern with a rapid increase during the first 30 min and after that the reaction rate steadily increased up to 60 min. The pattern had more than one inflection point indicating various reaction rates over the test period (Table 4.1). It was reported that the effectiveness of carotenoids as DPPH radical scavengers increased by the length of the effective conjugated double-bond system and by the addition of hydroxyl groups on the terminal rings (Jiménez-Escrig et al. 2000). In addition to the conjugated double-bond system, lutein contains 2 hydroxyl groups that could contribute to the DPPH scavenging property of bread products.

4.5.2. Antioxidant properties measured by ABTS assay

The ABTS assay measures the ability of antioxidant compounds to scavenge ABTS free radical cation based on electron-transfer donated by antioxidant compounds (Brand-Williams et al. 1995). The ABTS radical cation has a relatively stable blue-green color which is in the presence of an antioxidant such as 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) or potential antioxidants in material extracts, the color production will be suppressed to a certain extent in proportional to the concentration of antioxidants (Ragaee et al. 2012). The ABTS scavenging capacities of bread crust, top crumb and center crumb are presented in Figure 4.3A, B and C, respectively. There were no significant differences among the bread crusts of lutein-enriched and control breads made from both
formulas, basic and enriched, except for the crust from bread enriched with lutein in an oil emulsion in the enriched formula. This treatment was the lowest compared to other treatments. The ABTS scavenging capacity of top crumb showed insignificant differences between non-enriched (control) and enriched bread among all treatments except for the top crumb from bread enriched with lutein in an oil emulsion in the enriched formula. A similar trend was also found for the ABTS scavenging capacity of center crumb. Once again, only the center crumb from bread enriched with lutein in an oil emulsion in enriched formula significantly had lower ABTS scavenging capacity compared to the other treatments. The addition of lutein in an oil emulsion system may result in lowering the antioxidant reaction rate leading to lower ability to scavenge ABTS radical cation. The kinetics of scavenging capacity of ABTS in the presence of non-enriched and enriched wholegrain crust, top crumb and center crumb are shown in Figure 4.4A, B and C. The reaction is not linear and rapidly increased during the first 26 min and then the rate of reaction steadily increased up to 60 min. The ABTS reaction showed more than one inflection points indicating various reaction rates (Table 4.1). Abdel-Aal and Rabalski (2008) reported that scavenge capacity against ABST radical cation by wheat antioxidants would vary depending upon concentration of individual bioactive compounds in wheat extracts and their synergic effects.

4.5.3. Antioxidant properties measured by ORAC assay

ORAC assay measures the ability of compounds to react with physiologically relevant peroxyl radicals (Moore et al. 2009). It measures scavenging capacity of peroxyl radical (ROO•) based on calculating the net protection area under the time recorded for the fluorescein decay curve in the presence of antioxidants. The ORAC values, similar to DPPH and ABTS, are expressed as micromole trolox per gram. The ORAC values of the crust of breads made from enriched formula were significantly higher than that made with the basic formula (Figure 4.5A). Similar trends were found for top crumb and center crumb samples
(Figure 4.5B and C). For the enriched formula, the lutein-enriched bread crust, top crumb and center crumb had significantly higher ORAC values. This was also true for the top crumb of breads from the basic formula (Figure 4.5B). It seems that the addition of lutein to the enriched formula improve exposure of lipophilic antioxidants (e.g. lutein) to the peroxyl radicals. Yu and Nanguet (2013) reported that the increasing of antioxidant capacity after bread making process using ORAC assay is because that phytochemicals are more likely to be detected by ORAC method. Another reason for the increase in antioxidant capacity, measured by ORAC assay, after baking could be due to Millard reactions (Yilmaz and Toledo 2005). The study also reported that the antioxidants properties could be affected by the temperature, pH, water activity, reactants type and availability of oxygen.

4.6. Conclusion

This study investigated the antioxidant capacity of the breads (crust, top crumb and center crumb) enriched with three different forms of lutein. The bread crusts showed the highest DPPH scavenging capacity compared with top and center crumbs. The crust and both crumbs of enriched formula breads, in general, provide higher antioxidant capacity than the parts of basic formula breads due to Millard reaction. The ABTS scavenging capacity, in general, did not show significant differences among several enrichment breads’ parts and formulas. The ORAC values in bread crust and both crumbs in enriched formula presented better scavenging capacity of peroxyl radical compared with basic formula. The lutein enrichment of bread by using enriched formula could improve the consumption of dietary antioxidants and thus promote human health.
Table 4.1: Regression equations, determination coefficients and inflection points of DPPH and ABTS reactions with wholegrain bread samples

<table>
<thead>
<tr>
<th>Assay</th>
<th>Regression Equation and determination coefficients</th>
<th>Inflection points (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2(^{nd}) order</td>
<td>3(^{rd}) order</td>
</tr>
<tr>
<td>DPPH</td>
<td>$y = 7 \times 10^{-5} x^2 - 0.008x + 0.833$</td>
<td>$y = 3 \times 10^{-6} x^3 + 0.0003x^2 - 0.0131x + 0.8471$</td>
</tr>
<tr>
<td></td>
<td>$R^2 = 0.9712$</td>
<td>$R^2 = 0.9953$</td>
</tr>
<tr>
<td>ABTS</td>
<td>$y = 0.0002x^2 - 0.0217x + 0.7025$</td>
<td>$y = 8 \times 10^{-6} x^3 + 0.001x^2 - 0.0386x + 0.8471$</td>
</tr>
<tr>
<td></td>
<td>$R^2 = 0.9305$</td>
<td>$R^2 = 0.9784$</td>
</tr>
</tbody>
</table>
Figure 4.1: DPPH scavenging capacity of crust (A), top crumb (B) and center crumb (C) of breads made from basic and enriched formulas. LP-B, LOE-B, LES-B, LP-E, LOE-E and LES-E stand for lutein powder, lutein in oil emulsion, and lutein in ethanol suspension with basic baking formula; and lutein powder, lutein in oil emulsion and lutein in ethanol suspension with enriched baking formula, respectively. Different letters indicate significant differences between means at p<0.05 (standard deviation values presented by error bars).
Figure 4.2: Kinetics of DPPH with non-enriched and enriched wholegrain bread crust (A), top crumb (B) and center crumb (C) in breads made from basic and enriched formulas. LP-B, LOE-B, LES-B, LP-E, LOE-E and LES-E stand for lutein powder, lutein in oil emulsion, and lutein in ethanol suspension with basic baking formula; and lutein powder, lutein in oil emulsion and lutein in ethanol suspension with enriched baking formula, respectively.
Figure 4.3: ABTS scavenging capacity of crust (A), top crumb (B) and center crumb (C) of breads made from basic and enriched formulas. LP-B, LOE-B, LES-B, LP-E, LOE-E and LES-E stand for lutein powder, lutein in oil emulsion, and lutein in ethanol suspension with basic baking formula; and lutein powder, lutein in oil emulsion and lutein in ethanol suspension with enriched baking formula, respectively. Different letters indicate significant differences between means at p<0.05 (standard deviation values presented by error bars).
Figure 4.4: Kinetics of ABTS with non-enriched and enriched wholegrain bread crust (A), top crumb (B) and center crumb (C) in breads made from basic and enriched formulas. LP-B, LOE-B, LES-B, LP-E, LOE-E and LES-E stand for lutein powder, lutein in oil emulsion, and lutein in ethanol suspension with basic baking formula; and lutein powder, lutein in oil emulsion and lutein in ethanol suspension with enriched baking formula, respectively.
Figure 4.5: ORAC scavenging capacity of crust (A), top crumb (B) and center crumb (C) of breads made from basic and enriched formulas. LP-B, LOE-B, LES-B, LP-E, LOE-E and LES-E stand for lutein powder, lutein in oil emulsion, and lutein in ethanol suspension with basic baking formula; and lutein powder, lutein in oil emulsion and lutein in ethanol suspension with enriched baking formula, respectively. Different letters indicate significant differences between means at p<0.05 (standard deviation values presented by error bars).
CHAPTER 5:

CONCLUSIONS AND FUTURE WORK

Wholegrain is a good source of dietary fiber and bioactive compounds such as dietary fiber, β-glucan, tocopherols, tocotrienols, phenolic acids, anthocyanins, and phytosterols and carotenoids (Ragaee et al. 2012). Wholegrain foods such as bread are rich in minerals, vitamins and protein that contribute to human health benefits. They also contain a large number of phytochemicals that some of them have been linked with reduced risk of cardiovascular diseases (Osganian et al. 2003), type II diabetes (Slavin et al. 2001), cancer (Michaud et al. 2000), and other chronic diseases. Carotenoids, especially lutein and zeaxanthin, are considered the main yellow pigment found in wheat (Abdel-Aal et al. 2010). Lutein plays significant roles in the health of eyes and skin. It is also associated with the prevention or reducing the risk of age-related macular degeneration, cancer and cataracts (Mares-Perlman et al. 2002). Lutein and zeaxanthin are found in the yellow spot of the human retina providing many protective functions such as protection of the macula from damage by blue light and scavenging harmful reactive oxygen species (Handelman et al. 1999). They also inhibit the oxidation of vulnerable molecules in food products or human body. They have to be delivered in the diet because human body can’t generate them. The development of high lutein staple foods such as bread would increase the consumption of this important carotenoid.

The stability of lutein in processed food products is critical and warrants investigation. Lutein is sensitive component to heat and baking process that, in turn, affect its content and antioxidant properties. It is a lipophilic compound that could be degraded during baking due to isomerization and oxidization. The great loss of lutein occurred in high fat baked products such as cookie and muffin (Abdel-Aal et al. 2010). In a high fat product
lutein becomes more accessible to baking conditions (e.g. high temperature and oxygen) resulting in more lutein degradation. Wholegrain bread is considered as a low-fat product where lutein is less prone to oxidization and isomerization. Therefore, different approaches are being researched to improve incorporation and stability of lutein in wholegrain bread as well as increasing its antioxidant properties. The different forms of lutein (powder versus liquid), the method of adding lutein, baking ingredients and storage period were expected to affect incorporation and stability of lutein in wholegrain bread which consequently would affect the antioxidant capacity of the bread.

The hypothesis was proved to be true through this study. Wholegrain breads enriched with lutein in a dry mix or liquid form showed that the distribution and concentration of lutein in final product were affected by the lutein form. Lutein is well preserved in bread crumb compared to crust. The crust in both formulas (basic and enriched) exhibits more degradation of lutein content than top and center crumb. This indicates that more lutein degradation occurs in the crust than crumb, perhaps due to the higher temperature of the crust as compared to crumb during baking. The use of basic formula with lutein powder enrichment in a dry mix was effective as compared with the enriched formula. The presence of fat in the enriched formula could make lutein more easily accessible resulting in a significant reduction in the concentration of lutein. The lutein content in enriched and non-enriched breads was not affected by storage at room temperature up to 7 days.

Dietary antioxidants are essential to combat reactive oxygen species and free radical in human body. The lutein-enriched and non-enriched wholegrain breads are considered good sources of antioxidants. This research evaluates antioxidant capacity of lutein-enriched bread products based on DPPH, ABTS and peroxyl free radical. Bread portions were examined to determine their contribution to antioxidants properties. The crust showed higher ability to scavenge DPPH radicals although lutein content in bread crust was the lowest compared to
top and center crumb. Millard reaction products could contribute to the DPPH scavenging capacity of crusts. Center crumb of breads had the lowest contribution to DPPH scavenging capacity, which means that the scavenging ability increased from the bread center to the crust during baking. In general, enriched formula bread parts exhibited higher DPPH scavenging capacity compared to basic formula bread. For ABTS scavenging capacity bread parts, in general, showed no significant differences between lutein-enriched and non-enriched obtained from both formulas (basic and enriched). Regarding ORAC, the addition of lutein in different forms to enriched formula helped to improve the antioxidant capacity of crust, top crumb and center crumb of the bread. Yu and Nanguet (2013) indicated that phytochemicals are more likely to be detected by ORAC assay.

This study has shown a good potential for improving lutein stability in functional wholegrain bread. Wholegrain bread as a low-fat product could hold a promise for developing high lutein functional bread and boost the daily intake of lutein. For lutein to deliver its anticipated health benefits, it has to be bioavailable. Therefore, lutein content as well as its bioavailability is required to determine the efficacy of high-lutein functional foods. In this regard more research is needed to determine bioavailability of lutein-enriched low-fat pan bread in vitro and in vivo. In terms of antioxidant properties, wholegrain bakery products are good sources of antioxidants but further research to evaluate antioxidant activity using cell-based assays and human studies should be considered.
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