

**Evaluation of antioxidant capacity and vitamin E  
content in barley grains (*Hordeum vulgare L.*) and  
the impact of processing and storage**

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## Abstract

Antioxidants, including vitamin E, may have a positive effect on human health but may be affected during storage, malting and/or other processing such as making pita bread. Those components are reported to be higher in barley compared to wheat and other grains. Therefore, as well as being a source of fibre, barley has potential for use in foods. However, there is limited knowledge about how vitamin E and antioxidant capacity might change in the barley food chain; and; whether there is genetic variability for these and what the genetic basis of differences between barley genotypes might be. Therefore, this research aimed to address the following questions: Do different barley genotypes have different antioxidant capacity, vitamin E content and isomers? What is the genetic basis of any differences in vitamin E content and antioxidant capacity observed? How are vitamin E and antioxidant capacity affected by common industrial practices for storage and processing (pearling, malting and baking) for barley genotypes high in those components?

Vitamin E content and antioxidant capacity were measured in 25 barley genotypes using high performance liquid chromatography (HPLC) and ability to scavenge DPPH radicals, respectively. Vitamin E comprises eight isomers:  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocopherol (T) and  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocotrienol (T3). As expected,  $\alpha$ -tocotrienol ( $\alpha$ -T3) and  $\alpha$ -tocopherol ( $\alpha$ -T) were the predominant tocol isomers. Vitamin E content and antioxidant capacity varied significantly among those genotypes. Vitamin E ranged from 8.5 to 30.8  $\mu\text{g/g}$  dry weight (DW) while ascorbic acid equivalent antioxidant capacity (AEAC) varied from 57.2 to 158.1 mg AEAC/100 g fresh weight (FW). Generally, lower vitamin E content or antioxidant capacity was observed in all

coloured (Jet, Sumire mochi, ICARDA 16, ICARDA 19, ICARDA 26, ICARDA 35 and ICARDA 39) or hullless genotypes (Jet, Sumire mochi and Macumba) except for the hullless variety Finniss. Results suggest some genotypes are potential candidates for breeding of barley cultivars with high vitamin E content or antioxidant capacity.

To determine the genetic basis of differences in vitamin E content and antioxidant capacity, the measurement of these compounds was conducted across two years for the Amaji nijo x WI2585 and Tadmor x ERApm mapping populations respectively. Quantitative trait loci (QTLs) were detected for vitamin E, two major isomers and two minor isomers. QTLs were identified on chromosome 7H for vitamin E (LOD=3.4 and 4.2), 7H for  $\alpha$ -T (LOD=3.32), 5H and 7H for  $\alpha$ -T3 (LOD=3.74 and 3.90 respectively), 2H for  $\beta$ -T (LOD=3.27), 4H and 5H for  $\beta$ -T3 (LOD=3.76 and 3.50 respectively) and 2H for  $\gamma$ -T (LOD=3.10). Some QTLs overlapped each other and associated markers have also been linked to other traits such as frost, salt and black point tolerance, all of which have all been correlated to antioxidant capacity. However, no QTL was found for antioxidant capacity in the Tadmor x ERApm mapping population.

Four months storage at 10°C decreased antioxidant capacity in all genotypes except the coloured genotypes (ICARDA 19, ICARDA 26, ICARDA 35, ICARDA 39, Sumire mochi and Tadmor) but vitamin E content was increased in all genotypes. Vitamin E content was significantly lower in the steeping, germination and kilning stages of malting for all genotypes compared to the unprocessed samples. However, the antioxidant capacity in the malt was higher than in the unprocessed samples for the majority of the genotypes. A strong correlation ( $r=0.9$ ,  $n=14$ ,  $p<0.05$ ) between

antioxidant capacity before and after malting indicated that barley varieties which have higher antioxidant capacity at harvest retain their antioxidants after malting.

The same trend was observed in pita bread made with a substitution of wheat flour with barley flour made from whole grain, pearled grain (at 10%, 15% or 20% pearling) or malt. Higher antioxidant capacity and vitamin E content were observed in barley flour, even when made from pearled grain, and consequently they remained higher in barley pita after baking. Pitas made with barley flour from malt, Finniss whole grain or 15% pearled WI2585 were acceptable to consumers and retained similar physical and sensory properties to those in the control pita indicating the potential use as functional food.

In summary, this study has shown that (i) certain barley genotypes have potential for use in pita bread due to their high antioxidant capacity and/or vitamin E content at harvest and the maintenance or increase of that during storage and processing; and (ii) the identification of QTL and genetic variability in vitamin E will allow barley breeding programs to develop varieties with greater vitamin E content. Further research could identify candidate genes responsible for vitamin E and antioxidant capacity in barley grains and should also ensure the bioavailability of vitamin E and antioxidant capacity to confirm its functionality in the human diet.

## **Declaration of Authorship**

I declare that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma, in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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**Thi Thu Dung Do**

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## Abbreviations

AEAC	ascorbic acid equivalent antioxidant capacity
ANOVA	analysis of variance
ATR	attenuated total reflectance
CAT	catalase
c-TMT	c-tocopherol methyltransferase
DH	doubled-haploid
DHAR	dehydroascorbate reductase
DMPBQ	2,3-dimethyl-5-phytyl-1,4-benzoquinone
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DW	dry weight
FW	fresh weight
GGPP	geranylgeranyl pyrophosphate
GSH	glutathione
GSSG	glutathione disulphide
GPX	glutathione peroxidase
GR	glutathione reductase
HGA	homogentisic acid
HPLC	high performance liquid chromatography
HPT	homogentisate phytyltransferase
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
LSD	least significant difference
MIR	middle infrared

MPBQ	2-methyl-6-phytyl-1,4-benzoquinone
MPBQ MT	MPBQ methyltransferase
n/a	not applicable
NIR	near infrared
NHMRC	national health and medical research council of Australia
NP-HPLC-FLD	normal phase HPLC using fluorescence detector
NS	no significant difference
PC	principal component
PCA	principal component analysis
PDP	phytyldiphosphate
PLE	pressurized liquid extraction
PLS-DA	partial least squares discriminant analysis
PRESS	predicted residual error sum of squares
PP	phytylphosphate
QTL	quantitative trait loci
RSD	relative standard deviation
SFE	supercritical fluid extraction
T	tocopherol
T3	tocotrienol
TC	tocopherol cyclase
TE	$\alpha$ -tocopherol-equivalents
$t_r$	retention times
WG	whole grain

# **Chapter 1 LITERATURE REVIEW**

## 1.1 Barley and its uses

Barley (*Hordeum vulgare L.*) belongs to the Poaceae family, the tribe Triticeae, the genus *Hordeum* and originated from the Middle East (Bhatty 1993). Barley can be classified by vegetative stage, flowering stage, maturity, head characters or grain characters. However, classification is usually based on whether the head type is two or six-rowed (depending on the kernels' physical arrangement) and the intended use for that (malt or animal feed).

Barley is one of the most important crops in the world. Its annual production (129 million tonnes, 2002-2006 mean) ranks fifth behind maize (*Zea mays*, 610 million tons), wheat (*Triticum spp*, 551 million tons), rice (*Oryza sativa*, 429 million tons) and soybean (*Glycine max*, 180 million tons), and ahead of sugar cane (*Saccharum spp.*, 93 million tons), potato (*Solanum tuberosum*, 60 million tons) and sorghum (*Sorghum bicolor*, 50 million tons) (Ullrich *et al.* 2008). The Food Agricultural Organisation (FAO) also reported that in 2007, with an average yield of 2.4 tons per ha, world barley production reached 136.2 tons and in 2008 barley output worldwide increased significantly by some 10% (nearly to 148 million tons) (Ullrich *et al.* 2008). Australia is one of the top ten countries contributing to this barley production as barley is the second most important cereal crop after wheat in this country (Murray and Brennan 2010). According to Fitzsimmons and Wrigley (2000), since 1990, barley growth has increased dramatically in Australia, with a tenfold increase in New South Wales, for example. Punda (2009), also reported that Australia is the highest barley exporter world-wide (24%), followed by France (22%), Germany (11%), Canada (8%) and Russia (5%). However, the application of barley for food world-wide in general, and Australia in particular is limited. About two-thirds of the barley crop has been used for

animal feed and one-third for malting, with only about 2-3% used directly for human consumption (Akar *et al.* 2004). Along with global trends, Australians consume only 0.3 kg per capita annually even though they produce a huge amount of barley (Punda 2009).

Barley is mainly consumed by livestock. In Spain, France and Germany, for instance, feed consumption accounts for 87, 85 and 72% of total production, respectively (Akar *et al.* 2004). Due to its lower protein content and uniform grain size, two-row barley is more suitable for malt production while six-row barley is used for animal feed (Punda 2009). However, both are used for animal feed with commonly grown two-row varieties in Australia including Brindabella, Bandulla, Cantala, Chebec, Ulandra and Yambla; and common six-row varieties including Abyssinian, Beecher and Yerong (Fitzsimmons and Wrigley 2000; Menz 2010).

Barley is also used for malting which is a source for various processes such as brewing, distilling and vinegar production (MacGregor and Bhatta 1993). For brewing, barley is the best material among other grains like wheat and maize because of its germination, filtering and firm texture (Akar *et al.* 2004). The selection of malting barley is based on the combination of low protein, high malt extract, high yield, low modification, and high germination ability. Other factors involved are starch content, enzyme activity and flavour compounds for distilling and aroma of beer making (Punda 2009). For these features, the cultivars Bass, Baudin, Granger, Scope, Schooner, Navigator and Westminster were used commonly as malting barley in Australia (Paynter *et al.* 2015; Wheeler 2015). More recently cultivars such as Gairdner, Flagship and Commander, Buloke and Vlamingh are used in the malting and brewing industries.

A minor use of barley grain is for food where both hulled and hull-less are utilised. Hulled barley has grains with adhering hulls, while hull-less barley (husk-less or naked) has grains without adhering hulls. Hull-less barley is preferred and used over hulled barley in food production due to several reasons: (1) less processing being required (Elsayed and Peter 2005); (2) the grain containing more protein, starch and total soluble fibre due to the need not to remove hulls (Bhatty 1999); and (3) the grain can be added directly to food without preparing extract and syrups (Elsayed and Peter 2005). Hull-less barley is ideal for the human diet but could also be used for feed for livestock. However, Bhatty (1999) reported that crude fibre is higher in hulled seeds compared to hull-less seeds (3-6% vs 1.5-3%). Hence, in order to provide a rich crude fibre feed for animals, feed barley should be made from hulled grains rather than hull-less grains. In terms of malting barley, hulled grains should be utilised because they contain husk which can control germination in malting (Punda 2009). According to Quinde *et al.*, (2004), both hulled and hull-less varieties are used for food in Australia including cultivars such as Crest, Morex and Kold (hulled barley); Condor and Finnis (hull-less barley). Hindmarsh is reported as a more recently cultivar grown in Western and Southern Australia (Paynter *et al.* 2015; Wheeler 2015). In conclusion, not only hulled barley grain but also hull-less grain has great potential for use in human food.

### ***1.1.1 Barley food uses***

Barley has been used as a food source world-wide, due not only to its taste but also natural flavour and colour. In Asian countries, including Japan, India, Tibet and Korea, barley has been popularly used for traditional dishes (Elsayed and Peter 2005). The preferred drink, Sattu, consumed widely in India, for example, is made from ground

toasted barley with sugar and water (Paras and Gujral 2011). In Western countries, barley has been an ingredient of many products such as cakes, bread, breakfast cereals, snacks, soups, noodles and baby food (Bhatty 1993). With regards to baby food, malt is an excellent material due to high enzyme activity, particularly  $\alpha$ - and  $\beta$ -amylases. These enzymes can convert starch to maltose which can be digested more easily than other types of starch (Bhatty 1999). For this reason, barley malt flour and extracts are also utilised for bread with the further advantage of yeast activity promotion (Bhatty 1999). A ratio of either 50:50 or 65:35 barley-rice is suggested for nutritious ready-to-eat cereal with good sensory characteristics of shape and crispness (Berglund *et al.* 1994), whereas 100% barley flour is suitable for quality cookies (Baik and Ullrich 2008). However, in normal bread (also called loaf bread or U.S pan bread) and instant noodles, a high amount of barley causes limited loaf volume leading to a hard texture of bread, and darkness, hardness and chewiness of noodles. Therefore, only 15-30% (Trogh *et al.* 2005) and 20-30% barley flour (Baik and Czuchajowska 1997) are acceptable for normal bread and noodles, respectively.

An alternative to normal bread may be the flat breads which include pita bread (also called pocket bread or Egyptian Balady bread) and Turkish flat bread (also called Bazlama). Pita bread forms pockets during baking while Turkish flat bread does not. This pocket allows food to be easily added inside pita bread making it a popular choice with consumers (Morad *et al.* 1984; Basman and Koksel 1999; Blandino *et al.* 2015). Pita bread also requires less leavening than normal bread therefore providing the possibility to increase the ratio of barley used (Blandino *et al.* 2015).



### ***1.1.2 Barley product processing***

Processes such as steaming, toasting, germination, fermentation and extrusion cooking are applied to produce popular products such as bread, cereal and baby food (Fernandez-Artigas *et al.* 2001). Extrusion cooking is a process by which food is heated under high pressure then slowly forced through fine pores. As this process takes place, water evaporates rapidly and food is dried, cooked and textured. To make bread, mixed flour will be followed by fermentation and baking steps (Hoseney 1994). To make ready-to-eat cereal, grain will be either extruded or steamed. During extrusion, flour is put into the extruder at 123-125°C and extruded cereal is subsequently toasted at 176°C for 15 min (Berglund *et al.* 1994). With steaming, the whole kernel is pressure steamed and then flaked to make smooth rolls (Hoseney 1994). Both extrusion and steaming are suitable for making baby food. However, the difference between commercial cereal and infant cereal is enzyme addition and product shape (Angle 1994). Baby foods based on ground cereals must also have  $\alpha$ - and  $\beta$ -amylases added, due to babies' limited digestive ability (Fernandez-Artigas *et al.* 2001). Therefore, barley malt flour and extracts are potentially ideal material for baby cereal-based food.

### ***1.1.3 Barley as a functional food***

Functional food can be defined as a conventional food that meets consumer needs for general health and wellbeing, and the prevention and management of compromised health conditions (Tapsell *et al.* 2005). As a possible constituent of so many food products, barley has potential for use in functional food for a number of reasons. A major advantage of barley is its high dietary fibre content (14-24% including 11-14% insoluble and 3-10% soluble dietary fibre) which has been credited with promoting

heart, cholesterol and digestive health (Panfili *et al.* 2008; Levitsky and Pacanowski 2011). In comparison with other cereals, barley also has a high level of  $\beta$ -glucan (4-9%) which contributes to the dietary fibre content. Extensive research has shown that high  $\beta$ -glucan content is linked to lower blood cholesterol level and cancer risk (Panfili *et al.* 2008). Another function of  $\beta$ -glucan is that it may aid in weight loss as a consequence of induction of satiety after barley consumption (Levitsky and Pacanowski 2011).

In addition, the type of barley starch is an important contributor to improving human health. With a 3:1 ratio of amylose to amylopectin in starch, high amylose-barley (BARLEYmax, for example) can prevent and manage type II diabetes, heart disease, colon and rectal cancers, obesity, constipation and diverticular disease (Topping *et al.* 2003). Jenkins *et al.*, (2008) also indicated that high resistant starch in rich amylose-barley results in a low glycaemic index (a measure of the effects of carbohydrates on blood sugar levels). For this reason, BARLEYmax was produced commercially as a primary resistant starch product (Matthews and McCaffery 2011).

Furthermore, barley is considered to be a good source of protein and contains more of the essential amino acid lysine compared to other cereals (John *et al.*, 2011). The total protein content in barley (13.2%) is comparable with wheat (13.5%) and higher than that of rice (12.2%), sorghum (11.0%) and corn (8.8%). Within protein, a higher content of lysine is observed in barley (0.5%) in comparison with wheat (0.35%), rice (0.4%), sorghum (0.27%) and corn (0.21%) (John *et al.*, 2011).

Food may also be functional due to antioxidant activity. Although the potential roles of antioxidants need further study (Bjelakovic *et al.* 2007), clinical trials of antioxidants suggested that they can prevent and combat diseases (Block 1991;

Serafini *et al.* 1996; Temple 2000; Young and Woodside 2001; Axelsen *et al.* 2011; Ostertag *et al.* 2011). Antioxidants are highly concentrated in barley, especially vitamin E whose content reportedly ranges from 30.5 mg/kg to 80.6 mg/kg (Elsayed and Peter 2005). Zhao *et al.*, (2008) also reported from 14 barley varieties, the antioxidant activity or value of DPPH radical scavenging activity ranged from 9.33 to 11.78  $\mu\text{mol}$  Trolox equivalents (TE) per g dry weight (DW). In comparison with other rich antioxidant source, antioxidant activity in barley was higher than each fruit cedar bay cherry (9.57  $\mu\text{mol}$  TE/ g DW), Illawarra plum (8.85  $\mu\text{mol}$  TE/ g DW) and molucca raspberry (5.29  $\mu\text{mol}$  TE/ g DW) (Netzel *et al.* 2006). For this reason, the future of barley for use in food as an antioxidant source is therefore promising provided they are bioavailable. The term bioavailability refers to the proportion of a compound absorbed and utilised from the intestine by the body (Ball 2006). Vitamin E bioavailability differs among products (Reboul *et al.* 2006), hence, the understanding of whether the vitamin E in barley is readily bioavailable needs to be ascertained.

## **1.2 Antioxidants**

### ***1.2.1 Free radical reaction and disease***

Free radicals are defined as any atom which contains at least one unpaired electron in the outermost shell. This unpaired electron makes them extremely reactive with other components within the living cell, allowing damage to occur. Free radicals including the hydroxyl radical ( $\text{OH}^\cdot$ ), superoxide and its protonated form, the perhydroxyl radical ( $\text{O}_2^-/\text{HO}_2^\cdot$ ), singlet oxygen ( $\text{O}^\cdot$ ) and the nitric oxide radical ( $\text{NO}^\cdot$ ) are produced naturally during metabolism or by toxic environments such as pollution, cigarette smoke, herbicides and radiation (Young and Woodside 2001). The hydroxyl radical is

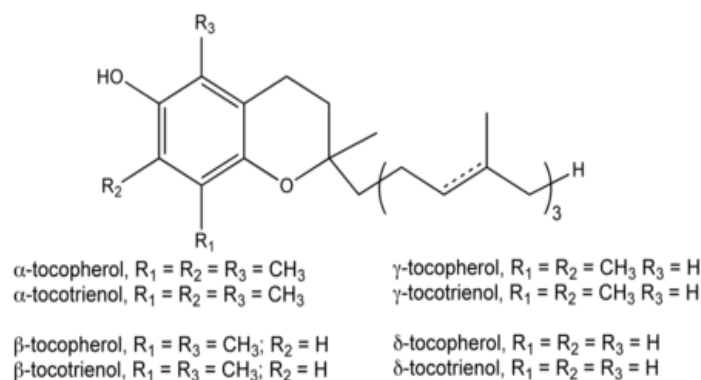
generated when electromagnetic radiation splits water in the body but  $O_2^-/HO_2^-$  and  $NO^\cdot$  can be byproducts of respiration and therefore unavoidable (Gutteridge and Halliwell 2000). Even though some  $O_2^-/HO_2^-$  and  $NO^\cdot$  are necessary for signaling within physiological processes (Young and Woodside 2001), free radicals are generally considered toxic because they react so readily with compounds in the cell. Another free radical, the peroxy radical ( $ROO^\cdot$ ) is formed when the H atom is replaced by an organic group (Marnett 1987). This radical is formed in biological systems in which its principal pathway is auto-oxidation such as lipid peroxidation (Zeev 1997).

Epidemiological studies have suggested free radicals cause chronic diseases such as heart disease, diabetes (Willet 1994), atherosclerosis, low density lipoprotein (Young and Woodside 2001), cancer, cardiovascular (Layton *et al.* 1992), rheumatoid arthritis, inflammatory bowel and Parkinson's disease (Halliwell 1996). In order to avoid these diseases, antioxidants are therefore considered to be an important protectant.

### ***1.2.2 Antioxidant defence***

Antioxidants can prevent cell damage because they act as scavengers, preventing the formation of free radicals or promoting their decomposition (Young and Woodside 2001). Antioxidants include vitamin E, vitamin C, carotenoids, phenolics and antioxidant enzymes. Each type of antioxidant has a different mechanism for scavenging free radicals.

Vitamin E has eight different forms:  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ - tocopherol (T) and tocotrienol (T3), collectively called tocopherols. Vitamin E is the most important lipid phase chain breaking antioxidant due to its unsaturated tail (Figure 1.1).



**Figure 1.1 The structure of eight tocol homologues (Burton and Ingold 1981)**

For this reason, the peroxy radicals ( $\text{ROO}^\cdot$ ) quickly react with vitamin E instead of polyunsaturated fatty acids. Hence, polyunsaturated fatty acids are protected and lipid peroxidation is prevented. Tocopheroxyl radical, the product of the reaction between vitamin E and  $\text{ROO}^\cdot$ , then combines with another tocopheroxyl radical to become stable. Tocopheroxyl radical can also join with tocopherol quinone or vitamin C to transfer back to tocopherol (Burton and Ingold 1981).

The crucial function of vitamin E in the role as an antioxidant is indicated not only by this mechanism but also in epidemiological studies. For instance, vitamin E is mentioned as a main preventative of cardiovascular disease (Reboul *et al.* 2006). The dietary recommendation for vitamin E is 15 mg of  $\alpha$ -T daily, and the estimated average requirement is 12 mg as a preventative factor for cardiovascular disease, cancer and Alzheimer's disease (Gassmann 2000). However, there is some controversy about this since Bjelakovic *et al.*, (2007) reported that vitamin E supplements significantly increased mortality.

Moreover, the bioavailability value of vitamin E in bread has been demonstrated to be higher than that in other products. Bread appears to have much

more accessible values than others (almost 100% in wheat bread versus less than 1% in apple and below 30% in dairy products) (Table 1.1). This can be explained by the dependence on chemical forms and other gathered components of the ingested food matrix such as fibre and lipids which can interfere or stimulate vitamin E utilisation by the body (Reboul *et al.* 2006).

**Table 1.1 Bioaccessibility of vitamin E from food**

Products	$\alpha$ -T (%)	$\beta$ - T (%)	References
Apples (fresh)	0.47 $\pm$ 0.13	6.54 $\pm$ 2.60	(Reboul <i>et al.</i>
Bread (white wheat bread )	99.62 $\pm$ 11.30	8.36 $\pm$ 4.60	2006)
Cheese	28.67 $\pm$ 6.22	59.09 $\pm$ 9.17	(Leonard <i>et al.</i>
Milk	21.95 $\pm$ 1.60	6.76 $\pm$ 2.59	2004)

While vitamin E seems to be the most efficient antioxidant which breaks the chain reaction of lipid peroxidation, vitamin C is considered to be an aqueous phase chain breaking antioxidant for two reasons. Firstly, vitamin C may inhibit nitrosamine caminogenesis which are the mutated compounds made when nitrite reacts with amine. In other words, vitamin C can prevent DNA damage. In addition, vitamin C plays an important role in combating lipid peroxidation by assisting  $\alpha$ -T. The tocopheryl radical can be recycled by combining with vitamin C to form tocopherol quinone (Halliwell 1994). In fact, epidemiological data shows that vitamin C intake has been associated with a decreased risk of oesophagus, stomach and lung cancers (Block 1991; Byers and Guerrero 1995; Temple 2000).

Similar to vitamin E, carotenoids are within the lipid soluble group and might have the same function in inhibiting lipid peroxidation.  $\beta$ -carotenoid traps the peroxy

radical and reacts with singlet oxygen (Fukuzawa *et al.* 1998). Other reports also indicate that carotenoids may have an antioxidant function *in vivo* under certain conditions (Gottlieb *et al.* 1993; Krinsky 1993; Dixon *et al.* 1994). Although there is a hypothesis that carotenoids may use a similar mechanism to scavenge free radicals as vitamin E, its bioavailability is not clear. Bioaccessibilities measured for vitamin E were much higher than those for carotenoids (Reboul *et al.* 2006). However, Halliwell (1996) provided evidence of no change in health promotion of smokers who consumed carotenoids. This is in agreement with a clinical trial which demonstrated no evidence of a relationship between cancer prevention and carotenoid consumption (Hennekens *et al.* 1996; Heinonen *et al.* 1998).

In contrast to carotenoids, there is strong epidemiological evidence that phenolic compounds can help to prevent chronic diseases such as coronary heart disease exists (Hertog *et al.* 1993; Rimm *et al.* 1996). According to Podsedek *et al.*, (2000) phenolics might also prevent brittleness and excessive permeability of capillary blood vessels. Lee *et al.*, (2003) also showed that phenolics can protect cells from damage caused by free radicals. However, phenolic compounds are probably only efficient with a high intake because only high concentrations of some phenolic compounds (more than 100  $\mu\text{M}$ ) show the inhibition of platelet activation and aggregation *in vitro* (Ostertag *et al.* 2011). Moreover, the understanding of how phenolics act as antioxidants is limited. Some reports show that the bioavailability of phenolics is poor (Hollman *et al.* 1995; McAnlis *et al.* 1999), and most phenolic compounds were found in human urine (Ostertag *et al.* 2011), whereas other findings suggest intake of phenolics is important to enhance total antioxidant level (Serafini *et al.* 1996).

Besides phenolics and vitamins, various antioxidant enzymes including catalase, glutathione peroxidase and superoxide dismutase efficiently protect against oxidants in biological systems. Catalase and peroxidase remove hydrogen peroxide ( $H_2O_2$ ) within cells to prevent the damage normally caused by its reaction with other compounds leading to the formation of  $OH^\cdot$  (Halliwell and Gutteridge 1989). Catalase catalyses the reaction from  $H_2O_2$  to oxygen and water, while glutathione peroxidase catalyses the reaction between  $H_2O_2$  and glutathione (GSH) to form water and glutathione disulfide (GSSG) (Jones 2002). These antioxidant enzymes are present in all major body organs and their enzyme capacity follows the descending order liver, erythrocytes, brain, heart and skeletal muscle (Halliwell and Gutteridge 1989). The antioxidant enzymes cannot be taken up from food like antioxidant phenolics and the vitamins (Young and Woodside 2001). Therefore, for the purpose of functional food consumption (and in particular, using barley), only phenolics and vitamins would be considered.

### **1.3. Antioxidants in barley**

#### ***1.3.1 Antioxidant composition in barley***

Tocols (vitamin E), carotenoids, phenolics and enzymes are the main antioxidants found in grain, as the vitamin C content is negligible or not detected (US Department of Agriculture, 2011). Goupy *et al.*, (1999) reported that the antioxidant compounds in barley can be divided into two types, either apolar or polar compounds. The former are the apolar compounds combining tocols and carotenoids (especially lutein and zeaxanthin). The latter are the polar compounds or the phenolics. There are many different phenolics, of which flavan-3-ols can contribute between 70 and 80% of the



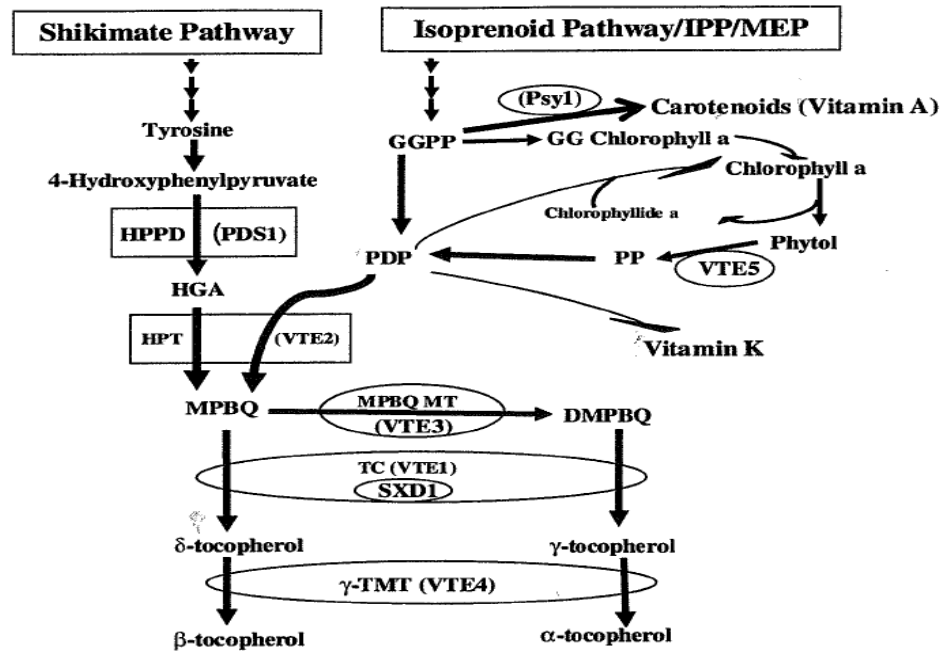
total phenolics in barley. Flavan-3-ols appear in both monomer and polymer form including catechin, epicatechin and gallic catechin (Goupy *et al.* 1999). Flavonol and phenolic acid are also present. Anthocyanins have also been reported as responsible for differences in barley colour (Abdel-Aal *et al.* 2006) with the main forms being cyanidin-3-glucose and delphinidin-3-glucose. Furthermore, proanthocyanidin, another structure observed in barley, also has antioxidant activity (Abdel-Aal *et al.* 2006).

In terms of vitamin E, the main source for dietary intake is from plants, especially cereal grains that are also rich in high-potency oils (Ball 2006). According to Yang (2003), vitamin E sources in food are primarily oil, cereal, nuts and green leaf vegetables. Barley has high vitamin E content (136 mg/100 g) behind wheat germ (254 mg/100 g), sea buckthorn seed/ gooseberry (247 mg/100 g), cloudberry (200 mg/100 g) and ahead of many nuts and seed oils such as soybean (121 mg/100 g), rice bran (98 mg/100 g), sunflower (64 mg/100 g) and peanut (37 mg/100 g) (Yang 2003). However, vegetable oils cannot be consumed in the same quantity as grains and grains are stable suggesting they are a potentially greater source of vitamin E. The comparison of vitamin E in terms of serves may therefore be much better. Among 1000 food serves in different groups (including cereal, nuts, sea food, vegetable, fruit dairy products, chips and meat), the ready-to-eat cereals or whole grain products were reported as containing the highest vitamin E (13.5 mg/serve), followed by almonds (7.43 mg/serve), oil for salad (4.64 mg/serve) and mixed vegetables (0.69 mg/serve) (U.S. Department of Agriculture 2011). Among grains, Holasova *et al.* (1995) found that the content of vitamin E in barley caryopses was significantly higher (21.9 – 25.5 mg/kg)

compared to triticale (4.8 – 18.8 mg/kg), wheat (13.5 – 17.6 mg/kg), oat (13.6 – 17.6 mg/kg) and rye (15.5 – 18.7 mg/kg).

Bramley *et al.*, (2000) reported that in barley, tocopherols are present as  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ -T and  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ -T3. The T3 content is higher than that of T, with approximately 63 and 37% of total tocopherols respectively (Holasova *et al.*, 1998, cited by Panfili, 2008). The concentration of each isomer in barley grain in descending order is:  $\alpha$ -T3 (42-63%),  $\alpha$ -T (11-19%),  $\gamma$ -T3 (10-22%),  $\beta$ -T3 (7-20%),  $\gamma$ -T (2-10%),  $\beta$ -T (0.6-2%),  $\delta$ -T (0.4-1.2%),  $\delta$ -T3 (not detected to 1.7%) (Panfili *et al.* 2008). However, isomer contents are not parallel with their antioxidant activity. According to Ball (2006), the order of *in vitro* antioxidant activities of the tocopherols conforms to their oxidation potentials and parallels their biological activities, that is  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  - tocopherols. Sheppard *et al.*, (1993) also found that among T and T3, the descending order of antioxidant activity is:  $\alpha$ -T,  $\beta$ -T,  $\alpha$ -T3,  $\gamma$ -T,  $\beta$ -T3, and  $\delta$ -T ( $\gamma$ -T3 and  $\delta$ -T3 have no function). In contrast, some studies reported that  $\alpha$ -T3 is more effective than  $\alpha$ -T (Therriault *et al.* 2002).

The variation in vitamin E content in barley may be due either to the effects of the environment as observed in (Lampi *et al.* 2010) or have a genetic basis. The enzymes responsible for the production of vitamin E and its various forms (Figure 1.2) (Chander *et al.* 2008) are likely to be responsible for differences between varieties. Differences may occur in the genes that encode for the enzymes (thereby affecting amino acid sequence and function) or in the regulation of their production (affecting content).



**Figure 1.2: Tocopherol (T) biosynthesis pathway in plants (maize)** (Chander *et al.* 2008). *Geranylgeranyl pyrophosphate* (GGPP); *phytylphosphate* (PP); *phytyldiphosphate* (PDP); *homogentisic acid* (HGA); *2-methyl-6-phytyl-1,4-benzoquinone* (MPBQ); *2,3-dimethyl-5-phytyl-1,4-benzoquinone* (DMPBQ). T pathway enzymes include: *HPP dioxygenase* (HPPD) at PDS1 locus; *homogentisate phytyltransferase* (HPT) at VTE2 locus; *MPBQ methyltransferase* (MPBQ MT) at VTE3 locus; *tocopherol cyclase* (TC) at VTE1 or SXD1 locus; *c-tocopherol methyltransferase* (c-TMT) at VTE4; *phytyl kinase* at VTE5 locus.

Tocols are synthesized only in photosynthetic organisms and mainly in plastids: in chloroplast membranes and in the oil bodies of seeds. T are widely distributed in higher plants, whereas T3 occurs mainly in some non-photosynthetic tissues, such as seeds and latex, and principally in the embryo of monocotyledonous seeds (including barley) (Lampi *et al.* 2010).

At the genetic or molecular level, only limited information is available. Genetic dissection of tocol content and composition in barley has not occurred but corresponding information is available for maize (Figure 1.2). Wong *et al.*, (2003)

mapped several quantitative trait loci (QTL) for T in maize using a segregating population. Subsequently, Chander *et al.*, (2008) (Figure 1.2) found six candidate genes located on individual loci in the T biosynthesis pathway. Finding the locus (loci) corresponding to tocol production is crucial for the enrichment of vitamin E in grain through breeding or molecular methods. An example of this is the 80-fold increase of  $\alpha$ -T level that has been observed after over-expression of *VTE4* (vitamin E on chromosome 4) in *Arabidopsis* (Shintani and DellaPenna 1998). Hence, determining QTL for tocols in barley may provide knowledge about the genetic basis for use in future breeding strategies for the over production of a natural source of vitamin E.

### ***1.3.2 Antioxidant activity in different barley fractions***

Each barley fraction differs in terms of weight proportion and tocol antioxidant activity (Table 1.2). In general, starchy endosperm has been found to have a lower amount of tocols than other fractions. In contrast, the outer layers including the germ and hull are rich in tocols. In addition, (Salomonsson *et al.* 1980) showed that phenolics were lowest in the centre of the barley kernel and increased dramatically in the outer layers, including lignified husk. However, information about total antioxidant capacity in barley fractions is limited.

**Table 1.2 Antioxidant activity in different barley fractions**

<b>Barley fractions</b>	<b>Fraction mass (%)</b>	<b>Total tocots (mg/kg)</b>	<b>References</b>
Whole grain	100	40.3	(Peterson 1994) (Ko <i>et al.</i> 2003) (Wang <i>et al.</i> 1993)
Hull	17.5 - 25	28.9 - 34.1	(Peterson 1994) (Ko <i>et al.</i> 2003) (Wang <i>et al.</i> 1993)
Germ	0.2 - 0.5	134 - 206.5	(Peterson 1994) (Ko <i>et al.</i> 2003) (Wang <i>et al.</i> 1993)
Endosperm	73 - 75.1	11.6 - 33.3	(Peterson 1994) (Ko <i>et al.</i> 2003) (Wang <i>et al.</i> 1993)

### ***1.3.3 Antioxidants during storage and processing of barley products***

#### ***1.3.3.1 Antioxidants during storage***

Newly harvested barley will often be in storage for between 4 and 18 months before processing (Idaho Barley Commission 2011). This storage time may have an effect on total tocol content and antioxidant capacity in both whole grain and flour. Barley flour seems to lose more tocots than whole grain during storage. Tyopponen and Hakkarainen (1985) cited by Liu and Moreau (2008) found that the tocol content in barley flour decreased by 5% weekly during 8 weeks of storage at 25°C. At the same temperature, however, the degradation is less in intact barley with only 1% loss of tocots monthly during 11 months of storage (Hakkarainen *et al.* 1983). In terms of the eight isomers of tocots (Figure 1.1), the influence of storage is not the same. Wang *et al.*, (1993) reported that after storing at 27°C, T remained stable, while T3 significantly

changed (specifically, decreasing  $\alpha$ -T3 and increasing  $\delta$ -T3). In contrast, a similar content of T3 and a slight increase of T were observed after holding barley at 35°C (Liu and Moreau, 2008).

Although Tyopponen and Hakkarainen (1985), cited by (Liu and Moreau 2008), showed the tocol loss from barley flour exposed to light, the reason for tocol degradation was not explained. According to Ball (2006), T and T3 may be destroyed fairly rapidly by sunlight and artificial light containing wavelengths in the UV region. However, light was not the only factor in this case because tocol reduction was also observed in the dark at 27°C (Wang *et al.* 1993). Wang assumed that the isoforms are slowly oxidised by atmospheric oxygen to form mainly biologically inactive quinones. The oxidation was accelerated by light, alkalinity, certain trace metals and heat. Heat may be also an important factor in grain preservation (Metz 2006) (Table 1.3).

From these data, heat combined with moisture negatively affects barley shelf life. Barley should be stored at 10-20°C and at less than 11.5% moisture to be kept for a period of more than 6 months. Newman (2002), also suggested that storage between 4.4°C and 15.5°C with moisture below 12.5% are safe conditions for barley. On an industry scale, barley has been stored in silos with cooling aeration systems to cool grains to less than 15°C and ideally 10°C (Viljoen 2001). Although the optimal conditions for storage have been determined, research on the influence of temperature and moisture on tocols and antioxidant capacity is limited. There is no report on the change of tocols and antioxidants during industrial storage.

**Table 1.3 The effect of storage temperature on barley shelf life at different grain moisture (Metz 2006)**

<b>Barley moisture %</b>	<b>Storage temperature</b>	<b>Potential storage period</b>
<10.5	10°C-20°C	Very long (12-18 months)
	20°C-30°C	Moderate (6 months)
	> 30°C	Short (3 months)
10.5 - >11.5	10°C-20°C	Long (12 months)
	20°C-30°C	Moderate (6 months)
	> 30°C	Short (3 months)
11.5 - > 12.5	10°C-20°C	Moderate (6 months)
	20°C-30°C	Short (3 months)
	> 30°C	Very short (< 3months)
> 12.5	10°C-20°C	Short (3 months)
	20°C-30°C	Very short (< 3 months)
	> 30°C	Perhaps (1 month)

In addition, antioxidants are considered to be preservatives in processed food and to lengthen shelf life of food during storage (Ronald *et al.* 1991). For example, Pereira de Abreu *et al.*, (2010) demonstrated the efficacy of the packaging film containing natural antioxidants from barley in slowing down lipid damage in Atlantic salmon and lengthening the storage time of salmon. Vitamin E, therefore, may be able to maintain barley and its products during storage. In other words, the grains with more vitamin E and antioxidant capacity might store for longer than other grains.

### 1.3.3.2 Antioxidants during pearling

Pearling refers to the procedure that separates the fractions of grain (pearled grain) and pearling flour (hull, aleurone, sub-aleurone and germ). Because, tocols are more concentrated in outer layer fractions rather than the starchy endosperm (Table 1.2), pearling flour was reported to have higher tocol content than both the whole grain and remaining flour (Wang *et al.* 1993; Peterson 1994). The pearling fraction might have a possible use as a nutrient-rich source (2.7, 3.5, 4.4 and 8.0 times higher in  $\alpha$ -T3,  $\gamma$ -T3,  $\alpha$ -T and  $\beta$ -T content respectively compared to the whole grain) (Wang *et al.* 1993; Peterson 1994). In a comparison between pearling flour and hulled grain, Panfili *et al.*, (2008) reported four-, seven- and five-fold increases in tocols total, each T and T3 content, respectively. Hence, the pearled barley has a significant lower tocol concentration when compared to the whole kernel. Bhatti (1999) also reported that removing bran (hull, aleurone and sub-aleurone) caused a significant loss of tocols (74.8 – 116.6 mg/kg in bran versus only 40.3 mg/kg in whole grain). Phenolics have also been reported to be removed during pearling because it is located in the outer layers of kernel. Gong *et al.* (2012) reported that phenolic content of the first fraction (10% pearling) was double that of the second fraction (20% pearling) in hullless barley.

Although pearling might reduce vitamin E content and antioxidant capacity, the percentage of pearling flour removed was different between studies most likely due to the different fraction mass between genotypes (Table 1.2). The concentration of vitamin E and antioxidants in outer layers also varied among genotypes (Evers *et al.* 1999). In addition, the removal of hull by pearling contributed to lower tocols in pearled grain suggesting the potential of hull-less barley (Bhatti 1999). However, the comparison in terms of tocol content in hulled and hullless barley is required.



### 1.3.3.3 Antioxidants during malting

The findings among studies regarding the change of vitamin E or antioxidants during malting including steeping, germination and kilning are not uniform. While there is no significant change in tocopherols (or individual isomers) after malting according to Peterson (1994), other studies have shown an effect of malting on tocopherol levels. For example, upon evaluation of the total tocopherol levels in nine barley varieties and their corresponding malt, Goupy *et al.*, (1999) demonstrated that five varieties lost between 12 and 32% tocopherols after malting but that tocopherol content increased significantly in four other varieties (by 4-9%). The enrichment in tocopherols in malted barley was also reported in Ilona *et al.*, (2011) with a 38% increase compared to raw hull-less barley. This increase might be due to an increase in tocopherol synthesis during germination as has been observed for lupin (Frias *et al.* 2005). Antioxidant activity has been shown to increase during wheat (Yang *et al.* 2001) and lupin germination (Frias *et al.* 2005). Antioxidant activity was also reported to be higher in barley samples steeped for one day compared to two days steeping due to lack of adequate oxygen (Ilona *et al.* 2011).

Any decrease of tocopherols might be attributed to high temperature impact in kilning. For instance, there is no effect of low kilning temperature (75-80°C) on tocopherol content in Pilsener malt, whereas a significant influence has been detected at the higher temperatures used to produce Munich (100°C), Caramel (150°C) and Black (230°C) malt (Ilona *et al.* 2011). Although the unstable tocopherol level was ascribed to the range of kilning temperatures (dependent on type of malt), the cultivar may also contribute because at the same kilning temperature, the content of vitamin E in hulled commercial (Pilsener, Munich, Caramel and Black) malt type was lower than in hull-less experimental malt (Klass, 3528, L-400, 3475, 3537) (Ilona *et al.* 2011). This might

indicate the important interaction between genotypes (or genetic basis) and type of malt. However, a detailed analysis of the effect of malting on antioxidants in different barley genotypes is needed.

#### *1.3.3.4 Antioxidants during thermal processing of barley*

According to Ball (2006), all fat soluble vitamins are degraded during thermal processing except for vitamin K. However, these descending levels depended upon the type of processing and temperature. Firstly, extrusion cooking and hydrothermal processing used for breakfast cereal (or flakes and chips) and dried barley respectively were analysed. The rate of vitamin E loss depended upon the temperature but temperatures from 80°C to 200°C used in extrusion cooking have also been shown to negatively affect tocol capacity in barley (Zielinski *et al.* 2001). Earlier studies, Millauer *et al.*, (1984) cited by (Zielinski *et al.* 2001) suggested losses up to 66%. In hydrothermal processing, barley was soaked and then dry steeped at 120-200°C and the reported decrease in tocols was 63%-94% (Zielinski *et al.* 2001). Besides extrusion and hydrothermal cooking, the effect of baking on tocols (for bread and biscuit) and extrusion combined to dry (for pasta) were examined. The average tocol losses were 21.4%, 28.2% and 41.1% in bread, biscuit and pasta, respectively (Hidalgo and Brandolini 2010). However, these products were made from wheat, and no information is available in the literature for barley.

In addition, the degradation of tocols might be reduced if either a steaming or a microwave treatment step is applied before processing (Hakansson and Jagerstad 1990). The explanation for this may be related to enzymic (enzyme lipoxygenase is active immediately when cereals mixed with water) or non-enzymic lipid oxidation

(peroxidized lipids can be formed due to the non-enzymic catalysts copper and iron derived from cooking water or processing equipment) (Hakansson and Jagerstad 1990). Moreover, according to Gassmann (2000), antioxidants can prevent lipid peroxidation thus if there is more antioxidant, cereals might be protected during processing.

#### **1.4 Literature summary**

Barley is a very important crop worldwide with a huge annual yield but only 2-3% of barley grains are consumed by people in products such as breakfast cereal, bread, noodles, biscuits and soup. However, barley is a food ingredient rich in antioxidants especially vitamin E which scavenges free radicals in the human body and may therefore combat diseases such as cardiovascular disease and cancer. Although vitamin E level and antioxidant capacity has been shown to be different among some varieties, little is known for most Australian barley varieties or the genetic basis for those differences. No one study has followed the vitamin E content of different genetic backgrounds through the food chain to the consumer. Vitamin E content is affected by storage but knowledge of the effect of industry conditions is limited. After storage, barley is passed through processing such as pearling, malting and thermal processing into products such as pita bread. Pearling reduces vitamin E content and antioxidant capacity in barley because this component concentrates in the hull. Thermal processing may negatively affect vitamin E level due to lipid degradation. However, the influence of malting on vitamin E and antioxidants is unclear. In addition, the vitamin E content and antioxidant capacity remaining in potential food products is unknown.

## 1.5 Research questions and aims of the project

As summarised above, there is limited knowledge about how vitamin E content and antioxidant capacity might change in the barley food chain and the genetic basis of differences between barley varieties. This research therefore aimed to address the following questions: Do different barley genotypes have different antioxidant capacity and vitamin E content? What is the genetic basis of any differences in antioxidant capacity and vitamin E content observed? How are antioxidant capacity and vitamin E content affected by common practices for storage and processing (pearling, malting and baking) for barley genotypes high in those components? In particular, this research has established

- Optimised methods for analysis of the contents of vitamin E and its individual isomers as well as antioxidant capacity of vitamin E (Chapter 2);
- The effect of genotypes and storage on antioxidant capacity and vitamin E content (and its isomers) [Chapter 3, (Do *et al.* 2015a)];
- The effect of malting on antioxidant capacity and vitamin E content in different barley genotypes [Chapter 4, (Do *et al.* 2015b)];
- The potential of pita bread containing barley with enriched antioxidant capacity and vitamin E content and the impact of pearling [Chapter 5, (Do *et al.* 2016)] and;
- The genomic regions (QTLs) responsible for differences in vitamin E content [Chapter 6, (Do *et al.* to be submitted Crop and Pasture Science)].

**Chapter 2: OPTIMISATION OF METHODS  
FOR THE ANALYSIS OF VITAMIN E  
CONTENT AND ANTIOXIDANT CAPACITY**

## 2.1. Introduction

Prior to this thesis, antioxidant capacity and vitamin E content in barley had been analysed in several studies [including Panfili *et al.* (2003), Omwamba and Hu (2009), Lampi *et al.* (2010)]. However, these existing studies had only included a limited number of barley genotypes and had not directly compared covered with hullless genotypes or waxy with non-waxy genotypes. Moreover, the specific contribution of vitamin E to total antioxidant capacity in barley had not been measured previously. Therefore, one of the central aims of this thesis was to measure vitamin E and its antioxidant capacity in addition to total antioxidant capacity in barley grain from a wide range of different genotypes, including both hulled and hullless and waxy and non-waxy varieties.

While a number of methods for assessment of vitamin E content had been reported in previous studies, they have all involved one of two general approaches. The first approach involves hot or cold saponification of the sample followed by liquid extraction with organic solvents such as petroleum ether, heptane or hexane (Lampi *et al.* 2010). In the second approach, there is no saponification of the sample prior to extraction, and the vitamin E is extracted using solvents such as ethanol (Omwamba and Hu 2009), methanol (Panfili *et al.* 2003) or a mixture of ethanol:acetone:water (Ilona *et al.* 2011); or using supercritical fluid extraction (SFE) (Carlucci *et al.* 2001) or pressurized liquid extraction (PLE) (Delgado-Zamarreno *et al.* 2009). For the studies in this thesis, the original intention was to extract vitamin E via the non-saponification method using ethanol, since the resulting extract could then also be used to analyse antioxidant capacity (Omwamba and Hu 2009). However, a previous study by Panfili *et al.* (2003) had reported that the results obtained for vitamin E content

using the non-saponification method were significantly lower than those obtained using the hot saponification method on the same sample, suggesting that there was an improved extraction efficiency when using saponification. Therefore, in order to select the most efficient method for the studies presented in this thesis, the two approaches described earlier (saponification and non-saponification) were compared prior to the start of experiments.

This Chapter describes how the methods used for the measurement of vitamin E content and antioxidant capacity were adapted and optimised to: (a) allow for the measurement of antioxidant capacity and vitamin E content in the same sample at the same time; (b) enable the same method to be used reliably across a range of genotypes; (c) shorten the time required for the measurements; and; (d) minimise losses of antioxidant capacity and vitamin E content, both of which are relatively unstable, during the analysis.

## **2.2 Methods**

### ***2.2.1 Comparison of the saponification and non-saponification method***

For the saponification methods, hexane, rather than heptane or petroleum ether, was selected as the solvent to use post-saponification to separate vitamin E from the lipids. This decision was based on the lower cost and higher purity of hexane compared to these other reagents, as well as its suitability for use in High-Performance Liquid Chromatography (HPLC) analysis as a mobile phase (Panfili *et al.* 2003). For the non-saponification method, ethanol was selected as the solvent, due to its suitability for the measurement of antioxidant capacity in vitamin E by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) which is soluble in ethanol (Omwamba and Hu 2009). Ethanol had also

successfully been used in previous studies as a mobile phase solvent in HPLC for the quantification of vitamin E content (Chiou *et al.* 1977). The relative efficiency of the saponification and non-saponification methods in extracting vitamin E from the same sample were determined by comparison of the peak area obtained by HPLC for  $\alpha$ -tocopherol (the major vitamin E isomer) in the extracts from each of the procedures.

#### *2.2.1.1. Saponification method*

The method for vitamin E extraction with saponification previously described in detail by Lampi and colleagues (Lampi *et al.* 2004) was used in this experiment (Figure. 2.1A). Briefly, flour (0.5 g), from the barley genotypes Sloop (hulless, non-waxy) and Finniss (hulled, non-waxy) was accurately weighed into a 30 mL Pyrex glass tube with a Teflon screw cap. Ascorbic acid (0.1 g), 100% ethanol (5 mL) and nanopure water (2 mL) were then added. After mixing with a vortex mixer, the tube was flushed with nitrogen before the addition of KOH (0.5 mL, 10.7M). The tube was then capped and transferred to a boiling water bath for 25 min. The tube was mixed with the vortex mixer after 10 min of boiling and then returned to the water bath for the remaining 15 min. The tubes were then removed and cooled in an ice-water bath. After cooling, 5 mL of 50% ethanol was added to the tubes. The unsaponified lipids remaining in the tubes were extracted by the addition of three volumes (each 10 mL) of n-hexane:ethyl acetate (8:2). Tubes were shaken for 10 min at 500 rpm, which resulted in the separation of the samples into two distinct phases.

The clear organic layers were collected in a separation funnel and these organic extracts were washed three times with NaCl (10 mL, 1M) to prevent the formation of emulsions. The washed extract was then transferred to a 250 mL round bottomed flask,

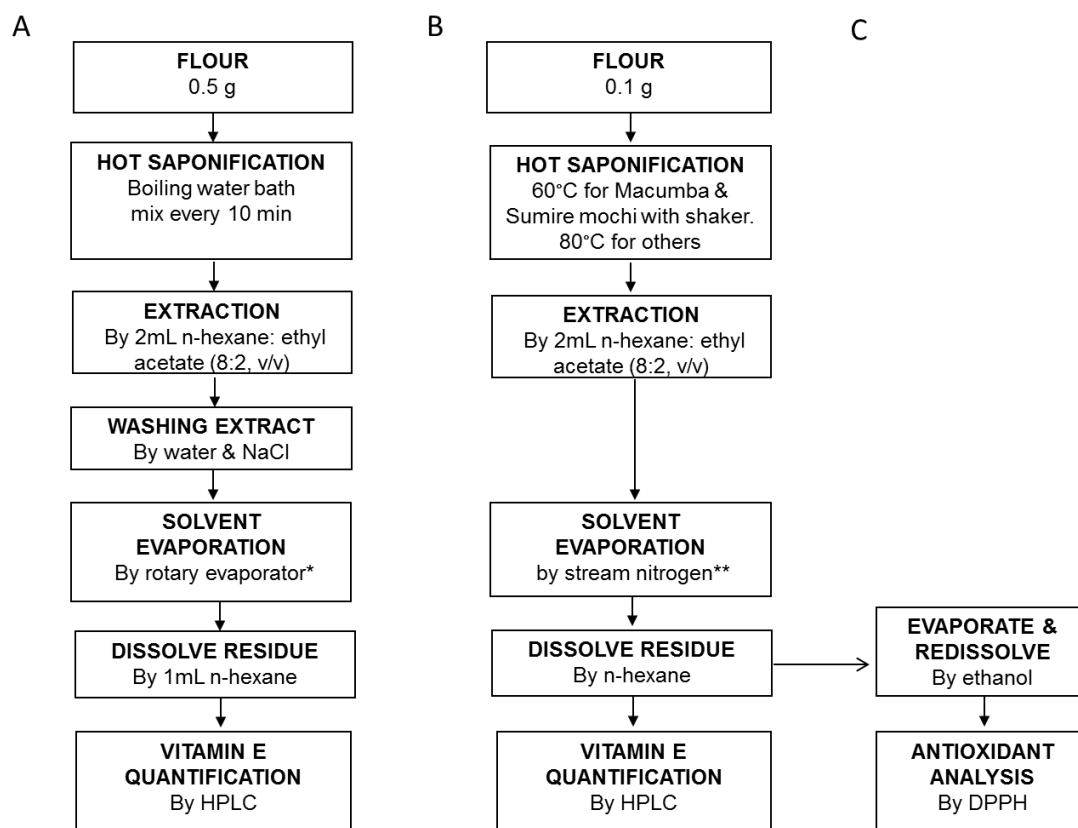


and the organic phase was evaporated by rotary evaporation under pressure (100mm Hg). Ethanol (2 mL) and n-hexane (2 mL) were added and the sample evaporated again to remove any remaining water. The residue was dissolved in 3 mL n-hexane and transferred to a 5 mL volumetric flask. Sample extracts were stored in Kimax test tubes in the dark at 70°C. Prior to HPLC analysis, extracts were filtered through a cellulose acetate 0.45 µm filter (Micro Analytix Pty Ltd, Australia) to ensure optimum purity.

Concentrations of  $\alpha$ -tocopherol in the extracts were quantified according to the method described by Lampi *et al.* (2004) with some modifications. The tocopherols were separated by a normal phase HPLC using a GRACE Altima HP Silica 150 x 3 mm, 3 micron column and quantified with a fluorescence detector (NP-HPLC-FLD) with an excitation wavelength of 290 nm and an emission wavelength of 325 nm. The mobile phase was 1,4-dioxane/n-hexane (3:97, v/v) at a flow rate of 2 mL/min. Based on retention time (Panfili *et al.* 2003), the peak area for  $\alpha$ -tocopherol was determined.

#### 2.2.1.2. Non-saponification method

The method of extracting vitamin E with ethanol was conducted as previously described (Omwamba and Hu 2009). Briefly, barley flour (1 g) was extracted in 80% ethanol (10 mL) in a shaking water bath for 2 h in the dark at 45°C. The supernatant and residue were then separated by vacuum filtration. The residue was then re-extracted using the same conditions and the two extraction solutions combined. These final extraction solutions were stored in the dark at -20°C and analysed within 24 h.  $\alpha$ -tocopherol was separated by a normal phase HPLC using a GRACE Platinum EPS C18 column and quantified with a fluorescence detector (NP-HPLC-FLD) with an



**Figure 2.1 Method for vitamin E analysis.** (A) The original method for vitamin E extraction and quantification as described in Lampi *et al.* (2004). (B) The optimised method for vitamin E extraction and quantification used in this study. (C) The optimised method for measurement of antioxidant capacity in the vitamin E extractions used in this study. \* One sample for several hours. \*\* 24 samples for 20 minutes.

excitation wavelength of 290 nm and an emission wavelength of 325 nm. The mobile phase was 95% ethanol at a flow rate of 2 mL/min. The levels of  $\alpha$ -tocopherol in the extracts were quantified using the same approach as for the saponification method (section 2.2.1.1).

### 2.2.2. Optimisation of the saponification method

The saponification method was more efficient than the non-saponification method and was therefore chosen to measure vitamin E and optimised further (Figure 2.1B) by

altering the saponification temperature and drying step with the purpose of increasing the extraction yield and shortening the extraction time.

#### *2.2.2.1. Temperature of saponification*

While hot saponification has been reported to extract more bound lipids from the sample in comparison to cold saponification (Fратиanni *et al.* 2002; Panfili *et al.* 2003), in the initial experiments placing the samples into the boiling water bath was observed to be associated with a high risk of the sample drying out during this process, as the ethanol was easily evaporated in the smaller tubes used during the drying step. Thus, the efficiency of the saponification and subsequent extraction was compared at three different temperatures [100°C (original method), 90°C and 80°C] by comparing the amount of  $\alpha$ -tocopherol extracted from the same samples of the barley genotypes, Finnis (hulless, non-waxy) and Sloop (covered, non-waxy) under these different conditions.

In later experiments, although the waxy, covered genotypes (Macumba and Sumire mochi) did not dry out when incubated at 80°C, they tended to stick together which made them difficult to extract and resulted in low extraction yield. Therefore the temperature in the water bath was further reduced to 70°C or 60°C, coupled with continuous mixing of the samples throughout the incubation period, until the samples did not stick together.

#### *2.2.2.2. Drying of extracts*

Two of the main disadvantages of the original method described by Lampi and colleagues were the limited number of samples that could be processed in each run and

the lengthy extraction time. This was largely due to the fact that this method used a rotary evaporator, which meant that only one sample could be evaporated at a time and that each individual sample took several hours to dry. Therefore, an unpublished method (Robert Asenstorfer, personal communication) using vacuum drying that reduced the sample volume and allowed processing of multiple samples at any one time was trialled. The amount of flour sample was reduced from 0.5 g to 0.1 g and the amount of solvent from 10 mL to 2 mL, thus making the use of a 10 mL Pyrex glass tube with a Teflon screw cap possible (instead of a 30 mL tube). Samples were then dried using a SpeediVac (Univapor 150H, Uniequip, Germany). The use of vacuum drying and the smaller sample size meant that 24 extracts could be dried at one time, compared to only one in the original method. The evaporation of the samples in the SpeediVac was also significantly quicker (one hour compared to several hours), thus reducing the total time required for the procedure. An alternative method for drying samples was subsequently identified: the use of stream nitrogen, which had been described as an inexpensive and fast method of evaporating organic solvents (Nevins *et al.* 2005). In the current study, this approach was therefore compared with the use of the SpeediVac in relation to the extraction yield and extraction time.

### ***2.2.3. Antioxidant capacity***

Total antioxidants were extracted from barley grain using an adaptation of the methods described by (Omwamba and Hu 2009). In their original method, the mixture of sample (1 g) and ethanol (10 mL) was placed in an incubator at 45°C and shaken for two hours. The sample was then filtered through Whatman No.1 filter paper, before re-extraction of the solid component with 10 mL ethanol for an additional 2 h. In initial

experiments using this approach, however, a significant proportion of the flour was observed to be lost through the filter paper during the filtering process. In order to avoid this, the volume of ethanol used was doubled (to 20 mL) and the extraction time extended to 4 h, so that the sample was extracted once, rather than twice. In addition, a syringe filter with a 0.45 $\mu$ m pore size (Millex Syringe-driven Fylter Unit, Millipore Ireland Ltd, Carrigtwohill, Ireland) was used to filter the solution following the paper filtration step and prior to DPPH analysis to ensure that the sample extract was free from any residual particles of flour. Alternatively, a Filtropur BT25, 250 mL, 0.45  $\mu$ m (SARSTEDT, USA) was used to replace both the filter paper and syringe filter.

Two methods of measuring the antioxidant capacity in vitamin E were also compared. The first method directly analysed the antioxidant capacity of the vitamin E extract (dissolved in hexane) while the second method involved first evaporating the hexane using stream nitrogen and then dissolving the extraction in ethanol before analysis (Figure 2.1C). Antioxidant capacity was measured as per the DPPH free radical method reported by Omwamba and Hu (2009). For each extract, 0.1 mL was added to 2.9 mL of 112  $\mu$ M DPPH in a 3 mL polystyrene cuvette (SARSTEDT, Numbrecht, Germany). After mixing, the samples were allowed to stand in the dark for 20 min at 23°C. The absorbance value was measured at 517 nm after 20 min using a spectrophotometer (UV/VIS SP 8001, Metertech, Taiwan) and compared for the extracts from the two methods.

#### ***2.2.4. Statistical analysis***

The data are presented as means of three determinations. Student's *t* test was used to determine the differences among two means using GenStat 14. One-way Analysis of

Variance (ANOVA) was performed using GenStat 14 to determine the differences between more than two means, with the Least Significant Difference (LSD) test applied post-hoc to determine significant differences between groups at  $P < 0.05$ .

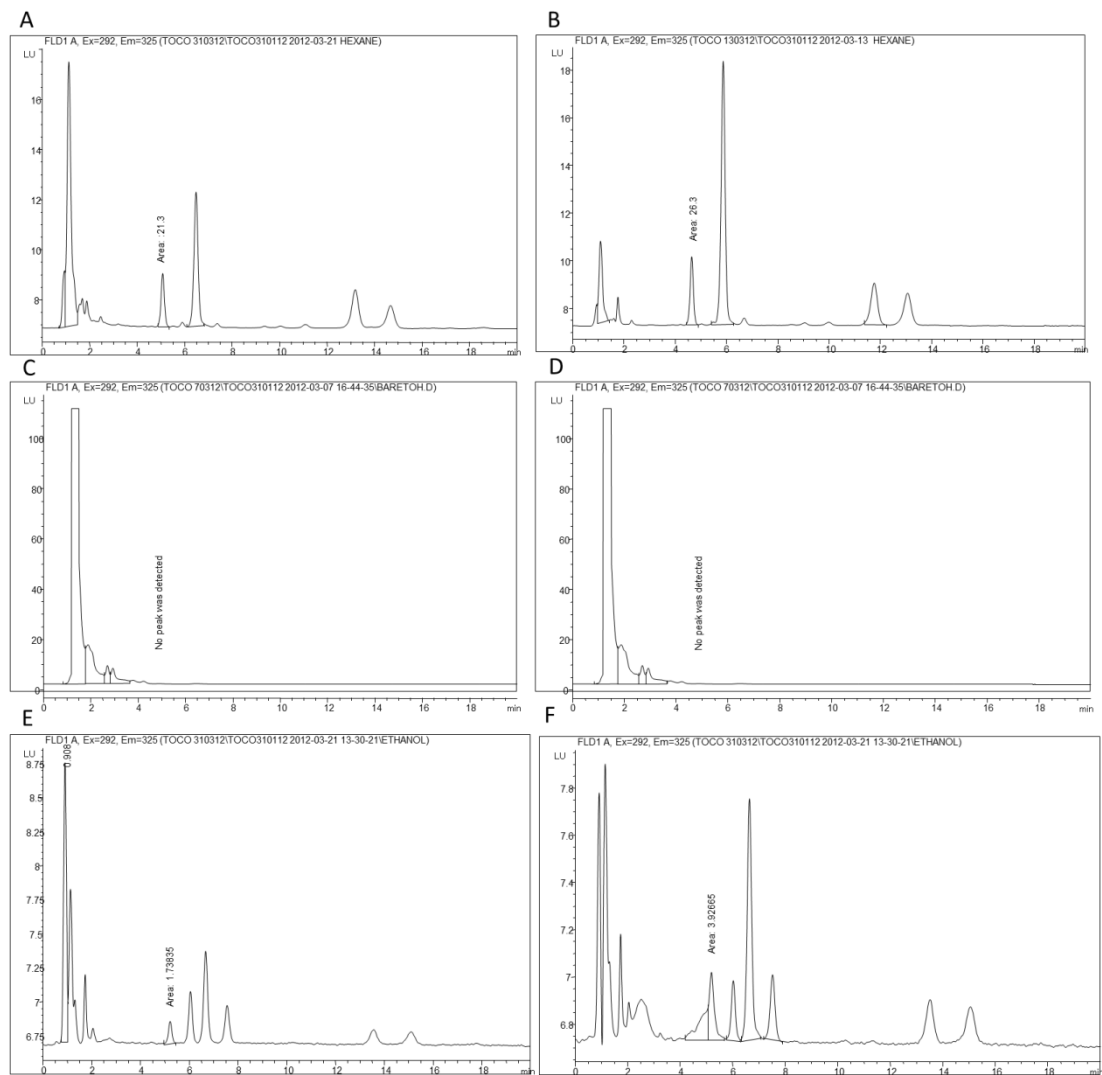
## 2.3. Results

### 2.3.1. Comparison of the saponification and non-saponification method

The  $\alpha$ -tocopherol was readily detectable by HPLC in both samples (Finniss and Sloop genotypes) extracted by hexane (Figure. 2.2A, 2.2B) while this isomer was not detected in samples extracted using ethanol (Figure. 2.2C, 2.2D). Therefore, the samples extracted by ethanol were concentrated by evaporating in a SpeediVac until the volume of sample was reduced four-fold (Figure. 2.2E, 2.2F) prior to quantification by HPLC. After repeating the analysis, the retention times ( $t_r$ ) of  $\alpha$ -tocopherol, as confirmed by the standard, were  $4.8 \pm 0.2$  min for the hexane extraction and  $5.1 \pm 0.1$  min for the ethanol extraction. However, the peak areas of  $\alpha$ -tocopherol in samples extracted by hexane were significantly higher than those in ethanol extraction (Table 2.1).

**Table 2.1 Peak areas of  $\alpha$ -tocopherol extracted by hexane and ethanol in Finniss and Sloop.** The number of biological replicates was  $n=3$  for both Finniss and Sloop. Values were expressed as mean  $\pm$  SE. Different superscripts indicate significant differences between the  $\alpha$ -tocopherol peak area within each variety as determined using Student's  $t$  test.

Samples	Peak areas	
	Extract by hexane	Extract by ethanol
Finniss	21.43 $\pm$ 0.07 <sup>a</sup>	6.86 $\pm$ 0.18 <sup>b</sup>
Sloop	26.27 $\pm$ 0.14 <sup>a</sup>	15.72 $\pm$ 0.08 <sup>b</sup>



**Figure 2.2 Representative chromatograms of  $\alpha$ -tocopherol derived from two genotypes of barley by different extraction methods.  $\alpha$ -tocopherol is the peak with the details of the area over it. (A) and (B)  $\alpha$ -tocopherol in Finnis and Sloop extracted by hexane (saponification), respectively; (C) and (D)  $\alpha$ -tocopherol in Finnis and Sloop extracted by ethanol (non-saponification), respectively; (E) and (F)  $\alpha$ -tocopherol in Finnis and Sloop extracted by ethanol after concentrating, respectively.**

### 2.3.2. Optimisation of saponification method

#### 2.3.2.1. Temperature

The peak areas of  $\alpha$ -tocopherol extracted from Finniss and Sloop at three different temperatures [100°C (original method), 90°C and 80°C] were compared. Reducing the temperature of the water bath to 80°C prevented the samples from drying out and consequently produced a higher  $\alpha$ -tocopherol content compared to other temperatures (Table 2.2). Therefore, incubation of the samples in an 80°C water bath with mixing every 10 min was applied for the majority of the barley genotypes in future experimentation.

**Table 2.2 Peak areas of  $\alpha$ -tocopherol extracted by hexane in Finniss and Sloop at different saponification temperatures.** The number of biological replicates was n=3 for Finniss and Sloop. Values were expressed as mean  $\pm$  SE. Different superscripts indicate significant differences between the  $\alpha$ -tocopherol peak area as determined using the Least Significant Difference (LSD) ( $P < 0.05$ ) for between temperatures and within each genotype. LSD=1.33 and 1.58 for Finniss and Sloop, respectively.

Temperature Samples	80°C		90°C		100°C	
	Peak area	Dry	Peak area	Dry	Peak area	Dry
Finniss	24.68 $\pm$ 0.34 <sup>a</sup>	No	22.29 $\pm$ 0.16 <sup>b</sup>	Yes	20.91 $\pm$ 0.54 <sup>c</sup>	Yes
Sloop	27.72 $\pm$ 0.38 <sup>a</sup>	No	25.87 $\pm$ 0.51 <sup>b</sup>	Yes	25.08 $\pm$ 0.45 <sup>b</sup>	Yes

In later experiments involving waxy, covered genotypes (Macumba and Sumire mochi), they were observed to stick together when incubated at 80°C, making extraction difficult (Figure.2.3). Therefore, the efficiency of the extraction obtained was compared when the temperature was lowered further (to 70°C and 60°C) in comparison to 80°C. The peak areas of  $\alpha$ -tocopherol extracted from Macumba and Sumire mochi at these three different temperatures (80°C, 70°C and 60°C) are shown in Table 2.3. At 60°C, the flour did not become sticky and there was a greater



extraction yield than that at 80°C. Therefore, incubation at 60°C with continual mixing of the samples was utilised for Macumba and Sumire mochi varieties in subsequent experiments.

**Table 2.3 Peak areas of  $\alpha$ -tocopherol extracted by hexane in Sumire mochi and Macumba at different saponification temperatures.** The number of biological replicates was n=3 for both Sumire mochi and Macumba. Values were expressed as mean  $\pm$  SE. Different superscripts indicate significant differences between the  $\alpha$ -tocopherol peak area as determined using the Least Significant Difference (LSD) ( $P<0.05$ ) for between temperatures and within each genotype. LSD=1.07 and 0.93 for Sumire mochi and Macumba, respectively.

Temperature Samples	60°C		70°C		80°C	
	Peak area	Sticky	Peak area	Sticky	Peak area	Sticky
Sumire mochi	18.44 $\pm$ 0.25 <sup>a</sup>	No	12.70 $\pm$ 0.40 <sup>b</sup>	Yes	10.02 $\pm$ 0.23 <sup>c</sup>	Yes
Macumba	21.0 $\pm$ 0.25 <sup>a</sup>	No	16.89 $\pm$ 0.13 <sup>b</sup>	Yes	13.63 $\pm$ 0.36 <sup>c</sup>	Yes

#### 2.3.2.2. Drying of extracts

The peak areas of  $\alpha$ -tocopherol extracted from Finniss and Sloop using drying methods including stream nitrogen and SpeediVac as well as the drying time are shown in Table 2.4. There was no difference in the results obtained for  $\alpha$ -tocopherol content for the different drying methods for the Sloop genotype ( $P<0.05$ ). However, the Finniss sample that was dried by nitrogen stream had significantly higher yield ( $P<0.05$ ) of  $\alpha$ -tocopherol compared with that dried by SpeediVac. Given that the stream nitrogen method only took 20 min compared to 1 h for the SpeediVac, the stream nitrogen drying approach was used in all subsequent experiments.

**Table 2.4 Peak areas of  $\alpha$ -tocopherol extracted by hexane in Finniss and Sloop using the two different drying methods (stream nitrogen and SpeediVac).** The number of biological replicates was n=3 for Finniss and Sloop. Values were expressed as mean  $\pm$  SE. Different superscripts indicate significant differences between the  $\alpha$ -tocopherol peak area within each variety as determined using Student's *t* test.

Drying method Samples	Stream nitrogen		SpeediVac	
	Peak area	Time (min)	Peak area	Time (min)
Finniss	25.84 $\pm$ 0.47 <sup>a</sup>	20	24.21 $\pm$ 0.12 <sup>b</sup>	60
Sloop	27.76 $\pm$ 0.13 <sup>a</sup>	20	27.04 $\pm$ 0.35 <sup>a</sup>	60

### 2.3.3. Antioxidant capacity in vitamin E

The absorbance readings using the spectrophotometer were compared between Finniss extracted by hexane and Finniss extracted by hexane but re-evaporated and re-dissolved in ethanol. The result indicated that in the former method the measurements varied significantly between replicates (the mean was  $0.352 \pm 0.020$ , relative standard deviation (RSD)  $>5\%$ , n=9). In contrast, the later method provided absorbance readings which were more consistent between replicates (the mean was  $0.364 \pm 0.007$ , relative standard deviation (RSD)  $<2\%$ , n=9). Therefore, for future experimentation, the hexane was removed from vitamin E extracts by evaporation before dissolving in ethanol for measurements of vitamin E antioxidant capacity.

## 2.4. Discussion

This Chapter has described the steps that were followed for the optimisation of methods for the measurement of vitamin E content, vitamin E antioxidant capacity and total antioxidant capacity, which were subsequently applied to all measurements reported in this thesis. The main steps involved in these procedures are illustrated in Figure. 2.1. For the vitamin E content analysis, the saponification method by hexane

was found to be more efficient at extracting vitamin E than the non-saponification method using ethanol. This is in accordance with the findings of Panfili *et al.* (2003), which showed the contents of all eight vitamin E isomers in barley extracted by hot saponification were higher than those in barley extracted by the non-saponification method using methanol. The low content of all isomers obtained using the non-saponification method may be attributed to the fact that these isomers exist in ester form or are bound to the matrix, and are only released when saponification is applied (Fratianni *et al.* 2002; Panfili *et al.* 2003).

In terms of the saponification methods, the optimised method (Figure.2.1B) provided a higher extraction yield than the original method (Figure. 2.1A), due to the reduction of the extraction temperature which prevents loss due to evaporation, and provided a shorter extraction time due to the reduction of sample volume and the replacement of the rotary evaporator by the stream nitrogen. In addition, the optimised method enabled a range of barley genotypes, including waxy and non-waxy genotypes, to be analysed. While the majority of the genotypes were extracted at 80°C, waxy varieties, including Sumire mochi and Macumba, were prone to clumping at this temperature and the extraction efficiency was improved by reducing the temperature to 60°C. There have been no previous studies which have specifically reported methods for extracting waxy barley genotypes for the purpose of measuring vitamin E content/antioxidant capacity, however waxy varieties are likely to be gelatinous when heated at higher temperatures since they contain high amounts of amylopectin and less than 0.5% of amylose (Banks *et al.* 1970). According to Jane *et al.* (1999), the high-amylose maizes displayed a much higher gelatinisation temperature (~71°C) than waxy and normal maize starch (~64°C) while waxy rice has a gelatinisation

temperature at 62°C compared with 70°C for non-waxy rice. In this study, the waxy barley genotypes seemed to have the same behaviour as waxy maize and rice, such that a lower temperature was required for the saponification during extraction.

With regards to the analysis method of antioxidant capacity in vitamin E (Figure. 2.1C), the vitamin E extracted by hexane without re-evaporation and re-dissolution in ethanol prior to measurement with DPPH resulted in inconsistent readings. This is likely to be due to the low solubility of ethanol, a polar molecule in hexane, a nonpolar molecule (Cichos *et al.* 1997). Therefore, the vitamin E extracted by hexane but re-evaporated and re-dissolved in ethanol prior to measurement with DPPH was utilised in subsequent experiments.

The optimised methods for measurement of vitamin E content, total antioxidant capacity and that in vitamin E were used for the remaining experiments presented in this thesis.

**Chapter 3. Manuscript 1: ANTIOXIDANT  
CAPACITY AND VITAMIN E IN BARLEY:  
EFFECT OF GENOTYPE AND STORAGE**

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Statement of Authorship is located in Appendix A.



## Antioxidant capacity and vitamin E in barley: Effect of genotype and storage



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### ABSTRACT

Antioxidants, including vitamin E, may have a positive effect on human health and prolong storage of food items. Vitamin E content and antioxidant capacity were measured in 25 barley genotypes before and after 4 months storage at 10 °C using high performance liquid chromatography (HPLC) and ability to scavenge DPPH radicals, respectively. As expected,  $\alpha$ -tocotrienol ( $\alpha$ -T3) and  $\alpha$ -tocopherol ( $\alpha$ -T) were the predominant tocol isomers. Vitamin E content and antioxidant capacity varied significantly among genotypes. Vitamin E ranged from 8.5 to 31.5  $\mu$ g/g dry weight (DW) while ascorbic acid equivalent antioxidant capacity (AEAC) varied from 57.2 to 158.1 mg AEAC/100 g fresh weight (FW). Generally, lower vitamin E content or antioxidant capacity was observed in hullless or coloured genotypes. These results suggest that some genotypes are potential candidates for breeding of barley cultivars with high vitamin E content or antioxidant capacity at harvest, even after storage.

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### 1. Introduction

Barley (*Hordeum vulgare* L.) has historically been used for malt and livestock feed. Recently, however, there has been growing interest in the potential use of barley in human foods largely due to its high content of fibre,  $\beta$ -glucan and antioxidants (Ehrenbergerova, Belcrediova, Havlova, et al., 2006). Barley is likely to be a good dietary source of antioxidants not only because of its high antioxidant capacity but also because of the relatively larger amounts consumed in typical diets compared to fruit and vegetables (Kim et al., 2007). Antioxidants are crucial in maintaining the health of tissues and organs because of their ability to slow tissue damage by preventing the formation of free radicals, scavenging them, or by promoting their decomposition (Young & Woodside, 2001). Antioxidant capacity has been reported to be higher in coloured than white grains, including rice (Htwe et al., 2010) and barley germplasm (Kim et al., 2007). In addition, the antioxidants concentrate in the outer layers of the grain (Peterson, 1994). Thus, removal of these layers (the hull, aleurone

and germ) in covered barley by the process of pearling, used to make flour whiter, significantly reduces antioxidant capacity (Bhatty, 1999). Hullless genotypes, therefore, may have an enriched antioxidant capacity, since they can be used without the requirement for pearling. The different genotypes across coloured and white barley; covered and hullless barley; food, malting and feed barley are also likely to differ in antioxidant capacity.

Antioxidants are generally accepted as including vitamin E (tocotrienols and tocopherols), ascorbic acid (vitamin C), enzymes (catalase, glutathione peroxidase and superoxide dismutase), phenolic compounds, and carotenoids (Goupy, Hugues, Boivin, & Amiot, 1999). Vitamin E is a lipid phase chain-breaking antioxidant that appears effective in improving health outcomes in clinical trials with diminished risk of cancer and cardiovascular disease, especially in smokers (Reboul et al., 2006), although benefits have not been reported in all studies (Bjelakovic, Nikolova, Gluud, Simonetti, & Gluud, 2007). Vitamin E has eight isomers:  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocopherol (T) and  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocotrienol (T3). Tocopherol and tocotrienol are also called tocols. Ball (2006) reported the descending order of their antioxidant capacity to be  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -tocols. Sheppard, Pennington, and Weihrach (1993) however found that among T and T3, the descending order of antioxidant activity was:  $\alpha$ -T,  $\beta$ -T,  $\alpha$ -T3,  $\gamma$ -T,  $\beta$ -T3, and  $\delta$ -T ( $\gamma$ -T3 and  $\delta$ -T3 had no function). However, some studies reported that  $\alpha$ -T3 is a more effective antioxidant than  $\alpha$ -T (Packer, 1995).

Among different cereals, vitamin E has been reported to be higher in barley compared to other grains (Panfili, Fratianni, & Irano, 2003)

**Abbreviations:** DPPH, 2,2-diphenyl-1-picrylhydrazyl; MIR, middle infrared; NIR, near infrared; ATR, attenuated total reflectance; AEAC, ascorbic acid equivalent antioxidant capacity.

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suggesting that barley may have potential applications as a functional food providing vitamin E. However, the reported content of vitamin E in barley differs between studies, for example 51.6 µg/g DW in [Ehrenbergerova, Belcrediova, Havlova, et al. \(2006\)](#), 59.0 µg/g DW in [Bhatty \(1999\)](#) and 69.1 µg/g DW in [Panfili, Fratianni, Criscio, Marconi \(2008\)](#). The contribution to vitamin E content of the eight isomers in these studies was also different. The discrepancies between studies could be due, at least in part, to the fact that different genotypes were studied, as different genotypes may contain different amounts of vitamin E. These studies also differed in the extraction methods used, that is, whether saponification was used and which solvent. Methods using saponification and hexane as a solvent are currently regarded as the most reliable for tocopherol extraction in cereals because esterification is prevented and recovery is greatest ([Panfili et al., 2003](#)).

Another issue to be considered is storage. Antioxidants and vitamin E, in particular, can be easily destroyed by light, water and heat ([Wang, Xue, Newman, & Newman, 1993](#)). Newly harvested barley will often be in storage for between 4 and 18 months before processing ([Idaho Barley Commission, 2011](#)). Even though barley is usually stored in silos with cooling aeration systems at less than 15 °C and ideally to 10 °C ([Viljoen, 2001](#)); storage may have an effect on vitamin E content and antioxidant capacity. Indeed, storage at 25, 27 or 35 °C led to a significant loss of vitamin E content ([Liu & Moreau, 2008](#); [Wang et al., 1993](#)). However, research has not been published on the influence of storage on both antioxidant capacity and vitamin E content in barley at the usual temperatures used by industry.

This study focused not only on the antioxidant capacity of barley genotypes but also on the content of vitamin E, which is often claimed to be an important antioxidant compound. The objectives of this study were to evaluate these components in a range of barley genotypes and the impact on them of storage under conditions similar to those used in industry. Barley genotypes were chosen based on their grain colour, whether hullless or not, and their use in food, malt or animal feed.

## 2. Materials and methods

### 2.1. Materials

Materials in this study, provided by the University of Adelaide Barley Breeding Program, were 25 common food, malting and animal feed genotypes including hullless and covered, coloured and white barley (which can be categorised by genotype as Food, Coloured, Hullless: Jet, Sumire mochi; Food, White, Hullless: Finniss, Macumba; Food, Coloured, Covered: Tadmor; Food, White, Covered: Adagio, Er/Apm; Malting, White, Covered: Flagship, WI2585, Vlamingh, Amaji Nijo, Harrington, ND24260-1, Commander, Alexis, Dhow, Sloop, Buloke; Feed, Coloured, Covered: ICARDA 16, ICARDA 19, ICARDA 26, ICARDA 35, ICARDA 39; Feed, White, Covered: Fleet, Chebec) ([Supplementary information, Table S1](#)). All 25 genotypes were grown from June 2011 to December 2011 as a single plot in a complete randomised design at Charlick Experimental Research Station, Strathalbyn, South Australia (35°19'46.26"S, 138°52'42.39"E). After harvesting, the grains were screened using a 2.5 mm slotted ISO 5223 sieve. The grains were kept at –20 °C until their moisture content, antioxidant capacity and vitamin E content were analysed for at least three individual biological replicates.

### 2.2. Storage

After harvesting, 1 kg of barley grains from all genotypes except Jet, ICARDA 26 and Buloke, which were not available to plant, was cleaned and placed in a 300 × 200 × 100 mm plastic box with lid.

Beakers of silica gel (50 mL) were placed in the containers to ensure humidity was maintained, as confirmed by humidity meters. The grain boxes were stored at 10 °C in the dark for 4 months, as is common industrial practice ([Idaho Barley Commission, 2011](#)). The moisture content, vitamin E content and antioxidant capacity were analysed for at least three individual biological replicates for each genotype before and after 4 months of storage.

### 2.3. Grinding

Barley grain (10 g) for each genotype was ground to a fine powder using an IKA Mill (Germany) with running water to avoid heat increases during the milling.

### 2.4. Moisture content

The moisture content of barley flour was determined in triplicate according to the procedure described in American Association of Cereal Chemists-AACC (2000) Method No. 44-15A. Two grams of sample was weighed into a pre-weighed dish and dried in an air forced oven at a temperature of 105 ± 5 °C until the weight was constant.

### 2.5. Determination of vitamin E content

#### 2.5.1. Vitamin E extraction

To extract tocopherols from barley flour and avoid degradation of the isomers, an optimised method using hot saponification was adapted from that utilised by [Lampi, Ryyanen, Salo-Vaananen, Ollilainen, and Piironen \(2004\)](#). Flour (0.1 g) was added to a solution of 1 mL 100% (v/v) ethanol, 0.4 mL water and 20 mg ascorbic acid in a 15 mL Pyrex glass tube with a Teflon screw cap. After addition of 100 µL 10.7 M potassium hydroxide solution and thorough mixing, the tube was capped and transferred to a boiling water bath for 25 min. During saponification, the sample was mixed every 10 min to improve the hydrolysis. The tube was cooled in an ice water bath for 10 min and 0.5 mL 50% (v/v) ethanol was added. To extract tocopherols and other unsaponifiable lipids, three portions (each 2 mL) of n-hexane:ethyl acetate (8:2, v/v) were used. After shaking samples and solvent for 10 min and separation of the phases, the upper organic layers were collected with a disposable glass pipette and placed into a new glass test tube. This process was repeated three times with n-hexane:ethyl acetate (8:2, v/v), and the extract was then dried using stream nitrogen. The residue was dissolved in 1 mL n-hexane and filtered through a 0.45 µm syringe filter before transfer to a 12 × 32 GRACE glass HPLC (high-performance liquid chromatography) vial with an amber screw cap for analysis.

#### 2.5.2. HPLC analysis

The tocopherols were quantified according to the method reported by [Lampi et al. \(2004\)](#) with some modification. Tocopherols were separated by a normal phase HPLC using a GRACE Altima HP Silica 150 × 3 mm, 3 micron column and quantified with a fluorescence detector (NP-HPLC-FLD) with an excitation wavelength of 290 nm and an emission wavelength of 325 nm. The mobile phase was 1,4-dioxane/n-hexane (2:98, v/v) at a flow rate of 1 mL/min. Separation of tocopherols was based on isocratic elution ([Lampi et al., 2004](#)).

The quantity of the individual vitamin E isomers in samples was calculated by the use of calibration curves for all standard isomers. Tocopherol (T) standards (α-, β-, γ-, δ-T) and tocotrienol (T3) standards (α-, β-, γ-, δ-T3) were purchased from Cayman Chemical, USA, while n-hexane, ethyl acetate, ascorbic acid and DPPH (2,2-diphenyl-1-picrylhydrazyl) were from Chem-Supply Pty. Ltd.,



Australia. Standard stock solutions were prepared to a concentration of 500 µg/mL in hexane. Calibration curves for all isomers were prepared over the concentration range of 1.0–25.0 µg/mL using GenStat 14 (Lawes Agricultural Trust; VSN International, Ltd., Hemel Hempstead, UK).

Barley extractions and standard isomers were injected into the HPLC machine separately. Identification of peaks in barley samples was made based on the retention time when compared with standard peaks. Curves between standard peaks and standard contents were used to calculate contents of isomers in barley samples.

The vitamin E content, expressed in mg of  $\alpha$ -tocopherol-equivalents (TE), was calculated according to Mclaughlin and Weihrach (1979) using biological activities of 1.0 for  $\alpha$ -T, 0.3 for  $\alpha$ -T3, 0.4 for  $\beta$ -T, 0.05 for  $\beta$ -T3, 0.1 for  $\gamma$ -T, 0.01 for  $\gamma$ -T3 and 0.01 for  $\delta$ -T ( $\alpha$ -TE =  $\alpha$ -T \* 1.0 +  $\alpha$ -T3 \* 0.3 +  $\beta$ -T \* 0.4 +  $\beta$ -T3 \* 0.05 +  $\gamma$ -T \* 0.1 +  $\gamma$ -T3 \* 0.01 +  $\delta$ -T \* 0.01).

## 2.6. Determination of antioxidant capacity

### 2.6.1. Ethanol extraction of antioxidants in whole grain

Barley flour (1 g) was extracted with 20 mL 80% ethanol at 45 °C in a flask placed in a 200 rpm shaking water bath for 4 h under dark conditions. Vacuum filtration was then used to separate the supernatant, which was stored in the dark at –20 °C and analysed within 24 h using the DPPH free radical method (Omwamba & Hu, 2009).

### 2.6.2. DPPH free radical method

Barley flour extract (0.1 mL) was added to 2.9 mL of DPPH (112 µM). After mixing, the sample was allowed to stand at 23 °C in the dark for 20 min. Reduction in absorbance was measured at 517 nm after 20 min using a spectrophotometer (UV/VIS SP 8001, Metertech, Taiwan). Antioxidant capacity was then determined using a standard curve for ascorbic acid and prediction models provided by GenStat 14 and expressed as mg ascorbic acid equivalent antioxidant capacity per 100 g of fresh weight of grain (mg AEAC/100 g FW).

### 2.6.3. Determination of antioxidant capacity of vitamin E

The vitamin E was extracted as described previously (Section 2.6.1). After extraction and drying using stream nitrogen, 1 mL n-hexane was replaced by 1 mL of 100% (v/v) ethanol to dissolve the residue. The antioxidant capacity of the vitamin E extracts was then determined using the DPPH free radical method (Section 2.6.2).

## 2.7. Mid-infrared (MIR) measurement

To determine whether there were any identifiable biochemical differences between samples that differed in vitamin E or antioxidant capacity after storage, MIR was applied. Barley flour, from fresh and stored samples, was scanned using a platinum diamond ATR single reflection sampling module cell mounted in a Bruker Alpha instrument (Bruker Optics GmbH, Ettlingen, Germany) and spectra were recorded on OPUS software version 7.0 provided by Bruker Optics (average of 64 scans at a resolution of 8 cm<sup>-1</sup>, between 4000 and 375 cm<sup>-1</sup>) (Cozzolino, Roumeliotis, & Eglinton, 2013). The samples were held against the ATR crystal using the pressure applicator or sample clamp mechanism supplied by the instrument manufacturer to ensure that the same and constant pressure was applied for all replicates. Air was used as reference background spectra.

## 2.8. Statistical analysis

One-way and two-way Analysis of Variance (ANOVA) was performed using GenStat 14 to determine the differences between

means using the Least Significant Difference (LSD) at  $P < 0.05$ . Spectra were exported from the OPUS software into The Unscrambler X software (version X, CAMO ASA, Oslo, Norway) for chemometric analysis, data processed using the second derivative Savitzky–Golay and then Principal Component Analysis (PCA) was used to interpret the changes in the MIR spectra of the samples related to storage, as per Cozzolino et al. (2013).

## 3. Results

### 3.1. Tocol and antioxidant capacity are genotype-dependent in barley grain

The isomers were easily distinguishable in the order of  $\alpha$ -T,  $\alpha$ -T3,  $\beta$ -T,  $\gamma$ -T,  $\beta$ -T3,  $\gamma$ -T3 (Supplementary information, Fig. S1). The retention times ( $t_r$ ) as confirmed by standards were as follows:  $\alpha$ -T  $t_r = 5.5 \pm 0.1$  min,  $\alpha$ -T3  $t_r = 7.0 \pm 0.1$  min,  $\beta$ -T  $t_r = 9.8 \pm 0.2$  min,  $\gamma$ -T  $t_r = 11.0 \pm 0.2$  min,  $\beta$ -T3  $t_r = 13.0 \pm 0.2$  min,  $\gamma$ -T3  $t_r = 14.7 \pm 0.3$  min,  $\delta$ -T  $t_r = 17.5 \pm 0.2$  min and  $\delta$ -T3  $t_r = 21.6 \pm 0.2$  min. This order and retention times of all peaks were as expected for barley (Panfili et al., 2003). Amounts of  $\delta$ -T3 and  $\delta$ -T were considered to be negligible in all barley genotypes since their minor peaks were not measurable.

The main tocols of the 25 genotypes detected, in descending order, were  $\alpha$ -T3,  $\alpha$ -T, ( $\gamma$ -T3 and  $\beta$ -T3), ( $\gamma$ -T and  $\beta$ -T),  $\delta$ -T3 and  $\delta$ -T (Table 1).  $\alpha$ -T3 was the main homologue, accounting for approximately 58% of total tocols and 73% of total tocotrienol. All genotypes also had significant amounts of  $\alpha$ -T (~16% of total tocols), followed by  $\gamma$ -T3 (~12%) and  $\beta$ -T3 (~10%). Smaller amounts of  $\gamma$ -T and  $\beta$ -T than other isomers were found in all samples. The percentage of total tocotrienol (~80% of total tocols) was much higher than that of total tocopherol (~19%).

The content of the six individual vitamin E isomers differed significantly between genotypes with the 25 genotypes being easily categorised into three content groups: low, medium and high. Jet, the ICARDA lines, Macumba, Sumire mochi and Tadmor had the lowest content for the two main isomers,  $\alpha$ -T and  $\alpha$ -T3. Jet contained the lowest level of all isomers except  $\beta$ -T. The high tocol content group comprised Harrington, ND24260-1, Commander and Adagio. Harrington was richest in  $\alpha$ -T3 and  $\gamma$ -T3 but also had relatively high content of the other isomers. The remaining 12 genotypes had isomer contents that were intermediate between the low and high groups.

There was a considerable range of vitamin E content between genotypes (Fig. 1a). The genotype with the highest vitamin E content was Harrington (white, covered) with 31.5 µg/g DW, which is more than three times that of the lowest, Jet (hullless, black) with 8.5 µg/g DW. The hullless and coloured genotypes comprising Jet, Macumba, Finniss, Sumire mochi, the ICARDA group and Tadmor were generally observed to have lower vitamin E content than covered and white genotypes. When comparing between food, malt and feed groups; the malting genotypes were richest in vitamin E content (~26–32 µg/g DW). In the food group, Adagio was the best source of vitamin E with ~27 µg/g DW.

The average antioxidant capacity in the barley genotypes was ~109 mg AEAC/100 g FW and the capacity ranged widely, from ~57 mg AEAC/100 g FW in ICARDA 39 to ~158 mg AEAC/100 g FW in WI2585 (Fig. 1b). WI2585 and Harrington had the highest antioxidant capacity and were also high in vitamin E, whereas the ICARDA lines were low in both. As with vitamin E content, all coloured genotypes were lower in antioxidant capacity than white genotypes (~57–104 mg AEAC/100 g FW compared with ~106–158 mg AEAC/100 g FW). The malting genotypes tended to represent the best sources of antioxidants (124.3 ± 5.0 mg AEAC/100 g FW), followed by food (108.0 ± 5.1 mg AEAC/100 g FW) and then feed genotypes (84.6 ± 9.2 mg AEAC/100 g FW).

**Table 1**  
Mean content of tocopherols and tocotrienols in 25 barley genotypes at harvest (for  $n = 6$  biological replicates except  $n = 9$  for Jet and ICARDA 16 and  $n = 3$  for Buloke).

Genotype	Content ( $\mu\text{g/g DW}$ )								
	$\alpha$ -T	$\beta$ -T	$\gamma$ -T	Total T	$\alpha$ -T3	$\beta$ -T3	$\gamma$ -T3	Total T3	Tocols T + T3
Jet	4.62 <sup>a</sup>	0.34 <sup>efgh</sup>	0.29 <sup>a</sup>	5.25	12.18 <sup>a</sup>	1.55 <sup>a</sup>	1.31 <sup>a</sup>	15.04	20.29
Sumire mochi	8.60 <sup>b</sup>	0.40 <sup>efghi</sup>	1.04 <sup>b</sup>	10.04	38.36 <sup>de</sup>	3.45 <sup>c</sup>	8.24 <sup>fg</sup>	50.05	60.09
Macumba	8.93 <sup>b</sup>	0.40 <sup>efghi</sup>	0.40 <sup>a</sup>	9.73	35.96 <sup>d</sup>	3.51 <sup>c</sup>	8.10 <sup>efg</sup>	47.57	57.30
Finniss	9.55 <sup>c</sup>	0.42 <sup>efghij</sup>	0.32 <sup>a</sup>	10.29	43.59 <sup>gh</sup>	7.92 <sup>lm</sup>	7.47 <sup>e</sup>	58.98	69.27
ICARDA 16	13.15 <sup>k</sup>	0.25 <sup>abcd</sup>	2.60 <sup>jk</sup>	16.00	44.01 <sup>h</sup>	7.31 <sup>jk</sup>	4.50 <sup>c</sup>	55.82	71.82
ICARDA 19	14.47 <sup>l</sup>	0.26 <sup>bcde</sup>	5.70 <sup>q</sup>	20.43	32.34 <sup>c</sup>	2.84 <sup>b</sup>	4.33 <sup>c</sup>	39.51	59.94
ICARDA 26	12.59 <sup>ji</sup>	0.13 <sup>a</sup>	2.38 <sup>hi</sup>	15.10	27.38 <sup>b</sup>	4.00 <sup>d</sup>	4.37 <sup>c</sup>	35.75	50.85
ICARDA 35	11.00 <sup>de</sup>	0.33 <sup>cdefg</sup>	1.63 <sup>d</sup>	12.96	33.42 <sup>c</sup>	4.14 <sup>d</sup>	4.13 <sup>c</sup>	41.69	54.65
ICARDA 39	12.17 <sup>ghi</sup>	0.20 <sup>ab</sup>	2.78 <sup>k</sup>	15.15	28.70 <sup>b</sup>	6.41 <sup>gh</sup>	2.73 <sup>b</sup>	37.84	52.99
Tadmor	10.03 <sup>c</sup>	0.44 <sup>ghij</sup>	1.90 <sup>f</sup>	12.37	40.71 <sup>ef</sup>	7.70 <sup>kl</sup>	6.49 <sup>d</sup>	54.90	67.27
Er/Apm	10.59 <sup>d</sup>	0.29 <sup>bcde</sup>	1.30 <sup>c</sup>	12.18	43.91 <sup>h</sup>	6.81 <sup>hi</sup>	11.37 <sup>jk</sup>	62.09	74.27
Vlamingh	11.83 <sup>fg</sup>	0.31 <sup>bcdef</sup>	2.19 <sup>gh</sup>	14.33	51.89 <sup>ij</sup>	6.18 <sup>g</sup>	8.48 <sup>g</sup>	66.55	80.88
Commander	12.39 <sup>hij</sup>	0.49 <sup>ij</sup>	2.06 <sup>fg</sup>	14.94	55.40 <sup>k</sup>	8.38 <sup>n</sup>	9.88 <sup>h</sup>	73.66	88.60
Buloke	12.06 <sup>gh</sup>	0.46 <sup>hij</sup>	5.03 <sup>p</sup>	17.55	44.56 <sup>h</sup>	10.06 <sup>p</sup>	13.36 <sup>m</sup>	67.98	85.53
Fleet	12.10 <sup>ghi</sup>	0.43 <sup>fg hij</sup>	1.89 <sup>ef</sup>	14.42	45.84 <sup>h</sup>	10.50 <sup>pq</sup>	11.40 <sup>jk</sup>	67.74	82.16
Flagship	11.33 <sup>ef</sup>	0.29 <sup>bcde</sup>	2.08 <sup>fg</sup>	13.70	46.06 <sup>h</sup>	7.56 <sup>kl</sup>	8.04 <sup>efg</sup>	61.66	75.36
Sloop	10.79 <sup>d</sup>	0.40 <sup>efghi</sup>	1.13 <sup>bc</sup>	12.32	51.11 <sup>i</sup>	13.56 <sup>r</sup>	10.21 <sup>i</sup>	74.88	87.20
Chebec	12.80 <sup>jk</sup>	0.63 <sup>k</sup>	2.07 <sup>fg</sup>	15.50	45.93 <sup>h</sup>	17.77 <sup>s</sup>	12.52 <sup>l</sup>	76.22	91.72
Amaji nijo	13.29 <sup>k</sup>	0.48 <sup>ij</sup>	4.58 <sup>n</sup>	18.35	41.27 <sup>fg</sup>	4.60 <sup>e</sup>	14.81 <sup>n</sup>	60.68	79.03
Harrington	12.21 <sup>ghi</sup>	0.45 <sup>ghij</sup>	2.54 <sup>ij</sup>	15.20	60.43 <sup>l</sup>	10.74 <sup>q</sup>	16.06 <sup>o</sup>	87.23	102.43
WI2585	11.70 <sup>fg</sup>	0.66 <sup>k</sup>	3.74 <sup>l</sup>	16.10	44.35 <sup>h</sup>	7.01 <sup>ij</sup>	11.61 <sup>k</sup>	62.97	79.07
Dhow	12.00 <sup>gh</sup>	0.23 <sup>abcd</sup>	1.70 <sup>de</sup>	13.93	54.05 <sup>jk</sup>	9.48 <sup>o</sup>	10.89 <sup>ij</sup>	74.42	88.35
ND24260-1	12.58 <sup>ij</sup>	0.35 <sup>efgh</sup>	3.74 <sup>l</sup>	16.67	50.36 <sup>i</sup>	6.06 <sup>g</sup>	7.62 <sup>ef</sup>	64.04	80.71
Adagio	12.75 <sup>jk</sup>	0.54 <sup>jk</sup>	4.69 <sup>no</sup>	17.98	45.93 <sup>h</sup>	8.22 <sup>mn</sup>	11.99 <sup>kl</sup>	66.14	84.12
Alexis	12.45 <sup>hij</sup>	0.21 <sup>abc</sup>	4.23 <sup>m</sup>	16.89	51.08 <sup>i</sup>	5.07 <sup>f</sup>	10.45 <sup>i</sup>	66.60	83.49
Mean	11.44	0.38	2.48	14.30	42.56	7.23	8.81	58.60	72.90
LSD	0.52	0.12	0.19		2.51	0.45	0.68		

The same letter indicates no difference between genotypes for individual isomers (within column) as determined using the Least Significant Difference (LSD) ( $P < 0.05$ ).  $\delta$ -T3 and  $\delta$ -T were not detected.

Along with the total antioxidant capacity analysis, the antioxidant capacity of vitamin E was also examined (Fig. 1c) and found to be much lower than total antioxidant capacity ( $\sim 0.06$ – $7$  mg AEAC/100 g FW compared with  $\sim 57$ – $158$  mg AEAC/100 g FW), indicating that vitamin E contributes a low proportion of the total antioxidant capacity in barley. In addition, vitamin E and total antioxidant capacity were not significantly correlated ( $n = 25$ ,  $r = 0.46$ ).

### 3.2. Effect of storage on vitamin E and antioxidant capacity

As expected, all six major isomers ( $\alpha$ -T,  $\alpha$ -T3,  $\beta$ -T,  $\beta$ -T3,  $\gamma$ -T and  $\gamma$ -T3) were detected in barley genotypes after 4 months storage (Fig. 2), but the amounts of  $\delta$ -T and  $\delta$ -T3 were negligible (data not shown). The ranking order for the concentration of isomers was similar to that of non-stored samples, which was  $\alpha > \gamma > \beta$ . No significant storage effect was observed for  $\alpha$ -T in Macumba (Fig. 2a);  $\alpha$ -T3 in ICARDA 19, Vlamingh, Tadmor, Commander or Flagship (Fig. 2b);  $\beta$ -T in ICARDA 19, Sumire mochi, Macumba, Commander, Harrington, Er/Apm or WI2585 (Fig. 2c); and  $\gamma$ -T in Sumire mochi (Fig. 2e). In this study, the change of  $\alpha$ -T among genotype genotypes ranged from  $\sim 7\%$  to  $34\%$ , whereas that of  $\alpha$ -T3 was  $\sim 0.7$ – $25\%$  (Fig. 2a and b). As a result of these changes, overall vitamin E content changed by between  $6\%$  and  $30\%$  after 4 months of storage (Fig. 3).

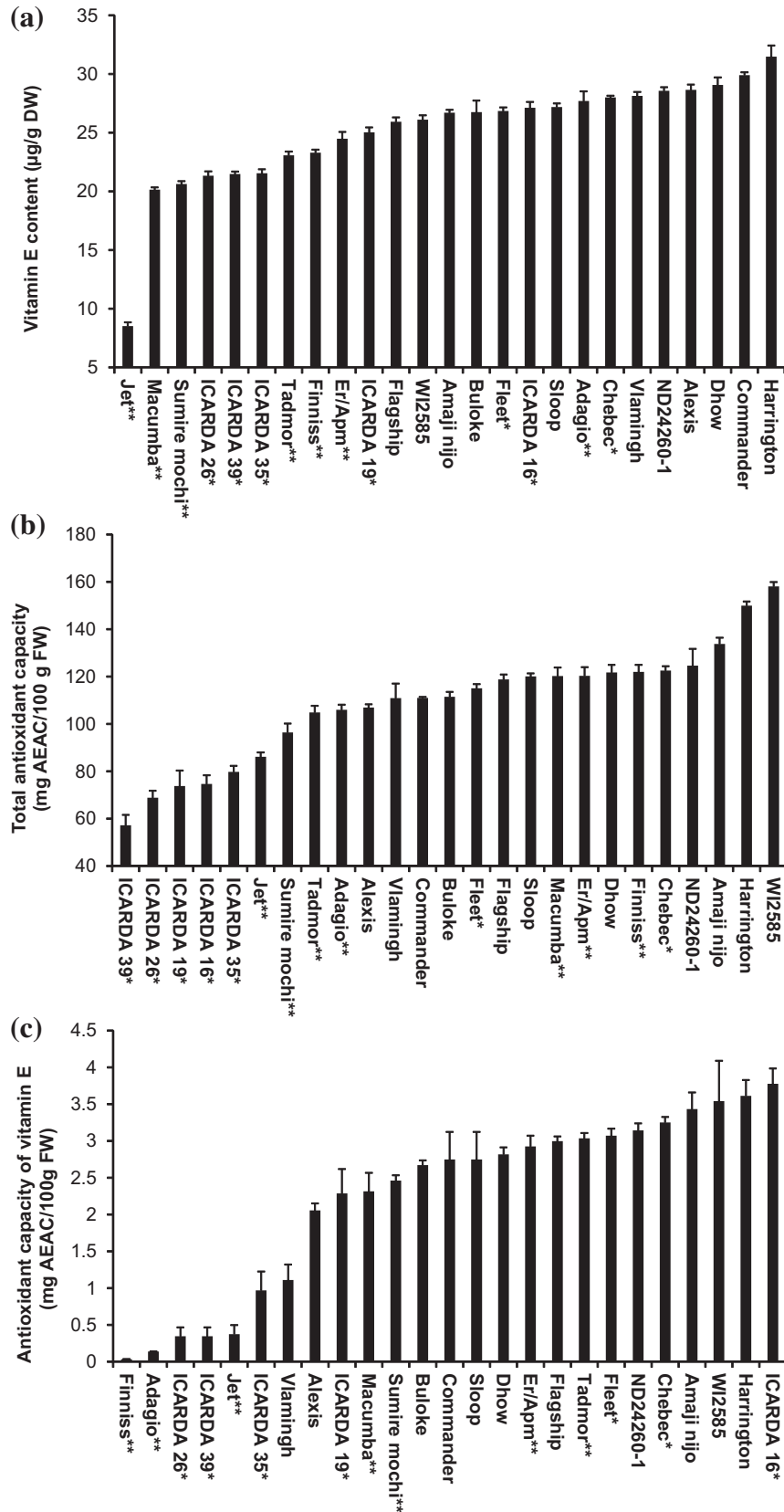
The antioxidant capacity for most genotypes appeared lower in stored grains than grains at harvest (Fig. 4). The reductions in antioxidant capacity in grains after storage were lowest in Finniss ( $\sim 6\%$ ) and highest in ND24260-1 ( $\sim 16\%$ ). However, a significant increase in antioxidant capacity was observed in the coloured genotypes ICARDA 26 and ICARDA 39. No difference was observed for ICARDA 19, ICARDA 35, Sumire mochi or Tadmor (coloured genotypes) or Macumba, Flagship and Adagio (white genotypes).

PCA plots derived from the ATR-MIR analysis revealed that grains at harvest can be differentiated from grain samples stored for 4 months (Fig. 5) using the first two principal components, PC1 and PC2. The observed discrimination into different clusters based on storage can be explained by the main MIR regions for each of the principal components (Supplementary information, Table S2). For the purpose of this paper only the lipid region between frequencies  $2900$  to  $2800\text{ cm}^{-1}$  as well as  $1750$  to  $1700\text{ cm}^{-1}$  was analysed and explained more than  $90\%$  of the variance in the PCA score plot.

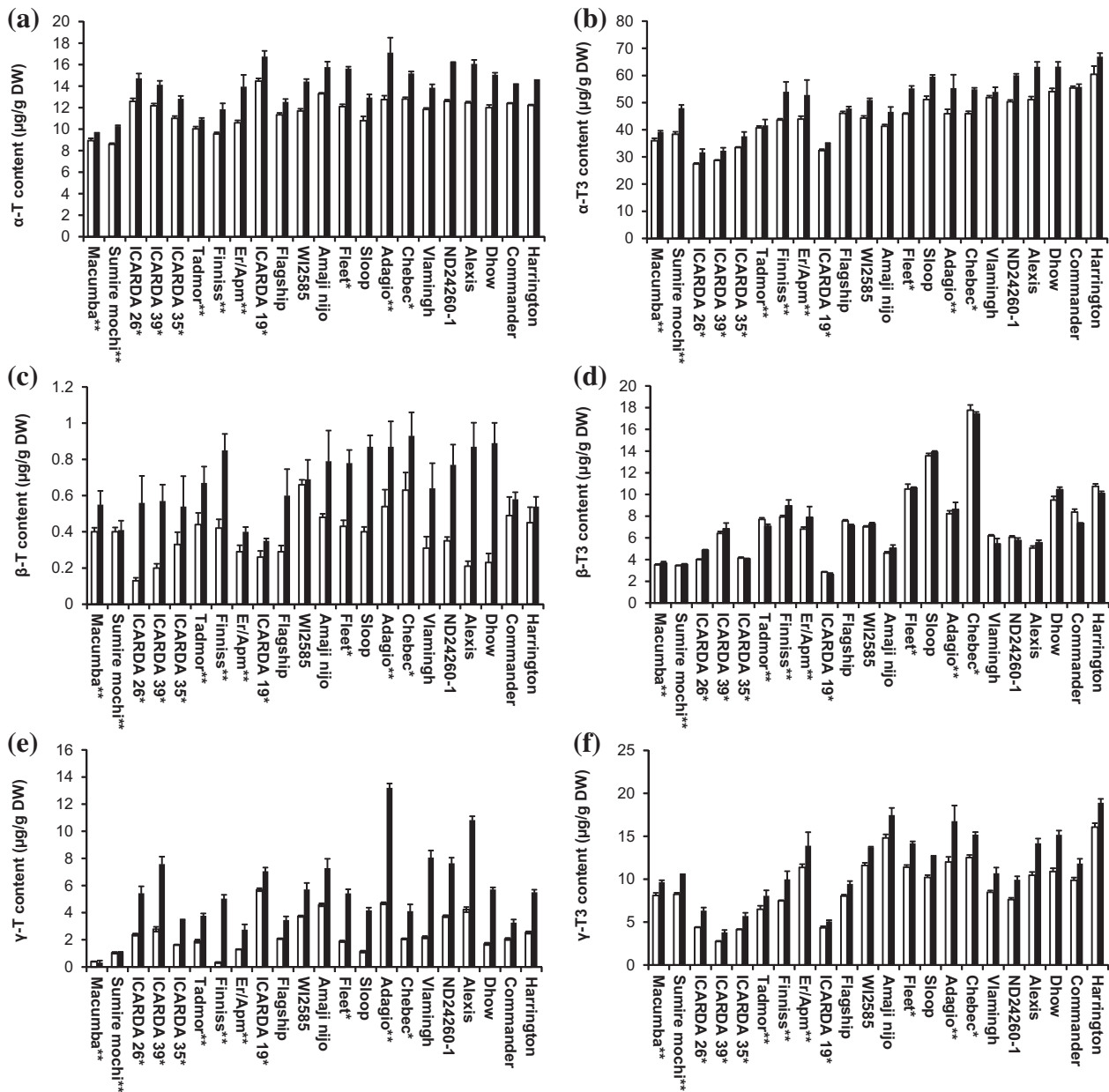
## 4. Discussion

While a limited number of studies have investigated both antioxidant capacity and vitamin E content in barley, no previous study has assessed these parameters across a number of different genotypes. Our findings demonstrate that both vitamin E content and antioxidant capacity are variable and genotype-dependent. Furthermore, declines in antioxidant capacity and increases in vitamin E content were observed in most genotypes after 4 months of storage at an industry relevant temperature.

The mean content of tocols for all barley genotypes was  $72.9\text{ }\mu\text{g/g DW}$ , which is in good agreement with those previously found by Peterson and Qureshi (1993) ( $58\text{ }\mu\text{g/g DW}$ ), Cavallero, Gianinetti, Finocchiaro, Delogu, and Stanca (2004) ( $54.5\text{ }\mu\text{g/g DW}$ ) and Panfili et al. (2008) ( $69.1\text{ }\mu\text{g/g DW}$ ). The average vitamin E content ( $24.2\text{ }\mu\text{g/g DW}$ ) was also in good agreement with Panfili et al. (2008) ( $21.9\text{ }\mu\text{g/g DW}$ ). However, the range of total tocol amount varied from  $20.29\text{ }\mu\text{g/g DW}$  for Jet (hullless/black/food type) to  $102.43\text{ }\mu\text{g/g DW}$  for Harrington (covered/white/malting type). This was much wider than that reported previously ( $42$ – $80\text{ }\mu\text{g/g DW}$  (Peterson & Qureshi, 1993);  $51.0$ – $61.4\text{ }\mu\text{g/g DW}$  (Cavallero et al., 2004) and  $50.3$ – $88.6\text{ }\mu\text{g/g DW}$  (Panfili et al., 2008)). However, these previous studies included a much more



**Fig. 1.** Vitamin E contents (a), total antioxidant capacity (b) and antioxidant capacity of vitamin E (c) in 25 barley genotypes at harvest, shown in ascending order. The names marked with \* or with \*\* are feed or food genotypes, respectively. The others are malting genotypes. Vitamin E is expressed in mg of  $\alpha$ -tocopherol-equivalents (TE) and antioxidant capacity is expressed as mg ascorbic acid equivalent antioxidant capacity (AEAC) per 100 g of fresh weight (FW) of grain. Bars represent the mean  $\pm$  SE. For (a),  $n = 6$  except  $n = 9$  for Jet and ICARDA 16 and  $n = 3$  for Buloke. The Least Significant Difference (LSD) ( $P < 0.05$ ) = 1.03. For (b),  $n = 4$  except  $n = 7$  for Finiss, Adagio, Sumire mochi, Macumba, Tadmor, Jet, Commander, Chebec, Harrington, Sloop, Er/Apm, Flagship and  $n = 3$  for Buloke. The LSD ( $P < 0.05$ ) = 7.66. For (c),  $n = 3$  for all genotypes. The LSD ( $P < 0.05$ ) = 0.61.

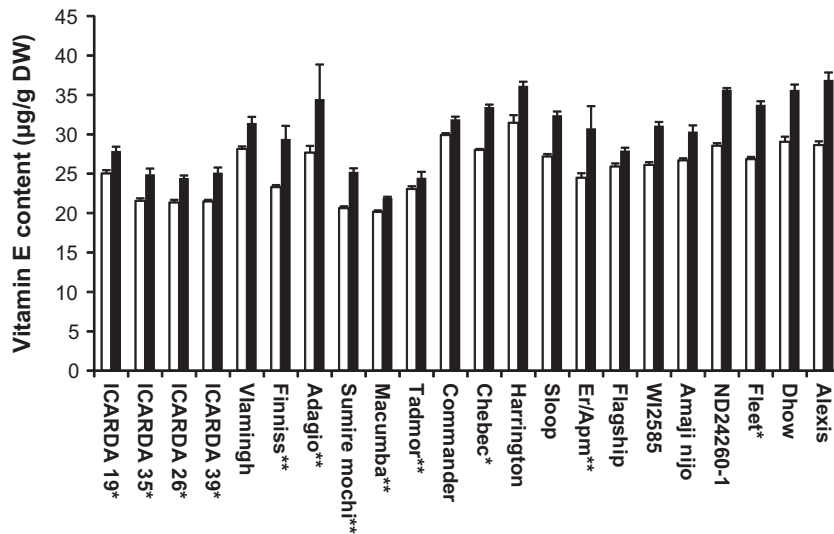


**Fig. 2.** Effect of storage on the profile of tocopherol and tocotrienol isomers in different barley genotypes.  $\alpha$ -T (a),  $\alpha$ -T3 (b),  $\beta$ -T (c),  $\beta$ -T3 (d),  $\gamma$ -T (e), and  $\gamma$ -T3 (f). The genotype names marked with \* or with \*\* are feed or food genotypes, respectively. The others are malting genotypes.  $\square$ , content before storage,  $n = 6$  except for Jet and ICARDA 16, and  $n = 3$  for Buloko;  $\blacksquare$ , content after storage,  $n = 3$  for all stored genotypes. The bars represent means ( $\pm$ SE). The Least Significant Difference ( $LSD_{\text{sample,time}}$ ) ( $P < 0.05$ ) for  $\alpha$ -T = 0.65;  $LSD_{\text{sample,time}}$  for  $\alpha$ -T3 = 3.11;  $LSD_{\text{sample,time}}$  for  $\beta$ -T = 0.18;  $LSD_{\text{sample,time}}$  for  $\beta$ -T3 = 0.57;  $LSD_{\text{sample,time}}$  for  $\gamma$ -T = 0.39;  $LSD_{\text{sample,time}}$  for  $\gamma$ -T3 = 0.95.

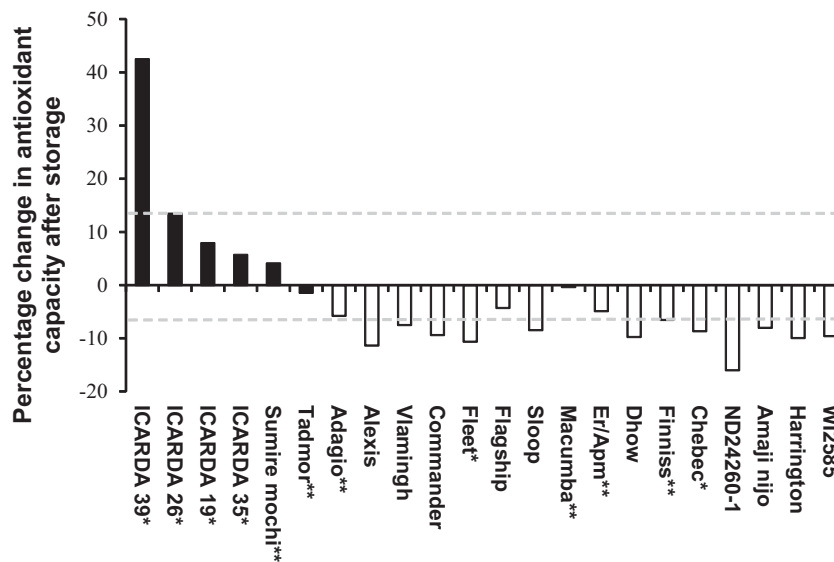
limited range of genotypes, and did not always include different classes. Therefore, our results provide evidence that tocol content in barley is highly genotype-dependent. The contribution of individual isomers to tocol content was similar to that found in other studies (Ehrenbergerova, Belcrediova, Pryma, Vaculova, & Newman, 2006; Panfili et al., 2008).  $\alpha$ -T3 and  $\alpha$ -T were most dominant in all barley genotypes, followed by  $\beta$ - and  $\gamma$ -tocols. The  $\delta$ -tocols, undetected isomers in the present study, were also not detected in other studies, or made up less than 2  $\mu\text{g/g DW}$  of content (Ehrenbergerova, Belcrediova, Pryma, et al., 2006; Panfili et al., 2008). In the vitamin E pathway,  $\alpha$ -tocols are derived from  $\gamma$ -tocols while  $\beta$ -tocols are derived from  $\delta$ -tocols (Hunter & Cahoon, 2007) which may explain the higher content of  $\alpha$ -tocols (compared to  $\gamma$ -tocols) and  $\beta$ -tocols (compared to  $\delta$ -tocols).

Similar to tocol content, antioxidant capacity varied between the genotypes. Within food genotypes, even though antioxidant capacity has been reported to be concentrated in the husk (Peterson, 1994), the hullless genotypes used in this study, Macumba and Finnis, also had a relatively high antioxidant capacity (120 and 122 mg AEAC/100 g FW, respectively) compared with the lowest antioxidant capacity genotype, Jet (57.2 mg AEAC/100 g FW) and the highest antioxidant capacity genotype WI2585 (158.1 mg AEAC/100 g FW). This suggests that both Macumba and Finnis may have potential for use as a source of antioxidants in food, particularly as they do not need to be pearled during processing.

Regardless of genotype, vitamin E only contributed a low proportion of the total antioxidant capacity in barley grain. There



**Fig. 3.** The vitamin E content of grains from different barley genotypes before and after storage. The names marked with \* or with \*\* are feed or food genotypes, respectively. The others are malting genotypes. □, content before storage,  $n = 6$  except  $n = 9$  for Jet and ICARDA 16, and  $n = 3$  for Buloke; ■, content after storage,  $n = 3$  for all stored genotypes. Bars present the mean  $\pm$  SE. The Least Significant Difference samples by time ( $LSD_{\text{sample,time}}$ ) ( $P < 0.05$ ) = 1.40.

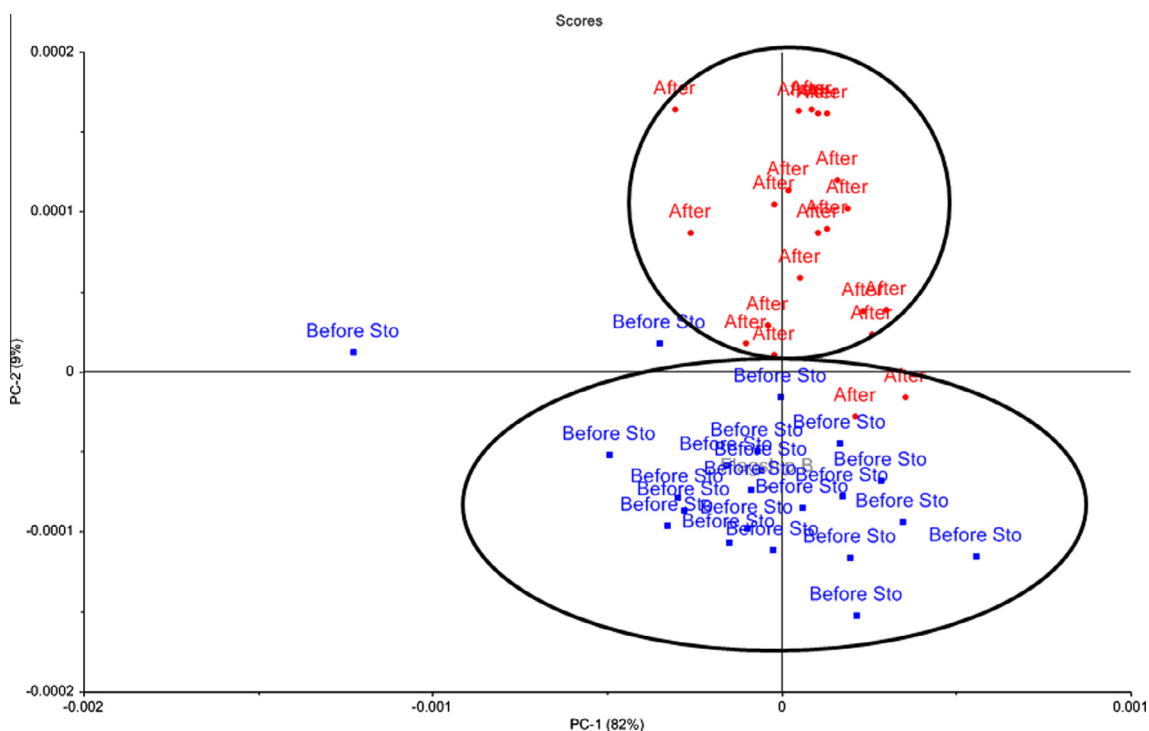


**Fig. 4.** The percentage change of antioxidant capacity of grains from different barley genotypes after storage. The names marked with \* or with \*\* are feed or food genotypes, respectively. The others are malting genotypes. ■, colour genotypes; □, non-colour genotypes. Bars not within dotted lines represent genotypes with significantly different percentage change of antioxidant capacity after storage.

was no correlation between antioxidant capacity and the contents of five of the individual vitamin E isomers, however a significant correlation was observed between antioxidant capacity and  $\alpha$ -T3 content ( $n = 25$ ,  $r = 0.7$ ,  $p < 0.05$ ). For example, Harrington had high antioxidant capacity and  $\alpha$ -T3 content while ICARDA 39 had low antioxidant capacity and  $\alpha$ -T3 content. Although  $\alpha$ -T has historically been reported as the most efficient antioxidant (McLaughlin & Weihrauch, 1979),  $\alpha$ -T3 has recently been shown to be at least threefold more efficient as a scavenger of peroxy radicals than  $\alpha$ -T (Packer, 1995).  $\alpha$ -T3 was the main vitamin E isomer in barley grain, regardless of genotype, and the correlation with antioxidant capacity supports this observation.

All the coloured genotypes used in this study (the ICARDA lines, Jet, Sumire mochi and Tadmor) showed lower levels of vitamin E than the white genotypes. These coloured barley genotypes were

also poor in antioxidant capacity (ICARDA lines > Jet > Sumire mochi > Tadmor). Recent studies indicate that antioxidant capacity is high in coloured cereal grains due to the contribution of pigment compounds such as proanthocyanidin and anthocyanins, with the main forms being cyanidin-3-glucose and delphinidin-3-glucose (Abdel-Aal, Young, & Rabalski, 2006). However, all eight coloured genotypes in this study had a lower antioxidant capacity than white genotypes, suggesting that non-pigmented compounds may be more likely to contribute to antioxidant capacity in barley. Indeed, barley has previously been reported to contain flavonol, phenolic acids and flavan-3-ols (Goupy et al., 1999). Furthermore, antioxidant enzymes and apolar compounds combining tocals and carotenoids (especially lutein and zeaxanthin) also appear to contribute to antioxidant capacity in barley (Goupy et al., 1999). Given that vitamin E was probably not the main contributor to



**Fig. 5.** Score plot of the two principal components of barley flour samples before and after storage analysed using attenuated total reflectance and mid infrared spectroscopy. PC1, principal component 1; PC2, principal component 2.

antioxidant capacity in barley grain, the contribution of these other compounds in the genotypes used in this study should be investigated further in future studies.

Vitamin E has recently been shown to be unstable in the presence of light, water, oxygen and heat, particularly during extended periods of exposure (Wang et al., 1993). In the current study, the changes observed after storage were different among genotypes, as well as among their isomers, similar to the findings of Park, Kim, Park, and Lee (2004) and Peterson (1995). For example, the change in T was lower than that of T3. This can likely be attributed to the difference in their molecular structures; T3 may be more susceptible to oxidation because of its unsaturated side chains, whereas T has saturated side chains (Liu & Moreau, 2008). Macumba and Commander were more stable than the other tested genotypes. Stability of tocol isomer composition in barley has also been observed after storage for 11 months in a conventional silo (Hakkarainen, Työppönen, & Bengtsson, 1983), at ambient temperature for 8 weeks and 90 °C for 48 h (Työppönen & Hakkarainen, 1985). The  $\gamma$ -T content in Macumba declined after storage, even though the other isomers remained unchanged. Given that  $\gamma$ -T is the precursor to  $\alpha$ -T in the vitamin E pathway, its decline suggests that the stability of  $\alpha$ -T was due to increased conversion of  $\gamma$ -T to  $\alpha$ -T. All other genotypes showed increases in all individual isomers after storage.

The changes in vitamin E content after storage differed between the genotypes, and these differences are likely to be explained by the different initial content of vitamin E in the respective genotypes before storage. Furthermore, each genotype appeared to have a different fatty acid and lipid profile, indicating different ratios between unsaturated and saturated fatty acids (data not shown). Indeed, the discrimination between samples before and after storage observed in the PCA plots derived from the ATR-MIR analysis (Fig. 5) appears to be due to differences in the frequencies associated with methyl groups, particularly in lipids. Biochemical reactions changing the frequencies of methyl groups in lipids and the

tolcols possibly associated with them may therefore occur during storage. This may lead to differences in the extractability of lipids (Pomeranz & Chang, 1977) and therefore vitamin E. In stored grain, reactions such as esterification may also prevent loss of vitamin E due to reduction of vitamin E oxidation (Church & Pond, 1977).

Our findings are in good agreement with Liu and Moreau (2008) who found that tocopherol contents increased in barley stored at 35 °C as intact whole grains for 3 weeks or 25 °C for 6 months. Wang et al. (1993) reported increasing  $\delta$ -T3, while all T isomers remained stable in barley after storage at 27 °C. In contrast, some studies observed a decrease in tocol isomers during storage. Työppönen and Hakkarainen (1985) found that the tocol content of barley flour which was stored and exposed to light at 25 °C, decreased by 5% every week over an 8 week period. At the same temperature, however, the degradation was less in intact barley, with only 1% loss of tocols each month during 11 months of storage (Hakkarainen et al., 1983). This discrepancy may be explained by differences in temperature, light and moisture during storage. Heat may be an important factor in grain preservation (Metz, 2006), and 10 °C is considered to be an ideal storage temperature (Viljoen, 2001). According to Ball (2006), T and T3 may be destroyed fairly rapidly by sunlight and artificial UV light. The higher the moisture content, the shorter the shelf life of barley grains, for example, barley grains with 11.5–12.5% moisture content stored for 3 months but barley grains with 10.5–11.5% stored for 6 months (Metz, 2006). Furthermore, compared with barley flour, tocols in intact barley grain degraded at a slower rate (Hakkarainen et al., 1983). Storage time may also influence tocols. Interestingly, the content of  $\alpha$ -T and  $\alpha$ -T3 increased during the first 2–3 months of storage but declined after 11 months storage in silos, from 92 to 20  $\mu\text{g/g}$  DW (at 28% moisture content) or 80–15  $\mu\text{g/g}$  DW (at 23% moisture content) (Hakkarainen et al., 1983).

Our study showed that most of the barley genotypes, with the exception of coloured genotypes, lost their antioxidant capacity after storage. This loss of antioxidant capacity after storage has also

been reported previously in wheat bran stored at 25 °C (38% lost) or 60 °C (47% lost) after 9 days (Cheng et al., 2006) while the enzymatic activities of dehydroascorbate reductase, glutathione reductase, glutathione peroxidase and catalase decreased when grains were stored at 10 °C (Spano, Bottega, Lorenzi, & Grilli, 2011). The increase in antioxidant capacity observed in coloured barely genotypes after storage in the present study is in agreement with the findings of Htwe et al. (2010) in black and red rice during storage at 20, 30 and 40 °C for up to 4 months. This result also indicated that  $\beta$ -carotene in black rice increased whereas total anthocyanin remained stable and both free soluble conjugated and insoluble bound forms of polyphenols decreased significantly. Therefore, the relationship between increase in  $\beta$ -carotene content and higher antioxidant capacity in coloured barley genotypes after storage needs further study.

As free radicals have been widely indicated as the major cause of seed deterioration (Lehner et al., 2008), the genotypes with higher antioxidant capacity may be more likely to be preserved during storage and processing. Other authors have previously shown that processed oat products were more stable when the oat genotype contained higher antioxidant levels (Peterson, 2001). Antioxidants have also been shown to act as a preservative when added to various foodstuffs. This is the case not only for pure antioxidants but also for extracted antioxidants; oat hull extract, for example, can inhibit fungal and bacterial growth (Peterson, 2001). Therefore, grains with inherent antioxidants may have more potential to protect themselves in storage. This is in accordance with the results of a previous study of stored wheat, which showed a negative correlation between the efficiency of the antioxidant enzymatic machinery and the age of grain (Lehner et al., 2008; Spano et al., 2011). Possible future research could be aimed at investigating the relationship between high antioxidant genotypes and their shelf life during storage.

## 5. Conclusions

The genotypic differences identified here will allow genotypes with high antioxidant capacity and/or vitamin E content to be chosen for breeding purposes. The genotypes that maintain antioxidant capacity and/or vitamin E content after storage have the greatest potential for functional food products. We are now determining the genetic basis of differences in antioxidant capacity and/or vitamin E content by evaluating mapping populations derived from parents with low or high antioxidant capacity or vitamin E content.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2015.04.028>.

## References

Abdel-Aal, E. S. M., Young, J. C., & Rabalski, I. (2006). Anthocyanin composition in black, blue, pink, purple, and red cereal grains. *Journal of Agricultural and Food Chemistry*, 54(13), 4696–4704.

Ball, G. F. M. (2006). Vitamins in foods: Analysis, bioavailability and stability. In G. F. M. Ball (Ed.), *Vitamins in foods: Analysis, bioavailability and stability* (pp. 785). Boca Raton, USA: CRC Press LLC.

Bhatty, R. S. (1999). The potential of hull-less barley. *Cereal Chemistry*, 76(5), 589–599.

Bjelakovic, G., Nikolova, D., Glud, L. L., Simonetti, R. G., & Glud, C. (2007). Mortality in randomized trials of antioxidant supplements for primary and secondary prevention – Systematic review and meta-analysis. *Journal of the American Medical Association*, 297(8), 842–857.

Cavallero, A., Gianinetti, A., Finocchiaro, F., Delogu, G., & Stanca, A. M. (2004). Tocols in hull-less and hulled barley genotypes grown in contrasting environments. *Journal of Cereal Science*, 39(2), 175–180.

Cheng, Z., Su, L., Moore, J., Zhou, K., Luther, M., Yin, J.-J., et al. (2006). Effects of postharvest treatment and heat stress on availability of wheat antioxidants. *Journal of Agricultural and Food Chemistry*, 54(15), 5623–5629.

Church, D. C., & Pond, W. G. (1977). *Basic animal nutrition and feeding*. New York: Wiley.

Cozzolino, D., Roumeliotis, S., & Eglinton, J. (2013). Prediction of starch pasting properties in barley flour using ATR–MIR spectroscopy. *Carbohydrate Polymers*, 95(1), 509–514.

Ehrenbergerova, J., Belcrediova, N., Pryma, J., Vaculova, K., & Newman, C. W. (2006). Effect of cultivar, year grown, and cropping system on the content of tocopherols and tocotrienols in grains of hulled and hullless barley. *Plant Foods for Human Nutrition*, 61(3), 145–150.

Ehrenbergerova, J., Belcrediova, N., Havlova, P., Pryma, J., Vaculova, K., & Vejrazka, K. (2006). Barley grain as source of natural antioxidants and nutraceuticals beneficial to health. In *Proceedings of the 3rd international congress flour–bread '05 and 5th Croatian Congress of Cereal Technologists* (pp. 188–194).

Goupy, P., Hugues, M., Boivin, P., & Amiot, M. J. (1999). Antioxidant composition and activity of barley (*Hordeum vulgare*) and malt extracts and of isolated phenolic compounds. *Journal of the Science of Food and Agriculture*, 79(12), 1625–1634.

Hakkarainen, R. V. J., Työppönen, J. T., & Bengtsson, S. G. (1983). Relative and quantitative changes in total vitamin-E and isomer content of barley during conventional and airtight storage with special reference to annual variations. *Acta Agriculturae Scandinavica*, 33(4), 395–400.

Htwe, N. N., Srilaong, V., Tanprasert, K., Tongchitpakdee, S., Kanlayanarat, S., & Uthairatanakij, A. (2010). Effects of storage time and temperature on radical scavenging activities and bioactive compounds in colored rice varieties. *Journal of Food Agriculture & Environment*, 8(3–4), 26–31.

Hunter, S. C., & Cahoon, E. B. (2007). Enhancing vitamin E in oilseeds: Unraveling tocopherol and tocotrienol biosynthesis. *Lipids*, 42(2), 97–108.

Idaho Barley Commission. (2011). *Malt barley storage*. <[http://barley.idaho.gov/pdf/malt\\_barley\\_presentations/grain\\_storage.pdf](http://barley.idaho.gov/pdf/malt_barley_presentations/grain_storage.pdf)> Accessed 11.09.11.

Kim, M.-J., Hyun, J.-N., Kim, J.-A., Park, J.-C., Kim, M.-Y., Kim, J.-G., et al. (2007). Relationship between phenolic compounds, anthocyanins content and antioxidant activity in colored barley germplasm. *Journal of Agricultural and Food Chemistry*, 55(12), 4802–4809.

Lampi, A. M., Ryyanen, M., Salo-Vaananen, P., Ollilainen, V., & Piironen, V. (2004). A small-scale sample preparation method with HPLC analysis for determination of tocopherols and tocotrienols in cereals. *Journal of Food Composition and Analysis*, 17(6), 749–765.

Lehner, A., Mamadou, N., Poels, P., Come, D., Bailly, C., & Corbineau, F. (2008). Changes in soluble carbohydrates, lipid peroxidation and antioxidant enzyme activities in the embryo during ageing in wheat grains. *Journal of Cereal Science*, 47(3), 555–565.

Liu, K. S., & Moreau, R. A. (2008). Concentrations of functional lipids in abraded fractions of hullless barley and effect of storage. *Journal of Food Science*, 73(7), C569–C576.

Mclaughlin, P. J., & Weihrauch, J. L. (1979). Vitamin-E content of foods. *Journal of the American Dietetic Association*, 75(6), 647–665.

Metz, N. (2006). *The WA guide to high moisture harvest management, grain storage and handling*. Australia: CBH Group and South East Premium Wheat Growers Association.

Omwamba, M., & Hu, Q. (2009). Antioxidant capacity and antioxidative compounds in barley (*Hordeum vulgare* L.) grain optimized using response surface methodology in hot air roasting. *European Food Research and Technology*, 229(6), 907–914.

Packer, L. (1995). Nutrition and biochemistry of the lipophilic antioxidants, vitamin E and carotenoids. In L. Packer, E. Niki, & A. S. H. Ong (Eds.), *Nutrition, lipids, health and disease* (pp. 8–35). Champaign: AOCS Press.

Panfili, G., Fratianni, A., Criscio, T. d., & Marconi, E. (2008). Tocol and beta-glucan levels in barley varieties and in pearling by-products. *Food Chemistry*, 107(1), 84–91.

Panfili, G., Fratianni, A., & Irano, M. (2003). Normal phase high-performance liquid chromatography method for the determination of tocopherols and tocotrienols in cereals. *Journal of Agricultural and Food Chemistry*, 51(14), 3940–3944.

Park, S. R., Kim, Y. H., Park, H. J., & Lee, Y. S. (2004). *Stability of tocopherols and tocotrienols extracted from unsaponifiable fraction of rice bran under various temperature and oxygen conditions*. Brisbane, Australia: 4th International Crop Science Congress.

Peterson, D. M. (1994). Barley tocals: Effects of milling, malting, and mashing. *Cereal Chemistry*, 71(1), 43–44.

Peterson, D. M. (1995). Oat tocals: Concentration and stability in oat products and distribution within the kernel. *Cereal Chemistry*, 72(1), 21–24.

Peterson, D. M. (2001). Oat antioxidants. *Journal of Cereal Science*, 33(2), 115–129.

Peterson, D. M., & Qureshi, A. A. (1993). Genotype and environment effects on tocals of barley and oats. *Cereal Chemistry*, 70(2), 157–162.

Pomeranz, Y., & Chang, O. K. (1977). Interactions of lipids with proteins and carbohydrates in bread-making. *Journal of the American Oil Chemists' Society*, 55, 285–289.

- Reboul, E., Richelle, M., Perrot, E., Desmoulins-Malezet, C., Pirisi, V., & Borel, P. (2006). Bioaccessibility of carotenoids and vitamin E from their main dietary sources. *Journal of Agricultural and Food Chemistry*, 54(23), 8749–8755.
- Sheppard, A. J., Pennington, J. A. T., & Weihrauch, J. L. (1993). Analysis and distribution of vitamin E in vegetable oils and foods. In L. Packer & J. Fuchs (Eds.), *Vitamin E in health and disease* (pp. 9–31). New York: Marcel Dekker Inc.
- Spano, C., Bottega, S., Lorenzi, R., & Grilli, I. (2011). Ageing in embryos from wheat grains stored at different temperatures: Oxidative stress and antioxidant response. *Functional Plant Biology*, 38(7), 624–631.
- Työppönen, J. T., & Hakkarainen, R. V. J. (1985). Thermal stability of vitamin E in barley. *Acta Agriculturae Scandinavica*, 35(2), 136–138.
- Viljoen, J. (2001). Select grain stores carefully to control costs. *Farming Ahead*, 119, 44–45.
- Wang, L., Xue, Q., Newman, R. K., & Newman, C. W. (1993). Enrichment of tocopherols, tocotrienols, and oil in barley fractions by milling and pearling. *Cereal Chemistry*, 70(5), 499–501.
- Young, I. S., & Woodside, J. V. (2001). Antioxidants in health and disease. *Journal of Clinical Pathology*, 54(3), 176–186.



**Supplementary Information,** Do TDT, Cozzolino D, Muhlhausler B, Box A, Able AJ (2015) *Antioxidant capacity and vitamin E in barley: effect of genotype and storage.*

**Table S1. Barley genotypes and their characteristics**

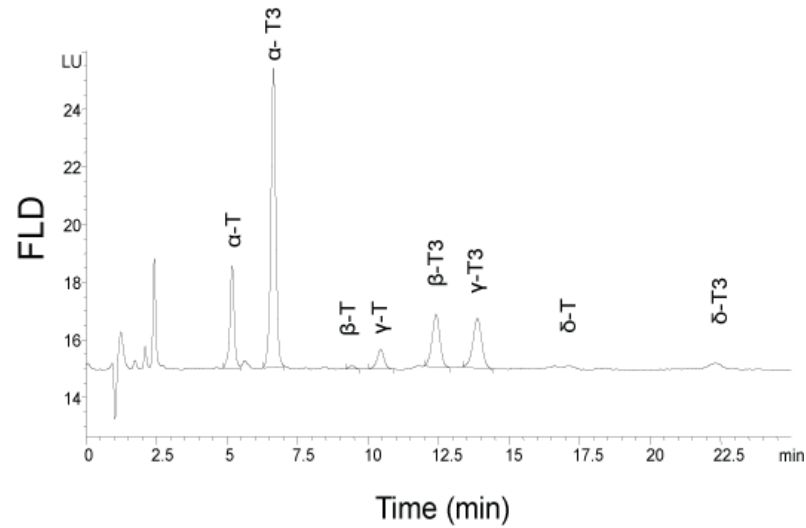
	Food		Malting		Feed	
	Coloured	White	Coloured	White	Coloured	White
Hulless	Jet Finniss Sumire mochi Macumba					
Covered	Tadmor	Adagio Er/Apm		Flagship WI2585 Vlamingh Amaji nijo Harrington ND24260-1 Commander Alexis Dhow Sloop Buloke	ICARDA 16 ICARDA 19 ICARDA 26 ICARDA 35 ICARDA 39	Fleet Chebec

**Supplementary Information,** Do TDT, Cozzolino D, Muhlhausler B, Box A, Able AJ (2015) *Antioxidant capacity and vitamin E in barley: effect of genotype and storage.*

**Table S2. The loadings in the first two principal components at wavenumbers associated with certain molecules.** PC, principal component; +, positive; -, negative; na, not applicable.

Wavenumbers	Molecules	PC1	PC2
2970	CH <sub>3</sub> (Lipids)	na	+
2960	CH <sub>3</sub> (Lipids)	-	na
2923	CH <sub>3</sub> (Lipids)	+	na
2911	CH <sub>3</sub> (Lipids)	na	-
2898	CH <sub>3</sub> (Lipids)	-	na
2872	CH <sub>3</sub> (Lipids)	na	+
2853	CH <sub>3</sub> (Lipids)	+	na
2841	CH <sub>3</sub> (Lipids)	na	-
2826	CH <sub>3</sub> (Lipids)	-	na
1708-1710	Lipids	-	-
1742	Lipids	+	-

**Supplementary Information,** Do TDT, Cozzolino D, Muhlhausler B, Box A, Able AJ (2014) *Antioxidant capacity and vitamin E in barley: genotypic dependence and effect of storage.*



**Figure S1** Representative chromatogram of tocopherols in barley in this study.

**Chapter 4. Manuscript 2: EFFECT OF  
MALTING ON ANTIOXIDANT CAPACITY  
AND VITAMIN E IN DIFFERENT BARLEY  
GENOTYPES**

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Statement of Authorship is located in Appendix B.

# Effect of malting on antioxidant capacity and vitamin E content in different barley genotypes

Thi Thu Dung Do, Daniel Cozzolino, Beverly Muhlhausler, Amanda Box and Amanda J. Able\*

Unprocessed barley is known to contain relatively high levels of antioxidants, which play a critical role in human health and the preservation of food and drink products. However, there are limited data on how the antioxidant levels in barley are affected by malting, and whether the level of antioxidants in the processed malt differs between barley varieties. This study aimed to determine the levels of individual vitamin E isomers, total vitamin E content and total antioxidant capacity before, during and after malting in 12 covered and two hulless barley genotypes. Vitamin E content and antioxidant capacity were determined by high-performance liquid chromatography (HPLC) and ability to scavenge DPPH radicals, respectively. The vitamin E content of most genotypes was reduced after steeping, germination and kilning compared with the unprocessed samples. However, the antioxidant capacity in the malt was higher than in the unprocessed samples for the majority of the genotypes. While there was variation in the percentage change in antioxidant capacity between varieties, the antioxidant capacity of samples after malting was directly correlated with their antioxidant capacity before processing ( $r = 0.9$ ,  $n = 14$ ,  $p < 0.05$ ). These results indicate that barley varieties that have higher antioxidant capacity at harvest retain their antioxidants after malting. Thus, these varieties are likely to be the most suitable for producing malts with the added health benefits and anti-spoiling properties associated with greater antioxidant content. Copyright © 2015 The Institute of Brewing & Distilling

**Keywords:** barley; genotypes; vitamin E; antioxidant capacity; malting

## Introduction

Barley (*Hordeum vulgare* L.) contains a number of beneficial nutritional components, including  $\beta$ -glucan (4–9%) and the essential amino acid, lysine (0.5%), and has a high fibre content (14–24%) (1). We have recently reported that barley has a relatively high antioxidant content, and that this is genotype dependent (2). Antioxidants have a number of important health benefits, which are due to their ability to slow tissue damage by preventing the formation of free radicals or scavenging them, or by promoting their decomposition (3). Antioxidants also play a role in the preservation of foods and beverages, and are therefore likely to help protect foods and beverages containing barley products from spoilage (4,5). For example, the major antioxidant in barley, vitamin E, is a lipid phase chain-breaking antioxidant that inhibits lipid peroxidation to limit the formation of *trans*-2-nonenal, one of the main compounds responsible for the stale taste of beer (6). Therefore, using malts with a higher antioxidant capacity may have commercial benefits for the brewing industry, as well as providing health benefits.

A significant proportion of barley harvested worldwide is used for the production of malt, a key ingredient in beer. Malt is also now increasingly used in food products to enhance flavour and provide nutritional benefits, including increased digestibility, making malt an ideal ingredient for baby foods (1,7). Barley malt is also commonly utilized for bread making, with the further advantage of promoting yeast activity (7). Both the health benefits and preserving actions of antioxidants mean that a higher antioxidant content in malts would be beneficial. To date, however, most of the studies assessing antioxidant content in barley have been conducted using unprocessed samples, and the effect of processing on antioxidant capacity is unclear.

This is particularly important since malting is a thermal process, and high temperatures have the potential to impact antioxidant capacity negatively (8,9). However, the results of previous studies that have investigated changes in the content of the key antioxidant, vitamin E, during malting have been inconsistent. Peterson and colleagues (10) found no significant change in vitamin E content after malting, while other studies have shown both positive and negative effects of malting on levels of vitamin E and individual vitamin E isomers (11,12). However, the studies to date have not included all malting stages and have been restricted to some specific vitamin E isomers, such that total vitamin E content, which is calculated by  $\alpha$ -T equivalence involving all isomers –  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ -tocopherol (T) and  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ -tocotrienol (T3) – could not be assessed. Thus, no previous studies have determined the effect of malting stage on all individual isomers, total vitamin E content or antioxidant capacity in barley.

In addition, the studies to date have only included a limited number of barley varieties. This is important, since we have previously reported that both vitamin E content and antioxidant capacity at harvest can vary significantly between barley genotypes (2). However, whether this variation between genotypes persists after malting and whether any changes that occur during malting are consistent between genotypes is unknown. Expanding the use of

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hulless malt is of particular interest because it can be directly added to a variety of products after milling without needing to process and dispose of the hull, or prepare malt extract (13). Hulless malt can also provide benefit to brewing industries owing to its shorter steeping time and higher extraction yield in comparison to hulled varieties, which is due to a finer grind of the malt and higher content of fermentable materials (13).

The objectives of this study were to determine the impact of malting on the content of vitamin E isomers, total vitamin E and antioxidant capacity in a range of barley genotypes, with different antioxidant capacities at harvest, including hulless varieties.

## Materials and methods

### Materials

The barley varieties included in this study were provided by the University of Adelaide Barley Breeding Program and consisted of 14 common malting genotypes including 12 covered (Sloop, Flagship, WI2585, Vlamingh, Dhow, Buloke, Harrington, Commander, Alexis, ND24260-1, Chebec and Amaji nijo) and two hulless (Sumire mochi and Finniss) genotypes. All 14 genotypes were grown from June 2011 to December 2011 in a single plot in a complete randomized design at Charlick Experimental Research Station, Strathalbyn, South Australia (35°19'46.26" S, 138°52'42.39" E).

### Malting

The barley grain from all 14 genotypes was micromalted in a Phoenix Automatic Micromalting System, in accordance with the standard protocol used by the Barley Quality Laboratory at The University of Adelaide (14). Three separate malts using 60 g of grain each were prepared for Vlamingh, Finniss, Commander, Sloop, Flagship and Buloke; one malt of 60 g for WI2585, Dhow, Commander, Alexis, ND24260-1, Chebec and Amaji nijo; and one malt of 30 g for Harrington. The micromalting schedule used comprised three main stages: (a) steeping and air rest [7:8:9 h (wet:dry:wet) at 17°C, followed by another air rest, 9:0:5 h (dry:wet)]; (b) germination (94.5 h at 17°C); and (c) kilning (50–55°C, for 9 h followed by 55–60°C for 4 h, 60–70°C for 2 h and 70–80°C for 4.5 h). Samples of each genotype, except Harrington, were collected at harvest and after each of the three stages of malting (steeping, germination and kilning) for the measurement of vitamin E content and antioxidant capacity. Samples of Harrington were only collected after kilning owing to limited availability of seed. The malts were kept at –20°C until their moisture content, antioxidant capacity and vitamin E content were analysed. At least three biological replicates were analysed for each barley genotype at each malting stage for each parameter.

### Grinding

For all measurements, 10 g of barley grains were ground to a fine powder using an IKA Mill (Germany) with running water to avoid overheating during milling.

### Moisture content

The moisture content of barley flour was determined in triplicate using near infrared spectroscopy (Unity Scientific SpectraStar 2500, Unity Scientific Asia Pacific, Australia) according to the methods reported in Pomeranz (15).

### Determination of vitamin E content

To extract tocopherols from barley and avoid degradation of the isomers, an optimized method using hot saponification was adapted from Lampi et al. (16). Briefly, flour (0.1 g) was added to a solution of 1 mL 100% (v/v) ethanol, 0.4 mL water and 20 mg ascorbic acid in a 15 mL Pyrex glass tube with a Teflon screw cap. After the addition of 100 µL 10.7 M potassium hydroxide solution and thorough mixing, the tube was capped and transferred to a water bath at a temperature of 60°C for Sumire mochi and 80°C for the remaining genotypes and incubated for 25 min. During saponification, the sample was mixed every 10 min to improve hydrolysis. The tubes were then cooled in a water bath containing ice for 10 min and 0.5 mL 50% (v/v) ethanol was then added. To extract tocopherols and other unsaponifiable lipids, three portions (each 2 mL) of *n*-hexane–ethyl acetate (8:2, v/v) were added to each sample. After shaking the samples and solvent for 10 min and allowing them to separate into phases, the upper organic layers were collected with a disposable glass pipette and transferred to a new glass tube. This process was repeated three times with *n*-hexane–ethyl acetate (8:2 v/v), and the extracted samples were then dried under nitrogen. The residue was dissolved in 1 mL *n*-hexane and filtered through a 0.45 µm syringe filter before being transferred to a 12 × 32 GRACE glass HPLC (high-performance liquid chromatography) vial with an amber screwcap for analysis.

The tocopherols were quantified according to the method reported by Lampi et al. (16) with some modifications: (a) tocopherols were separated by a normal-phase HPLC using a GRACE Altima HP Silica 150 × 3 mm, 3 µm column and quantified using a fluorescence detector (NP-HPLC-FLD) with an excitation wavelength of 290 nm and an emission wavelength of 325 nm; (b) the mobile phase was 1,4-dioxane/*n*-hexane (2:98, v/v) at a flow rate of 1 mL/min; and (c) separation of tocopherols was based on isocratic elution (16).

The quantity of individual vitamin E isomers in the samples was determined by comparison of the retention time with the calibration curves of standards, which were prepared in hexane over the concentration range of 1.0–25.0 µg/mL using GenStat 14 (Lawes Agricultural Trust; VSN International Ltd, Hemel Hempstead, UK). The vitamin E content, expressed in milligrams of  $\alpha$ -tocopherol equivalents (TE), was calculated using the equation:  $\alpha\text{-TE} = \alpha\text{-T} \times 1.0 + \alpha\text{-T3} \times 0.3 + \beta\text{-T} \times 0.4 + \beta\text{-T3} \times 0.05 + \gamma\text{-T} \times 0.1 + \gamma\text{-T3} \times 0.01 + \delta\text{-T} \times 0.01$  as described by McLaughlin and Weihs (17) and using biological activities of 1.0 for  $\alpha$ -T, 0.3 for  $\alpha$ -T3, 0.4 for  $\beta$ -T, 0.05 for  $\beta$ -T3, 0.1 for  $\gamma$ -T, 0.01 for  $\gamma$ -T3 and 0.01 for  $\delta$ -T.

### Determination of antioxidant capacity

Antioxidants were extracted from barley flour (1 g) with 20 mL of 80% ethanol, which was placed in a flask in a 200 rpm shaking water bath for 4 h in the dark at 45°C. Vacuum filtration was used to separate the supernatant, which was stored in the dark at –20°C and analysed within 24 h (18). Antioxidant capacity was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical method (18). Briefly, barley flour extract (0.1 mL) was added to 2.9 mL of DPPH (112 µM). After mixing, the sample was allowed to stand at 23°C in the dark for 20 min. Reduction in absorbance was measured at 517 nm after 20 min using a spectrophotometer (UV-vis, SP 8001, Metertech, Taiwan). Antioxidant activity was then determined using a standard curve for ascorbic acid and prediction models provided by GenStat 14 and expressed as milligrams ascorbic acid equivalent antioxidant capacity per 100 g fresh weight of grain (mg AEAC/100g FW).

## Middle infrared measurement

Middle infrared (MIR) analysis was applied to determine whether there were any identifiable biochemical differences between samples from the different malting stages. Barley flour of all genotypes in each stage was scanned using a platinum diamond attenuated total reflectance (ATR) single reflection sampling module cell mounted in a Bruker Alpha instrument (Bruker Optics GmbH, Ettlingen, Germany) and spectra were recorded on OPUS software version 7.0 provided by Bruker Optics (average of 64 scans at a resolution of  $8\text{ cm}^{-1}$ , between  $4000$  and  $375\text{ cm}^{-1}$ ) (19). The samples were held against the ATR crystal using the pressure applicator or sample clamp mechanism supplied by the instrument manufacturer to ensure that the pressure applied was consistent for all replicates.

The MIR spectra was exported in csv format into The Unscrambler X software (version 10.1, CAMO ASA, Oslo, Norway) for principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA). Full cross validation (leave-one-out) was used to validate both PCA and PLS-DA models (20). In order to avoid over fitting in the models, the optimum numbers of terms in both PCA and PLS-DA models were indicated by the lowest number of factors that gave the minimum value for the predicted residual error sum of squares (PRESS) in cross-validation.

Discriminant models were developed using PLS-DA regression as described elsewhere (21). In this pattern recognition technique, each sample is assigned a dummy variable as reference value, which is an arbitrary number designating whether the sample belongs to a particular group or not; in this case barley samples sourced before malting were assigned a numeric value of 4, after kilning a value of 3, after germination a value of 2 and for samples sourced from steeping a value of 1.

## Statistical analysis

In order to determine the differences between means using the least significant difference (LSD) at  $p < 0.05$ , one-way and two-way analysis of variance (ANOVA) was performed using Genstat 14. Correlation analysis was performed using Microsoft Excel.

## Results and discussion

### Vitamin E content during malting

Both total vitamin E content and that of its main isomer,  $\alpha$ -T, decreased significantly (57–79% and 49–66%, respectively) after steeping compared with levels at harvest in all barley genotypes (Fig. 1). The same pattern was seen for the other vitamin E isomers; however, the degree of loss varied, with decreases in the content of 54–72% for  $\alpha$ -T3, 34–61% for  $\beta$ -T3, 30–87% for  $\gamma$ -T and 42–82% for  $\gamma$ -T3. In the case of  $\beta$ -T, the levels after steeping were below the limit of detection in all genotypes.

The percentage decrease in both the total vitamin E content and the content of individual vitamin E isomers after steeping also varied between genotypes. Sumire mochi, which had the lowest vitamin E content prior to steeping, also exhibited the greatest percentage loss in total vitamin E content and the content of all isomers with the exception of  $\alpha$ -T (100% for  $\alpha$ -T3,  $\beta$ -T3,  $\gamma$ -T and 82% for  $\gamma$ -T3). Amaji nijo exhibited the next most substantial decline in vitamin E content after steeping (70%), and the greatest percentage decline in the content of the main vitamin E isomer,

$\alpha$ -T (66%). Consequently, this genotype had one of the lowest vitamin E contents after steeping, despite being among the genotypes with the highest vitamin E content at harvest.

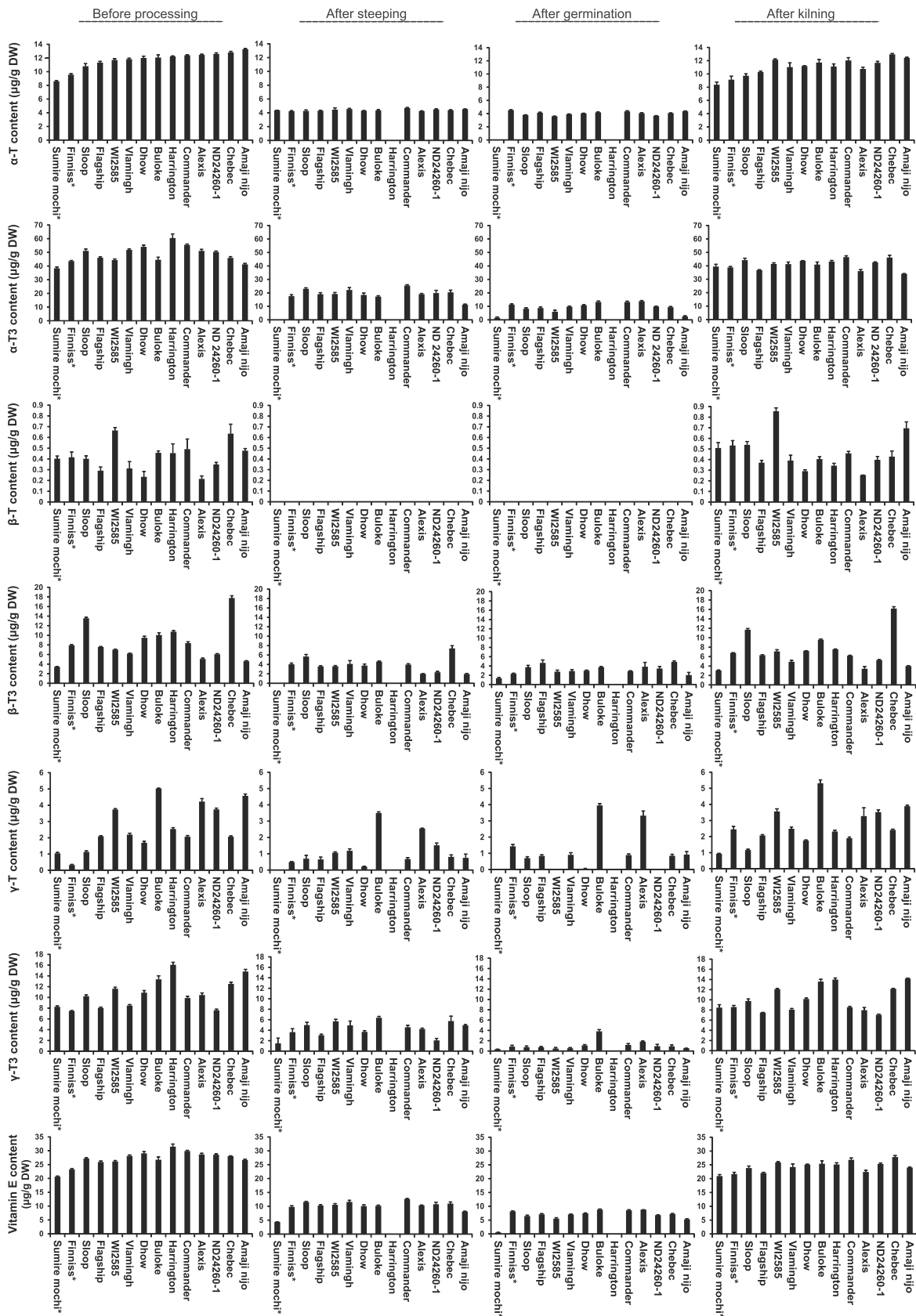
In contrast, the reduction in both total vitamin E content and the content of the individual isomers after steeping was lowest in Sloop (58% for total vitamin E, 60, 55, 58 and 51% for  $\alpha$ -T,  $\alpha$ -T3,  $\beta$ -T3,  $\gamma$ -T and  $\gamma$ -T3, respectively). This is likely to be a result of the lower water sensitivity of Sloop in comparison to all other genotypes included in this study (data not shown). This is due to the fact that, during steeping, the moisture content of barley increases to a level (~45%) at which the enzymes produced during germination can diffuse throughout the starchy endosperm and vitamin E can leach from the grain into the water (22). As a result, barley varieties that are less sensitive to water, such as Sloop, would be expected to exhibit a lower increase in their moisture content during steeping, and therefore retain a greater proportion of their original vitamin E content.

Similar to the effect of steeping, the content of vitamin E and its isomers was lower after germination compared with after steeping for the majority of the genotypes (Fig. 1). However, there were some exceptions, with increases in the content of specific isomers observed for  $\alpha$ -T in Finniss (7% increase) and  $\beta$ -T3 in Alexis, ND14260-1, Flagship and Amaji nijo (91, 48, 30 and 3% increase, respectively). The content of  $\gamma$ -T also increased during the germination stage for the majority of genotypes, from 13% (Buloke) to 190% (Finniss). Again, however, the change in  $\gamma$ -T content was not consistent across genotypes, with Sloop and Chebec exhibiting no change in  $\gamma$ -T content, and levels actually being lower after germination in Vlamingh (24%) and Dhow (78%).

There have been limited previous studies evaluating the changes in the content of either total vitamin E or specific vitamin E isomers during malting, and these have produced conflicting results. In the only prior study to date which has been conducted in barley, Dabina-Bicka et al. (23) found that the vitamin E content was increased by 4.1- and 4.5-fold after germination in the two out of three genotypes they studied, which they suggested was due to the beginning of biochemical reactions in the grains during the germination stage. However, this study was limited to three genotypes and the method used to assess vitamin E content, the AOAC Official Method 971.30 ' $\alpha$ -tocopherol and  $\alpha$ -tocopheryl acetate in foods and feeds' standard colorimetric method (1971–1972), is no longer considered optimal and has been replaced by other much more sensitive methods (24). Thus, whether those results are reliable is not clear.

Other studies, conducted in wheat (25), legumes (26) and lupins (27,28) have also reported an increase in the content of specific vitamin E isomers after germination. Koga and Terao (28) suggested that the increase in vitamin E content of lupin grains during germination could be related to the degradation of liposome membranes, leading to release of vitamin E, which could then be consumed in the process of scavenging phospholipid peroxy radicals. In wheat, for example, the contents of specific vitamin E isomers, including  $\alpha$ -T and  $\gamma$ -T, were reported to increase from 4.37 to 10.92  $\mu\text{g/g}$  and from 0.91 to 1.50  $\mu\text{g/g}$ , respectively, after 7 days of germination (25). However, there were only two isomers analysed in their study while other isomers such as  $\alpha$ -T3, which is considered to be the dominant vitamin E isomer, were not included. A similar study on lupin (27) showed that germination brought about an increase in the content of  $\alpha$ -T, which resulted in an increase of 238% in the vitamin E activity after 9 days of germination. This same study also reported, however, that germination caused a decrease in the content of  $\gamma$ -T and did not affect the content of





**Figure 1.** The content of isomers and vitamin E in 14 genotypes during malting. The genotypes marked with an asterisk are hullless genotypes. The others are covered genotypes. Vitamin E is expressed in milligrams of  $\alpha$ -tocopherol-equivalents (TE). Bars represent the mean  $\pm$  SE. Before processing,  $n = 6$  except  $n = 3$  for Buloke. After steeping,  $n = 9$  except  $n = 3$  for Chebec, Dhow, Sumire mochi, ND24260-1, WI2585, Alexis and Amaji nijo. After germination,  $n = 9$  except  $n = 3$  for Chebec, Dhow, Sumire mochi, ND24260-1, WI2585, Alexis and Amaji nijo. After kilning,  $n = 9$  except  $n = 3$  for Chebec, Dhow, Sumire mochi, ND24260-1, WI2585, Alexis, Amaji nijo, and Harrington. Harrington was only sampled before processing and after kilning due to limited availability of seed.

$\delta$ -T in the lupin. Significant differences in the effect of germination on the content of different vitamin E isomers have also been reported in studies in mung beans and cowpeas (14), which showed an increase in  $\alpha$ -T content, but a decrease in  $\gamma$ -T content after germination.

In the current study, a decline in the content after germination was detected for the vitamin E isomers  $\alpha$ -T from 4% (Buloke) to 19% (WI2585),  $\alpha$ -T3 from 23% (Buloke) to 78% (Amaji nijo),  $\beta$ -T3 from 19% (Buloke) to 42% (Finniss) and  $\gamma$ -T3 from 40% (Buloke) to 91% (WI2585). In addition, the presence of  $\beta$ -T was not detected after germination, which contributed to the decline in total vitamin E content of 14–47% and 65–80% in comparison to steeping and harvesting, respectively. Differences in the finding between this study and previous studies may relate to the shorter period time of germination in the present study, which has used standard malting protocols, compared with previous studies (4 and 7–9 days, respectively). According to Yang et al. (25), the  $\alpha$ -T content of wheat increased after 4 days of germination, while  $\gamma$ -T content increased after 4 or 5 days germination, depending on genotypes, and reached a peak on day 8. This finding is in agreement with a study in lupin that showed a significant decrease in vitamin E content of samples in the first 2 days of germination compared with steeping, followed by gradual increase up to day 9 (27). Thus, the determination of changes in all vitamin E isomers, as well as total vitamin E content, across the germination period would be interesting to explore in more detail in future studies.

Kilning, the final stage of the malting process, was associated with a significant increase in both total vitamin E content and the content of individual isomers, for most genotypes compared with levels at the completion of the germination stage (Fig. 1). This resulted in substantially higher vitamin E contents at the end of the malting process compared with the steeping and germination stages (from 164% in Finniss to 354% in Amaji nijo). The differences in the content of vitamin E and its isomer contents between germination and kilning varied both between isomers and between the genotypes. The most substantial changes in isomer content were observed for  $\gamma$ -T3, followed by  $\gamma$ -T,  $\alpha$ -T3,  $\alpha$ -T and  $\beta$ -T3. Interestingly,  $\beta$ -T was not detected in any genotypes at the completion of the steeping and germination stages, but returned to levels comparable to those in unprocessed barley after kilning. Biosynthesis of  $\beta$ -T in seeds may therefore take place during kilning.

While there was a sharp rise in contents of vitamin E and its isomers after kilning compared with germination, the levels attained in malts were still lower than those in unprocessed barley for the majority of genotypes (Fig. 2). The  $\alpha$ -T content was significantly lower after malting compared with before processing in eight genotypes (Dow, Vlamingh, Amaji nijo, ND24260-1, Flagship, Sloop and Alexis), but was not different between unprocessed barley and the malt for the remaining genotypes (Fig. 2A). In the case of  $\alpha$ -T3 content, all genotypes, with the exception of Chebec and Sumire mochi, exhibited a significant decrease after malting compared with before processing (Fig. 2B). All genotypes, except for WI2585, also had significantly lower levels of  $\beta$ -T3 after malting (Fig. 2D). In contrast, most genotypes had higher levels of  $\beta$ -T in malt than in the unprocessed samples, but this was only significant for Amaji nijo, Sloop, WI2585 and Finniss (Fig. 2C). Interestingly, malting seemed to have little or no effect on  $\gamma$ -T (Fig. 2E) and  $\gamma$ -T3 (Fig. 2F) in most genotypes, with the exception of Finniss, which exhibited a considerable increase in  $\gamma$ -T content after malting (656%). This was not, however, sufficient to increase total vitamin E content to levels greater than those in unprocessed Finniss.

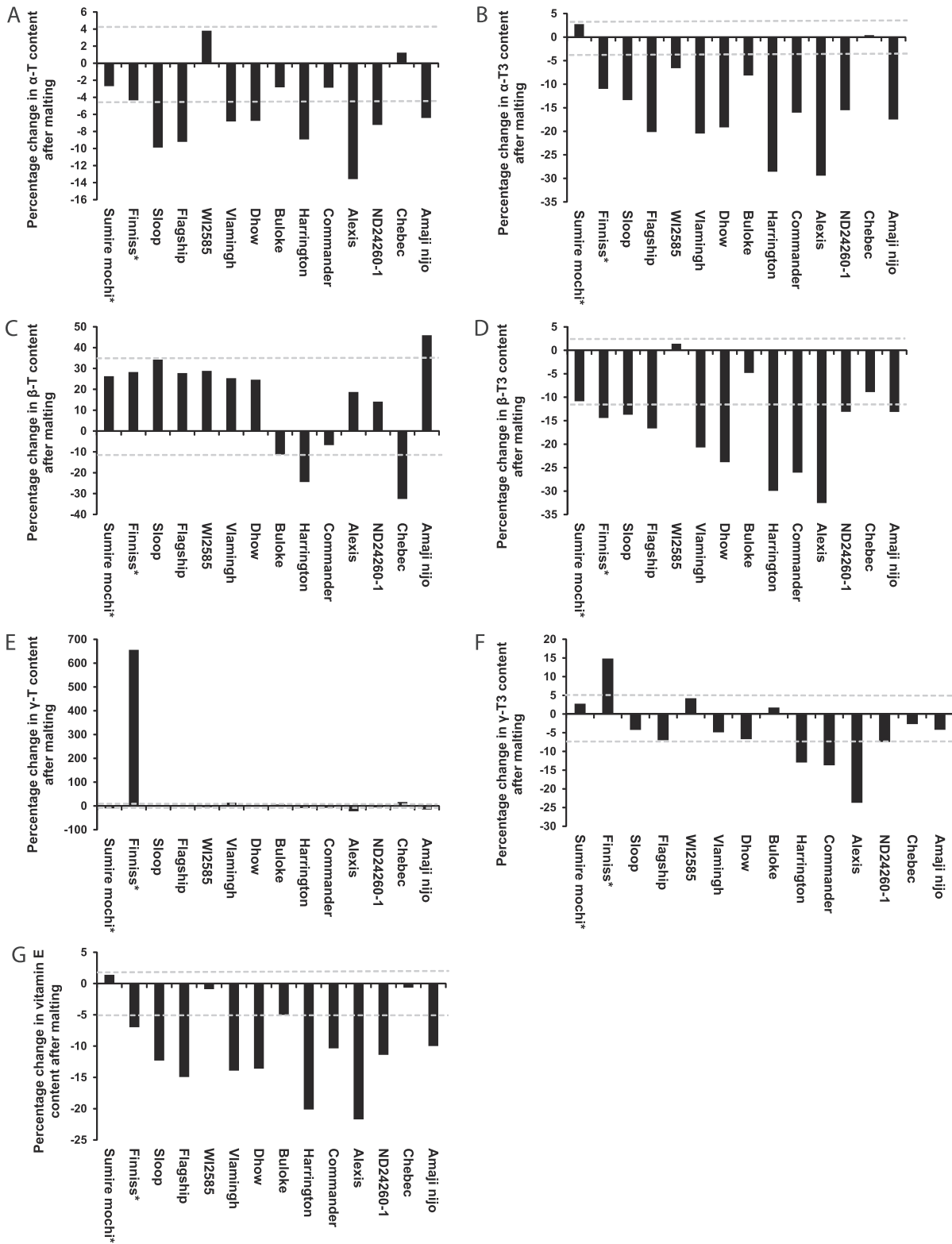
In relation to total vitamin E content, levels were lower in the malt than in the unprocessed barley for the majority of the genotypes, although this difference was relatively small [between 6% (Finniss) and 22% (Alexis)], and was not significantly different for Sumire mochi, Chebec, WI2585 and Buloke (Fig. 2G). Vitamin E is known to be unstable in the presence of light, water, oxygen and heat, all of which are applied to the samples during processing, and this may explain the decline in its content during malting (29). However, both the levels of vitamin E in the unprocessed samples and the stability of this compound during malting clearly varied between genotypes as observed previously for stored barley grain (2). Therefore, in order to obtain malts with higher vitamin E contents, genotypes containing higher levels of vitamin E before processing, and in which this compound remains relatively stable during malting (such as Chebec, WI2585 and Commander), are likely to be more suitable than other varieties.

### Antioxidant capacity during malting

Similar to vitamin E, antioxidant capacity was reduced after steeping by between 49% (WI2585) and 74% (Sumire mochi) (Fig. 3). Importantly, the decline in antioxidant capacity was directly correlated ( $r=0.7$ ,  $n=14$ ,  $p<0.05$ ) with the decrease in total vitamin E content after steeping. This suggests that the loss of antioxidant capacity after steeping is likely to be largely ascribed to the loss of vitamin E. However, since the loss of vitamin E did not explain 100% of the reduction in total antioxidant capacity after steeping, other compounds appear to also contribute to the overall antioxidant activity of the barley. Compounds, in particular phenolic compounds such as caffeic acid, vanillic acid and gallic acid, may have leached from the pericarp and testa of the barley or formed insoluble complexes with proteins during steeping (6).

Interestingly, while vitamin E was generally reduced after malting compared with before processing, antioxidant capacity was increased in the majority of genotypes, with the exception of Sloop, Vlamingh, Flagship and WI2585 (Fig. 3). This finding is in accordance with a previous report in barley that polyphenol,  $\beta$ -carotenoid and vitamin C increased after germination, which was explained by the synthesis of these compounds in the seeds (23). This suggests that the vitamin E content of the malt may not be a reliable indicator of its overall antioxidant capacity at this stage.

Malting was associated with an increase in antioxidant capacity in comparison with before processing in most genotypes, which was significant in the case of Alexis, Flagship, Sloop and Amaji nijo (8, 12, 13 and 22%, respectively; Fig. 4). The increase in antioxidant capacity after malting is likely to be due to the collective influence of three processes: (a) release of phenolic compounds bound to cellular structures; (b) better extraction; and (c) the formation of Maillard reaction products (30). The phenolic compounds, mainly phenolic acids, which were bound to lignin and arabinoxylans in the unprocessed samples, would be expected to be released in the presence of enzymes that are synthesized and/or activated during either the final stages of germination or the early stages of kilning (30). The change in tissue structure caused by kilning, in particular the increased friability of the grain, has been shown to increase the ease of extracting compounds located in the outer layers of the grain, such as the phenolic compounds, resulting in improved efficiency of extraction of antioxidant compounds (30). In addition, reducing sugars and amino acids that are reported to be released during germination can react to produce Maillard products such as melanoidins, which also have antioxidant capacity (30,31). Interestingly, while almost all genotypes exhibited a

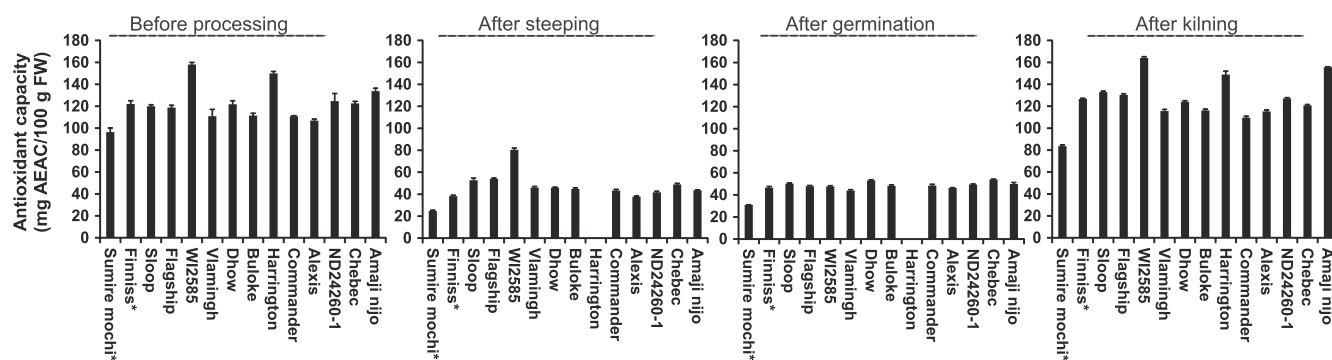


**Figure 2.** The percentage change after malting in content of  $\alpha$ -T (A),  $\alpha$ -T3 (B),  $\beta$ -T (C),  $\beta$ -T3 (D),  $\gamma$ -T (E),  $\gamma$ -T3 (F) and vitamin E (G) in different barley genotypes. The genotypes marked with an asterisk are hullless genotypes. The others are covered genotypes. Vitamin E is expressed in milligrams of TE. Bars represent the mean  $\pm$  SE. Bars not within dotted lines represent genotypes with significantly different percentage change of antioxidant capacity after malting.

significant increase in antioxidant content, Sumire mochi exhibited a significant decrease of 12.8%.

Since free radicals have been widely indicated as the major cause of seed deterioration (32), those genotypes with higher

antioxidant capacity may be more likely to be preserved during processing. In support of this, we found significant correlations between the antioxidant capacity in unprocessed barley and that after kilning when data from all genotypes were combined



**Figure 3.** The antioxidant capacity in 14 genotypes during malting. The genotypes marked with an asterisk are hulless genotypes. The others are covered genotypes. Antioxidant capacity is expressed as milligrams ascorbic acid equivalent antioxidant capacity (AEAC) per 100 g of fresh weight (FW) of grain. Bars represent the mean  $\pm$  SE. Before processing,  $n = 6$  except  $n = 3$  for Buloke. After steeping,  $n = 9$  except  $n = 3$  for Chebec, Dhow, Sumire mochi, ND24260-1, WI2585, Alexis and Amaji nijo. After germination,  $n = 9$  except  $n = 3$  for Chebec, Dhow, Sumire mochi, ND24260-1, WI2585, Alexis and Amaji nijo. After kilning,  $n = 9$  except  $n = 3$  for Chebec, Dhow, Sumire mochi, ND24260-1, WI2585, Alexis, Amaji nijo and Harrington. Harrington was only sampled before processing and after kilning owing to limited availability of seed.

( $r = 0.9$ ,  $n = 14$ ,  $p < 0.05$ ). Similar correlations were present for all vitamin E isomers and total vitamin E content,  $r = 0.9$  (for  $\alpha$ -T,  $\beta$ -T3,  $\gamma$ -T,  $\gamma$ -T3), 0.7 (for  $\beta$ -T and total vitamin E) and 0.5 (for  $\alpha$ -T3), all with  $n = 14$ ,  $p < 0.05$ . These findings therefore suggest that a higher antioxidant capacity and/or vitamin E content in unprocessed barley may protect the samples from losses in antioxidant capacity and/or vitamin E during processing. Furthermore, these data suggest that barley genotypes that are naturally high in antioxidant capacity and/or vitamin E content are likely to be the most suitable for producing malt that also has a high antioxidant capacity, and therefore provide potential associated health and anti-spoiling/staling benefits. However, further research is required to establish whether antioxidant capacity and vitamin E content are maintained in the wort or other products from malt and whether they impact quality.

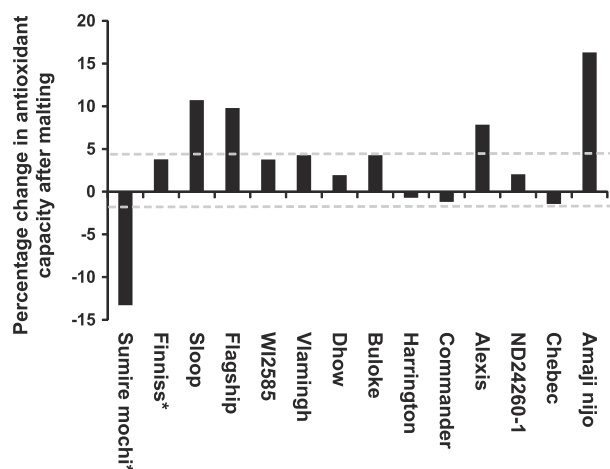
Expanding the use of hulless barley in the production of malt is a particular interest, since malts produced from hulless varieties can be directly added to a variety of products after milling without the need for hull disposal or malt extraction and processing times are generally shorter than for covered genotypes (13). Of the two hulless genotypes investigated in this study, the malt produced

from one, Sumire mochi, was lowest in both vitamin E content and antioxidant capacity. Finniss malt was the second lowest in vitamin E content; however, its antioxidant capacity was ranked sixth among 14 genotypes, indicating that this attribute was comparable to that of the hulled, covered genotypes. This suggests that the Finniss hulless barley might be useful for malt with higher antioxidant content with short steeping time and high extraction yield in brewing industries as well as in food industries when added directly to products.

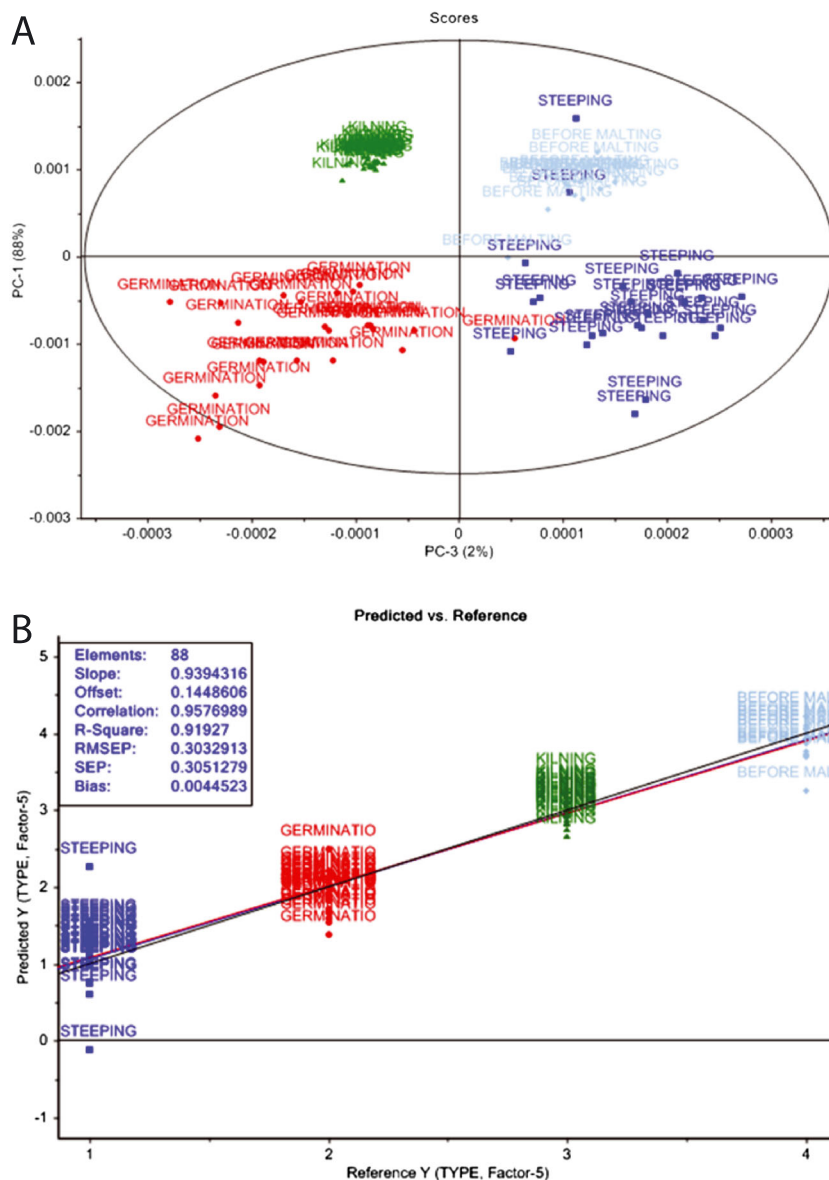
### Investigating compositional changes during processing by MIR

In an attempt to gain further insights into the compositional changes that occurred within the different barley genotypes during processing, samples were analysed by MIR before and after each of the malting stages (Fig. 5A). PCA analysis of the data acquired from the MIR spectra produced clear clustering of the samples according to the processing stage. The distribution pattern of the genotypes, however, varied according to the processing stage, and the different genotypes were much more tightly clustered before processing and after kilning than after the steeping and germination stages. The tight clustering of the unprocessed barley samples is expected given that they had not yet gone through any processing, thus no chemical reactions had occurred, and no new products had been formed. Similarly, the close clustering of the genotypes after malting is probably due to the fact that the chemical reactions that would have occurred during processing were stopped by the high temperatures applied during kilning, since previous studies have demonstrated that enzymes in grains were inactivated and moisture content reduced to 2–6% after the kilning stages (31).

In contrast, there was a considerably wider spread of the genotypes for the samples collected at the completion of the steeping and germination stages. This is likely to be due to variation between the genotypes in their moisture content and/or water sensitivity (data not shown), both of which would affect the rate at which biochemical reactions stimulated by the increasing oxygen/water content of the grains, or by swelling of grains, proceeded during the steeping stage (22). Similarly, the rate and extent of the biosynthesis of new enzymes/compounds during the germination stage is also likely to be affected by the water content and physical characteristics of the grain, and therefore to vary between genotypes. In the steeping and germination stages,



**Figure 4.** The percentage change after malting in antioxidant capacity in different barley genotypes. The genotypes marked with an asterisk are hulless genotypes. The others are covered genotypes. Antioxidant capacity is expressed as milligrams AEAC per 100 g FW of grain. Bars not within dotted lines represent genotypes with significantly different percentage change of antioxidant capacity after malting.



**Figure 5.** Score plot of the two principal components of barley genotypes during malting. (A) classification using partial least squares discriminant analysis (PLS-DA). (B) Using score plot of the two principal components based on the attenuated total reflectance and mid infrared spectroscopy (ATR-MIR). PC1, principal component 1; PC3, principal component 3. This figure is available in colour online at [wileyonlinelibrary.com/journal/brewing](http://wileyonlinelibrary.com/journal/brewing).

the two hulless varieties, Finnis and Sumire mochi, were clustered closely together, and at a distance from the covered varieties, which is likely to be indicative of inherent differences in composition/physical characteristics between covered and hulless varieties of barley.

The ability of the classification models based on MIR to identify the process is based on the molecular information provided by the MIR spectra, and the greater the variability in composition between the various malting stages (kilning, germination, etc.), the better the accuracy of the model. The combination and interactions of several compositional characteristics of the sample therefore provide the necessary information for discrimination. In this study, the combination of spectroscopy and chemometrics as an analytical tool gave the advantage of being able to rapidly visualize the changes occurring during the processing of barley without the need for quantitative data.

The classification statistics obtained when the PLS-DA classification model was developed using the ATR-MIR spectra are shown in (Fig. 5B). The coefficient of determination ( $R^2$ ) obtained for the PLS-DA models was 0.9 (standard error of cross-validation 0.3), indicating that these models accounted for 90% of the variability in composition related to the processing stage. The number of PLS loadings used to develop the models was 5, and the optimal PLS-DA loadings derived from the calibration model are shown in Table 1. The analysis of the optimal loadings indicated that MIR regions corresponding to compounds containing nitrogen (e.g. proteins), carbohydrates and lipids explain the variability observed between the barley samples related to the processing stage. These findings are consistent with existing knowledge of the changes that occur during the malting process, which include (a) the formation of insoluble phenolic complexes with proteins (6); (b) the synthesis of amylases, proteases and  $\beta$ -glucanases,

**Table 1.** Optimal loadings derived from the PLS-DA analysis at wavenumbers associated with certain molecules

Wavenumbers (cm <sup>-1</sup> )	Molecules	Effect
2939	Lipids	–
1716	Lipids	–
1636	Amide group and water	+
1542–1539	Amide groups/protein	–
1427		–
1396		+
1267	P=O phospholipids	–
985	<i>Trans</i> fatty acids	+
771	<i>Trans</i> fatty acids	–

+, Positive; –, negative.

causing polymer degradation (33); and (c) degradation of lipids owing to the presence of water and oxygen and high temperature (34).

The highest and negative loadings were observed at 2939, 1716, 1427 and 1396 cm<sup>-1</sup> (Table 1) corresponding to lipids and carbohydrates (35). Specifically, the 2939 and 1427 cm<sup>-1</sup> bands are associated with CH stretching and bending, respectively, while the 1716 cm<sup>-1</sup> band corresponds to the carbonyl group. These bands indicated the presence of triglycerides, diglycerides, acyl group carbonyls and hydrocarbon chains in the barley samples and they change as a consequence of the processing. The diethyl ether soluble polar fractions around 1267 cm<sup>-1</sup> might be also associated with the P=O band of phospholipids. In addition, the 985 and 771 cm<sup>-1</sup> regions have been reported by other authors to correspond to *trans* fatty acids in cereals products (15). While the unprocessed and processed samples were clearly distinct from all other malting stages in the PLS-DA and PCA analyses, there was some overlap in the models for the steeping and germination stages. This can be explained by overlap in the lipid composition in some of the varieties at these two stages.

## Conclusions

While there were variations between genotypes in their vitamin E content and antioxidant capacity at harvest and during and after processing, there were significant positive correlations between both these parameters in the unprocessed barley and those in the malts. The change in antioxidant capacity after malting was also closely related to that of vitamin E, indicating that vitamin E makes a significant contribution to overall antioxidant capacity during malting. Genotypes that have high vitamin E content and/or antioxidant capacity at harvest retain these properties after malting, and are therefore likely to be the most appropriate varieties to select in order to produce malts with high antioxidant potential. Furthermore, different growing environments are unlikely to greatly affect vitamin E content and antioxidant capacity based on a previous study (36) and our general observation that malt samples from two varieties of barley grown in different years and different locations are consistent (data not shown). Finniss, a hullless barley variety, had an antioxidant capacity comparable to that of hulled varieties that were tested. This variety therefore has potential applications in the production of high antioxidant malts, and we are currently investigating the potential use of the malt derived from this hullless variety in food products.

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## References

- Ehrenbergerova, J., Belcrediova, N., Havlova, P., Pryma, J., Vaculova, K., and Vejrazka, K. (2006) Barley grain as source of natural antioxidants and nutraceuticals beneficial to health, in *Proceedings of the 3rd International Congress on Flour – Bread '05 and 5th Croatian Congress on Cereal Technology*, pp. 188–194.
- Do, T. T. D., Cozzolino, D., Muhlhausler, B., Box, A., and Able, A. J. (2015) Antioxidant capacity and vitamin E in barley: Effect of genotype and storage, *Food Chem.* **187**, 65–74.
- Young, I. S., and Woodside, J. V. (2001) Antioxidants in health and disease, *J. Clin. Pathol.* **54**, 176–186.
- Peterson, D. M. (2001) Oat antioxidants, *J. Cereal Sci.* **33**, 115–129.
- Pereira de Abreu, D. A., Paseiro Losada, P., Maroto, J., and Cruz, J. M. (2010) Evaluation of the effectiveness of a new active packaging film containing natural antioxidants (from barley husks) that retard lipid damage in frozen Atlantic salmon (*Salmo salar* L.), *Food Res. Int.* **43**, 1277–1282.
- Lu, J., Zhao, H., Chen, J., Fan, W., Dong, J., Kong, W., Sun, J., Cao, Y., and Cai, G. (2007) Evolution of phenolic compounds and antioxidant activity during malting, *J. Agric. Food Chem.* **55**, 10994–11001.
- Bhatty, R. S. (1999) The potential of hull-less barley, *Cereal Chem.* **76**, 589–599.
- Sharma, P., Gujral, H. S., and Singh, B. (2012) Antioxidant activity of barley as affected by extrusion cooking, *Food Chem.* **131**, 1406–1413.
- Gallegos-Infante, J. A., Rocha-Guzman, N. E., Gonzalez-Laredo, R. F., and Pulido-Alonso, J. (2010) Effect of processing on the antioxidant properties of extracts from Mexican barley (*Hordeum vulgare*) cultivar, *Food Chem.* **119**, 903–906.
- Peterson, D. M. (1994) Barley tocots: Effects of milling, malting, and mashing, *Cereal Chem.* **71**, 43–44.
- Goupy, P., Hugues, M., Boivin, P., and Amiot, M. J. (1999) Antioxidant composition and activity of barley (*Hordeum vulgare*) and malt extracts and of isolated phenolic compounds, *J. Sci. Food Agric.* **79**, 1625–1634.
- Dabina-Bicka, I., Karklina, D., and Kruma, Z. (2011) Polyphenols and vitamin E as potential antioxidants in barley and malt, in *Proceedings of 6th Baltic Conference on Food Science and Technology: Innovations for Food Science and Production, FOODBALT*, 2011, 5, pp. 121–126.
- Bhatty, R. S. (1996) Production of food malt from hull-less barley, *Cereal Chem.* **73**, 75–80.
- Cozzolino, D., Degner, S., and Eglinton, J. (2014) A novel approach to monitor the hydrolysis of barley (*Hordeum vulgare* L.) malt: A chemometrics approach, *J. Agric. Food Chem.* **62**, 11730–11736.
- Pomeranz, Y. (1973) Interaction between glycolipids and wheat flour macromolecules in breadmaking, *Adv. Food Res.* **20**, 153–188.
- Lampi, A. M., Ryyanen, M., Salo-Vaananen, P., Ollilainen, V., and Piironen, V. (2004) A small-scale sample preparation method with HPLC analysis for determination of tocopherols and tocotrienols in cereals, *J. Food Compos. Anal.* **17**, 749–765.
- McLaughlin, P. J., and Weihrauch, J. L. (1979) Vitamin E content of foods, *J. Am. Diet. Assoc.* **75**, 647–665.
- Omwamba, M., and Hu, Q. (2009) Antioxidant capacity and antioxidative compounds in barley (*Hordeum vulgare* L.) grain optimized using response surface methodology in hot air roasting, *Eur. Food Res. Technol.* **229**, 907–914.
- Cozzolino, D., Roumeliotis, S., and Eglinton, J. (2013) Prediction of starch pasting properties in barley flour using ATR-MIR spectroscopy, *Carbohydr. Polym.* **95**, 509–514.
- Næs, T., Isaksson, T., Fearn, T., and Davies, T. (2002) *A User-friendly Guide to Multivariate Calibration and Classification*, NIR Publications, Chichester.
- Esbensen, K. H. (2002) *Multivariate Data Analysis in Practice*, CAMO, Oslo.

22. Kirsop, B. H., and Polloc, J. R. A. (1957) Study in barley and malt. XI. Steeping in relation to water-sensitivity in malting barleys, *J. Inst. Brew.* 63, 383–385.
23. Dabina-Bicka, I., Karklina, D., Rakcejeva, T., Sniedzane, R. and Kviesis, J. (2010) The dynamics of vitamins C and E in barley products during malting, *Annual 16th International Scientific Conference Proceedings, 'Research for rural development 2010'*, Jelgava, Latvia, 19–21 May 2010, Vol. 1, pp. 111–115.
24. Fratianni, A., Caboni, M. F., Irano, M., and Panfilì, G. (2002) A critical comparison between traditional methods and supercritical carbon dioxide extraction for the determination of tocopherols in cereals, *Eur. Food Res. Technol.* 215, 353–358.
25. Yang, F., Basu, T. K., and Ooraikul, B. (2001) Studies on germination conditions and antioxidant contents of wheat grain, *Int. J. Food Sci. Nutr.* 52, 319–330.
26. Marero, L. M., Payumo, E. M., Aguinaldo, A. R., Homma, S., and Igarashi, O. (1991) Vitamin E constituents of weaning foods from germinated cereals and legumes, *J. Food Sci.* 56, 270–271.
27. Frias, J., Miranda, M. L., Doblado, R., and Vidal-Valverde, C. (2005) Effect of germination and fermentation on the antioxidant vitamin content and antioxidant capacity of *Lupinus albus* L. var. Multolupa, *Food Chem.* 92, 211–220.
28. Koga, T., and Terao, J. (1996) Antioxidant behaviors of vitamin E analogues in unilamellar vesicles, *Biosci. Biotechnol. Biochem.* 60, 1043–1045.
29. Leskova, E., Kubikova, J., Kovacikova, E., Kosicka, M., Porubska, J., and Holcikova, K. (2006) Vitamin losses: Retention during heat treatment and continual changes expressed by mathematical models, *J. Food Compos. Anal.* 19, 252–276.
30. Maillard, M. N., Soum, M. H., Boivin, P., and Berset, C. (1996) Antioxidant activity of barley and malt: Relationship with phenolic content, *Food Sci. Technol.* 29, 238–244.
31. Barrett, J., Griffiths, C. M., and Kirsop, B. H. (1967) Some effects of malt kilning on wort properties, *J. Inst. Brew.* 73, 445–450.
32. Lehner, A., Mamadou, N., Poels, P., Come, D., Bailly, C., and Corbineau, F. (2008) Changes in soluble carbohydrates, lipid peroxidation and antioxidant enzyme activities in the embryo during ageing in wheat grains, *J. Cereal Sci.* 47, 555–565.
33. Olkku, J., Reinikainen, P., and Cores, C. (1991) A technology of steeping and germination, *Ferment* 4, 248–251.
34. Maillard, M. N., and Berset, C. (1995) Evolution of antioxidant activity during kilning – role of insoluble bound phenolic-acids of barley and malt, *J. Agric. Food Chem.* 43, 1789–1793.
35. Stuart, B. (1996) Modern infrared spectroscopy, in *Analytical Chemistry by Open Learning Series* (Ando, D. J., Ed.), p. 180, Wiley, Chichester.
36. Peterson, D. M., and Qureshi, A. A. (1993) Genotype and environment effects on tocopherols of barley and oats, *Cereal Chem.* 70, 157–162.

**Chapter 5. Manuscript 3: ENRICHMENT OF  
ANTIOXIDANT CAPACITY AND VITAMIN  
E IN PITA MADE FROM BARLEY**



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# Enrichment of Antioxidant Capacity and Vitamin E in Pita Made from Barley

Thi Thu Dung Do, Beverly Muhlhausler, Amanda Box, and Amanda J. Able

**Abstract:** This study aimed to enhance total antioxidant and vitamin E content of pita bread, by replacing 50% of the standard baker's flour with flours milled from covered (WI2585 and Harrington) or hullless (Finniss) barley genotypes, previously shown to have high antioxidant and vitamin E levels at harvest. Pita breads were made from either 100% baker's flour (control) or 50% malt flour, whole-grain flour, or flour from barley grains pearled at 10%, 15%, and 20% grain weight. Antioxidant capacity and vitamin E content of flours and pitas were determined by their ability to scavenge 2,2-diphenyl-1-picrylhydrazyl radicals and high performance liquid chromatography, respectively. The physical and sensory properties of the pitas were also assessed. All pitas made from either whole grain or pearled barley flour had a higher antioxidant capacity and most also had higher vitamin E content than standard pita. The antioxidant and vitamin E levels were reduced in pearled compared to whole grains, however the extent of that reduction varied among genotypes. The greatest antioxidant and vitamin E levels were found in pita made from malt flour or Finniss whole grain flour. Furthermore, sensory analysis suggested these pitas were acceptable to consumers and retained similar physical and sensory properties to those in the control pita.

**Keywords:** antioxidant capacity, barley, pearling, pita bread, vitamin E

**Practical Application:** Bread is a staple food and providing breads which are a source of fiber and bioactive compounds has the potential to provide health benefits. Here, we show that malt flour, whole-grain flour, or pearled-grain flour from covered or hullless barley with high antioxidant and vitamin E levels at harvest can be used to produce pitas with higher antioxidant and vitamin E level than standard pitas, and whose sensory properties are acceptable to consumers.

## Introduction

Wheat is a staple food around the world and is consumed in many forms including flat or pan style leavened bread (Pomeranz 1987). Due to the rising world population and greater awareness of a healthy lifestyle, bread containing multigrains, whole grain, or other functional ingredients is becoming more popular among consumers (Vulicevic and others 2004). A number of previous studies have demonstrated that including barley in bread improves the natural nutritional value, by increasing levels of  $\beta$ -glucan, minerals, and antioxidants (Newman and Newman 2006).

Antioxidants in food may have a number of important health benefits, which are primarily due to their ability to slow tissue damage by preventing the formation of free radicals, scavenging them, or by promoting their decomposition (Young and Woodside 2001). In a previous study, vitamin E, a lipid phase chain-breaking antioxidant, was found at highest levels in barley caryopses compared to wheat, oats, and rye (Holasova and others 1995). We have also recently demonstrated that antioxidant capacity and vitamin E content varies substantially among different barley genotypes at harvest (Do and others 2015a) and during storage and malting (Do and others 2015b). Although health claims for barley grain have been approved by the U.S. Food and Drug Administration (USFDA 2003), there is currently no published research which has determined whether and to what extent the antioxidant capacity and vitamin E content is maintained in final food products. Thus,

whether breads made from barley genotypes with high antioxidant capacity and vitamin E content at harvest can be a good dietary source of these factors is unknown.

Barley is typically polished (also known as pearling) before consumption because whitened grain is generally preferred by consumers and food manufacturers (Gong and others 2012). The process of pearling removes the hull (also known as the husk), and the bran, which is firmly attached to the inner layers of the hull, is consequently abraded (Blandino and others 2015). The husk and bran, both of which are rich in antioxidant capacity, and vitamin E, are either discarded or utilized for animal feed (Maillard and Berset 1995). Although several studies have been conducted on the effect of pearling on either antioxidant capacity or vitamin E content on the barley grain, little work has been performed on either of these components in barley products (Ko and others 2003; Panfili and others 2008; Blandino and others 2015).

Hullless barley does not require pearling and is preferred in food production as less processing is required (Elsayed and Peter 2005); the grain contains more protein, starch, and total soluble fiber (Bhatty 1999); and the grain can be added directly to food (Elsayed and Peter 2005). Malt made from hullless barley is of particular interest because of the same advantages (Bhatty 1996). Barley malt is also ideal for bread manufacture due to high  $\alpha$ - and  $\beta$ -amylase enzyme activity allowing starch to be converted to maltose which can be more easily digested and also promote yeast activity (Bhatty 1999). A further advantage was demonstrated in our previous study (Do and others 2015b) with an increase of antioxidant capacity in malt compared to unprocessed barley.

Little has been published on the benefits of adding different types of barley-derived materials on the antioxidant capacity and vitamin E contents of pita bread. These could include flour made

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from the whole grain, pearled grain, or malt; and that is derived from covered or hullless barley genotypes. Although there will probably be greater antioxidant capacity and vitamin E content in pita bread made with higher percentages of barley, the impact on the sensory quality must be positive. The appropriate combination of sensory properties together with the health benefits therefore needs to be considered (Biloukha and Utermohlen 2000).

The objectives of this study therefore were to determine antioxidant capacity and vitamin E content in pita bread supplemented with barley flour made from whole-grain, pearled-grain, or malt from covered or hullless barley genotypes with high antioxidant and vitamin E levels at harvest; and to determine their impact on the physical and sensory properties of pita bread.

## Materials and Methods

### Materials

The barley varieties used in this study (the hullless genotype Finnis and the covered genotypes WI2585 and Harrington) were previously identified as being high in antioxidant capacity and vitamin E content (Do and others 2015a). Grain from each variety was used immediately after harvest to make flour, either from whole grains (0% pearling), or with 10%, 15%, or 20% pearling. Flour was also prepared from malt prepared from Finnis (Do and others 2015b) and after storage at 10 °C for 4 mo. The barley varieties, provided by the Univ. of Adelaide Barley Breeding Program, were grown from June to December 2014 as a single plot in a complete randomized design at Charlick Experimental Research Station, Strathalbyn, South Australia (35°19'46.26"S, 138°52'42.39"E). The grain was hand sieved using a 2.5 mm slotted ISO 5223 sieve as per U.S. Dept. of Agriculture (2013).

To pearl grain, a Satake grain tester (model TM05, Tokyo, Japan) using a procedure adapted from Takenouchi Barley Processing Co., Ltd., Kagoshima-shi, Japan (Washington and others 2003) was set at 1150 rpm with a 36 grit size wheel and was warmed up by pearling a 180 g control sample twice for 12 min each. The removed husk weight was obtained by weighing the collected pearl dust and pearling was stopped at levels of 10%, 15%, and 20% (w/w) of husk removed.

Malt (8 cans, 60 g each) was obtained from Finnis using a Phoenix Automatic Micromalting System<sup>®</sup> in accordance with the standard protocol used by the Barley Quality Laboratory at the Univ. of Adelaide (Cozzolino and others 2014).

All samples (malt, whole grain, and pearled grain) were ground to a fine powder using a UDY Cyclone Sample Mill (Udy Corp., Boulder, Colo., U.S.A.). The resultant flour was used for pita bread preparation.

### Pita preparation

Pitas were prepared and cooked as per Bailey (2007) with some modifications. Flour (50 g), instant dry yeast (0.4%; Defiance Quality Food, Byford, Australia), salt (1.8%), sugar (1%), and water at 30 °C (60% to 65%) were mixed using a 50 g micro-mixer (Natl. MFG Co., Lincoln, NE, U.S.A.) for 15 min. Control samples were made with 100% commercial baker's flour (Defiance Quality Food) although in the other samples, 50% of the flour was replaced with barley flour prepared from malt, whole grain, or pearled grain as per Malcolmson and others (2011). Each dough was rounded into a ball, placed in a 75 × 50 × 32 mm mini-loafing tin and left to ferment in a sealed plastic bowl for 90 min at 30 ± 1 °C and 70% relative humidity (RH). Dough balls were then sheeted to 4 mm thick using a bench sheeter (RONDO GmbH

& Co. KG, Burbach, Germany) and then cut to 12 cm diameter using a circular pastry cutter. These dough rounds were rested in a fermentation cabinet at 30 ± 1 °C for 15 min and subsequently fried in a nonstick pan (Kambrook, Botany, Australia) for 8 min at 180 °C with gentle flipping every 1 min using a wooden spatula. Cooked pitas were cooled at room temperature for 30 min and photographed (Canon, Tokyo, Japan) before analysis of physical parameters or sensory properties. Pita bread samples were ground with an IKA analytical mill (IKA, Selangor, Malaysia) to a fine powder and stored at -80 °C until vitamin E and antioxidant analysis.

### Determination of vitamin E content and antioxidant capacity

Tocols were extracted using saponification as per Do and others (2015a) and individual vitamin E isomers ( $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -tocopherol [T] and tocotrienol [T3]) quantified using HPLC (Do and others 2015a). The vitamin E content, expressed in mg of  $\alpha$ -tocopherol equivalents (TE), was calculated using the equation:  $\alpha\text{-TE} = \alpha\text{-T} \times 1.0 + \alpha\text{-T3} \times 0.3 + \beta\text{-T} \times 0.4 + \beta\text{-T3} \times 0.05 + \gamma\text{-T} \times 0.1 + \gamma\text{-T3} \times 0.01 + \delta\text{-T} \times 0.01$  (McLaughlin and Weihrauch 1979).

Antioxidants were extracted using 80% ethanol and antioxidant capacity measured using 2,2-diphenyl-1-picrylhydrazyl as per Do and others (2015a). Antioxidant activity was expressed as mg ascorbic acid equivalent antioxidant capacity per 100 g fresh weight of grain.

### Physical parameters

Dough height was measured after fermentation using Digimax digital calipers (Camlab Limited, Cambridge, U.K.), whereas the extent of pocket formation (or puffing) was observed during baking (full, three-fourth, half, or not at all). After baking, an image of each pita was captured and the thickness measured using the calipers. If the pita was fully puffed, the thickness of the upper and lower layers was also measured. The upper layer was defined as the top layer of dough during the resting time after sheeting and was placed first in the pan for cooking.

Pita firmness was determined using a compression test according to the AACC approved method 74-09 (AACC, 2000) with some modifications. A food texture analyzer (Mecmesin Imperial 1000 Motorised Test Stand, West Sussex, U.K.) equipped with a 100 N load cell was used to compress the pita with a 25-mm-flat aluminum plunger up to 40% maximum strain at a speed of 1.7 mm/s at 20 °C. Pretest and posttest speeds were 1.0 and 10.0 mm/s, respectively. The bread was laid on the texture analyzer platform, and the distance between the platform and the plunger set to 5 cm. Although compression tests are usually conducted on crustless high or medium volume bread, the crust in the case of flat bread is very thin and difficult to remove without taking parts of the crumb. For this reason, the 1st 25% of the analysis was discarded and firmness was defined as the force at 40% strain minus the force at 25% strain (Alhajji 2011).

The color of flour and pita samples was measured (Minolta Colorimeter CR-300, Ramsey, N.J., U.S.A.) and data recorded using the  $L^*$  (lightness),  $a^*$  (green [-a] to red [+a]), and  $b^*$  (blue [-b] to yellow [+b]) color system.

### Sensory analysis

Sensory evaluation (Univ. of Adelaide Human Research Ethics Committee approval number H-2015-156) was conducted by 52 consumers (28 females and 24 males; 18 to 60 y old; and

students or staff of the Univ. of Adelaide). After providing written informed consent, each consumer was provided with a tray containing 4 samples (from pita substituted with flour milled from malt, Finniss whole grain or 15% pearled WI2585, and control pita made from baker's flour). All samples were coded with randomly selected 3-digit numbers and presented together with a scorecard in a randomized order and room temperature water for mouth cleansing between testing samples (Meilgaard and others 2007). Consumers were asked to record the acceptability for appearance, texture, flavor, and taste on a 9-point hedonic scale (1 = dislike extremely; 5 = neither dislike nor like, and 9 = like extremely; Shewfelt and others 2015) as well as the intensity scores for color (1 = dark and 9 = light), texture (1 = firm and 9 = soft), and flavor and taste (1 = none and 9 = high).

### Statistical analysis

In order to determine the differences between means using the least significant difference at  $P < 0.05$ , 1- and 2-way analysis of variance was performed using Genstat 14 (VSN Intl. Ltd., Hemel Hempstead, U.K.). Correlation analysis was performed using Microsoft Excel.

## Results and Discussion

### Pearling removes antioxidant capacity and vitamin E content from barley grain

Regardless of genotype, the levels of vitamin E and its isomers were significantly higher in the flour made from whole grain than in flour made from pearled grain, even in the case of the lowest amount of pearling (10% ; Figure 1A). The extent of this reduction was greatest for the hullless variety (Finniss), which exhibited an approximately 2-fold decrease for  $\alpha$ -T and  $\beta$ -T3 and 3-fold decrease for  $\alpha$ -T3 and  $\delta$ -T3 isomers, accounting for the observation that flour made from 10% pearled grain was 3-fold lower in vitamin E content than the flour made from whole grain (Table 1). The levels of vitamin E and the majority of isomers were further reduced in the flour made from 20% pearled grain, suggesting that most isomers decreased progressively from the external to the internal layers. This was especially obvious in the covered genotypes, WI2585 and Harrington, and was associated with a roughly 2-fold decrease in vitamin E content in the flour at 20% pearling. The only 2 isomers which appeared to be stable during all pearling stages were  $\beta$ -T and  $\gamma$ -T; however, they were only present at very low levels ( $<1 \mu\text{g/g DW}$ ) in most samples.

Tocol content in the hullless Finniss variety decreased most after the 1st pearling step whereas in the covered genotypes, Harrington and WI2585, tocol content decreased most after the 2nd and 3rd pearling steps, respectively. According to Ko and others (2003), the hull accounts for approximately 10.1% of whole grain weight whereas bran layers, which are richest in tocotrienols, account for approximately 12.6% and germ, rich in tocopherols, accounts for approximately 0.6% of whole grain weight. Thus, the extent of the reduction in vitamin E observed after pearling in this study indicates that the pearling levels abraded not only the hull but also the bran and part of the germ but in a genotype-dependent manner. For the hullless Finniss, more bran and germ might be removed with 20% pearling than for covered varieties. For WI2585, 15% pearled grain was significantly lower in vitamin E than 10% pearled grain with further significant reductions at 20% pearling. However, for Harrington, vitamin E reduced most at the 15% pearling stage with limited further reduction at the 20% pearling stage, suggesting that the thickness of the outer layers may vary

between these 2 genotypes. Even though Ko and others (2003) reported that the relative weight of the hull, bran, and germ can be influenced by growing conditions and location, all genotypes in our study were grown under the same environmental conditions, suggesting that any variation in this parameter was due to genotype.

Even though pearling reduced vitamin E content, the vitamin E content of pearled grain from the covered genotypes (WI2585 and Harrington), regardless of amount of pearling, was still significantly greater than the vitamin E content in the standard baker's flour (Figure 1A). The vitamin E content of flour made from the 10% pearled grain from Finniss and flour from malt was also significantly greater than the standard flour.

Storage had no effect on the vitamin E content in flour made from the whole grain from any of the genotypes, as expected (Do and others 2015a). Flour from Finniss whole grain was richest in vitamin E content among the samples but malt prepared from Finniss had a reduced level of vitamin E. This finding confirms our previous study that vitamin E decreases during malting because of leakage into the water during steeping and high temperature during kilning (Do and others 2015b).

Although the control sample ranked 7th out of 14 samples in terms of  $\alpha$ -T content in the flour, its  $\alpha$ -T3 content was lowest ( $2.2 \mu\text{g/g DW}$ ; Table 1) resulting in a low content of vitamin E ( $26.7 \mu\text{g/g DW}$ ; Figure 1A). In contrast, the highest level of  $\alpha$ -T was found in flour made from the whole grain of Harrington and WI2585, followed by flour from the 10% pearled WI2585. The level of  $\alpha$ -T3 was 20 to 30 times greater in flour prepared from whole grains of Finniss, Harrington, and WI2585 as well as 10% pearled grain of Harrington compared to standard baker's flour. Flour prepared from malt had a significantly lower  $\alpha$ -T content than control flour but was 19 times higher in  $\alpha$ -T3 content resulting in a high vitamin E content, which was 2-fold greater than the control. Although  $\alpha$ -T has historically been reported as the most efficient antioxidant (McLaughlin and Weihrauch 1979),  $\alpha$ -T3 has recently been shown to be at least 3-fold more efficient as a scavenger of peroxy radicals than  $\alpha$ -T (Packer 1995). In our previous study (Do and others 2015a),  $\alpha$ -T3 was the main vitamin E isomer in barley grain, regardless of genotype, and the correlation of  $\alpha$ -T3 with antioxidant capacity supports this observation. Storage increased content of  $\beta$ -T3 and  $\gamma$ -T3 in flour prepared from whole grain of WI2585 whereas  $\gamma$ -T3 was significantly greater in Finniss whole grain. However, no significant change was observed in the 2 main isomers,  $\alpha$ -T and  $\alpha$ -T3, and consequently the vitamin E content in flour prepared from stored grain, regardless of genotype.

Similar to what was observed for vitamin E content, a progressive decrease in the antioxidant capacity of flour was also observed with pearling for all genotypes (Figure 2A). At 10% pearling, the loss of antioxidant capacity in descending order of flour made from Finniss, Harrington, and WI2585 was approximately 48%, 23%, and 3%, respectively, whereas at 15% pearling the antioxidant capacity lost in those genotypes was approximately 52%, 38%, and 15%, respectively. When 20% of the grain was pearled, the highest percentage decrease of antioxidant capacity was observed in flour from the hullless variety, Finniss (55%) followed by Harrington (approximately 49%) and WI2585 (28%). The decrease in antioxidant capacity for hullless Finniss and covered Harrington primarily occurred with 10% pearling, suggesting that the 10% pearl fraction contains the majority of antioxidants, including both vitamin E (Figure 1A) and other phenolic compounds (Goupy and others 1999). However, we previously found that vitamin E was

Table 1-Tocopherol and tocotrienol content (µg/g DW) in flour and pita after processing.

Samples <sup>a</sup>	α-T			β-T			γ-T			α-T3			β-T3			γ-T3			
	Flour	Pita	Flour	Pita	Flour	Pita	Flour	Pita	Flour	Pita	Flour	Pita	Flour	Pita	Flour	Pita	Flour	Pita	
Control (baker's flour)	9.1 ± 0.3 <sup>f</sup>	3.0 ± 0.1 <sup>a</sup>	0.4 ± 0.0 <sup>*</sup>	0.4 ± 0.0	2.8 ± 0.5 <sup>d</sup>	0.4 ± 0.0 <sup>a</sup>	2.2 ± 0.2 <sup>a*</sup>	1.0 ± 0.1 <sup>a</sup>	12.2 ± 0.2 <sup>g</sup>	1.7 ± 0.1 <sup>a</sup>	0.0 ± 0.0 <sup>**</sup>	0.0 ± 0.0 <sup>**</sup>	0.0 ± 0.0 <sup>**</sup>	8.3 ± 0.4 <sup>e</sup>	2.3 ± 0.1 <sup>ef</sup>	0.0 ± 0.0 <sup>**</sup>	0.0 ± 0.0 <sup>**</sup>	0.0 ± 0.0 <sup>**</sup>	
Malt	7.1 ± 0.3 <sup>c</sup>	5.5 ± 0.3 <sup>d</sup>	0.3 ± 0.0 <sup>*</sup>	0.4 ± 0.0	0.6 ± 0.2 <sup>bc</sup>	1.4 ± 0.1 <sup>c</sup>	42.0 ± 1.6 <sup>b</sup>	12.2 ± 0.4 <sup>g</sup>	5.5 ± 0.2 <sup>d</sup>	7.1 ± 0.4 <sup>b</sup>	8.3 ± 0.4 <sup>e</sup>	2.3 ± 0.1 <sup>ef</sup>	8.3 ± 0.4 <sup>e</sup>	14.4 ± 0.5 <sup>i</sup>	5.6 ± 0.2 <sup>i</sup>	8.3 ± 0.4 <sup>e</sup>	2.3 ± 0.1 <sup>ef</sup>	8.3 ± 0.4 <sup>e</sup>	
Fresh Finnis (whole grain)	12.3 ± 0.4 <sup>b</sup>	4.5 ± 0.1 <sup>c</sup>	0.0 ± 0.0 <sup>*</sup>	0.0 ± 0.0	0.6 ± 0.0 <sup>bc</sup>	1.9 ± 0.3 <sup>f</sup>	63.8 ± 2.1 <sup>1</sup>	11.8 ± 0.6 <sup>fg</sup>	12.2 ± 0.5 <sup>g</sup>	9.6 ± 0.4 <sup>f</sup>	14.4 ± 0.5 <sup>i</sup>	5.6 ± 0.2 <sup>i</sup>	14.4 ± 0.5 <sup>i</sup>	14.4 ± 0.5 <sup>i</sup>	5.6 ± 0.2 <sup>i</sup>	14.4 ± 0.5 <sup>i</sup>	5.6 ± 0.2 <sup>i</sup>	14.4 ± 0.5 <sup>i</sup>	
Fresh Finnis (10% pearling)	5.2 ± 0.1 <sup>c</sup>	3.7 ± 0.0 <sup>bc</sup>	0.3 ± 0.0 <sup>*</sup>	0.3 ± 0.0	0.4 ± 0.1 <sup>ab</sup>	1.9 ± 0.0 <sup>f</sup>	19.7 ± 0.4 <sup>e</sup>	0.8 ± 0.2 <sup>a</sup>	7.2 ± 0.4 <sup>e*</sup>	7.1 ± 0.6 <sup>h</sup>	4.5 ± 0.1 <sup>b</sup>	1.7 ± 0.1 <sup>cde</sup>	4.5 ± 0.1 <sup>b</sup>	4.5 ± 0.1 <sup>b</sup>	1.7 ± 0.1 <sup>cde</sup>	4.5 ± 0.1 <sup>b</sup>	1.7 ± 0.1 <sup>cde</sup>	4.5 ± 0.1 <sup>b</sup>	
Fresh Finnis (15% pearling)	4.4 ± 0.0 <sup>b</sup>	3.1 ± 0.1 <sup>ab</sup>	0.3 ± 0.0 <sup>*</sup>	0.3 ± 0.0	0.0 ± 0.0 <sup>a</sup>	1.2 ± 0.2 <sup>de</sup>	14.8 ± 0.1 <sup>c</sup>	0.4 ± 0.0 <sup>a</sup>	6.6 ± 0.0 <sup>e</sup>	5.2 ± 0.2 <sup>fg</sup>	3.1 ± 0.1 <sup>a</sup>	1.0 ± 0.0 <sup>bc</sup>	3.1 ± 0.1 <sup>a</sup>	3.1 ± 0.1 <sup>a</sup>	1.0 ± 0.0 <sup>bc</sup>	3.1 ± 0.1 <sup>a</sup>	1.0 ± 0.0 <sup>bc</sup>	3.1 ± 0.1 <sup>a</sup>	
Fresh Finnis (20% pearling)	3.6 ± 0.1 <sup>a*</sup>	3.1 ± 0.1 <sup>ab</sup>	0.3 ± 0.0 <sup>*</sup>	0.3 ± 0.0	0.0 ± 0.0 <sup>a</sup>	0.8 ± 0.0 <sup>abcd</sup>	11.5 ± 0.2 <sup>b</sup>	0.4 ± 0.0 <sup>a</sup>	6.7 ± 0.3 <sup>c</sup>	5.0 ± 0.1 <sup>f</sup>	2.3 ± 0.0 <sup>a</sup>	0.8 ± 0.0 <sup>ab</sup>	2.3 ± 0.0 <sup>a</sup>	2.3 ± 0.0 <sup>a</sup>	0.8 ± 0.0 <sup>ab</sup>	2.3 ± 0.0 <sup>a</sup>	0.8 ± 0.0 <sup>ab</sup>	2.3 ± 0.0 <sup>a</sup>	
Fresh W12585 (whole grain)	13.1 ± 0.1 <sup>i</sup>	4.6 ± 0.2 <sup>c</sup>	0.0 ± 0.0 <sup>*</sup>	0.4 ± 0.0	0.8 ± 0.1 <sup>bc*</sup>	0.7 ± 0.1 <sup>bc*</sup>	44.4 ± 1.1 <sup>1</sup>	6.4 ± 0.1 <sup>cd</sup>	4.4 ± 0.6 <sup>bc</sup>	3.5 ± 0.5 <sup>bcd</sup>	10.2 ± 1.3 <sup>g</sup>	2.7 ± 0.3 <sup>f</sup>	10.2 ± 1.3 <sup>g</sup>	10.2 ± 1.3 <sup>g</sup>	2.7 ± 0.3 <sup>f</sup>	10.2 ± 1.3 <sup>g</sup>	2.7 ± 0.3 <sup>f</sup>	10.2 ± 1.3 <sup>g</sup>	
Fresh W12585 (10% pearling)	12.9 ± 0.0 <sup>i</sup>	4.5 ± 0.6 <sup>c</sup>	0.3 ± 0.0 <sup>*</sup>	0.0 ± 0.0	0.8 ± 0.1 <sup>bc</sup>	1.2 ± 0.4 <sup>de</sup>	39.3 ± 0.5 <sup>g</sup>	3.3 ± 0.8 <sup>b</sup>	4.2 ± 0.5 <sup>ab**</sup>	4.0 ± 0.7 <sup>de</sup>	9.3 ± 1.0 <sup>f</sup>	2.7 ± 0.6 <sup>f</sup>	9.3 ± 1.0 <sup>f</sup>	9.3 ± 1.0 <sup>f</sup>	2.7 ± 0.6 <sup>f</sup>	9.3 ± 1.0 <sup>f</sup>	2.7 ± 0.6 <sup>f</sup>	9.3 ± 1.0 <sup>f</sup>	
Fresh W12585 (15% pearling)	10.7 ± 0.4 <sup>g</sup>	3.6 ± 0.0 <sup>b</sup>	0.3 ± 0.0 <sup>*</sup>	0.4 ± 0.0	0.8 ± 0.1 <sup>bc*</sup>	0.6 ± 0.0 <sup>ab</sup>	29.9 ± 0.3 <sup>f</sup>	3.2 ± 0.1 <sup>b</sup>	3.7 ± 0.7 <sup>ab**</sup>	3.1 ± 0.1 <sup>bc</sup>	7.1 ± 1.2 <sup>d</sup>	1.3 ± 0.2 <sup>bcd</sup>	7.1 ± 1.2 <sup>d</sup>	7.1 ± 1.2 <sup>d</sup>	1.3 ± 0.2 <sup>bcd</sup>	7.1 ± 1.2 <sup>d</sup>	1.3 ± 0.2 <sup>bcd</sup>	7.1 ± 1.2 <sup>d</sup>	
Fresh W12585 (20% pearling)	6.9 ± 0.6 <sup>c</sup>	3.4 ± 0.0 <sup>ab</sup>	0.0 ± 0.0 <sup>*</sup>	0.3 ± 0.0	0.5 ± 0.0 <sup>bc*</sup>	0.6 ± 0.0 <sup>ab</sup>	17.6 ± 2.7 <sup>d</sup>	1.6 ± 0.3 <sup>ab</sup>	3.5 ± 0.5 <sup>a*</sup>	3.0 ± 0.0 <sup>b</sup>	4.5 ± 0.6 <sup>b</sup>	1.2 ± 0.0 <sup>bcd</sup>	4.5 ± 0.6 <sup>b</sup>	4.5 ± 0.6 <sup>b</sup>	1.2 ± 0.0 <sup>bcd</sup>	4.5 ± 0.6 <sup>b</sup>	1.2 ± 0.0 <sup>bcd</sup>	4.5 ± 0.6 <sup>b</sup>	
Fresh Harrington (whole grain)	13.2 ± 0.5 <sup>i</sup>	4.6 ± 0.1 <sup>c</sup>	0.3 ± 0.0 <sup>*</sup>	0.4 ± 0.0	1.1 ± 0.3 <sup>c**</sup>	1.1 ± 0.0 <sup>cde</sup>	55.2 ± 0.5 <sup>1</sup>	6.5 ± 0.7 <sup>cd</sup>	9.4 ± 0.2 <sup>f</sup>	7.2 ± 0.1 <sup>h</sup>	18.0 ± 0.0 <sup>k</sup>	5.7 ± 0.2 <sup>i</sup>	18.0 ± 0.0 <sup>k</sup>	18.0 ± 0.0 <sup>k</sup>	5.7 ± 0.2 <sup>i</sup>	18.0 ± 0.0 <sup>k</sup>	5.7 ± 0.2 <sup>i</sup>	18.0 ± 0.0 <sup>k</sup>	
Fresh Harrington (10% pearling)	10.5 ± 0.3 <sup>g</sup>	4.1 ± 0.2 <sup>bc</sup>	0.3 ± 0.0 <sup>*</sup>	0.3 ± 0.0	0.8 ± 0.3 <sup>bc**</sup>	1.0 ± 0.0 <sup>bcde</sup>	43.4 ± 0.2 <sup>hi</sup>	6.9 ± 0.8 <sup>cd</sup>	8.7 ± 0.2 <sup>f</sup>	7.0 ± 0.5 <sup>h</sup>	14.7 ± 0.1 <sup>i</sup>	4.9 ± 0.2 <sup>def</sup>	14.7 ± 0.1 <sup>i</sup>	14.7 ± 0.1 <sup>i</sup>	4.9 ± 0.2 <sup>def</sup>	14.7 ± 0.1 <sup>i</sup>	4.9 ± 0.2 <sup>def</sup>	14.7 ± 0.1 <sup>i</sup>	
Fresh Harrington (15% pearling)	5.9 ± 0.3 <sup>d</sup>	3.2 ± 0.1 <sup>ab</sup>	0.3 ± 0.0 <sup>*</sup>	0.0 ± 0.0	0.0 <sup>a</sup>	0.8 ± 0.1 <sup>abc</sup>	18.0 ± 1.5 <sup>de</sup>	0.7 ± 0.2 <sup>a</sup>	4.4 ± 0.3 <sup>bc**</sup>	4.4 ± 0.4 <sup>ef</sup>	5.9 ± 0.5 <sup>c</sup>	1.9 ± 0.2 <sup>def</sup>	5.9 ± 0.5 <sup>c</sup>	5.9 ± 0.5 <sup>c</sup>	1.9 ± 0.2 <sup>def</sup>	5.9 ± 0.5 <sup>c</sup>	1.9 ± 0.2 <sup>def</sup>	5.9 ± 0.5 <sup>c</sup>	
Fresh Harrington (20% pearling)	5.2 ± 0.3 <sup>c</sup>	2.9 ± 0.0 <sup>a</sup>	0.3 ± 0.0 <sup>*</sup>	0.3 ± 0.0	0.0 ± 0.0 <sup>a</sup>	0.7 ± 0.1 <sup>abc</sup>	16.1 ± 0.0 <sup>cd</sup>	0.2 ± 0.0 <sup>a</sup>	5.2 ± 0.4 <sup>d</sup>	3.9 ± 0.2 <sup>de</sup>	4.9 ± 0.7 <sup>b</sup>	0.9 ± 0.1 <sup>bc</sup>	4.9 ± 0.7 <sup>b</sup>	4.9 ± 0.7 <sup>b</sup>	0.9 ± 0.1 <sup>bc</sup>	4.9 ± 0.7 <sup>b</sup>	0.9 ± 0.1 <sup>bc</sup>	4.9 ± 0.7 <sup>b</sup>	
Stored Finnis (whole grain)	12.1 ± 0.1 <sup>h</sup>	4.6 ± 0.0 <sup>c</sup>	0.3 ± 0.0 <sup>*</sup>	0.0 ± 0.0	0.6 ± 0.0 <sup>bc*</sup>	1.0 ± 0.0 <sup>bcd</sup>	63.0 ± 0.5 <sup>1</sup>	9.1 ± 0.2 <sup>e</sup>	12.4 ± 0.1 <sup>g</sup>	7.5 ± 0.2 <sup>h</sup>	15.6 ± 0.1 <sup>j</sup>	4.7 ± 0.1 <sup>h</sup>	15.6 ± 0.1 <sup>j</sup>	15.6 ± 0.1 <sup>j</sup>	4.7 ± 0.1 <sup>h</sup>	15.6 ± 0.1 <sup>j</sup>	4.7 ± 0.1 <sup>h</sup>	15.6 ± 0.1 <sup>j</sup>	
Stored W12585 (whole grain)	13.3 ± 0.0 <sup>i</sup>	4.4 ± 0.1 <sup>c</sup>	0.4 ± 0.0 <sup>*</sup>	0.4 ± 0.0	0.9 ± 0.0 <sup>c*</sup>	0.7 ± 0.0 <sup>abc</sup>	43.7 ± 0.0 <sup>hi</sup>	5.8 ± 0.5 <sup>c</sup>	5.1 ± 0.0 <sup>cd</sup>	3.8 ± 0.1 <sup>cde</sup>	11.8 ± 0.0 <sup>h</sup>	3.2 ± 0.1 <sup>g</sup>	11.8 ± 0.0 <sup>h</sup>	11.8 ± 0.0 <sup>h</sup>	3.2 ± 0.1 <sup>g</sup>	11.8 ± 0.0 <sup>h</sup>	3.2 ± 0.1 <sup>g</sup>	11.8 ± 0.0 <sup>h</sup>	
Stored Harrington (whole grain)	13.1 ± 0.3 <sup>i</sup>	4.2 ± 0.1 <sup>c</sup>	0.4 ± 0.0 <sup>*</sup>	0.4 ± 0.0	0.4 ± 0.0 <sup>ab*</sup>	0.7 ± 0.1 <sup>abc</sup>	56.6 ± 1.4 <sup>k</sup>	7.9 ± 0.3 <sup>de</sup>	8.9 ± 0.4 <sup>f</sup>	5.9 ± 0.8 <sup>g</sup>	18.1 ± 0.7 <sup>k</sup>	5.4 ± 0.4 <sup>hi</sup>	18.1 ± 0.7 <sup>k</sup>	18.1 ± 0.7 <sup>k</sup>	5.4 ± 0.4 <sup>hi</sup>	18.1 ± 0.7 <sup>k</sup>	5.4 ± 0.4 <sup>hi</sup>	18.1 ± 0.7 <sup>k</sup>	
LSD	0.5	0.5	NS	NS	0.4	0.4	1.9	1.9	0.7	0.7	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8

<sup>a</sup> Means ± SE are shown where n = 3 for all flour samples, n = 9 for pitas from control baker's flour, flour prepared from malt and flour from Finnis whole grain; and n = 3 for the remaining pita samples. Same letters (within column) or \* (within row) indicates no difference between samples for individual isomers or no difference between flour and pita as determined using the Least Significant Difference (LSD; P < 0.05). NS indicates there was no significant difference (P > 0.05). δ-T3 and δ-T were not detected.

not the main contributor to antioxidant capacity in barley (Do and others 2015a), and therefore the removal of other phenolic compounds by pearling is likely to have the greatest impact. Total soluble phenolic content of the 1st fraction (10% pearling) has previously been shown to be double that of the 2nd fraction (20% pearling) in 2 other hullless genotypes examined by Gong and others (2012). Previous studies of covered genotypes have shown that *P*-coumaric acid levels increased dramatically in the outer grain layers, especially in lignified husk (Salomonsson and others 1980), although ferulic acid was highest in the cell walls of the aleurone layer (McNeil and others 1975).

Although most antioxidants were removed in the 1st pearling stage for Finnis and Harrington, this appeared to occur in the 2 subsequent pearling stages for WI2585, which reflected the pattern observed for vitamin E. Environment affects antioxidant capacity in wheat (Moore and others 2006), but does not appear to do so in barley (Peterson and Qureshi 1993). However, barley was only

grown in one environment in this study and therefore the impact of environment on antioxidant capacity requires further investigation. The differences in the effect of pearling on antioxidant capacity in the different genotypes are more likely to be related to genotypic variation affecting the development of the outer layers of the grain and the concentration or types of antioxidant present. According to Evers and others (1999), the hull amount can range between 7% and 25% of grain weight depending upon genotype, growing conditions and grain size. Finnis is a hullless genotype and was therefore expected to lose antioxidant capacity more quickly with pearling. Harrington has a loose adhering husk and is highly susceptible to skinning (Menz 2010). In contrast, WI2585 has a thicker and more adhering husk, which might result in a higher percentage of husk per grain weight and explain the differences between these genotypes in terms of the loss of antioxidant capacity in flour from pearled grain. In addition, some researchers have reported that phenolic acids are concentrated in the cell walls

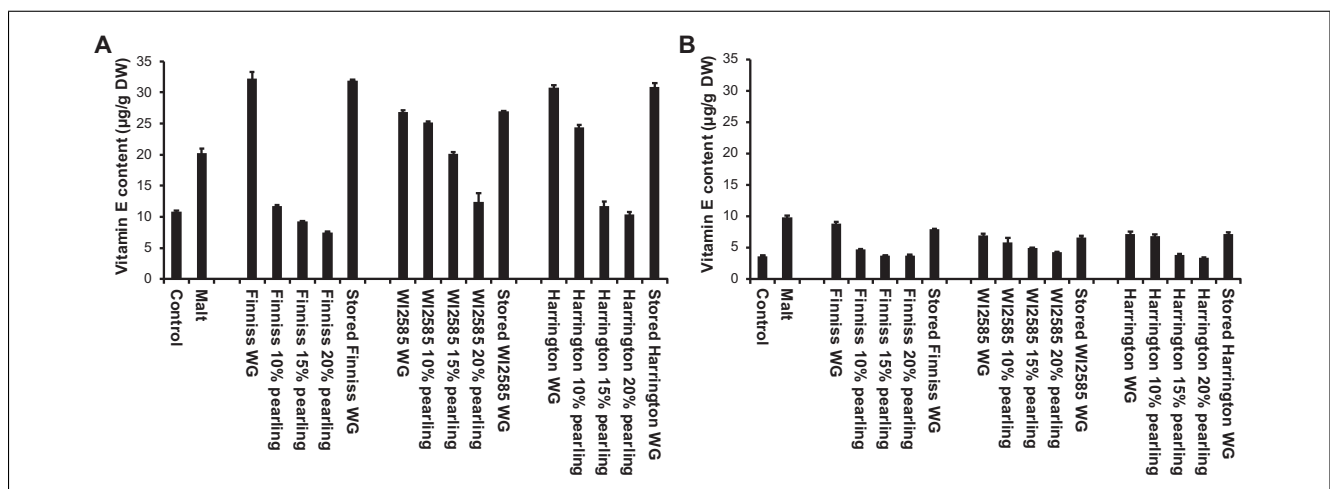


Figure 1—Vitamin E content ( $\mu\text{g/g}$  fresh weight [FW]) in flour and pita after processing. Vitamin E is expressed in mg of  $\alpha$ -tocopherol equivalents (TE). Bars represent the mean  $\pm$  SE. (A) vitamin E content in flour before processing,  $n = 3$  for all samples. (B) Vitamin E content in pita after processing,  $n = 9$  for the pita from baker's flour, malt flour, and Finnis whole grain flour,  $n = 3$  for the rest of pita samples. Difference between samples as determined using the least significant difference (LSD;  $P < 0.05$ ),  $\text{LSD}_{\text{sample-time}} = 0.9$ . WG indicates whole grain.

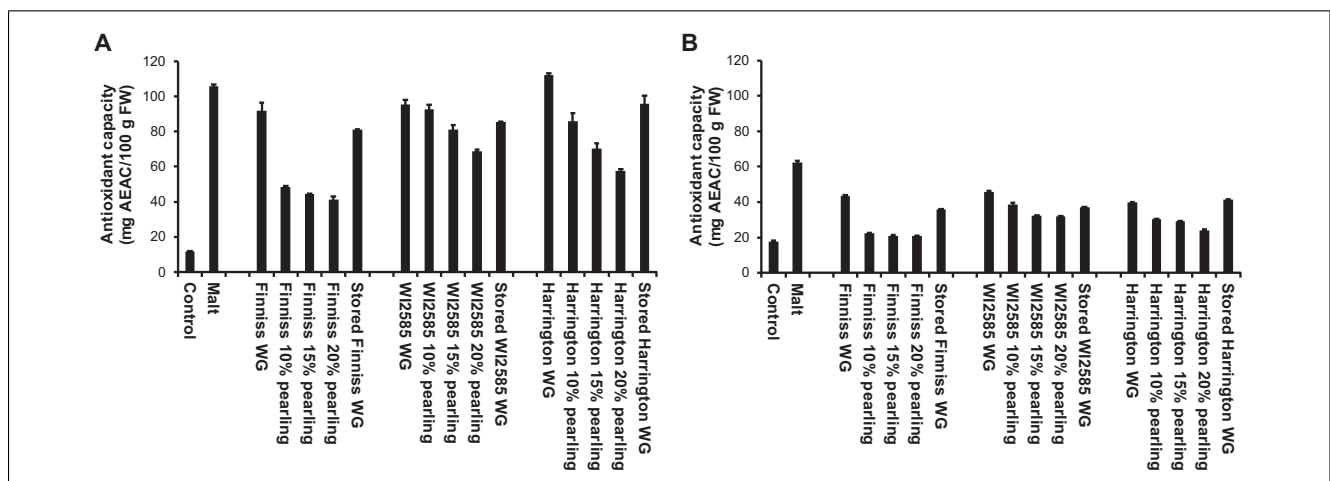


Figure 2—Antioxidant capacity in flour and pita after processing. Antioxidant capacity is expressed as mg ascorbic acid equivalent antioxidant capacity (AEAC) per 100 g of fresh weight (FW) of grain. Bars represent the mean  $\pm$  SE. (A) Antioxidant capacity in flour before processing,  $n = 3$  for all samples. (B) Antioxidant capacity in pita after processing,  $n = 9$  for pitas from baker's flour, malt flour, and Finnis whole grain flour,  $n = 3$  for the rest of pita samples. Difference between samples as determined using the least significant difference (LSD;  $P < 0.05$ ),  $\text{LSD}_{\text{sample-time}} = 2.8$ . WG indicates whole grain.

of outer layers (Maillard and Berset 1995), although others have indicated that phenolic acids were mainly present in the aleurone layer and endosperm due to genotypic differences (Goupy and others 1999). However, the relationship between phenolics and which layers they are concentrated in for these genotypes remains to be established as does the availability of phenolics (bound vs. free; Lu and others 2007).

As expected, the antioxidant capacity of the control wheat sample was 4 times lower than that of barley flour at the highest pearling level (20%) and 10 times lower than that of flour prepared from barley whole grains, regardless of genotype (Figure 2A). This finding is in agreement with a previous study which showed a lower antioxidant capacity in wheat grain compared to barley grain (Holasova and others 1995). In addition, wheat pearling to produce white baker's flour has been shown to reduce antioxidant capacity linearly as the degree of pearling increased (Liyana-Pathirana and others 2006). Flour from Harrington whole grain had the highest antioxidant capacity, followed by flour from malt, flour from WI2585 whole grain, and Finnis whole grain. Malt prepared from Finnis had a slightly higher antioxidant capacity compared to unprocessed Finnis, a finding which was consistent with our previous observations (Do and others 2015b). In contrast, storage reduced the antioxidant capacity by 10.5%, 11.8%, and 14.5% in whole grain of WI2585, Finnis, and Harrington, respectively, as per Do and others (2015a).

### Barley pitas contain higher antioxidant and vitamin E content

The reduction of vitamin E content and antioxidant capacity after pearling in raw samples resulted in reduction of these same components in pita breads (Figure 1B and 2B). In addition, the pita breads made from barley flour or malt had a significantly lower antioxidant capacity and vitamin E content than that in the flour; however, this was not the case for the pita made with 100% baker's flour. After cooking, the antioxidant capacity decreased by 41% to 59% and vitamin E content by 50% to 77%. Vitamin E content reduced by roughly 3-fold for Finnis and more than 4-fold for WI2585 and Harrington, suggesting that vitamin E might be more stable in Finnis during cooking. Pita cooking also caused a reduction in the content of most isomers especially the dominant types,  $\alpha$ -T and  $\alpha$ -T3. Even though  $\alpha$ -T3 contributed most to total tocol content, it was negligible in pitas made with flour from 20% pearling. The content of other vitamin E isomers was generally lower in the pitas made with flour from pearled grain (Table 1) except for  $\beta$ -T; however, this isomer was present at significantly lower levels than all others.

Similar to vitamin E content, the antioxidant capacity in pita made from whole-grain flour was much higher than in pita made from pearled-grain flour (Figure 2B). For Finnis, antioxidant capacity significantly decreased in pita made with flour from 10% pearled grain but did not decrease further in pitas made with higher percentage pearled grain. Because antioxidant capacity was reduced in stored barley whole grain compared to that at harvest, except in the case of Harrington, the antioxidant capacity in pitas prepared with flour milled from stored whole grains was lower than in pitas prepared from fresh whole-grain flour, except in the case of Harrington.

The flour from malt had a higher antioxidant capacity than all other flour samples, and also exhibited the lowest percentage change in antioxidant capacity during cooking. Consequently, pitas made from malt flour had the highest antioxidant capacity.

Although the vitamin E content of the pitas made with flour from 20% pearled grain of Finnis and Harrington were not significantly different from that observed for the wheat bakers' flour, all barley pita samples had significantly greater antioxidant capacity. This supports their potential use as functional food products as a source of antioxidants for consumers. However, even though phenolics are probably the main contributor to antioxidant capacity in barley grains (Goupy and others 1999), which compounds are responsible for the increased antioxidant capacity in the barley pita samples still requires investigation.

The losses in vitamin E content/antioxidant capacity observed in the cooked pita bread compared to the grains and flours are not unexpected, because they are known to be unstable, especially at high temperatures. However, at increased temperature, reducing sugars and amino acids can react to produce Maillard products such as melanoidins, which also have antioxidant capacity (Maillard and others 1996). This may explain why the antioxidant capacity was not reduced to the same extent as the vitamin E content in the pitas in this study. The antioxidant capacity also remained higher in pita made from flour of the whole grain and malt compared with that made with flour from pearled grains. Regardless of genotype, a high correlation between antioxidant capacity of the flour and the pitas made from that flour was found ( $r = 0.85$ ,  $P < 0.05$ ,  $n = 14$ ). This indicates that selecting the material with high antioxidant capacity enriches this component in pitas. A high correlation was also observed between vitamin E content in unprocessed grains or malt and in pita ( $r = 0.81$ ,  $P < 0.05$ ,  $n = 14$ ). Therefore, barley genotypes known to have high antioxidant capacity and vitamin E content can ensure much greater quantities of these components in the end product. However, the quality of the product needs to be confirmed by evaluating the physical and sensory properties as has been commonly done in other food studies (Alu'datt and others 2014; Blandino and others 2015).

### Physical quality attributes of barley pitas

There are no specific guidelines available to judge pita bread quality but puffing formation, ease of layer separation, crust, shape, and color are considered the most important parameters (Morad and others 1984). Similar compression values (as a measure of firmness) were found in control pita and barley pitas made with flour from Finnis (regardless of whether pearled or whole grain) and pitas made from flour from 15% or 20% pearled grain from WI2585 or Harrington (Table 2). Firmness was, however, significantly greater in pita containing flour prepared from malt; 10% pearled WI2585; 10% pearled Harrington; and whole grain of WI2585 and Harrington, both stored and fresh. Malt pita was twice as hard than the control pita whereas the covered whole grain pita was 3 times as hard.

The thickness of the pita was greatest in the control sample (15.3 mm) and lowest in pitas made from flour of the whole grains from covered genotypes (Table 2), and was negatively correlated with firmness across all samples ( $r = -0.9$ ,  $P < 0.05$ ,  $n = 17$ ). Only pita made with flour from 20% pearled Finnis and WI2585 had similar thickness to that of control pita. Control pita also showed better crumb pore uniformity (Figure S1) and even though the upper layer did not significantly differ between any of the samples, the lower layer was significantly greater in the control than in barley pitas (Table 2). Crust with adhering crumb was observed for all pitas except those made with flour from the covered whole grain, which seemed to only have a crust in their upper layer. This

**Table 2—Instrumental texture analysis values of different pita bread.**

Samples <sup>a</sup>	Dough height (mm)	Thickness (mm)	Upper layer thickness (mm)	Lower layer thickness (mm)	Compression (N)	Pocket formed
Control (baker's flour)	35.0 ± 0.6 <sup>h</sup>	15.3 ± 0.2 <sup>h</sup>	5.0 ± 0.2	7.8 ± 0.1 <sup>d</sup>	2.8 ± 0.4 <sup>a</sup>	Fully
Malt	28.1 ± 0.2 <sup>c</sup>	9.2 ± 0.1 <sup>ab</sup>	n/a	n/a	6.5 ± 0.3 <sup>b</sup>	3/4
Fresh Finnis (whole grain)	29.8 ± 0.1 <sup>d</sup>	12.8 ± 1.0 <sup>ef</sup>	3.9 ± 0.2	4.5 ± 0.2 <sup>abc</sup>	4.2 ± 0.8 <sup>a</sup>	Fully
Fresh Finnis (10% pearling)	32.0 ± 0.2 <sup>f</sup>	13.6 ± 0.2 <sup>efg</sup>	4.6 ± 0.3	4.8 ± 0.2 <sup>abc</sup>	3.5 ± 0.0 <sup>a</sup>	Fully
Fresh Finnis (15% pearling)	33.5 ± 0.0 <sup>g</sup>	13.9 ± 0.1 <sup>efg</sup>	4.1 ± 0.1	5.9 ± 0.4 <sup>c</sup>	3.3 ± 0.6 <sup>a</sup>	Fully
Fresh Finnis (20% pearling)	33.7 ± 0.1 <sup>g</sup>	14.1 ± 0.2 <sup>fgh</sup>	4.0 ± 0.1	4.8 ± 0.1 <sup>abc</sup>	3.3 ± 0.4 <sup>a</sup>	Fully
Fresh WI2585 (whole grain)	27.0 ± 0.4 <sup>b</sup>	8.6 ± 0.0 <sup>a</sup>	n/a	n/a	8.4 ± 1.3 <sup>cd</sup>	No
Fresh WI2585 (10% pearling)	27.9 ± 0.1 <sup>c</sup>	9.3 ± 0.1 <sup>ab</sup>	n/a	n/a	6.7 ± 0.3 <sup>b</sup>	1/2
Fresh WI2585 (15% pearling)	30.7 ± 0.0 <sup>c</sup>	13.9 ± 0.6 <sup>efg</sup>	4.5 ± 0.7	5.5 ± 1.2 <sup>bc</sup>	4.1 ± 0.2 <sup>a</sup>	Fully
Fresh WI2585 (20% pearling)	32.1 ± 0.2 <sup>f</sup>	14.2 ± 0.6 <sup>gh</sup>	4.3 ± 0.6	5.9 ± 1.0 <sup>c</sup>	3.2 ± 0.2 <sup>a</sup>	Fully
Fresh Harrington (whole grain)	26.0 ± 0.2 <sup>a</sup>	9.1 ± 1.0 <sup>ab</sup>	n/a	n/a	8.8 ± 0.4 <sup>d</sup>	No
Fresh Harrington (10% pearling)	27.0 ± 0.3 <sup>b</sup>	10.1 ± 0.6 <sup>bc</sup>	n/a	n/a	7.0 ± 0.1 <sup>bc</sup>	No
Fresh Harrington (15% pearling)	29.3 ± 0.2 <sup>d</sup>	10.7 ± 0.2 <sup>c</sup>	4.1 ± 0.5	4.2 ± 0.3 <sup>ab</sup>	3.9 ± 0.2 <sup>a</sup>	Fully
Fresh Harrington (20% pearling)	30.9 ± 0.0 <sup>c</sup>	11.3 ± 0.6 <sup>cd</sup>	4.2 ± 0.1	4.1 ± 0.2 <sup>ab</sup>	4.2 ± 0.4 <sup>a</sup>	Fully
Stored Finnis (whole grain)	31.0 ± 0.0 <sup>c</sup>	13.7 ± 0.3 <sup>efg</sup>	4.2 ± 0.6	3.7 ± 0.4 <sup>a</sup>	4.0 ± 0.7 <sup>a</sup>	Fully
Stored WI2585 (whole grain)	29.5 ± 0.0 <sup>d</sup>	12.6 ± 0.5 <sup>de</sup>	n/a	n/a	6.6 ± 0.3 <sup>b</sup>	3/4
Stored Harrington (whole grain)	28.0 ± 0.0 <sup>c</sup>	10.3 ± 0.3 <sup>bc</sup>	n/a	n/a	7.1 ± 0.4 <sup>bc</sup>	3/4
LSD	0.6	1.3	NS	1.6	1.5	

<sup>a</sup>Means ± SE are shown where  $n = 3$  for each sample. Same letters (within column) indicates no difference between samples as determined using the least significant difference (LSD;  $P < 0.05$ ). Not applicable (n/a), not measured. NS indicates no significant difference.

crust formation happened in the thin upper layer during puffing and consequently the pocket was not fully formed.

Pocket formation did not occur during the baking of pita breads which contained flour from whole grains or 10% pearled grains of WI2585 and Harrington. In addition, 3-quarter or half pockets were observed for pita made with flour from malt, stored WI2585 whole grain, stored Harrington whole grain, or 10% pearled WI2585. According to Faridi and Rubenthaler (1984), pocket formation occurs when the internal temperature reaches a point high enough to develop steam during baking, but the extent to which this occurs also depends on how many bubble cells are formed in the dough during fermentation. Fewer bubble cells were observed in dough from whole grain flour from covered genotypes, which may explain why pocket formation was reduced in these pitas. In addition, the higher water absorption of the husk may have caused lower water availability in the dough for starch to be gelatinized during baking, which would also act to inhibit pocket formation (Varrianomarston and others 1980).

The softness or loaf volume of bread in general is related to the properties of the dough (Wang and others 2002). A strong correlation between dough height and thickness/firmness in this study ( $r = 0.9$ ,  $P < 0.05$ ,  $n = 17$  for both) supports this observation. The height of the control dough after fermentation was higher than all barley doughs possibly due to the lack of gluten in barley, leading to lower gluten levels and consequent difficulties in dough handling, lower loaf volume and reduced softness (Wang and others 2002). Moreover, the high content of  $\beta$ -glucans in barley can tightly bind water in dough, thereby reducing the availability of water to develop a gluten network and rupture the bubble cells normally formed during fermentation (Wang and others 2002). Indeed, the height of the barley doughs from covered genotypes increased with increased pearling, probably due to the removal of the glucan-rich husk.

Dough height was significantly lower than the control for pitas made from malt flour although dough from Finnis whole-grain flour rose better than dough from covered whole-grain flour, indicating the advantage of hullless whole grain genotypes in food production. The differences in texture of pita made with covered genotypes, WI2585 and Harrington whole grain, could also be

attributed to the difference in their content of tannin and amylose. Tannins are known to bind with protein (Hulse 1979) and are likely to form a tannin-gluten complex which might change rheological properties while differences in amylose content may cause differences in dough stickiness and therefore differences in pasting properties (Izydorczyk and others 2008).

The use of flour prepared from stored whole grain significantly increased the dough height when compared to fresh whole grain flour, and produced better pocket formation. This is consistent with previous findings in pitas made from wheat flour (Pomeranz 1992; Suter and others 1995), which indicated that 2 to 4 mo storage following harvest increased loaf volume and overall baking quality. In these previous studies, the authors suggested that this was due to an improved balance of gluten properties, an increase in protein molecular mass and higher gas-retention capacity in baking (Pomeranz 1992) as well as improved dough strength due to oxidative polymerization of glutenins during storage (Suter and others 1995).

Lower dough and loaf volume have also been reported in previous studies on the effect of barley inclusion on properties of pita breads; however, data were provided through sensory analysis not physical testing (Alu'datt and others 2014). Reductions in loaf volume of 27% (Ragae and others 2011) and 65% (Gujral and others 2003) have also been reported when 20% barley flour was incorporated into western-style loaf bread. However, western-style bread differs significantly from pita bread in terms of texture and due to its lower leavening requirements, pita bread might better accommodate high fiber ingredients such as barley (Qarooni and others 1992; Blandino and others 2015).

Significant differences in the  $L^*$ ,  $a^*$ , and  $b^*$  values were observed between the control baker's flour and flour prepared from whole grain, pearled grain, or malt as well as their respective pita breads (Table 3). Control and pearled barley flour generally had higher  $L^*$  (lightness) values but lower  $b^*$  (blue–yellow components), and  $a^*$  (red components) when compared to the flour from whole grain and malt, and this same trend was also observed in the pita. An increase in the percentage of pearling resulted in a moderate increase in the  $L^*$  but a reduction in the  $b^*$  for both flour and pita. In a previous study, Sumner and others (1985) reported that



**Table 3— $L^*$ ,  $a^*$ , and  $b^*$  color values of different pita bread.**

Samples <sup>a</sup>	Flour			Pitas		
	$L^*$	$a^*$	$b^*$	$L^*$	$a^*$	$b^*$
Control (Baker flour)	94.1 ± 0.0 <sup>bc</sup>	0.6 ± 0.0 <sup>a</sup>	9.0 ± 0.0 <sup>fg</sup>	77.6 ± 0.6 <sup>g</sup>	1.2 ± 0.1 <sup>c</sup>	16.1 ± 0.3 <sup>k</sup>
Malt	86.6 ± 0.0 <sup>f</sup>	2.2 ± 0.0 <sup>d</sup>	10.1 ± 0.0 <sup>i</sup>	62.3 ± 0.2 <sup>n</sup>	7.6 ± 0.0 <sup>l</sup>	26.0 ± 0.1 <sup>v</sup>
Fresh Finnis (Whole grain)	92.7 ± 0.0 <sup>de</sup>	1.1 ± 0.0 <sup>bc</sup>	8.7 ± 0.0 <sup>efg</sup>	67.7 ± 0 <sup>ik</sup>	4.4 ± 0.0 <sup>j</sup>	20.8 ± 0.1 <sup>q</sup>
Fresh Finnis (10% pearling)	96.0 ± 0.0 <sup>a</sup>	0.6 ± 0.0 <sup>a</sup>	6.0 ± 0.0 <sup>bc</sup>	69.4 ± 1.3 <sup>i</sup>	8.0 ± 0.9 <sup>l</sup>	27.6 ± 0.8 <sup>w</sup>
Fresh Finnis (15% pearling)	95.8 ± 0.0 <sup>a</sup>	0.5 ± 0.0 <sup>a</sup>	5.8 ± 0.0 <sup>a</sup>	71.0 ± 0.3 <sup>h</sup>	2.6 ± 0.1 <sup>def</sup>	16.8 ± 0.3 <sup>lm</sup>
Fresh Finnis (20% pearling)	96.1 ± 0.0 <sup>a</sup>	0.5 ± 0.0 <sup>a</sup>	5.3 ± 0.0 <sup>a</sup>	71.6 ± 0.3 <sup>h</sup>	2.5 ± 0.1 <sup>de</sup>	17.2 ± 0.2 <sup>mn</sup>
Fresh WI2585 (Whole grain)	91.7 ± 0.0 <sup>e</sup>	0.9 ± 0.0 <sup>abc</sup>	9.2 ± 0.0 <sup>gh</sup>	64.1 ± 0.4 <sup>m</sup>	4.5 ± 0.0 <sup>j</sup>	25.0 ± 0.3 <sup>u</sup>
Fresh WI2585 (10% pearling)	93.7 ± 0.0 <sup>cd</sup>	0.8 ± 0.0 <sup>abc</sup>	8.4 ± 0.0 <sup>ef</sup>	67.9 ± 0.4 <sup>jk</sup>	3.9 ± 0.0 <sup>i</sup>	22.2 ± 0.2 <sup>r</sup>
Fresh WI2585 (15% pearling)	94.3 ± 0.0 <sup>bc</sup>	0.7 ± 0.0 <sup>ab</sup>	7.4 ± 0.0 <sup>d</sup>	67.3 ± 1.8 <sup>kl</sup>	3.2 ± 0.0 <sup>gh</sup>	19.4 ± 0.1 <sup>p</sup>
Fresh WI2585 (20% pearling)	95.1 ± 0.0 <sup>ab</sup>	0.7 ± 0.0 <sup>ab</sup>	6.6 ± 0.0 <sup>c</sup>	71.3 ± 0.4 <sup>h</sup>	2.6 ± 0.0 <sup>def</sup>	18.5 ± 0.1 <sup>o</sup>
Fresh Harrington (Whole grain)	92.0 ± 0.0 <sup>e</sup>	1.1 ± 0.0 <sup>bc</sup>	10.1 ± 0.0 <sup>i</sup>	59.8 ± 0.4 <sup>o</sup>	4.8 ± 0.0 <sup>jk</sup>	23.3 ± 0.0 <sup>s</sup>
Fresh Harrington (10% pearling)	94.1 ± 0.0 <sup>bc</sup>	0.8 ± 0.0 <sup>abc</sup>	8.2 ± 0.0 <sup>c</sup>	66.3 ± 0.6 <sup>l</sup>	3.5 ± 0.1 <sup>hi</sup>	19.8 ± 0.5 <sup>p</sup>
Fresh Harrington (15% pearling)	94.8 ± 0.0 <sup>abc</sup>	0.7 ± 0.0 <sup>ab</sup>	7.5 ± 0.0 <sup>d</sup>	67.3 ± 0.6 <sup>kl</sup>	2.9 ± 0.3 <sup>efg</sup>	18.5 ± 0.8 <sup>o</sup>
Fresh Harrington (20% pearling)	95.5 ± 0.0 <sup>ab</sup>	0.7 ± 0.0 <sup>ab</sup>	6.5 ± 0.0 <sup>c</sup>	68.9 ± 0.7 <sup>ij</sup>	3.0 ± 0.0 <sup>fg</sup>	17.7 ± 0.2 <sup>n</sup>
Stored Finnis (whole grain)	92.6 ± 0.0 <sup>de</sup>	1.1 ± 0.0 <sup>bc</sup>	8.9 ± 0.0 <sup>fg</sup>	67.6 ± 0.0 <sup>kl</sup>	4.4 ± 0.0 <sup>j</sup>	20.9 ± 0.0 <sup>q</sup>
Stored WI2585 (whole grain)	91.6 ± 0.0 <sup>e</sup>	0.9 ± 0.0 <sup>abc</sup>	9.6 ± 0.0 <sup>hi</sup>	64.0 ± 0.0 <sup>m</sup>	4.6 ± 0.0 <sup>jk</sup>	25.9 ± 0.0 <sup>v</sup>
Stored Harrington (whole grain)	91.4 ± 0.0 <sup>e</sup>	1.2 ± 0.0 <sup>c</sup>	10.7 ± 0.0 <sup>i</sup>	59.20.0 <sup>o</sup>	5.0 ± 0.0 <sup>k</sup>	24.1 ± 0.0 <sup>t</sup>
LSD	1.3	0.5	0.7	1.3	0.5	0.7

Note:  $L^*$ , lightness component;  $a^*$ , green (− $a$ ) to red (+ $a$ );  $b^*$ , blue (− $b$ ) to yellow (+ $b$ ).

<sup>a</sup>Means ± SE are shown for  $n = 9$  for each sample. Same letters (within column) indicates no difference between samples for individual parameters as determined using the least significant difference (LSD;  $P < 0.05$ ). LSD = 1.30; 0.47, and 0.67 for  $L^*$ ,  $a^*$ , and  $b^*$ , respectively.

**Table 4—Sensory evaluation of pita bread from baker's flour and barley flour.**

Samples <sup>b</sup>	Color intensity <sup>a</sup>	Appearance liking	Texture intensity <sup>a</sup>	Texture liking	Flavor and taste intensity <sup>a</sup>	Flavor and taste liking	Overall
	Control	7.1 ± 0.2 <sup>b</sup>	6.7 ± 0.2 <sup>b</sup>	5.7 ± 0.3 <sup>b</sup>	6.0 ± 0.2 <sup>b</sup>	6.0 ± 0.2 <sup>ab</sup>	6.2 ± 0.2 <sup>b</sup>
Malt	3.7 ± 0.2 <sup>c</sup>	5.5 ± 0.2 <sup>b</sup>	3.7 ± 0.2 <sup>c</sup>	5.1 ± 0.3 <sup>b</sup>	6.1 ± 0.2 <sup>b</sup>	6.1 ± 0.2 <sup>ab</sup>	5.4 ± 0.3 <sup>b</sup>
Fresh Finnis (whole grain)	4.8 ± 0.2 <sup>b</sup>	5.6 ± 0.2 <sup>b</sup>	4.8 ± 0.3 <sup>b</sup>	5.7 ± 0.3 <sup>ab</sup>	5.4 ± 0.3 <sup>bc</sup>	5.5 ± 0.3 <sup>b</sup>	5.5 ± 0.2 <sup>b</sup>
Fresh WI2585 (15% pearling)	5.2 ± 0.2 <sup>b</sup>	5.9 ± 0.2 <sup>b</sup>	5.0 ± 0.3 <sup>ab</sup>	6.0 ± 0.2 <sup>a</sup>	5.0 ± 0.2 <sup>c</sup>	5.6 ± 0.3 <sup>ab</sup>	5.8 ± 0.3 <sup>ab</sup>
LSD	0.61	0.63	0.72	0.69	0.65	0.69	0.66

<sup>a</sup>Indicates the intensity of parameter, from dark (1 score) to light (9 score) for color, from firm (1 score) to soft (9 score) for texture, from non (1 score) to high (9 score) for flavor and taste.

<sup>b</sup>Means ± SE are shown for  $n = 52$  for each sample. Same letters (within column) indicates no difference between samples for individual parameter as determined using the least significant difference (LSD;  $P < 0.05$ ).

removal of the outer kernel layers by pearling resulted in an increase in the  $L^*$  value of the pearled grain accompanied by a reduction in the red and yellow value, due to exposure of the endosperm. Interestingly, we found that, although the control baker's flour was not as light as some of the barley flours, including the flour from 15% and 20% pearled Finnis and 20% pearled WI2585 and Harrington, the control pita bread was significantly lighter than all barley pitas (Figure S1). Differences in gelatinization of wheat and barley starch related to moisture content (Faridi and Rubenthaler 1984) or even differences in caramelization of sugars that may occur during baking may account for these findings, but requires further study.

Pitas made from flour from Finnis that had been pearled at any level; WI2585 and Harrington at the highest pearling level (20%) were slightly darker than control pitas, although the Finnis whole-grain flour pitas were lighter than pitas made with covered whole-grain flour. Therefore, compared to covered genotypes, hulless Finnis is likely to provide a product which is closer in appearance to standard pita bread made from wheat baker's flour in terms of brightness (Morad and others 1984). However, more recently, consumer preference for white bread has reduced as the consumption of more healthy bread has increased (Vulicevic and others 2004). Therefore, the lower brightness of the barley pitas may not necessarily reduce their acceptance by consumers. Storage appeared to slightly reduce the whiteness of whole grain but the reduction was not statistically significant for both flour and pita.

### Sensory properties of pita bread

On the basis of the results above, pita substituted with flour milled from malt, Finnis whole grain, or 15% pearled WI2585 were chosen for sensory analysis and compared to control pita made from baker's flour. Pitas made from malt and Finnis whole grain flour were chosen because antioxidant capacity and vitamin E content were high (Figure 1B and 2B) and pocket formation was satisfactory. Among pearled-barley pitas, the pita containing the highest antioxidant and vitamin E level as well as better formation was that from 15% pearled WI2585. When comparing those samples, all pitas were rated as acceptable (>5) using hedonic scales (Table 4) even though barley pitas had lower hedonic scores than control pitas.

Pita bread containing barley flour had a lower rating for color intensity than the control indicating they were darker. This finding agreed with the observation that malt-pita had the lowest  $L^*$  value but highest  $a^*$  value, followed by Finnis whole-grain flour-pita, 15% pearled WI2585 flour-pita, and the control. The change from creamy white to brown has been previously observed when barley was added to pita (Alu'datt and others 2014).

In terms of sensory evaluation, consumer texture preference for pitas made from 15% pearled WI2585 flour and whole grain Finnis flour were similar to pitas made from baker's flour, and all were higher than for malt pitas. Firmness was highly correlated to texture liking ( $r = 0.96$ ,  $P < 0.05$ ,  $n = 9$ ) and is likely to explain the findings, because the malt pita was the firmest of the

bread, due to it not being fully formed during baking. Pita made from Finnis whole grain was similar to pita from 15% pearled WI2585 in both flavor intensity and liking of taste. Given that bitter-tasting phenolic compounds and tannins are usually found in the seedcoat (Abdelghafor and others 2011), hullless grain may be more ideal for making pita. In terms of overall preference, barley pita from 15% pearled WI2585 flour was the only barley pita not significantly different to the control pita but it also was not significantly different to the other barley pita. The acceptance of all samples was contributed by the liking of appearance, texture and flavor, and taste with correlations of 0.99; 0.79, and 0.41 respectively with overall liking indicating the promise for future development of products.

## Conclusions

Although antioxidant and vitamin E in barley grain was lost during pearling, those components were still richer in pita made from pearled barley compared to control pita. Pitas from malt flour, Finnis whole grain flour, and 15% pearled WI2585 flour contained high antioxidant capacity and vitamin E level and had satisfactory physical properties such as pocket formation. Sensory evaluation indicated that they were acceptable to consumers and had potential as a functional food for the bakery industry.

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## Authors' Contributions

Thi Thu Dung Do conducted this study and drafted the manuscript, Beverly Muhlhauser and Amanda Box helped with interpretation of results and drafting of the manuscript, Amanda Able designed this study, interpreted results, and helped with drafting of the manuscript.

## References

- Abdelghafor RF, Mustafá AI, Ibrahim AMH, Krishnan PG. 2011. Quality of bread from composite flour of sorghum and hard white winter wheat. *Adv J Food Sci Technol* 3(1):9–15.
- Alhajji LAA. 2011. Investigation of the factors affecting the staling of arabic flat bread. [PhD]. United Kingdom: Loughborough University. p 216.
- Alu'datt MH, Rababah T, Al-Rabadi GJ, Ereifej K, Gammoh S, Masadeh N, Torley PJ. 2014. Effects of barley flour and malt protein isolate addition on rheological and sensory properties of pita bread. *J Food Quality* 37(5):329–38.
- Bailey L. 2007. Bakery studies course manual. TAFE, SA: Regency International.
- Bhatty RS. 1996. Production of food malt from hull-less barley. *Cereal Chem* 73(1):75–80.
- Bhatty RS. 1999. The potential of hull-less barley. *Cereal Chem* 76(5):589–99.
- Biloukha OO, Utermohlen V. 2000. Correlates of food consumption and perceptions of foods in an educated urban population in Ukraine. *Food Qual Prefer* 11(6):475–85.
- Blandino M, Locatelli M, Sovrani V, Coisson DJ, Rolle L, Travaglia F, Giacosa S, Bordiga M, Scarpino V, Reyneri A, Arlorio M. 2015. Progressive pearling of barley kernel: chemical characterization of pearling fractions and effect of their inclusion on the nutritional and technological properties of wheat bread. *J Agric Food Chem* 63(25):5875–84.
- Cozzolino D, Degner S, Eglinton J. 2014. A novel approach to monitor the hydrolysis of barley (*Hordeum vulgare* L) malt: a chemometrics approach. *J Agric Food Chem* 62(48):11730–36.
- Do TTD, Cozzolino D, Muhlhauser B, Box A, Able AJ. 2015a. Antioxidant capacity and vitamin E in barley: effect of genotype and storage. *Food Chem* 187:65–74.
- Do TTD, Cozzolino D, Muhlhauser B, Box A, Able AJ. 2015b. Effect of malting on antioxidant capacity and vitamin E in different barley genotypes. *J Inst Brew* 121(4):531–40 doi: 10.1002/jib.271.
- Elsayed AA, Peter W. 2005. Specialty grains for food and feed. USA: American Association of Cereal Chemists.
- Evers AD, Blakeney AB, O'Brien L. 1999. Cereal structure and composition. *Aust J Agr Res* 50(5):629–50.
- Faridi HA, Rubenthaler GL. 1984. Effect of baking time and temperature on bread quality, starch gelatinization, and staling of Egyptian balady bread. *Cereal Chemistry* 61(2):151–4.
- Gong LX, Jin C, Wu LJ, Wu XQ, Zhang Y. 2012. Tibetan hull-less barley (*Hordeum vulgare* L.) as a potential source of antioxidants. *Cereal Chem* 89(6):290–5.
- Goupy P, Hugues M, Boivin P, Amiot MJ. 1999. Antioxidant composition and activity of barley (*Hordeum vulgare*) and malt extracts and of isolated phenolic compounds. *J Sci Food Agric* 79(12):1625–34.
- Gujral HS, Gaur S, Rosell CM. 2003. Note: Effect of barley flour, wet gluten, and ascorbic acid on bread crumb texture. *Food Sci Technol Intl* 9(1):17–21.
- Holasova M, Velisek J, Davidek J. 1995. Tocopherol and tocotrienol contents in cereal grains. *Potravnarske Vedy* 13(6):409–17.
- Hulse JP. 1979. Polyphenols in cereals and legumes. In Proceedings of the 36th Annual Meeting of the Institute of Food Technologists, St. Louis, MO.
- Izydorczyk MS, Chornick TL, Paulley FG, Edwards NM, Dexter JE. 2008. Physicochemical properties of hull-less barley fibre-rich fractions varying in particle size and their potential as functional ingredients in 2-layer flat bread. *Food Chem* 108(2):561–70.
- Ko S, Kim C, Kim H, Kim C, Chung S, Tae B, Kim I. 2003. Tocol levels in milling fractions of some cereal grains and soybean. *J Am Oil Chem Soc* 80(6):585–9.
- Liyana-Pathirana C, Dexter J, Shahidi F. 2006. Antioxidant properties of wheat as affected by pearling. *J Agric Food Chem* 54(17):6177–84.
- Lu J, Zhao H, Chen J, Fan W, Dong J, Kong W, Sun J, Cao Y, Cai G. 2007. Evolution of phenolic compounds and antioxidant activity during malting. *J Agric Food Chem* 55(26):10994–1001.
- Maillard MN, Berset C. 1995. Evolution of antioxidant activity during kilning - role of insoluble bound phenolic-acids of barley and malt. *J Agric Food Chem* 43(7):1789–93.
- Maillard MN, Soum MH, Boivin P, Berset C. 1996. Antioxidant activity of barley and malt: relationship with phenolic content. *Food Sci Technol-Leb* 29(3):238–44.
- Malcolmson L, Sarkar A, Sopiwnyk E, Fu BX, Tweed T. 2011. Opportunities for developing health promoting foods from barley. Manitoba, Canada: Canadian International Grains Institute.
- McLaughlin PJ, Weihrauch JL. 1979. Vitamin E content of foods. *J Am Diet Assoc* 75(6):647–65.
- McNeil M, Albersheim P, Taiz L, Jones RL. 1975. The structure of plant cell walls. VII. Barley aleurone cells. *Plant Physiol* 55:64–8.
- Meilgaard MC, Civille GV, Carr BT. 2007. Sensory evaluation techniques. Taylor & Francis Group.
- Menz I. 2010. Australian barley varieties: a reference guide. NSW, Australia.
- Moore J, Liu JG, Zhou KQ, Yu LL. 2006. Effects of genotype and environment on the antioxidant properties of hard winter wheat bran. *J Agric Food Chem* 54(15):5313–22.
- Morad MM, Doherty CA, Rooney LW. 1984. Effect of sorghum variety on baking properties of U.S. conventional bread, Egyptian pita, "Balady" bread and cookies. *J Food Sci* 49:1070–4.
- Newman CW, Newman RK. 2006. A brief history of barley foods. *Cereal Foods World* 51(1): 4–7.
- Packer L. 1995. Nutrition and biochemistry of the lipophilic antioxidants, vitamin E and carotenoids. In: Packer L, Niki E, Ong ASH, editors. Nutrition, lipids, health and disease. Champaign, IL: American Oil Chemists Society. p 8–35.
- Panfili G, Fratiani A, Criscio Td, Marconi E. 2008. Tocol and beta-glucan levels in barley varieties and in pearling by-products. *Food Chem* 107(1):84–91.
- Peterson DM, Qureshi AA. 1993. Genotype and environment effects on tocols of barley and oats. *Cereal Chem* 70(2):157–62.
- Pomeranz Y. 1987. Modern cereal science and technology. Weinheim, Germany: VCH Publishers.
- Pomeranz Y. 1992. Biochemical, functional, and nutritive changes during storage. In: Sauer DB, editor. Storage of cereal grains and their products. St. Paul, MN: Am Assoc Cereal chem. p 55–118.
- Qarooni J, Ponte JG, Posner ES. 1992. Flat Breads of the World. *Cereal Foods World* 37(12): 863–5.
- Ragae S, Guzar I, Dhull N, Seetharaman K. 2011. Effects of fiber addition on antioxidant capacity and nutritional quality of wheat bread. *LWT-Food Sci Technol* 44(10):2147–53.
- Salomonsson AC, Theander O, Aman P. 1980. Composition of normal and high-lysine barleys. *Swed J Agr Res* 10(1):11–16.
- Shewfelt RL, Orta RA, Clarke AD. 2015. Introducing food science. Boca Raton, FL: CRC Press, Taylor & Francis Group.
- Sunmer AK, Gebreegziabher A, Tyler RT, Rosnagel BG. 1985. Composition and properties of pearled and fines fractions from hulled and hull-less barley. *Cereal Chem* 62(2):112–16.
- Suter D, Oliver J, Bekes F. 1995. Anomalous flours from the 1994–95 harvest. In: Williams YA, Wrigley CW, editors. Proceedings of the 45th Australian Cereal Chemistry Conference. Adelaide, South Australia: Royal Australian Chemical Institute. p 116–19.
- U.S. Department of Agriculture. 2013. Chapter 2: Barley. Grain inspection handbook. USA: Agricultural Research Service, U.S. Department of Agriculture, p 2.1–2.32.
- USFDA. 2003. Food labeling: health claims; soluble fiber from certain foods and risk of coronary heart disease. Final rule 68(144):44207–09.
- Varrianomaron E, Ke V, Huang G, Ponte J. 1980. Comparison of methods to determine starch gelatinization in bakery foods. *Cereal Chem* 57(4):242–8.
- Vulicevic IR, Abdel-Aal ESM, Mittal GS, Lu X. 2004. Quality and storage life of par-baked frozen breads. *Lebensm-Wiss Technol* 37(2):205–13.
- Wang JS, Rosell CM, de Barber CB. 2002. Effect of the addition of different fibres on wheat dough performance and bread quality. *Food Chem* 79(2):221–6.
- Washington J, Roumeliotis S, Lim P, Kaczmarek R, Barr AR. 2003. Pearling and single kernel characterization system analysis of Australian barley for the Asian food market. Proceedings of the Australian Barley Technical Symposium, South Australia.
- Young IS, Woodside JV. 2001. Antioxidants in health and disease. *J Clinical Pathol* 54(3):176–86.

## Supporting Information

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Image of pita bread made from baker's flour and different barley flour.

**Supplementary Information,** Do TDT, Muhlhausler B, Box A, Able AJ (2016) *Enrichment of antioxidant capacity and vitamin E in Pita made from barley.*



Figure S1 **Image of pita bread made from baker's flour and different barley flour.** A and B, pitas from baker's flour and malt flour; C, D, E, F, G, pitas from fresh Finnis whole grain flour, fresh Finnis flour at 10% pearling, fresh Finnis flour at 15% pearling, fresh Finnis flour at 20% pearling and stored Finnis whole grain flour; H, I, K, L, M, pitas from fresh WI2585 whole grain flour, fresh WI2585 flour at 10% pearling, fresh WI2585 flour at 15% pearling, fresh WI2585 flour at 20% pearling and stored WI2585 whole grain flour; N, O, P, Q, S, pitas from fresh Harrington whole grain flour, fresh Harrington flour at 10% pearling, fresh Harrington flour at 15% pearling, fresh Harrington flour at 20% pearling and stored Harrington whole grain flour.

**Chapter 6. Manuscript 4: QUANTITATIVE  
TRAIT LOCI FOR ANTIOXIDANT  
CAPACITY AND VITAMIN E CONTENT IN  
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# **Quantitative trait loci for antioxidant capacity and vitamin E content in barley**

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**Running title:** QTLs for antioxidant and vitamin E in barley.

## **Abstract**

Antioxidants and vitamin E in particular, have been credited with promoting human health. Our previous study has shown variation in antioxidant capacity and the content of total vitamin E and its individual isomers among barley genotypes, however, the genetic basis of these traits is unknown. The objectives of this study were to identify quantitative trait loci (QTLs) useful for improving total antioxidant and vitamin E levels in barley. Antioxidant capacity and vitamin E content was determined in the mapping populations, Tadmor x ERApm and Amaji nijo x WI2585 respectively. QTLs were detected on chromosomes 4H, 5H and 7H for the two main vitamin E isomers ( $\alpha$ -T and  $\alpha$ -T3), two non-dominant isomers ( $\beta$ -T3 and  $\gamma$ -T) and for total vitamin E content in the Amaji nijo x WI2585 DH population (127 lines), explaining high percentage of total phenotypic variance (from 10.6% to 34.8% for most QTLs). However, no QTL was identifiable for antioxidant capacity in the Tadmor x ERApm population. The identified QTLs should be useful to increase vitamin E levels in barley.

**Additional keywords:** Barley; antioxidant; vitamin E; QTLs.

## **Introduction**

Antioxidants might enable better health by preventing cell damage because of their role as scavengers, preventing the formation of free radicals or promoting their decomposition (Young and Woodside 2001). Epidemiological studies have suggested free radicals cause chronic diseases such as heart disease, diabetes (Willet 1994), atherosclerosis, low density lipoprotein cholesterol (Young and Woodside 2001), cancer, cardiovascular disease (Layton *et al.* 1992), rheumatoid arthritis, inflammatory bowel syndrome and Parkinson's disease (Halliwell 1996). Antioxidants are therefore considered to be an

important protectant against these diseases. According to Martinez-Tome *et al.* (2004) the level of antioxidant activity in blood increases after the consumption of foods high in antioxidants. Increasing antioxidants in commonly consumed foods, such as cereal grains including barley, is therefore necessary to improve human health.

Vitamin E is the most important lipid phase chain breaking antioxidant due to its unsaturated tail which reacts with free radicals, consequently protecting polyunsaturated fatty acids (Burton and Ingold 1981). The dietary recommendation for vitamin E is 15 mg of  $\alpha$ -tocopherol ( $\alpha$ -T) daily, and the estimated average requirement is 12 mg as a preventative factor for cardiovascular disease, cancer and Alzheimer's disease (Gassmann 2000). Including  $\alpha$ -T, vitamin E has eight isomers (also called tocopherols):  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocopherol (T); and;  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -tocotrienol (T3). Ball (2006) reported the descending order of their antioxidant capacity to be  $\alpha$ -,  $\beta$ -,  $\gamma$ - and then  $\delta$ -tocopherols. Sheppard *et al.*, (1993), however, found that among T and T3, the descending order of antioxidant activity was:  $\alpha$ -T,  $\beta$ -T,  $\alpha$ -T3,  $\gamma$ -T,  $\beta$ -T3, and  $\delta$ -T ( $\gamma$ -T3 and  $\delta$ -T3 had no function). In contrast,  $\alpha$ -T3 has recently been shown to be at least three-fold more efficient as a scavenger of peroxy radicals than  $\alpha$ -T (Packer 1995) or to be the only isomer highly correlated with antioxidant activity in barley (Do *et al.* 2015). Because  $\alpha$ -T has been traditionally viewed as the main isomer and the biological activity of the individual isomers has been shown to differ substantially, the total vitamin E content is often expressed as  $\alpha$ -tocopherol-equivalents (TE) and calculated using biological activities of 1.0 for  $\alpha$ -T, 0.3 for  $\alpha$ -T3, 0.4 for  $\beta$ -T, 0.05 for  $\beta$ -T3, 0.1 for  $\gamma$ -T, 0.01 for  $\gamma$ -T3 and 0.01 for  $\delta$ -T (McLaughlin and Weihrauch 1979). We have previously shown that although  $\alpha$ -T and  $\alpha$ -T3 are the main isomers in barley grain, the vitamin E content and the amount of individual isomers varies significantly among barley genotypes (Do *et al.* 2015). However, the genetic basis for these differences is yet to be established.



Apart from human health benefits, antioxidants, and vitamin E in particular, may prolong the storage of food items by preventing the formation of free radicals which have been widely indicated as the major cause of food deterioration (Lehner *et al.* 2008). As a result, antioxidants are often added to various food stuffs as a preservative in pure form or as a major component of a plant extract. For example, the antioxidant activity of polyphenols extracted from tea has been demonstrated for oil stability (Wanasundara and Shahidi 1996) and for prevention of lipid oxidation in cooked red meat, poultry and fish (Tang *et al.* 2001). Vitamin E in barley has been reported to inhibit lipid peroxidation to limit the formation of trans-2-nonenal, one of the main compounds responsible for the stale taste of beer (Lu *et al.* 2007). In other products, vitamin E has also been demonstrated to prolong shelf life of oil (Lindley 1998) or protect against rancid flavour, odour and discoloration in packaged meat (Wood *et al.* 2004). Barley grain with high antioxidant activity and/or vitamin E content may therefore not only be useful for functional food products but for preservation of food products.

Significant genotypic variation has been reported for both antioxidant capacity and vitamin E content in barley (Do *et al.* 2015), but information on the inheritance of these traits is still lacking. Finding the locus (loci) corresponding to tocol production is crucial for the enrichment of vitamin E in grain through breeding or molecular methods. An example of this is the 80-fold increase of  $\alpha$ -T levels that were observed after over-expression of *VTE4* which encodes  $\gamma$ -T methyltransferase in the  $\alpha$ -T pathway in *Arabidopsis* (Shintani and DellaPenna 1998). Hence, determining QTL for tocols in barley may provide knowledge about the genetic basis for use in future breeding strategies for the production of a natural source of vitamin E. A similar methodology has been shown to be useful for identifying the genetic basis of antioxidant capacity and increasing antioxidants in rice grain (Jin *et al.* 2009).

This study therefore aimed to detect and map QTLs affecting antioxidant activity and vitamin E concentration (total vitamin E content and individual isomers) in barley populations derived from a cross between low- and high- antioxidant or vitamin E parents.

## **Materials and methods**

### *Grain materials*

Our previous observations that differences in vitamin E content existed between the parental variety Amaji nijo and parental breeding line WI2585 (Do et al., 2015) provided the basis for evaluating 127 doubled-haploid (DH) lines derived from a cross between Amaji nijo and WI2585 for total vitamin E content (and individual tocol isomers). Amaji nijo was significantly higher than WI2585 in content of  $\alpha$ -T (13.3 vs 11.7  $\mu\text{g/g DW}$ ),  $\gamma$ -T (4.6 vs 3.7  $\mu\text{g/g DW}$ ) and  $\gamma$ -T3 (14.8 vs 11.6  $\mu\text{g/g DW}$ ) but not of  $\alpha$ -T3 (41.3 vs 44.4  $\mu\text{g/g DW}$ ),  $\beta$ -T (0.5 vs 0.7  $\mu\text{g/g DW}$ ) and  $\beta$ -T3 (4.6 vs 7.0  $\mu\text{g/g DW}$ ) (Do *et al.* 2015). Similarly, differences in antioxidant capacity between Tadmor and ERApm provided the basis for evaluating 105 DH lines derived from a cross between Tadmor and ERApm for antioxidant capacity. ERApm had significantly higher antioxidant capacity than Tadmor (120.3 vs 104.8 mg AEAC/100 g FW) (Do *et al.* 2015). Amaji nijo is a high malting quality variety and was bred by Sapporo Breweries Ltd in Japan from the cross Seijo 17 x Fuji Nijo (Kihara *et al.* 1998). WI2585 is an Australian breeders' line which was bred by R.C.M. Lance and D.H.B. Sparrow in South Australia from the cross Zephyr/Ketch//WI2335 and has many desirable features for adaptation to southern Australia (Pallotta *et al.* 2003). The cross Amaji nijo x WI2585 was made at The University of Adelaide, Waite Campus in 1994. Tadmor is a black-seeded two-row variety selected by ICARDA from Arabi Aswad, a Syrian landrace, which is very well

adapted to the dry conditions. ERApm is a two-row breeding line adapted to moderate rainfall conditions and released in Tunisia. The cross was made at ICARDA in 1987 (Teulat *et al.* 1998; von Korff *et al.* 2008).

The full Amaji nijo x WI2585 population of 127 DH lines and the full Tadmor x ERApm population of 105 DH lines were grown from June 2012 to December 2012 as a single plot in a complete randomised design at Charlick Experimental Research Station, Strathalbyn, South Australia (35°19'46.26" S, 138°52'42.39" E) to use for QTL analysis. For validation, representative selective phenotypes from the Amaji nijo x WI2585 DH population (20 lines high in content for the dominant tocol isomers,  $\alpha$ -T and  $\alpha$ -T3 and 20 low-content lines) and from the Tadmor x ERApm DH population (20 lines high in antioxidant capacity and 20 low-antioxidant capacity lines) were evaluated in the following year. They were grown in the same field from June 2013 to December 2013.

After harvesting, the grains from both mapping populations and the parental varieties were screened using a 2.5 mm slotted ISO 5223 sieve. The grains were kept at -80°C until their moisture content, antioxidant capacity and vitamin E content (including individual tocol isomers) were analysed for at least three individual biological replicates. Just prior to analysis, barley grain was ground to a fine powder using an UDY Cyclone Sample Mill (Udy Corporation, Boulder, CO, USA).

#### *Tocol analysis*

As previously described (Do *et al.* 2015), tocols were extracted using an optimized hot saponification method adapted from Lampi *et al.* (2004). Briefly, flour (0.1 g) was added to a solution of 1 mL 100% (v/v) ethanol, 0.4 mL water and 20 mg ascorbic acid in a 15 mL Pyrex glass tube with a Teflon screw cap. After the addition of 100  $\mu$ L 10.7M potassium hydroxide solution and thorough mixing, the tube was capped and transferred

to a water bath at 80°C for 25 min. During saponification, the sample was mixed every 10 min to improve hydrolysis. The tubes were then cooled in a water bath containing ice for 10 min and 0.5 mL 50% (v/v) ethanol was then added. To extract tocopherols and other unsaponifiable lipids, three portions (each 2 mL) of n-hexane:ethyl acetate (8:2, v/v) were added to each sample. After shaking the samples and solvent for 10 min and allowing it to separate into phases, the upper organic layers were collected with a disposable glass pipette and transferred to a new glass tube. This process was repeated three times with n-hexane:ethyl acetate (8:2 v/v), and the extracted samples were then dried under nitrogen. The residue was dissolved in 1 mL n-hexane and filtered through a 0.45 µm syringe filter before being transferred to a 12x32 GRACE glass HPLC (high-performance liquid chromatography) vial with an amber screw cap for analysis.

The tocopherols were then quantified according to the method reported by Lampi *et al.* (2004) with some modifications: (i) tocopherols were separated by a normal phase HPLC using a GRACE Altima HP Silica 150 x 3 mm, 3 micron column and quantified using a fluorescence detector (NP-HPLC-FLD) with an excitation wavelength of 290 nm and an emission wavelength of 325 nm, (ii) the mobile phase was 1,4-dioxane/n-hexane (2:98, v/v) at a flow rate of 1 mL/min and (iii) separation of tocopherols was based on isocratic elution (Lampi *et al.* 2004).

The quantity of individual vitamin E isomers in the samples was determined by comparison of the retention time with the calibration curves of standards which were prepared in hexane over the concentration range of 1.0-25.0 µg/mL using GenStat 14 (Lawes Agricultural Trust; VSN International, Ltd., Hemel Hempstead, UK).

The moisture content of barley flour was determined by using near infrared (NIR) spectroscopy (Unity Scientific SpectraStar™ 2500, Unity Scientific Asia Pacific,

Australia) to calculate tocol content in dry matter (DM) according to the methods reported in Pomeranz (1973).

#### *Antioxidant analysis*

Antioxidants were extracted, as per Do et al., 2015, from barley flour (1 g) with 20 mL of 80% ethanol, which was placed in a flask in a 200 rpm shaking water bath for 4 h in the dark at 45°C. Vacuum filtration was used to separate the supernatant, which was stored in the dark at -20°C and analysed within 24 h (Omwamba and Hu 2009). Antioxidant capacity was measured using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical method (Omwamba and Hu 2009). Briefly, barley flour extract (0.1 mL) was added to 2.9 mL of DPPH (112 µM). After mixing, the sample was allowed to stand at 23°C in the dark for 20 min. Reduction in absorbance was measured at 517 nm after 20 min using a spectrophotometer (UV/VIS. SP 8001, Metertech, Taiwan). Antioxidant activity was then determined using a standard curve for ascorbic acid and prediction models provided by GenStat 15 and expressed as mg ascorbic acid equivalent antioxidant capacity (AEAC) per 100 g fresh weight (FW) of grain.

#### *Identification of QTL*

QTL analyses of total vitamin E content, α-T, α-T3, β-T, β-T3, γ-T and γ-T3 in the Amaji nijo x WI2585 population and of antioxidant capacity in the Tadmor x ERapm population was conducted using GenStat release 15. The basic phenotypic model for a single environment can be expressed as:

$$y_i = \mu + Gi + \epsilon_i$$

where  $y_i$  is the trait value of the genotype  $i$ ;  $\mu$  is overall mean;  $Gi$  is the genotypic main effect; and  $\epsilon_i$  is the random error effect across genotype in a year.

The molecular marker maps used for the Amaji nijo x WI2585 DH population (106 markers) and the Tadmor x ERApm DH population (180 markers) were previously published by Pallotta *et al.* (2003) and von Korff *et al.* (2008), respectively. GenStat was also used to determine the additive estimates and the phenotypic proportion of variance explained by each QTL found in each individual trait. Initial linkage grouping of markers was done at a LOD threshold of 3.12 for  $\alpha$ -T,  $\alpha$ -T3 and  $\gamma$ -T3; LOD threshold of 3.03 for vitamin E and the rest of the isomers; and a LOD threshold of 3.3 for antioxidant capacity. The R program (R Development Core Team 2010) was used to confirm the results generated by Genstat as well as to determine the support interval for each QTL. GGT (Graphical Genotypes) version 2.0 was used for the visualization of the graphical genotype (van Berloo 2007). Phenotypic data analysis was performed using SAS Enterprise Guide 7.1 (SAS Institute 2014), using a linear mixed model with effects for genotype and year. Significant effect of each term was declared if *P* value < 0.05. Due to incomplete replication across the years, interaction plots were generated in SAS to determine if an interaction between years and genotypes exists.

## **Results**

### *Tocol analysis*

All tocols were easily quantifiable in the full population and in the parental varieties except  $\delta$ -T and  $\delta$ -T3 (Fig. 1) which were negligible. Concentrations of  $\alpha$ -tocols were highest, followed by  $\gamma$ - and then  $\beta$ -tocols.  $\alpha$ -T, the second most abundant isomer, contributed 72.8% to total tocopherol content but only 16.4% to total tocol content whereas  $\alpha$ -T3 contributed significantly to both total tocotrienol content and total tocol content being the most abundant of all isomers.  $\alpha$ -T3 and  $\alpha$ -T3 were highly correlated with total vitamin E content ( $r=0.8$  and  $0.9$ , respectively  $n=127$ ,  $p<0.05$ ) while a moderate

correlation was found for  $\gamma$ -T ( $n=127$ ,  $r=0.5$ ,  $p<0.05$ ) and low correlations were found for the rest of the isomers ( $r=0.05$  for  $\beta$ -T;  $0.2$  for  $\beta$ -T3 and  $0.3$  for  $\gamma$ -T3,  $n=127$ ,  $p<0.05$ ). Between isomers,  $\alpha$ -T was highly correlated with  $\gamma$ -T ( $r=0.6$ ,  $n=127$ ,  $p<0.05$ ), while a small but positive correlation was found between  $\alpha$ -T3 and  $\beta$ -T3 ( $r=0.3$ ,  $n=127$ ,  $p<0.05$ ).

A wide range of concentrations (from 22.1 to 35.5  $\mu\text{g/g DW}$ ) was seen for vitamin E in the full Amaji nijo x WI2585 DH population of which the total vitamin E content of Amaji nijo (parent 1) and WI2585 (parent 2) were 29.2 and 30.1  $\mu\text{g/g DW}$  (Fig. 1A). The  $\alpha$ -T content ranged from 10.1  $\mu\text{g/g DW}$  to 18.2  $\mu\text{g/g DW}$  which exceeded the parental values of 13.6  $\mu\text{g/g DW}$  (Amaji nijo) and 14.3  $\mu\text{g/g DW}$  (WI2585) (Fig. 1B). A small difference was observed between the two parental varieties for the most abundant isomer,  $\alpha$ -T3 with Amaji nijo having 48.4  $\mu\text{g/g DW}$  and WI2585 having 37.5  $\mu\text{g/g DW}$ . However, the population had a much larger range from 31.8  $\mu\text{g/g DW}$  to 62.4  $\mu\text{g/g DW}$  (Fig. 1C). A wide range of tocol contents was also observed for the other isomers in the full population, especially when compared to the parental varieties: from 0.3 to 1.5  $\mu\text{g/g DW}$  for  $\beta$ -T (Fig. 1D); from 2.9 to 13.5  $\mu\text{g/g DW}$  for  $\beta$ -T3 (Fig. 1E), 1.6 to 6.3  $\mu\text{g/g DW}$  for  $\gamma$ -T (Fig. 1F) and 2.1 to 17.2  $\mu\text{g/g DW}$  for  $\gamma$ -T3 (Fig. 1G).

Representative selective phenotypes (20 lines high in content for the dominant tocol isomers,  $\alpha$ -T and  $\alpha$ -T3) were chosen for the second year QTL analysis (Fig. 2). In general, similar ranges of content were observed for total vitamin E content (from 22.7 to 39.4  $\mu\text{g/g DW}$ ) (Fig. 2A) and each of the individual isomers in the subset of the population (the second year). In the second year,  $\alpha$ -T and  $\alpha$ -T3 levels still ranged widely from 12.4  $\mu\text{g/g DW}$  to 20.0  $\mu\text{g/g DW}$  and from 28.9  $\mu\text{g/g DW}$  to 59  $\mu\text{g/g DW}$ , respectively (Fig. 2B, C). The contents of  $\beta$ -T (ranging from 0.0 to 1.5  $\mu\text{g/g DW}$ , Fig. 2D),  $\beta$ -T3 (ranging from 1.5 to 9.1  $\mu\text{g/g DW}$ , Fig. 2E),  $\gamma$ -T (ranging from 2.8 to 8.0  $\mu\text{g/g DW}$ , Fig. 2F) and  $\gamma$ -T3 (ranging from 7.4 to 15.1  $\mu\text{g/g DW}$ , Fig. 2G). Moderate positive

correlations between the two years were found for contents of vitamin E,  $\alpha$ -T3,  $\beta$ -T3 and  $\gamma$ -T ( $r=0.4$ ;  $0.54$ ;  $0.6$  and  $0.52$ , respectively) but low correlations were observed for the rest of isomers including  $\alpha$ -T,  $\beta$ -T and  $\gamma$ -T3 ( $r=0.25$ ;  $0.32$  and  $0.18$ , respectively) ( $n=40$ ,  $p<0.05$ ). Interestingly, the subset of the population (year two) contained a higher level of all tocopherol forms ( $\alpha$ -T,  $\beta$ -T and  $\gamma$ -T) but lower level of the tocotrienol isomers than in the entire population (year one). In addition, the parental varieties did not behave as in the first year or as observed previously (Do et al. 2015) especially for  $\alpha$ -T,  $\beta$ -T and  $\gamma$ -T3. However, year had no impact on the content of  $\alpha$ -T3 ( $p=0.84$ ) and  $\gamma$ -T3 ( $p=0.45$ ) but affected vitamin E content ( $p<0.005$ ),  $\beta$ -T ( $p<0.05$ ) and the remaining isomers ( $p<0.001$ ). No genotype x environment interaction was found for content of vitamin E or any isomer (Fig. S1. A, B, C, D, E, F and G).

#### *QTL analysis*

Using the full population (year 1 analysis), a total of eight significant QTLs ( $LOD>3$ ) were identified for vitamin E; the two main isomers,  $\alpha$ -T and  $\alpha$ -T3; as well as for another two non-dominant isomers,  $\beta$ -T3 and  $\gamma$ -T. In the second year, with the selective phenotype subset, only three QTLs were detected but mostly on the same chromosomes in similar positions (Fig. 3). Two QTLs for vitamin E content were mapped in the first year on 7H (*QVTE1.AmWI-7H.Y1* and *QVTE2.AmWI-7H.Y1*) explaining 7% and 10.7% of the total phenotypic variation, respectively. The corresponding marker detected on *QVTE1.AmWI-7H.Y1* was ABG497b at 45.9 cM. However, the support interval was 78 cM, from 16 cM to 94 cM, and included two other markers with significant LOD, CDO358 and BCD263. The support interval for *QVTE2.AmWI-7H.Y1* was much smaller (from 182.4 to 200.4 cM) with the corresponding marker, PSR680, at 197.5 cM. In the second year, only one QTL was detected for vitamin E content (*QVTE.AmWI-7H.Y2*) in



a similar position to *QVTE2.AmWI-7H.Y1* with the corresponding marker, ABG714b at 181.6 cM.

One significant QTL was identified for  $\alpha$ -T on 7H in the first year (*QaT.AmWI-7H.Y1*) explaining 19.8% of the total phenotypic variation. Although, no significant QTL was detected in the second year for this trait, an analysis peak was also observed for chromosome 7H with the LOD score of 2.2 (Table 1). Furthermore, the  $\alpha$ -T QTL is in a similar position to one of the vitamin E content QTL identified in the first year (*QVTE1.AmWI-7H.Y1*). For  $\alpha$ -T3, two QTLs were identified on 5H and 7H in the first year (*QaT3.AmWI-5H.Y1* and *QaT3.AmWI-7H.Y1*) explaining 10.6 and 12.9% of the total phenotypic variation, respectively. The corresponding marker detected on *QaT3.AmWI-7H.Y1* was C7P190 at the position of 189.6 cM while two other significant markers were also identified at 197.5 cM (PSR680) and 181.6 cM (ABG714b). Interestingly, this region corresponds to the vitamin E content QTLs *QVTE2.AmWI-7H.Y1* and *QVTE.AmWI-7H.Y2*. The *QaT3.AmWI-5H.Y1* QTL corresponds with the location of the marker CDO504 at 135.5 cM on 5H and is closely linked with the marker MWG514. Although the intervals are overlapping, the QTL identified for  $\alpha$ -T3 content on 5H in the subset (the second year; *QaT3.AmWI-5H.Y2*) strongly corresponded to another marker, X-I (at 172.6cM) and contributing to phenotypic variation by 25.8%.

For the minor isomers, one QTL was identified in year 1 for  $\beta$ -T3 content on chromosome 4H (*QbT3.AmWI-4H.Y1*) explaining only 4.7% of the total phenotypic variation, and most strongly corresponding to the marker EBmac788. Another QTL for  $\beta$ -T3 content was identified on chromosome 5H in the first year (*QbT3.AmWI-5H.Y1*) contributing to phenotypic variation by 11.4%. The support interval for *QbT3.AmWI-5H.Y1* corresponded with the location of 10 markers (from 92.8 cM to 154.9 cM) but most strongly correlated with the markers CDO504 and MWG514, both of which also

corresponded with the location of *QaT3.AmWI-5H.Y1*. However, using the subset of the population (year 2), a QTL for  $\beta$ -T3 content was only identified on chromosome 5H explaining 28.1% of the phenotypic variation and corresponding to the marker X1 which also corresponded with the location of *QaT3.AmWI-5H.Y2*. A QTL for  $\gamma$ -T content, identified on 7H (*QgT.AmWI-7H.Y1*) and only in the first year, corresponded strongly with two markers, C7P75 (74.8 cM) and C7P65 (65.2 cM). *QgT.AmWI-7H.Y1* explained 17.7% phenotypic variation and also overlapped with the QTL for  $\alpha$ T on 7H (*QaT.AmWI.7H.Y1*). No QTL were found for  $\beta$ -T and  $\gamma$ -T3 content. However, analytical peaks were observed on 2H for both isomers just below the significant threshold limit of 3.1 (LOD = 2.8 for both isomers). The favourable alleles came from parent 2 (WI2585) for QTLs associated with  $\alpha$ T3 (*QaT3.AmWI-5H.Y1* and *QaT3.AmWI-5H.Y2*) or  $\beta$ -T3 (*QbT3.AmWI-5H.Y1* and *QbT3.AmWI-5H.Y2*) on 5H in both the full population (year 1) and the subset (year 2). The favourable alleles came from parent 1 (Amaji nijo) for QTLs associated with vitamin E in both years on 7H (*QVTE1.AmWI-7H.Y1*, *QVTE2.AmWI-7H.Y1*, *QVTE.AmWI-7H.Y2*),  $\alpha$ -T in year 1 on 7H (*QaT.AmWI-7H.Y1*),  $\alpha$ -T3 in year 1 on 7H (*QaT3.AmWI-7H.Y1*),  $\gamma$ -T in year 1 on 7H (*QgT.AmWI-7H.Y1*) and  $\beta$ -T3 in year 1 on 4H (*QbT3.AmWI-4H.Y1*).

#### *Antioxidant analysis*

There was a wide range of antioxidant capacity in the mapping population Tadmor x ERApm in the first year which ranged from 59.2 mg AEAC/100g FW to 130.0 mg AEAC/100g FW (Fig. 4A). The parental lines had an antioxidant capacity of 96.7 mg AEAC/100g FW for ERApm and 80.1 mg AEAC/100gFW for Tadmor. In the second year, although lines were selected based according to antioxidant capacity in the first year, the range of antioxidant capacity was not as wide as in the first year (90.3 to 129.7

mg AEAC/100g FW; Fig. 4B). This range again exceeded the antioxidant levels of parental lines. However, ERApm had lower antioxidant capacity than Tadmor with levels of 95.9 and 113.0 mg AEAC/100g FW, respectively. No interaction was found between genotypes and years (Fig. S1. H). No QTL were detected for antioxidant capacity in either year (data not shown) but an analysis peak at 27.9 cM (CaaaccS marker) was observed on chromosome 5H (LOD=2.2, threshold=3.3).

## **Discussion**

Based on our previous finding of genotypic variation in vitamin E concentration and antioxidant capacity in barley (Do *et al.* 2015) as well as barley's potential functional food capabilities, our aim was to identify the genetic basis for vitamin E and antioxidant capacity. At the time of undertaking this study, nobody had identified QTL for total vitamin E content, individual tocol isomers or antioxidant capacity in barley. However, since our study, the genetic basis of some individual tocol isomers has been identified in a different mapping population (a Falcon x Azhul recombinant inbred line population) (Oliver *et al.* 2014) but the authors did not examine total vitamin E content. Our findings demonstrated the contribution of each tocopherol and tocotrienol isomer to total vitamin E content and we have also identified individual QTLs for two main isomers ( $\alpha$ -T and  $\alpha$ -T3), two non-dominant isomers ( $\beta$ -T3 and  $\gamma$ -T) and total vitamin E content in the Amaji nijo x WI2585 DH mapping population. However, no QTL was identifiable for antioxidant capacity in the mapping population used (Tadmor x ERApm).

In this study, the contribution of individual isomers to tocol content was similar to that found in other studies (Ehrenbergerova *et al.* 2006; Panfili *et al.* 2008).  $\alpha$ -T3 and  $\alpha$ -T were most dominant across all lines in the mapping population, followed by  $\beta$ - and  $\gamma$ -tocols. The  $\delta$ -tocols, undetected isomers in the present study, were also not detected in

other studies, or made up less than 2  $\mu\text{g/g}$  DW of content (Ehrenbergerova *et al.* 2006; Panfili *et al.* 2008). In the vitamin E pathway,  $\alpha$ -tocols are derived from  $\gamma$ -tocols (Hunter and Cahoon 2007) which may explain the positive correlation found between  $\alpha$ -T and  $\gamma$ -T ( $n=127$ ,  $r=0.6$ ,  $p<0.05$ ) and the corresponding localisation of QTL for both  $\alpha$ -T and  $\gamma$ -T content on chromosome 7H. The recent study by Oliver *et al.* (2014) also mapped individual isomers with QTLs for  $\beta$ -T3,  $\gamma$ -T3 and  $\delta$ -T3 on 7H but in the feed barley-mapping population, Falcon x Azhul. Using a comparative approach between barley and rice they suggested that the 7H QTL identified in their study probably contained two genes: the *VTE2* gene which encodes for homogentisate phytyl transferase, the first dedicated enzyme of the tocopherol biosynthetic pathway; and 3- phosphoshikimate 1-carboxyvinyltransferase, which synthesizes a distant precursor of the tocochromanol ring structure (Oliver *et al.* 2014). Therefore, based on the genes identified in that genomic region their 7H QTL may be associated with any tocol including  $\alpha$ -T,  $\gamma$ -T,  $\alpha$ -T3 and consequently affecting vitamin E content. Our study also detected QTL for  $\alpha$ -T3,  $\alpha$ -T,  $\gamma$ -T on 7H as well as QTLs for vitamin E content (in similar locations). The QTL identified by Oliver *et al.* (2014) were primarily in two quite separate regions of 7H (14.1-23.9 and 116.6-126.3 cM). The QTLs identified in this study on 7H were also overlapped and clustered in two regions (22.0-88.0 and 180.0-201.2 cM) similar in position to Oliver *et al.* (2014) suggesting that genotypic variation at the two locations on 7H is important for vitamin E content in barley. No information has been published for markers in these QTLs except PSR680 which was associated with seedling resistance QTLs against barley grass stripe rust (Kamino *et al.* 2015). Fine-mapping of the regions, therefore, needs to be addressed in further research.

Moreover, our study indicated that QTLs designated *QaT3.AmWI-5H.Y1* and *QaT3.AmWI-5H.Y2*, responsible for  $\alpha$ T3 in the full population and subset population

respectively, were overlapped on 5H. Two markers, CDO504 and MWG514, which corresponded with the QTL for both  $\alpha$ T3 and  $\beta$ T3 in the first year (*QaT3.AmWI-5H.Y1*, *QbT3.AmWI-5H.Y1*) were reported to associate with salt tolerance and frost-induced sterility in barley respectively (Reinheimer *et al.* 2004). Antioxidants have been found to be important in frost damage or salt toxicity possibly because of the damage by reactive oxygen species (Wang *et al.* 2013) which would be scavenged by the antioxidants (Shao *et al.* 2008). This finding, therefore, might suggest that vitamin E is important in controlling damage from frost and salinity. On 5H, Oliver *et al.* (2014) also identified genomic regions for different isomers,  $\beta$ -T and  $\delta$ -T3, but they were not significant in their study and they did not provide any information of markers or positions to allow comparison with our genetic map. Barley-rice orthologous relationships suggest the genomic region on 5H contains *VTE3* which synthesizes  $\alpha$ -forms but not  $\beta$ -forms of tocols (Oliver *et al.* 2014). As 5H has QTLs for  $\beta$ -T3 in our study, further investigation is needed to determine whether 5H contains other sequences associated with  $\beta$ -tocol synthesis such as *VTE2*, *VTE1* or *VTE4*.

A QTL for  $\beta$ -T3 content was also detected on chromosome 4H corresponding with EBmac788 which has also been linked to black point resistance in barley (Tah *et al.* 2010), a physiological disorder that has been associated with peroxidative processes during grain development (March *et al.* 2007) and therefore likely to be affected by antioxidants such as  $\beta$ -T3. Some peaks for  $\beta$ -T and  $\gamma$ -T3 just below the threshold limit were also observed indicating more markers would be helpful (Lande and Thompson 1990; Allison *et al.* 2002). If time permitted, this study would have used the entire population in year 2 instead of using selective phenotyping based on the main tocols. In addition, validation in a different mapping population would help to confirm the QTLs of individual isomers and vitamin E content. However, even using a subset of 40 lines in

the second year, some overlap of QTLs was observed suggesting the use of the entire population would have allowed validation.

Even though we have previously shown that there is variation across genotypes for antioxidant capacity (Do *et al.* 2015), no QTL were identified but an analysis peak was observed on chromosome 5H (LOD=2.2, threshold=3.3) associated with the CaaaccS marker. This marker has been shown to be linked to osmotic potential in a drought study in the same Tadmor x ERApm population (Diab *et al.* 2004). A difference in parental behaviour across years and the variety of antioxidant compounds that might be involved in antioxidant capacity may explain the lack of QTL and the strong effect of environment on antioxidant capacity. In the year the parents were chosen (2011), ERApm and Tadmor had an antioxidant capacity of 120.3 mg AEAC/100g FW and 104.8 mg AEAC/100gFW, respectively (Do *et al.* 2015). ERApm had an antioxidant capacity 14.8% higher than Tadmor. This greater antioxidant capacity was also observed in 2012 (the first year where the full mapping population was used) with ERApm having an antioxidant capacity 20.6% greater than Tadmor. However, in 2013 (where the subset of the mapping population was used) ERApm had a lower antioxidant capacity than Tadmor by 15.1%. Apart from these differences between parents, the data from the mapping population (offspring) across years also showed that year had a significant effect on antioxidant capacity ( $P < 0.0001$ ) which was in accordance with a previous study that indicated environmental effects on antioxidant capacity (Moore *et al.* 2006).

In addition, antioxidants are generally accepted to be a range of compounds including vitamin E (tocotrienols and tocopherols), ascorbic acid (vitamin C), enzymes (catalase, glutathione peroxidase and superoxide dismutase), phenolic compounds, and carotenoids (Halliwell 1994; Goupy *et al.* 1999; Reboul *et al.* 2006). Identifying QTLs for antioxidant capacity may be confounded by the fact that each antioxidant component

is likely to have their own QTL rather than contribute to a QTL for total antioxidant capacity necessarily. However, QTLs associated with total antioxidant capacity have been identified in rice (Jin *et al.* 2009). Further studies on the impact of the environment on antioxidant capacity and individual antioxidants are therefore required.

In conclusion, with further refinement of the positions for the QTL identified for individual tocol isomers and total vitamin E content in this study and subsequent identification of the genes underlying QTLs, our aim of breeding functional barley which is vitamin E-rich genotype will be achievable in the future.

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## Reference

- Allison, DB, Fernandez, JR, Heo, M, Zhu, SK, Etzel, C, Beasley, TM, Amos, CI (2002) Bias in estimates of quantitative-trait-locus effect in genome scans: Demonstration of the phenomenon and a method-of-moments procedure for reducing bias. *American Journal of Human Genetics* **70**, 575-585.
- Ball, GFM, 2006. Vitamins in foods: analysis, bioavailability and stability. Vitamins in foods: analysis, bioavailability and stability. CRC Press LLC: USA.
- Burton, GW, Ingold, KU (1981) Autoxidation of biological molecules. 1. The antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants *in vitro*. *Journal of the American Chemical Society* **103**, 6472-6477.
- Diab, AA, Teulat-Merah, B, This, D, Ozturk, NZ, Benscher, D, Sorrells, ME (2004) Identification of drought-inducible genes and differentially expressed sequence tags in barley. *Theoretical and Applied Genetics* **109**, 1417-1425.
- Do, TTD, Cozzolino, D, Muhlhausler, B, Box, A, Able, AJ (2015) Antioxidant capacity and vitamin E in barley: effect of genotype and storage. *Food Chemistry* **187**, 65-74.
- Ehrenbergerova, J, Belcrediova, N, Pryma, J, Vaculova, K, Newman, CW (2006) Effect of cultivar, year grown, and cropping system on the content of tocopherols and tocotrienols in grains of hulled and hullless barley. *Plant Foods for Human Nutrition* **61**, 145-150.
- Gassmann, B (2000) Dietary reference intakes, report 3: vitamin C and E, selenium, carotenoids. Overview, comments and comparison with the DACH reference values for nutrient intake. *Ernahrungs-Umschau* **47**, 265-268, 270.
- Goupy, P, Hugues, M, Boivin, P, Amiot, MJ (1999) Antioxidant composition and activity of barley (*Hordeum vulgare*) and malt extracts and of isolated phenolic compounds. *Journal of the Science of Food and Agriculture* **79**, 1625-1634.
- Halliwell, B (1994) Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *Lancet* **344**, 721-4.
- Halliwell, B (1996) Antioxidants in human health and disease. *Annual Review of Nutrition* **16**, 33-50.
- Hunter, SC, Cahoon, EB (2007) Enhancing vitamin E in oilseeds: Unraveling tocopherol and tocotrienol biosynthesis. *Lipids* **42**, 97-108.
- Jin, L, Xiao, P, Lu, Y, Shao, YF, Shen, Y, Bao, JS (2009) Quantitative trait loci for brown rice color, phenolics, flavonoid contents, and antioxidant capacity in rice grain. *Cereal Chemistry* **86**, 609-615.
- Kamino, LN, Singh, D, Pallotta, MA, Collins, NC, Park, RF (2015) Mapping of seedling resistance in barley to *Puccinia striiformis* f. sp. *pseudohordei*. *Journal of Applied Genetics* In Press.
- Kihara, M, Kaneko, T, Ito, K (1998) Genetic variation of  $\beta$ -amylase thermostability among varieties of barley, *Hordeum vulgare* L., and relation to malting quality. *Plant Breeding* **117**, 425-428.
- Lampi, AM, Rynananen, M, Salo-Vaananen, P, Ollilainen, V, Piironen, V (2004) A small-scale sample preparation method with HPLC analysis for determination of tocopherols and tocotrienols in cereals. *Journal of Food Composition and Analysis* **17**, 749-765.
- Lande, R, Thompson, R (1990) Efficiency of Marker-Assisted Selection in the Improvement of Quantitative Traits. *Genetics* **124**, 743-756.
- Layton, ME, Wagner, JK, Nelson, SR, Samson, FS, Pazdernik, TL (1992) Oxidative stress and antioxidant response in rat piriform cortex associated with kainic acid-induced seizures. *Society for Neuroscience Abstracts* **18**, 1603.
- Lehner, A, Mamadou, N, Poels, P, Come, D, Bailly, C, Corbineau, F (2008) Changes in soluble carbohydrates, lipid peroxidation and antioxidant enzyme activities in the embryo during ageing in wheat grains. *Journal of Cereal Science* **47**, 555-565.
- Lindley, MG (1998) The impact of food processing on antioxidants in vegetable oils, fruits and vegetables. *Trends in Food Science & Technology* **9**, 336-340.



- Lu, J, Zhao, H, Chen, J, Fan, W, Dong, J, Kong, W, Sun, J, Cao, Y, Cai, G (2007) Evolution of phenolic compounds and antioxidant activity during malting. *Journal of Agricultural and Food Chemistry* **55**, 10994-11001.
- March, TJ, Able, JA, Schultz, CJ, Able, AJ (2007) A novel late embryogenesis abundant protein and peroxidase associated with black point in barley grains. *Proteomics* **7**, 3800-3808.
- Martinez-Tome, M, Murcia, MA, Frega, N, Ruggieri, S, Jimenez, AM, Roses, F, Parras, P (2004) Evaluation of antioxidant capacity of cereal brans. *Journal of Agricultural and Food Chemistry* **52**, 4690-4699.
- Mclaughlin, P. J., & Weihrauch, J. L. (1979) Vitamin-E Content of Foods. *Journal of the American Dietetic Association* **75**(6), 647-665.
- Moore, J, Liu, JG, Zhou, KQ, Yu, LL (2006) Effects of genotype and environment on the antioxidant properties of hard winter wheat bran. *Journal of Agricultural and Food Chemistry* **54**, 5313-5322.
- Oliver, RE, Islamovic, E, Obert, DE, Wise, ML, Herrin, LL, Hang, A, Harrison, SA, Ibrahim, A, Marshall, JM, Miclaus, KJ, Lazo, GR, Hu, GS, Jackson, EW (2014) Comparative Systems Biology Reveals Allelic Variation Modulating Tocochromanol Profiles in Barley (*Hordeum vulgare* L.). *PLoS One* **9**,
- Omwamba, M, Hu, Q (2009) Antioxidant capacity and antioxidative compounds in barley (*Hordeum vulgare* L.) grain optimized using response surface methodology in hot air roasting. *European Food Research and Technology* **229**, 907-914.
- Packer, L (1995) Nutrition and biochemistry of the lipophilic antioxidants, vitamin E and carotenoids. In 'Nutrition, Lipids, Health and Disease.' (Eds L Packer, E Niki, ASH Ong.) pp. 8-35. (American Oil Chemists Society Champaign IL)
- Pallotta, MA, Asayama, S, Reinheimer, JM, Davies, PA, Barr, AR, Jefferies, SP, Chalmers, KJ, Lewis, J, Collins, HM, Roumeliotis, S, Logue, SJ, Coventry, SJ, Lance, RCM, Karakousis, A, Lim, P, Verbyla, AP, Eckermann, PJ (2003) Mapping and QTL analysis of the barley population Amagi Nijo x WI2585. *Australian Journal of Agricultural Research* **54**, 1141-1144.
- Panfili, G, Fratianni, A, Criscio, Td, Marconi, E (2008) Tocol and beta-glucan levels in barley varieties and in pearling by-products. *Food Chemistry* **107**, 84-91.
- Pomeranz, Y (1973) Interaction between glycolipids and wheat flour macromolecules in breadmaking. *Adv. Food Res* **20**, 153-188.
- R Development Core Team, 2010. R: A language and environment for statistical computing, R Foundation for Statistical Computing <https://www.r-project.org/>.
- Reboul, E, Richelle, M, Perrot, E, Desmoulins-Malezet, C, Pirisi, V, Borel, P (2006) Bioaccessibility of carotenoids and vitamin E from their main dietary sources. *Journal of Agricultural and Food Chemistry* **54**, 8749-8755.
- Reinheimer, JL, Barr, AR, Eglinton, JK (2004) QTL mapping of chromosomal regions conferring reproductive frost tolerance in barley (*Hordeum vulgare* L.). *Theoretical and Applied Genetics* **109**, 1267-1274.
- SAS Institute (2014) The SAS Enterprise Guide 7.1. SAS Institute, Cary, NC, USA.
- Shao, HB, Chu, LY, Lu, ZH, Kang, CM (2008) Primary antioxidant free radical scavenging and redox signaling pathways in higher plant cells. *International Journal of Biological Sciences* **4**, 8-14.
- Sheppard, AJ, Pennington, JAT, Weihrauch, JL (1993) Analysis and distribution of vitamin E in vegetable oils and foods. In 'Vitamin E in health and disease.' (Eds L Packer, J Fuchs.) pp. 9-31. (Marcel Dekker, Inc., New York:
- Shintani, D, DellaPenna, D (1998) Elevating the vitamin E content of plants through metabolic engineering. *Science* **282**, 2098-100.
- Tah, PR, Lehmensiek, A, Fox, GP, Mace, E, Sulman, M, Bloustein, G, Daggard, GE (2010) Identification of genetic regions associated with black point in barley. *Field Crops Research* **115**, 61-66.

- Tang, S, Kerry, JP, Sheehan, D, Buckley, DJ, Morrissey, PA (2001) Antioxidative effect of added tea catechins on susceptibility of cooked red meat, poultry and fish patties to lipid oxidation. *Food Research International* **34**, 651-657.
- Teulat, B, This, D, Khairallah, M, Borries, C, Ragot, C, Sourdille, P, Leroy, P, Monneveux, P, Charrier, A (1998) Several QTLs involved in osmotic adjustment trait variation in barley (*Hordeum vulgare* L.). *Theoretical and Applied Genetics* **96**, 688-698.
- van Berloo, R (2007) 'GGT: user manual Version 2.0.' Available at
- von Korff, M, Grando, S, Del Greco, A, This, D, Baum, M, Ceccarelli, S (2008) Quantitative trait loci associated with adaptation to Mediterranean dryland conditions in barley. *Theoretical and Applied Genetics* **117**, 653-669.
- Wanasundara, NU, Shahidi, F (1996) Stabilization of sea blubber and menhaden oils with green tea catechins. *Journal of American Oil Chemists' society* **42**, 1285-1290.
- Wang, M, Zheng, QS, Shen, QR, Guo, SW (2013) The Critical Role of Potassium in Plant Stress Response. *International Journal of Molecular Sciences* **14**, 7370-7390.
- Willet, W (1994) Coffee and coronary heart disease. A new problem with old brew? *Annals of epidemiology* **4**, 497-8.
- Wood, JD, Richardson, RI, Nute, GR, Fisher, AV, Campo, MM, Kasapidou, E, Sheard, PR, Enser, M (2004) Effects of fatty acids on meat quality: a review. *Meat Science* **66**, 21-32.
- Young, IS, Woodside, JV (2001) Antioxidants in health and disease. *Journal of Clinical Pathology* **54**, 176-186.

**Table 1. QTLs for individual tocol isomers measured in the Amaji Nijo x WI2585 population grown in 2012 and 2013. Year 1 (2012, full population), Year 2 (2013, subset).**

Isomer	Year	QTL	Chromosomes	Nearest marker	Position (cM)	Support interval* (cM)	LOD**	R <sup>2</sup> ***	Additive main effect****		
αT	Year 1	<i>QaT.AmWI-7H.Y1</i>	7H	C7P65	65.2	22.0-88.0	3.2	19.8	-0.7		
	Year 2	-	-(7H)	-	-	-	-(2.2)	-	-		
αT3	Year 1	<i>QaT3.AmWI-5H.Y1</i>	5H	CDO504	135.5	132.0-140.0	3.9	10.6	2.2		
			5H	MWG514	133.6	132.0-140.0	3.2				
			7H	C7P190	189.6	180.0-201.0	4.1	12.9	-2.4		
			7H	PSR680	197.5		4.0				
				7H	ABG714b	181.6		3.2			
	Year 2	<i>QaT3.AmWI-5H.Y2</i>	5H	X-I	172.6	84.0-182.0	3.3	25.8	3.9		
βT	Year 1	-	-(2H)	-	-	-	-(2.8)	-	-		
	Year 2	-		-	-	-	-	-	-		
βT3	Year 1	<i>QbT3.AmWI-4H.Y1</i>	4H	EBmac788	119.7	98.0-152.0	3.08	4.7	-0.5		
			5H	CDO504	135.5	92.0-142.0	5.14	11.4	0.7		
			5H	MWG514	133.6		4.71				
			5H	ABG702b	130.1		4.22				
			5H	C5P121	120.8		4.20				
			5H	C5P141	140.8		4.16				
			5H	C5P111	111.5		4.14				
			5H	ABG3a	131.8		4.02				
			5H	C5P102	102.1		3.98				
			5H	C5P93	92.8		3.64				
			5H	C5P155	154.9		3.21				
			Year 2	<i>QbT3.AmWI-5H.Y2</i>	5H	X-I	172.6	152.0-188.0	3.6	28.1	0.8
	γT	Year 1	<i>QgT.AmWI-7H.Y1</i>	7H	C7P65	65.2	28.0-88.0	3.32	17.7	-0.4	
7H				C7P75	74.8		3.31				
Year 2		-	-(7H)	-	-	-	-(2.6)	-	-		
γT3	Year 1	-	-(2H)	-	-	-	2.8;2.6	-	-		
	Year 2	-		-	-	-	-	-	-		
Vitamin E	Year 1	<i>QVTE1.AmWI-7H.Y1</i>	7H	ABG497b	45.9	16.0-94.0	3.4	7.0	-0.8		
			7H	CDO358	86.3		3.2				
			7H	BCD263	87.1		3.2				
			7H	PSR680	197.5	182.4-200.4	4.2	10.7	-1.0		
			7H	ABG714b	181.6		3.1				
	Year 2	<i>QVTE.AmWI-7H.Y2</i>	7H	ABG714b	181.6	166.1-190.1	3.1	34.8	-2.1		

\*Interval based on one LOD to each side of the peak.

\*\*QTL detection was based on a LOD threshold of 3.12 for  $\alpha$ -T,  $\alpha$ -T3 and  $\gamma$ -T3 and 3.03 for vitamin E and the remaining isomers

\*\*\*Percentage of the phenotypic variation explained by the QTL

\*\*\*\*A positive effect indicates that the allele from WI2585 contributes to higher vitamin E or isomer content, while a negative effect indicates that the allele from Amaji nijo contribute to higher vitamin E or isomer content.

## Figure Captions

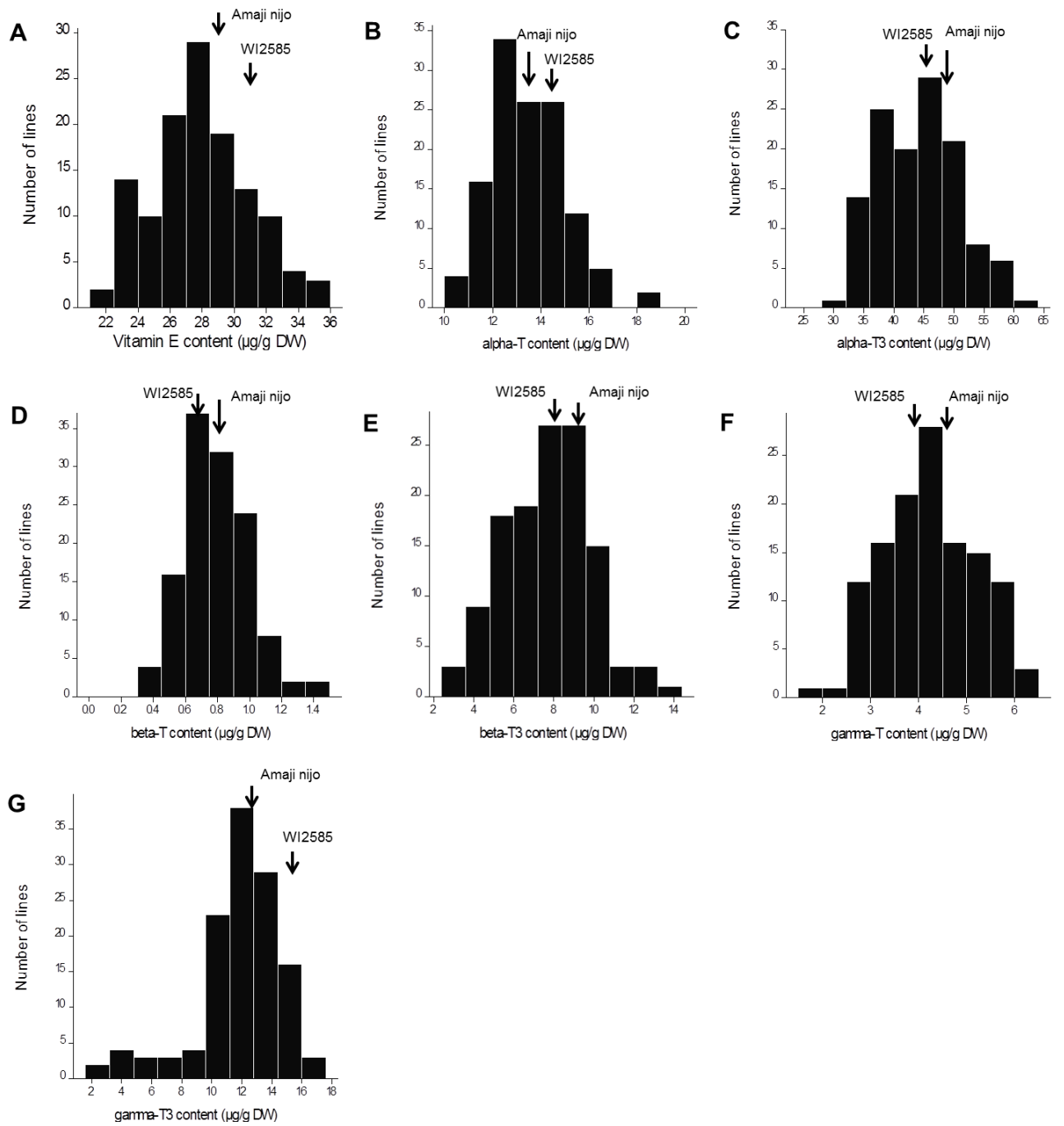
**Fig. 1** Frequency distribution in content of vitamin E and isomers in the Amaji nijo x WI2585 population in Year 1. Year 1 (2012, full population). A, vitamin E; B,  $\alpha$ -T; C,  $\alpha$ -T3; D,  $\beta$ -T; E,  $\beta$ -T3; F,  $\gamma$ -T; G,  $\gamma$ -T3 in 2012.

**Fig. 2** Frequency distribution in content of vitamin E and isomers in the Amaji nijo x WI2585 population in Year 2. Year 2 (2013, subset population). A, vitamin E; B,  $\alpha$ -T; C,  $\alpha$ -T3; D,  $\beta$ -T; E,  $\beta$ -T3; F,  $\gamma$ -T; G,  $\gamma$ -T3 in 2013.

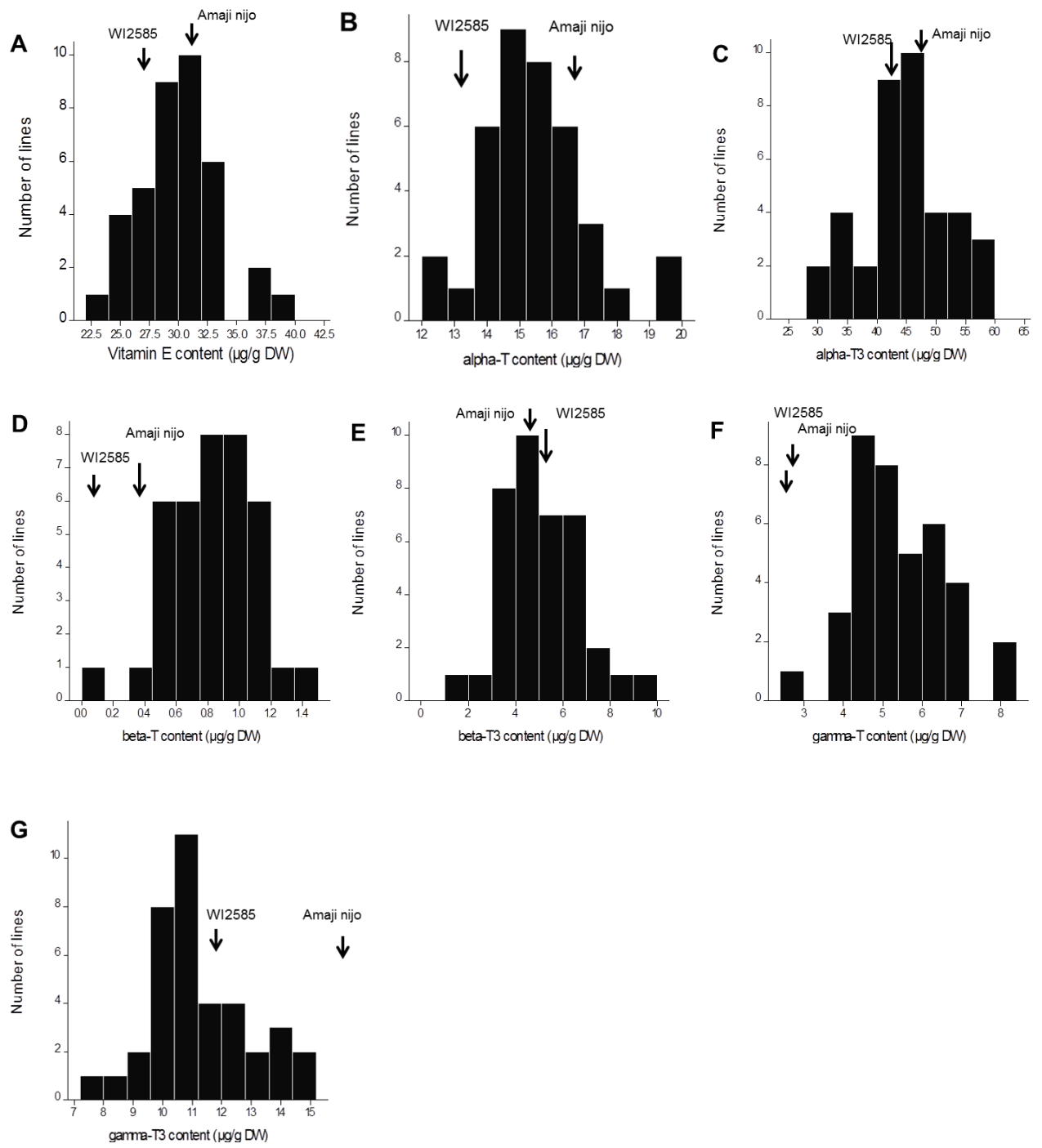
**Fig. 3** Chromosome locations of regions associated with the content of vitamin E and individual isomers in the Amaji nijo x WI2585 population.

**Fig. 4** Frequency distribution in antioxidant capacity of the Tadmor x ERApm population. A, variation in antioxidant capacity in 2012; B, variation in antioxidant capacity in 2013.

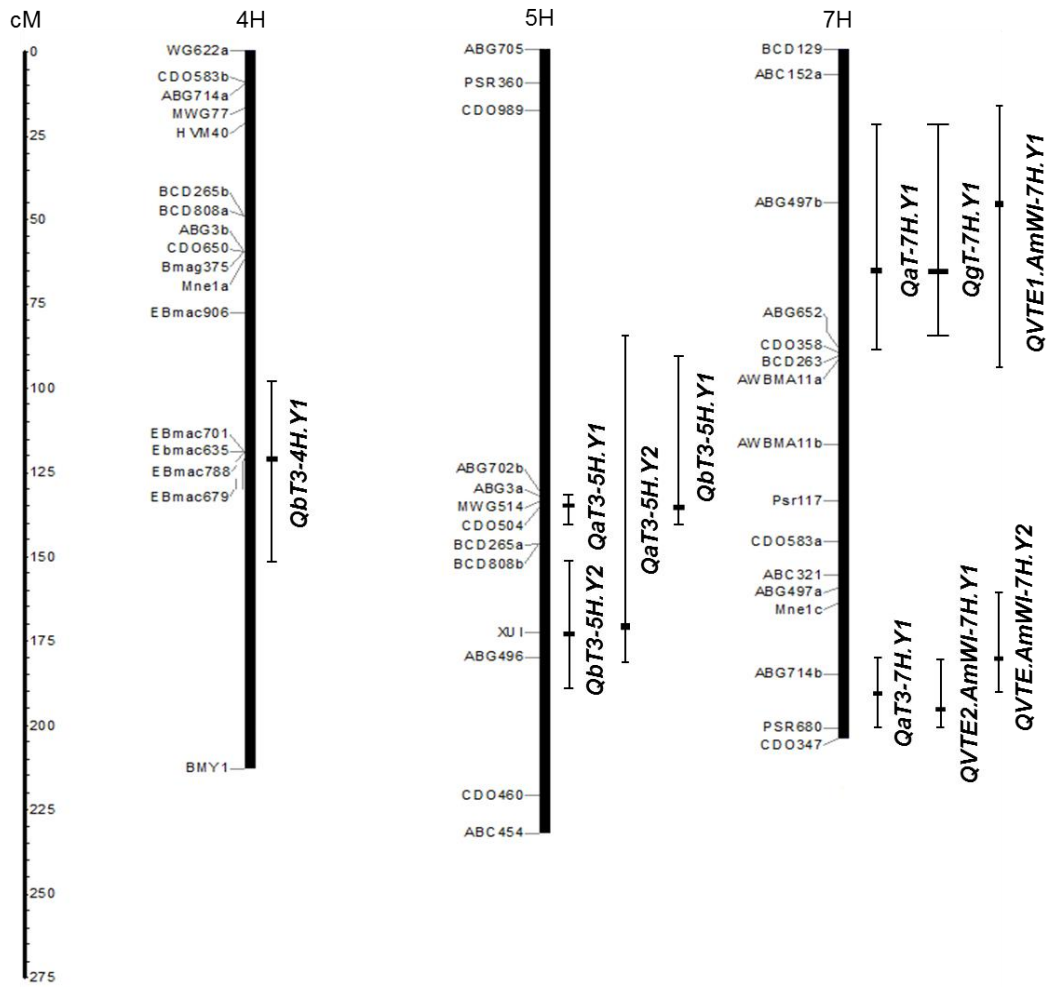
**Supplementary Fig. S1** Interaction plots for vitamin E and isomers in the WI2585/Amaji nijo population and for antioxidant in the Tadmor/ERApm population. A, vitamin E; B,  $\alpha$ -T; C,  $\alpha$ -T3; D,  $\beta$ -T; E,  $\beta$ -T3; F,  $\gamma$ -T; G,  $\gamma$ -T3; H, antioxidant capacity. No genotype x environment interaction was found if the lines are parallel.



**Fig. 1.**

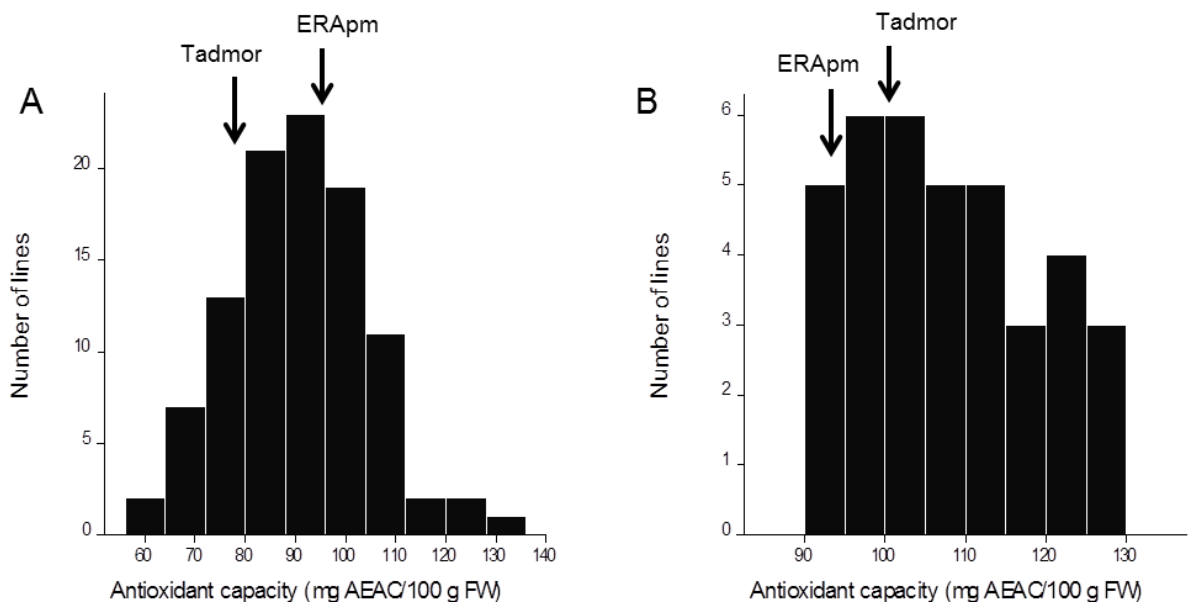


**Fig. 2**

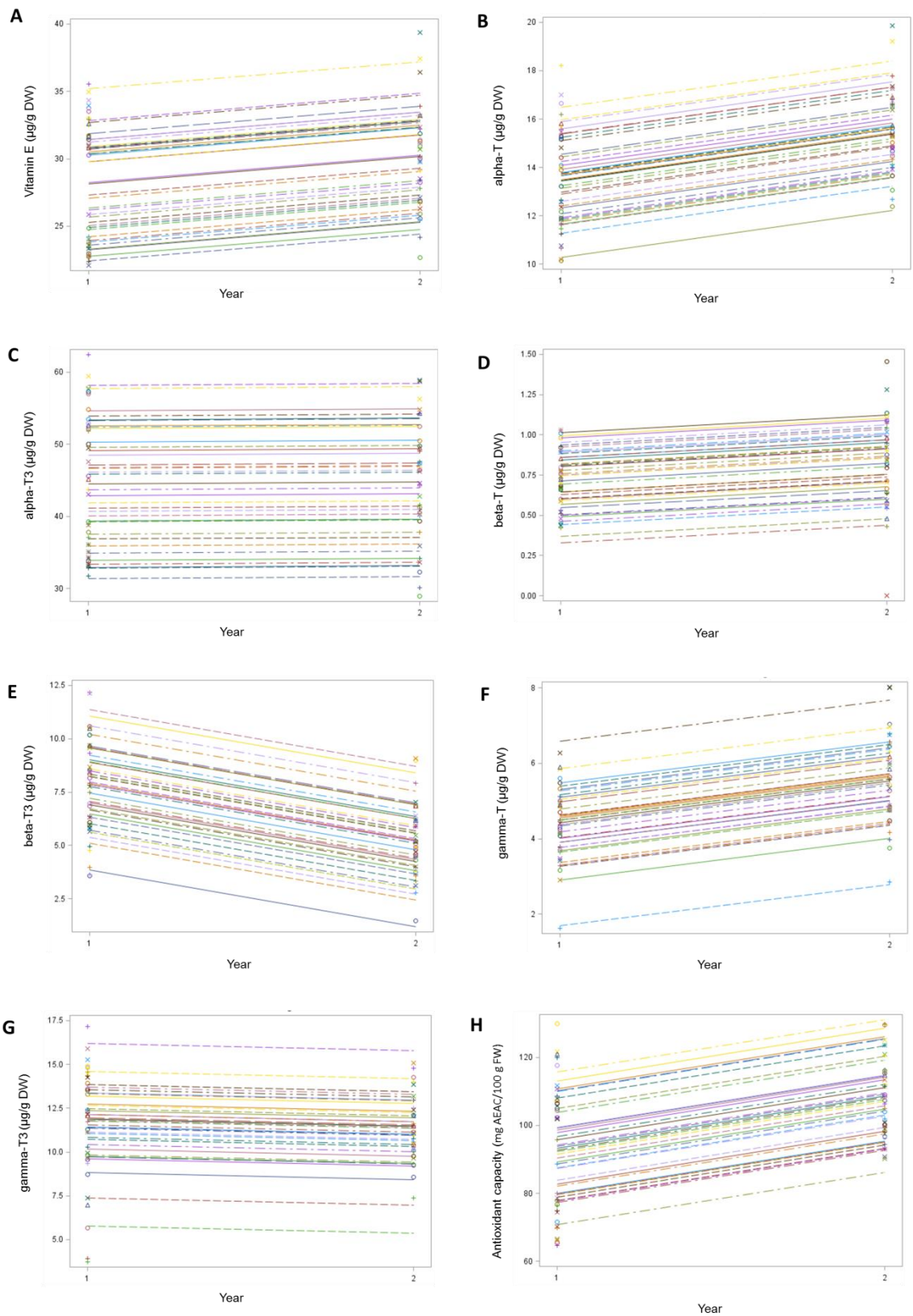


**Fig. 3**





**Fig. 4**



Supplementary Fig. S1

## **Chapter 7: GENERAL DISCUSSION**

## **7.1. Main outcomes of this study**

The important roles of antioxidants, including vitamin E, to human health are well known [reviewed by Frei (2012)]. This study successfully addressed the overall aim of determining whether these components are affected in the food chain, from barley grain to product. In particular, this study has established the genotypic basis for antioxidant capacity and vitamin E at (i) harvest; (ii) during storage and processing (making malt and pearling grain); and (iii) when making a food product (pita bread).

### *(i) Identifying the genetic basis of antioxidant capacity and vitamin E*

To allow accurate analysis of antioxidant capacity and vitamin E content, the methodology had to be optimised firstly (Chapter 2). The development of an optimised method in this study was also required to enable the large scale analysis of multiple genotypes in a timely manner with limited loss of material. In particular, the extraction temperature and sample volume was reduced; and rotary evaporation was replaced by the use of stream nitrogen. This optimised method can be used for vitamin E analysis on a range of barley genotypes (including non-waxy and waxy genotypes for which the ideal lower temperatures were established). It can also be used for measurement of antioxidant capacity in the vitamin E extraction by re-evaporating the hexane solvent and re-dissolving in ethanol prior to antioxidant analysis using DPPH.

Using this optimised method, antioxidant capacity and vitamin E content were measured in 25 barley genotypes including malting, food and feed types across colored or white, covered or hulless barley varieties. This coverage had not occurred in previous studies. Therefore, while this study showed a wide range of antioxidant capacity and vitamin E content levels, limited ranges were previously found because

of the limited genotypes (Peterson and Qureshi 1993; Cavallero *et al.* 2004; Panfili *et al.* 2008). There was a three-fold difference across the range of vitamin E content and a four-fold difference for antioxidant capacity indicating these components were genotypic dependent. Generally, malting genotypes were highest in both antioxidant and vitamin E, followed by food and feed genotypes. Among malting genotypes, WI2585 (white and covered) was highest in antioxidant capacity (158.1 mg AEAC/100g FW) and relatively high in vitamin E content (26.1  $\mu\text{g/g}$  DW) while Harrington (white and covered) was highest in vitamin E content (31.5  $\mu\text{g/g}$  DW) and relatively high in antioxidant (150.0 mg AEAC/100g FW). Mean levels of antioxidant capacity and vitamin E content in hullless genotypes was lower than that in covered genotypes but the hullless genotype Finnis contained these compounds at levels as high as covered genotypes. Thus the genotypes WI2585, Harrington and Finnis were used to make a food product (pita bread, Chapter 5). In addition, the contribution of vitamin E to total antioxidant capacity is relatively low with only a moderate correlation between these two components ( $n = 25, r = 0.46, p < 0.05$ ). This observation confirmed the previous findings (Goupy *et al.* 1999; Spano *et al.* 2011) that antioxidant capacity included not only vitamin E but also other compounds such as phenolics,  $\beta$ -carotenoid, vitamin C and enzymes such as dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione peroxidase (GPX) and catalase (CAT). While the ranking of antioxidant capacity of  $\alpha$ -T and  $\alpha$ -T3 has been controversial among studies (McLaughlin and Weihrauch 1979; Packer 1995), our finding showed that  $\alpha$ -T3 mainly contributed to total tocotrienol (73%) and total tocopherols (58%) and was the only isomer highly correlated to total antioxidant capacity ( $n=25, r=0.7, p<0.05$ ) which indicated that  $\alpha$ -T3 was the most efficient as a scavenger. The dominance of  $\alpha$ -T3 was

also confirmed in the QTL analysis (Chapter 6) which showed the genotypic contribution to the phenotypic variation for vitamin E and its isomers, particularly  $\alpha$ -T3, was significant.

To identify QTLs for content of total vitamin E and its isomers, the mapping population, WI2585 x Amaji niyo was chosen based on the amounts seen in the parents (Chapter 3). QTLs were found for two major isomers ( $\alpha$ -T and  $\alpha$ -T3), two minor isomers ( $\beta$ -T3 and  $\gamma$ -T) and total vitamin E content. Using the 127 offspring of the mapping population,  $\alpha$ -T3 content had the highest correlation with total vitamin E content ( $n=127$ ,  $r=0.9$ ,  $p<0.05$ ) followed by  $\alpha$ -T content ( $n=127$ ,  $r=0.8$ ,  $p<0.05$ ). These correlations were in agreement with the finding that QTLs associated with total vitamin E content overlapped with the QTL identified for  $\alpha$ -T3 at the end of 7H. Another QTL for vitamin E was identified at the top of 7H which also overlapped with QTL that corresponded for  $\alpha$ -T and  $\gamma$ -T. Content of  $\alpha$ -T were also highly correlated with that of  $\gamma$ -T ( $n=127$ ,  $r=0.6$ ,  $p<0.05$ ) (Chapter 3 and Chapter 6) probably because they are in the same pathway (Hunter and Cahoon 2007), which might also explain the overlapping QTL identified (Chapter 6). However, Oliver *et al.* (2014), the only other study to date, reported that 7H contained QTLs associated with  $\beta$ -T3,  $\gamma$ -T3 and  $\delta$ -T3 in the feed barley-mapping population, Falcon x Azhul. This difference between the two findings can be explained by two candidate genes, one encodes for homogentisate phytyl transferase, the first dedicated enzyme of the tocopherol biosynthetic pathway (the *VTE2* gene); and the other one synthesizes a distant precursor of the tocochromanol ring structure (3-phosphoshikimate 1-carboxyvinyltransferase) (Oliver *et al.* 2014). Therefore, based on the genes identified in that genomic region (7H), their QTL may be associated with any of the tocols. The QTLs detected by these authors

and in this study were in similar regions indicating they are important for vitamin E content in barley. Furthermore, our finding found a QTL on 4H for  $\beta$ -T3 and some QTLs for  $\alpha$ -T3 and  $\beta$ -T3 clustered together on 5H while no significant QTL were found in their study probably due to the different mapping population used. Interestingly, the markers associated with the total vitamin E content QTL were also linked to other traits such as frost, salt and black point tolerance which have all been linked to antioxidant capacity suggesting the importance that vitamin E could play during physiological processes that cause lipid damage such as these (Reinheimer *et al.* 2004; Wang *et al.* 2013). Unfortunately, no QTL was found for antioxidant capacity in the Tadmor x ERApm population. Although there was a significant difference in antioxidant capacities of parents the study of 25 genotypes (Chapter 3) and in the first year of QTL analysis (Chapter 6), antioxidant capacity did not behave similarly in the following year. Impact of environment might therefore be responsible for the non-identification of QTL. Since QTLs linked with antioxidant capacity have been found in rice (Jin *et al.* 2009), this should be addressed in barley by redoing the experiments or choosing other parents in a future study.

(ii) *Storage and processing affects antioxidant capacity and vitamin E content in a genotype-dependent manner*

The 25 barley genotypes across malting, food and feed; colored and white; covered and hullless barley varieties were stored at 10°C in the dark for 4 months and showed the different changes in between components as well as between genotypes. Of all isomers, the change in T was lower than that of T3 which may be attributed to unsaturated side chains of T3 being more susceptible to oxidation than saturated side

chains of T. Most of the genotypes showed an increase in vitamin E level after storage which was in accordance with some previous studies (Wang *et al.* 1993; Liu and Moreau 2008) but not all studies (Ball, 2006; Tyopponen & Hakkarainen, 1985). This controversy may be explained by different storage conditions such as temperature, light, grain moisture and storage time (Hakkarainen *et al.* 1983; Ball 2006; Metz 2006). For example Hakkarainen *et al.* (1983) indicated that two dominant isomers ( $\alpha$ -T and  $\alpha$ -T3) increased during the first 2 to 3 months of storage but declined after 11 months storage in silos. Therefore, the storage condition of 10°C in the dark for 4 months in this study seemed to be ideal for storage which was also suggested by Viljoen (2001). In contrast, antioxidant decreased in most genotypes except colored genotypes more than likely due to the reduction in total phenolic content (Pinto *et al.* 2005) or enzymes (Spano *et al.* 2011). The increase in antioxidant in colored genotypes may be attributed to an increase in  $\beta$ -carotene which was also observed in colored rice stored at 20, 30 and 40°C for up to four months (Htwe *et al.* 2010). Moreover, antioxidants and vitamin E have been reported as preservatives in food products (Wanasundara and Shahidi 1996; Tang *et al.* 2001) as well as possible protectants of seed from deterioration (Lehner *et al.* 2008). In this study, antioxidants and vitamin E before and after storage were highly correlated ( $n=25$ ,  $r>0.9$ ,  $p<0.05$ ) which may suggest the important role of antioxidant capacity and vitamin E content in shelf life of grain during storage.

The effect of malting stages on antioxidant capacity and vitamin E was examined in 14 genotypes including twelve covered and two hulless barley genotypes. After steeping, antioxidant capacity, content of total vitamin E and its isomers significantly decreased (more than 40%) probably because they were leached in the water (Su *et al.* 2011). After 4 days germination, the decrease was observed in vitamin



E isomers but antioxidant capacity increased which might be due to the synthesis of polyphenol and  $\beta$ -carotenoid in the seeds (Chiou *et al.* 1977). In contrast, vitamin E has been shown to increase when the germination period is longer (Yang *et al.* 2001; Frias *et al.* 2005), a parameter that could be studied for its effect on malting quality in the future. Although there was a sharp increase in the individual tocol isomers and total vitamin E content during kilning, this was not sufficient to increase the level in malt compared to the unprocessed grain in most genotypes except those that contained a high level at harvest such as WI2585. Finnis was a hullless genotype which had a lower decrease in vitamin E in the malt compared to other genotypes making it suitable for products that use malt. In contrast, antioxidant capacity in malt was higher than that in unprocessed grain which is likely to be due to the collective influence of three processes: (i) release of phenolic compounds bound to cellular structures; (ii) better extraction; and; (iii) the formation of Maillard reaction products (Morad *et al.* 1984). The phenolic compounds, mainly phenolic acids, which were bound to lignin and arabinoxylans in the unprocessed samples would be expected to be released in the presence of enzymes which are synthesised and/or activated during either the final stages of germination or the early stages of kilning (Morad *et al.* 1984). The change in tissue structure caused by kilning, in particular the increased friability of the grain, has been shown to increase the ease of extracting compounds located in the outer layers of the grain, such as the phenolic compounds, resulting in improved efficiency of extraction of antioxidant compounds (Morad *et al.* 1984). In addition, reducing sugars and amino acids which are reported to be released during germination can react to produce Maillard products such as melanoidins, which also have antioxidant capacity (Morad *et al.* 1984; Alex and Glenn 2012). Among genotypes, Finnis again had

comparable antioxidant capacity indicating the potential use for producing hullless malt. The antioxidant capacity and vitamin E content in malt highly correlated with those in unprocessed grain (Chapter 4) indicating genotypes with high levels of antioxidant and vitamin E are suitable for producing malt containing high levels of these compounds.

MIR was investigated to examine barley during storage and malting and had not been previously used in the context of examining possible linkages to antioxidant capacity and vitamin E content. ATR-MIR analysis showed the discrimination between samples before and after storage in the PCA plots which might be due to differences in the frequencies associated with methyl groups, particularly in lipids. Reactions may also result in the increase of vitamin E due to the reduction of vitamin E oxidation through esterification resulting in the formation of tocopherol acetate (Church and Pond 1977). During malting, ATR-MIR analysis showed samples before malting or after kilning clustered together probably because the chemical reactions which would have occurred during processing were stopped by the high temperatures applied during kilning. In contrast, samples after steeping or germination were considerably widespread which is likely to be due to variation between the genotypes in their moisture content and/or water sensitivity resulting in biochemical reactions stimulated by the increasing oxygen/water content of the grains, or by swelling of grains during the steeping stage (Su *et al.* 2011).

Pearling significantly reduced the antioxidant capacity and contents of vitamin E and its isomers in Finnis (hullless), WI2585 (covered genotype) and Harrington (covered genotype) which contained high levels of these components. The reduction was progressive from the external to the internal layers and different between

genotypes. Finniss lost the highest level of antioxidant capacity and vitamin E content at the first pearling stage (10%) while this loss happened in the second and third pearling stages for Harrington and WI2585 (15% and 20%, respectively). The bran and germ might have been more quickly abraded in Finniss than the other genotypes because it does not contain the hull fraction. Besides the variation in antioxidant and vitamin E between genotypes (Do *et al.* 2015a) (Chapter 3), Harrington has a loose/adhering husk and is highly susceptible to skinning (Menz 2010) in contrast to WI2585 which has a thicker and more adhering husk. Therefore, pearling might result in different levels of antioxidant and vitamin E being removed.

(iii) *Barley rich in antioxidant capacity and vitamin E content can be used in a product for consumers*

Flour to make pita bread was substituted with 50% barley flour from selected genotypes studied in Chapter 3, 4, 5 and then its antioxidant and vitamin E level, physical and sensory properties was compared to control pita made from baker's flour. Barley flour was made from Finniss malt, whole grain, pearled at 10%, 15% or 20% of Finniss, WI2585 or Harrington. Even though, pearling removed antioxidant capacity and vitamin E from whole grain flour, flour from most pearled grain was higher in vitamin E while all of them were higher in antioxidants than baker's flour. This can be explained by the observations of Holasova *et al.* (1995) that barley was richest in those components among cereal; and also the observations of (Beta *et al.* 2005) who reported the decrease of antioxidant and vitamin E during wheat pearling to make baker's flour. Consequently, pita made from barley contained higher antioxidant capacity and vitamin E content than control pita. However, not all of them

have similar physical properties. In general, the more outer layer removed in barley grain the better the physical properties in pita observed which were in good agreement with literature (Sumner *et al.* 1985; Wang *et al.* 2002). Pita made from whole grain barley flour had less satisfactory pocket formation, darker color, higher firmness, lower thickness and dough height than pita from pearled grain flour in the same genotype. This happened because the husk in the whole grain absorbed more water and caused lower water availability in the dough for starch to be gelatinised during baking such that pocket formation was adversely affected (Varrianomarston *et al.* 1980). Finnis was the best whole grain for making pita because it does not contain the hull layer. Pita made from WI2585 had better physical properties than Harrington pita at the same proportion of pearling which might be due to differences in their structure. WI2585 has a thicker and more adhering husk which might result in a higher percentage of husk per grain weight whereas Harrington has a loose adhering husk and is highly susceptible to skinning (Menz, 2010). Apart from structural differences, tannin and amylose might be involved (Hulse 1979; Izydorczyk *et al.* 2008). Tannins are known to bind with protein (Hulse 1979) and are likely to form a tannin-gluten complex which might be responsible for the change in rheological properties. Amylose content was reported to be different between barley genotypes causing differences in the stickiness of dough and therefore differences in pasting properties (Izydorczyk *et al.* 2008). Of all pita, those made from flour of control, malt, whole Finnis or 15% pearled WI2585 had higher antioxidant and vitamin E levels as well as physical properties especially pocket formation. These pitas were compared in terms of sensory value. Control pita had a higher score for consumers liking the appearance which correlated with the whiter color intensity of the control when compared with the barley

pita. However, overall, all of the pitas were acceptable to consumers indicating the potential use of barley to produce pita as a source of antioxidant and vitamin E.

## **7.2. Conclusions and future directions**

Antioxidant capacity and vitamin E content in barley were genotypic dependent which enables the choice of genotypes with high antioxidant and vitamin E content to determine the genetic basis for breeding purposes. Several QTLs were identified for total vitamin E, two major isomers ( $\alpha$ -T and  $\alpha$ -T3) and two minor isomers ( $\beta$ -T3,  $\gamma$ -T) which might also be associated with other traits such as frost, salt and black point tolerance which have all been linked to antioxidant capacity suggesting the importance that vitamin E could play during physiological processes that cause lipid damage such as these. Storage caused a decrease in antioxidant capacity in most genotypes except colored genotypes but caused an increase in vitamin E content after storing for 4 months at 10°C. In contrast, malting caused a decrease in vitamin E content but an increase in antioxidant capacity. The progressive decrease of both components was observed during pearling from external to internal layers. Antioxidant and vitamin E were higher in pita bread made with barley flour from malt, hullless Finnis whole grain and 15% pearled WI2585 compared to control pita made from baker's flour. These barley pitas also had acceptability from consumers indicating potential of barley pita as a source of antioxidant and vitamin E.

### **Future research should include:**

1. Although this study examined a larger number of genotypes including different classes compared to previous studies, validation by testing a wider diversity of barley

genotypes for antioxidant capacity, vitamin E and individual isomers is recommended. In particular, many different types of barley from different geographical origins could be tested.

2. More GxE studies with several environments (especially across years) are needed to see the influence of genotype and environment on expression of antioxidant capacity/vitamin E content and for QTL mapping. The refinement of the positions for the QTLs identified (Chapter 6) for vitamin E and four isomers and subsequent identification of the genes underlying these QTLs. In order to fine map, known markers and bioinformatics approaches could be applied. Another approach is to increase the density of markers and/or create near-isogenic lines which can eliminate the effect of genetic background on the expression of QTL and therefore facilitate fine map and isolation of the alleles of target QTLs.

3. Identification of QTLs associated with antioxidant capacity. Since environment may have an effect on antioxidant capacity of parents and offspring, the experiment needs to be repeated with more replicated trials and possibly across different environments. Alternatively, choosing mapping population with different parents that have a greater difference than Tadmor and ERApm would help to identify QTLs linked with antioxidant capacity.

4. Examining the relationship between high antioxidant genotypes and their shelf life during storage and processing. Antioxidants, both pure and extracted antioxidants have been shown to act as preservatives when added to various food stuffs suggesting that grains with inherent antioxidants may have more potential to protect themselves in storage. A range of genotypes (from low to high levels of antioxidant and vitamin

E) could be examined for their shelf life during storage and processing based on parameters such as weight loss and spoilage.

5. Determining the changes in antioxidant, vitamin E and its isomers across the germination stage to optimise the germination period during malting. The antioxidant and vitamin E level were reported to increase after 7 days of germination in wheat (Yang *et al.* 2001) or after 9 days of germination in lupin (Frias *et al.* 2005). The antioxidant and vitamin E level, therefore could be measured from day 0 to at least day 9 of germination and its impacts on malting determined. In addition, more GxE studies with several environments are needed to see the influence of genotype and environment on vitamin E/total antioxidant capacity/other antioxidant compounds in malt.

6. Investigating the use of high proportion of barley flour comprising flour from whole grain (hulless and covered), pearled grain and malt in other products such as normal bread and biscuits. Barley flour has been added in normal bread and biscuits (Sudha *et al.* 2007; Skrbic *et al.* 2009; Blandino *et al.* 2015) at less than 25% to total flour, however, the higher barley added in products the higher antioxidant and vitamin E level that products had. In addition, no one has used different types of barley such as whole grain, pearled grain, hulless, covered grain and malt. They should be mixed with baker's flour at more than 25% to make normal bread and biscuits. These products then could be analysed for the antioxidant, vitamin E level, physical and sensory parameters.

7. Testing bioavailability of pita bread to see how much antioxidant and vitamin E is observed in the human body. Antioxidant and vitamin E bioavailability differs among products (Reboul *et al.* 2006), hence, the understanding of whether the

antioxidant and vitamin E in barley is readily bioavailable needs to be ascertained. Bioavailability of pita bread can be examined using an *in vitro* digestion model or *in vivo* by determining antioxidant and vitamin E level in plasma or comparing antioxidant and vitamin E level intake and vitamin in the faeces (Ball 2006)



## Reference

- Abdel-Aal, ESM, Young, JC, Rabalski, I (2006) Anthocyanin composition in black, blue, pink, purple, and red cereal grains. *Journal of Agricultural and Food Chemistry* **54**, 4696-4704.
- Akar, T, Avci, M, Dusunceli, F (Ed. D Mejía (2004) 'Barley: post harvest operation.' (Food Agriculture Organisation: Ankara, Turkey)
- Alex, JN, Glenn, DR (2012) Genetic, morphometric, and behavioral factors linked to the midsagittal area of the corpus callosum. *Frontiers in Genetics* **3**, 1-12.
- Angle, G, 1994. European Patent Specification Euro Patent Office. France. 0453390B1:
- Axelsen, PH, Komatsu, H, Murray, IV (2011) Oxidative stress and cell membranes in the pathogenesis of Alzheimer's disease. *Physiology (Bethesda)* **26**, 54-69.
- Baik, BK, Czuchajowska, Z (1997) Barley in Udon noodles. *Food Science and Technology International* **3**, 423-435.
- Baik, BK, Ullrich, SE (2008) Barley for food: characteristics, improvement, and renewed interest. *Journal of Cereal Science* **48**, 233-242.
- Ball, GFM, 2006. Vitamins in foods: analysis, bioavailability and stability. Vitamins in foods: analysis, bioavailability and stability. CRC Press LLC: USA.
- Banks, W, Greenwood, CT, Walker, JT (1970) Studies on the starches of barley genotypes: The waxy starch. *Cereal Chemistry* **5**, 149-180.
- Basman, A, Koksels, H (1999) Properties and composition of Turkish flat bread (bazlama) supplemented with barley flour and wheat bran. *Cereal Chemistry* **76**, 506-511.
- Berglund, PT, Fastnaught, CE, Holm, ET (1994) Physicochemical and sensory evaluation of extruded high-fiber barley cereals. *Cereal Chemistry* **71**, 91-95.
- Beta, T, Nam, S, Dexter, JE, Sapirstein, HD (2005) Phenolic content and antioxidant activity of pearled wheat and roller-milled fractions. *Cereal Chemistry* **82**, 390-393.
- Bhatty, RS (Ed. AWBRS MacGregor (1993) 'Nonmalting uses of barley.'
- Bhatty, RS (1999) The potential of hull-less barley. *Cereal Chemistry* **76**, 589-599.
- Bjelakovic, G, Nikolova, D, Gluud, LL, Simonetti, RG, Gluud, C (2007) Mortality in randomized trials of antioxidant supplements for primary and secondary prevention - Systematic review and meta-analysis. *Journal of the American Medical Association* **297**, 842-857.
- Blandino, M, Locatelli, M, Sovrani, V, Coisson, DJ, Rolle, L, Travaglia, F, Giacosa, S, Bordiga, M, Scarpino, V, Reyneri, A, Arlorio, M (2015) Progressive pearling of barley kernel: Chemical characterization of pearling fractions and effect of their inclusion on the nutritional and technological properties of wheat bread. *J Agric Food Chem* **IN PRESS**,
- Block, G (1991) Vitamin C and cancer prevention- the epidemiologic evidence. *American Journal of Clinical Nutrition* **53**, S270-S282.
- Bramley, PM, Elmadfa, I, Kafatos, A, Kelly, FJ, Manios, Y, Roxborough, HE, Schuch, W, Sheehy, PJA, Wagner, KH (2000) Vitamin E. *Journal of the Science of Food and Agriculture* **80**, 913-938.
- Burton, GW, Ingold, KU (1981) Autoxidation of biological molecules. 1. The antioxidant activity of vitamin E and related chain-breaking phenolic

- antioxidants *in vitro*. *Journal of the American Chemical Society* **103**, 6472-6477.
- Byers, T, Guerrero, N (1995) Epidemiologic evidence for vitamin C and vitamin E in cancer prevention. *American Journal of Clinical Nutrition* **62**, S1385-S1392.
- Carlucci, G, Mazzeo, P, Governatore, Sd, Giacomo, Gd, Re, Gd (2001) Liquid chromatographic method for the analysis of tocopherols in malt sprouts with supercritical fluid extraction. *Journal of Chromatography, A* **935**, 87-91.
- Cavallero, A, Gianinetti, A, Finocchiaro, F, Delogu, G, Stanca, AM (2004) Tocols in hull-less and hulled barley genotypes grown in contrasting environments. *Journal of Cereal Science* **39**, 175-180.
- Chander, S, Guo, YQ, Yang, XH, Yan, JB, Zhang, YR, Song, TM, Li, JS (2008) Genetic dissection of tocopherol content and composition in maize grain using quantitative trait loci analysis and the candidate gene approach. *Molecular Breeding* **22**, 353-365.
- Chiou, WL, Peng, GW, Nation, RL (1977) Ethanol as mobile-phase solvent in high-performance liquid-chromatographic assays. *Clinical Chemistry* **23**, 2355-2355.
- Church, DC, Pond, WG (1977) 'Basic Animal Nutrition and Feeding.' (New York: Wiley:
- Cichos, F, Willert, A, Rempel, U, vonBorczykowski, C (1997) Solvation dynamics in mixtures of polar and nonpolar solvents. *Journal of Physical Chemistry A* **101**, 8179-8185.
- Delgado-Zamarreno, MM, Bustamante-Rangel, M, Sierra-Manzano, S, Verdugo-Jara, M, Carabias-Martinez, R (2009) Simultaneous extraction of tocotrienols and tocopherols from cereals using pressurized liquid extraction prior to LC determination. *Journal of Separation Science* **32**, 1430-1436.
- Dixon, ZR, Burri, BJ, Clifford, A, Frankel, EN, Schneeman, BO, Parks, E, Keim, NL, Barbieri, T, Wu, MM, Fong, AKH, Kretsch, MJ, Sowell, AL, Erdman, JW (1994) Effects of a carotene- deficient diet on measures of oxidative susceptibility and superoxide- dismutase activity in adult women. *Free Radical Biology and Medicine* **17**, 537-544.
- Do, TTD, Cozzolino, D, Muhlhausler, B, Box, A, Able, AJ (2015a) Antioxidant capacity and vitamin E in barley: effect of genotype and storage. *Food Chemistry* **187**, 65-74.
- Do, TTD, Cozzolino, D, Muhlhausler, B, Box, A, Able, AJ (2015b) Effect of malting on antioxidant capacity and vitamin E in different barley genotypes. *Journal of the Institute of Brewing* **121**, 531-540.
- Do, TDT, Muhlhausler, B, Box, A, Able, AJ (2016). Enrichment of antioxidant capacity and vitamin E in Pita made from barley. *Journal of Food Science* **81**, 777-785.
- Elsayed, AA, Peter, W (2005) 'Specialty grains for food and feed.' (American Association of Cereal Chemists, USA:
- Evers, AD, Blakeney, AB, O'Brien, L (1999) Cereal structure and composition. *Australian Journal of Agricultural Research* **50**, 629-650.
- Fernandez-Artigas, P, Guerra-Hernandez, E, Garcia-Villanova, B (2001) Changes in sugar profile during infant cereal manufacture. *Food Chemistry* **74**, 499-505.

- Fitzsimmons, RW, Wrigley, CW (2000) 'Australian barleys. Identification of varieties, grain defects and foreign seeds.' (Commonwealth scientific and industrial research organisation:
- Fratianni, A, Caboni, MF, Irano, M, Panfili, G (2002) A critical comparison between traditional methods and supercritical carbon dioxide extraction for the determination of tocochromanols in cereals. *European Food Research and Technology* **215**, 353-358.
- Frei, B (2012) 'Natural antioxidants in human health and disease.' (Academic Press Limited: London, United Kingdom)
- Frias, J, Miranda, ML, Doblado, R, Vidal-Valverde, C (2005) Effect of germination and fermentation on the antioxidant vitamin content and antioxidant capacity of *Lupinus albus* L. var. Multolupa. *Food Chemistry* **92**, 211-220.
- Fukuzawa, K, Inokami, Y, Tokumura, A, Terao, J, Suzuki, A (1998) Rate constants for quenching singlet oxygen and activities for inhibiting lipid peroxidation of carotenoids and alpha-tocopherol in liposomes. *Lipids* **33**, 751-756.
- Gassmann, B (2000) Dietary reference intakes, report 3: vitamin C and E, selenium, carotenoids. Overview, comments and comparison with the DACH reference values for nutrient intake. *Ernahrungs-Umschau* **47**, 265-268.
- Gong, LX, Jin, C, Wu, LJ, Wu, XQ, Zhang, Y (2012) Tibetan hull-less barley (*Hordeum vulgare* L.) as a potential source of antioxidants. *Cereal Chemistry* **89**, 290-295.
- Gottlieb, K, Zarling, EJ, Mobarhan, S, Bowen, P, Sugerman, S (1993) Beta-Carotene Decreases Markers of Lipid-Peroxidation in Healthy-Volunteers. *Nutrition and Cancer-an International Journal* **19**, 207-212.
- Goupy, P, Hugues, M, Boivin, P, Amiot, MJ (1999) Antioxidant composition and activity of barley (*Hordeum vulgare*) and malt extracts and of isolated phenolic compounds. *Journal of the Science of Food and Agriculture* **79**, 1625-1634.
- GRDC (2006) 'The WA Guide to High Moisture Harvest Management, Grain Storage and Handling.' (Group and South East Premium Wheat Growers Association:
- Gutteridge, JMC, Halliwell, B (2000) Free radicals and antioxidants in the year 2000 - A historical look to the future. In 'Reactive Oxygen Species: From Radiation to Molecular Biology: A Festschrift in Honor of Daniel L Gilbert.' (Ed. CC Chiueh.) Vol. 899 pp. 136-147.
- Hakansson, B, Jagerstad, M (1990) The effect of thermal inactivation of lipoxygenase on the stability of vitamin E in wheat. *Journal of Cereal Science* **12**, 177-185.
- Hakkarainen, RVJ, Tyopponen, JT, Bengtsson, SG (1983) Relative and quantitative changes in total vitamin-E and isomer content of barley during conventional and airtight storage with special reference to annual variations. *Acta Agriculturae Scandinavica* **33**, 395-400.
- Halliwell, B (1994) Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *Lancet* **344**, 721-4.
- Halliwell, B (1996) Antioxidants in human health and disease. *Annual Review of Nutrition* **16**, 33-50.
- Halliwell, B, Gutteridge, JMC (1989) 'Free Radicals in Biology and Medicine.' (Oxford University Press: New York)

- Heinonen, OP, Albanes, D, Virtamo, J, Taylor, PR, Huttunen, JK, Hartman, AM, Haapakoski, J, Malila, N, Rautalahti, M, Ripatti, S, Maenpaa, H, Teerenhovi, L, Koss, L, Virolainen, M, Edwards, BK (1998) Prostate cancer and supplementation with alpha-tocopherol and beta-carotene: Incidence and mortality in a controlled trial. *Journal of the National Cancer Institute* **90**, 440-446.
- Hennekens, CH, Buring, JE, Manson, JE, Stampfer, M, Rosner, B, Cook, NR, Belanger, C, LaMotte, F, Gaziano, JM, Ridker, PM, Willett, W, Peto, R (1996) Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. *New England Journal of Medicine* **334**, 1145-1149.
- Hertog, MGL, Feskens, EJM, Hollman, PCH, Katan, MB, Kromhout, D (1993) Dietary antioxidant flavonoids and risk of coronary heart- disease- the Zutphen elderly study. *Lancet* **342**, 1007-1011.
- Hidalgo, A, Brandolini, A (2010) Tocols stability during bread, water biscuit and pasta processing from wheat flours. *Journal of Cereal Science* **52**, 254-259.
- Holasova, M, Velisek, J, Davidek, J (1995) Tocopherol and tocotrienol contents in cereal grains. *Potravinarske Vedy* **13**, 409-417.
- Hollman, PCH, Devries, JHM, Vanleeuwen, SD, Mengelers, MJB, Katan, MB (1995) Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *American Journal of Clinical Nutrition* **62**, 1276-1282.
- Hoseney, RC (1994) 'Principles of cereal science and technology.' (American Association of Cereal Chemists: St. Paul, Minnesota, USA)
- Htwe, NN, Srilaong, V, Tanprasert, K, Tongchitpakdee, S, Kanlayanarat, S, Uthairatanakij, A (2010) Effects of storage time and temperature on radical scavenging activities and bioactive compounds in colored rice varieties. *Journal of Food Agriculture & Environment* **8**, 26-31.
- Hulse, JP (1979) 'Polyphenols in cereals and legumes, 36th Annual Meeting of the Institute of Food Technologists.' St. Louis, MO.
- Hunter, SC, Cahoon, EB (2007) Enhancing vitamin E in oilseeds: Unraveling tocopherol and tocotrienol biosynthesis. *Lipids* **42**, 97-108.
- Idaho Barley Commission (2011) 'Malt barley storage.' Available at [http://barley.idaho.gov/pdf/malt\\_barley\\_presentations/grain\\_storage.pdf](http://barley.idaho.gov/pdf/malt_barley_presentations/grain_storage.pdf) [Accessed 11.09.11].
- Ilona, B, Daina, K, Zanda, K (2011) 'Polyphenols and vitamin E as potential antioxidants in barley and malt, FOODBALT 2011.' Jelgava, Latvia.
- Izydorczyk, MS, Chornick, TL, Paulley, FG, Edwards, NM, Dexter, JE (2008) Physicochemical properties of hull-less barley fibre-rich fractions varying in particle size and their potential as functional ingredients in two-layer flat bread. *Food Chemistry* **108**, 561-570.
- Jane, J, Chen, II, Lee, LF, McPherson, AE, Wong, KS, Radosavljevic, M (1999) Effects of amylopectin branch chain-length distributions and amylose contents on the gelatinization and pasting properties of starches. *Cereal Chemistry* **76**, 629-637.
- Jenkins, DJA, Kendall, CWC, McKeown-Eyssen, G, Josse, RG, Silverberg, J, Booth, GL, Vidgen, E, Josse, AR, Nguyen, TH, Corrigan, S, Banach, MS, Ares, S, Mitchell, S, Emam, A, Augustin, LSA, Parker, TL, Leiter, LA (2008) Effect of a low-glycemic index or a high-cereal fiber diet on type 2 diabetes: a

- randomized trial. *JAMA, Journal of the American Medical Association* **300**, 2742-2753.
- Jin, L, Xiao, P, Lu, Y, Shao, YF, Shen, Y, Bao, JS (2009) Quantitative trait loci for brown rice color, phenolics, flavonoid contents, and antioxidant capacity in rice grain. *Cereal Chemistry* **86**, 609-615.
- Jones, DP (2002) Redox potential of GSH/GSSG couple: Assay and biological significance. *Protein Sensors and Reactive Oxygen Species, Pt B, Thiol Enzymes and Proteins* **348**, 93-112.
- Ko, S, Kim, C, Kim, H, Kim, C, Chung, S, Tae, B, Kim, I (2003) Tocol levels in milling fractions of some cereal grains and soybean. *Journal of the American Oil Chemists' Society* **80**, 585-589.
- Krinsky, NI (1993) Actions of carotenoids in biological systems. In 'Annual Review of Nutrition.' (Ed. RE Olson.) Vol. 13 pp. 561-587.
- Lampi, AM, Nurmi, T, Piironen, V (2010) Effects of the environment and genotype on tocopherols and tocotrienols in wheat in the HEALTHGRAIN diversity screen. *Journal of Agricultural and Food Chemistry* **58**, 9306-9313.
- Lampi, AM, Ryyanen, M, Salo-Vaananen, P, Ollilainen, V, Piironen, V (2004) A small-scale sample preparation method with HPLC analysis for determination of tocopherols and tocotrienols in cereals. *Journal of Food Composition and Analysis* **17**, 749-765.
- Layton, ME, Wagner, JK, Nelson, SR, Samson, FS, Pazdernik, TL (1992) Oxidative stress and antioxidant response in rat piriform cortex associated with kainic acid-induced seizures. *Society for Neuroscience Abstracts* **18**, 1603.
- Lehner, A, Mamadou, N, Poels, P, Come, D, Bailly, C, Corbineau, F (2008) Changes in soluble carbohydrates, lipid peroxidation and antioxidant enzyme activities in the embryo during ageing in wheat grains. *Journal of Cereal Science* **47**, 555-565.
- Leonard, SW, Good, CK, Gugger, ET, Traber, MG (2004) Vitamin E bioavailability from fortified breakfast cereal is greater than that from encapsulated supplements. *American Journal of Clinical Nutrition* **79**, 86-92.
- Levitsky, DA, Pacanowski, C (2011) Losing weight without dieting. Use of commercial foods as meal replacements for lunch produces an extended energy deficit. *Appetite* **57**, 311-317.
- Liu, KS, Moreau, RA (2008) Concentrations of functional lipids in abraded fractions of hullless barley and effect of storage. *Journal of Food Science* **73**, C569-C576.
- MacGregor, AW, Bhatta, RS (1993) 'Barley : chemistry and technology.' (American Association of Cereal Chemists: St. Paul, Minn.)
- Marnett, LJ (1987) Peroxyl free radicals: potential mediators of tumor initiation and promotion. *Carcinogenesis* **8**, 1365-73.
- Matthews, P, McCaffery, D (2011) Winter crop variety sowing guide 2011. Grain Research & Development Corporation, New South Wales.
- McAnlis, GT, McEneny, J, Pearce, J, Young, IS (1999) Absorption and antioxidant effects of quercetin from onions, in man. *European Journal of Clinical Nutrition* **53**, 92-96.
- McLaughlin, PJ, Weihrauch, JL (1979) Vitamin E content of foods. *Journal of the American Dietetic Association* **75**, 647-665.
- Menz, I (2010) 'Australian barley varieties. A reference guide.' NSW, Australia)

- Metz, N (2006) 'The WA Guide to High Moisture Harvest Management, Grain Storage and Handling.' (CBH Group and South East Premium Wheat Growers Association, Australia:
- Morad, MM, Doherty, CA, Rooney, LW (1984) Effect of sorghum variety on baking properties of U.S. conventional bread, Egyptian pita, "Balady" bread and cookies. *Journal of Food Science* **49**, 1070-1074.
- Murray, GM, Brennan, JP (2010) Estimating disease losses to the Australian barley industry. *Australasian Plant Pathology* **39**, 85-96.
- Netzel, M, Netzel, G, Tian, Q, Schwartz, S, Konczak, I (2006) Sources of antioxidant activity in Australian native fruits. Identification and quantification of anthocyanins. *Journal of Agricultural and Food Chemistry* **54**, 9820-9826.
- Nevins, CP, Vierck, JL, Bogachus, LD, Velotta, NS, Castro-Munozledo, F, Dodson, MV (2005) An inexpensive method for applying nitrogen evaporation to hexane-containing 24-or 96-well plates. *Cytotechnology* **49**, 71-75.
- Newman, CW (2002) Aeration - for preserving grain quality. Department of Agriculture Western Australia.
- Oliver, RE, Islamovic, E, Obert, DE, Wise, ML, Herrin, LL, Hang, A, Harrison, SA, Ibrahim, A, Marshall, JM, Miclaus, KJ, Lazo, GR, Hu, GS, Jackson, EW (2014) Comparative systems biology reveals allelic variation modulating tocopherol profiles in barley (*Hordeum vulgare* L.). *PLoS One* **9**,
- Omwamba, M, Hu, Q (2009) Antioxidant capacity and antioxidative compounds in barley (*Hordeum vulgare* L.) grain optimized using response surface methodology in hot air roasting. *European Food Research and Technology* **229**, 907-914.
- Ostertag, LM, O'Kennedy, N, Horgan, GW, Kroon, PA, Duthie, GG, de Roos, B (2011) In vitro anti-platelet effects of simple plant-derived phenolic compounds are only found at high, non-physiological concentrations. *Mol Nutr Food Res*
- Packer, L (1995) Nutrition and biochemistry of the lipophilic antioxidants, vitamin E and carotenoids. In 'Nutrition, Lipids, Health and Disease.' (Eds L Packer, E Niki, ASH Ong.) pp. 8-35. (American Oil Chemists Society Champaign IL)
- Panfili, G, Fratianni, A, Criscio, Td, Marconi, E (2008) Tocol and beta-glucan levels in barley varieties and in pearling by-products. *Food Chemistry* **107**, 84-91.
- Panfili, G, Fratianni, A, Irano, M (2003) Normal phase high-performance liquid chromatography method for the determination of tocopherols and tocotrienols in cereals. *Journal of Agricultural and Food Chemistry* **51**, 3940-3944.
- Paras, S, Gujral, HS (2011) Effect of sand roasting and microwave cooking on antioxidant activity of barley. *Food Research International* **44**, 235-240.
- Paynter, B, Hills, A, Gupta, S, Collins, S (2015) Barley varieties sowing guide for Western Australia. Department of Agriculture and Food. Western Australia.
- Pereira de Abreu, DA, Paseiro Losada, P, Maroto, J, Cruz, JM (2010) Evaluation of the effectiveness of a new active packaging film containing natural antioxidants (from barley husks) that retard lipid damage in frozen Atlantic salmon (*Salmo salar* L.). *Food Research International* **43**, 1277-1282.
- Peterson, DM (1994) Barley tocols: Effects of milling, malting, and mashing. *Cereal Chemistry* **71**, 43-44.
- Peterson, DM, Qureshi, AA (1993) Genotype and environment effects on tocols of barley and oats. *Cereal Chemistry* **70**, 157-162.

- Pinto, MdS, Lajolo, FM, Genovese, MI (2005) Effect of storage temperature and water activity on the content and profile of isoflavones, antioxidant activity, and *in vitro* protein digestibility of soy protein isolates and defatted soy flours. *Journal of Agricultural and Food Chemistry* **53**, 6340-6.
- Podsedeck, A, Wilska-Jeszka, J, Anders, B, Markowski, J (2000) Compositional characterisation of some apple varieties. *European Food Research and Technology* **210**, 268-272.
- Punda, I (Ed. D Prikhodko (2009) 'Barley Malt Beer.' (Food and Agriculture Organisation: New York)
- Quinde, Z, Ullrich, SE, Baik, BK (2004) Genotypic variation in color and discoloration potential of barley-based food products. *Cereal Chemistry* **81**, 752-758.
- Reboul, E, Richelle, M, Perrot, E, Desmoulins-Malezet, C, Pirisi, V, Borel, P (2006) Bioaccessibility of carotenoids and vitamin E from their main dietary sources. *Journal of Agricultural and Food Chemistry* **54**, 8749-8755.
- Reinheimer, JL, Barr, AR, Eglinton, JK (2004) QTL mapping of chromosomal regions conferring reproductive frost tolerance in barley (*Hordeum vulgare* L.). *Theoretical and Applied Genetics* **109**, 1267-1274.
- Rimm, EB, Katan, MB, Ascherio, A, Stampfer, MJ, Willett, WC (1996) Relation between intake of flavonoids and risk for coronary heart disease in male health professionals. *Annals of Internal Medicine* **125**, 384-389.
- Ronald, JM, Keller, DJ, Bibeau, LS, 1991. Process for preserving lemon juice utilizing a non-sulfite preservative. Borden, Tnc., Columbus, Ohio, United State. 5021251:
- Salomonsson, AC, Theander, O, Aman, P (1980) Composition of normal and high-lysine barleys. *Swedish Journal of Agricultural Research* **10**, 11-16.
- Serafini, M, Ghiselli, A, FerroLuzzi, A (1996) In vivo antioxidant effect of green and black tea in man. *European Journal of Clinical Nutrition* **50**, 28-32.
- Sheppard, AJ, Pennington, JAT, Weihrauch, JL (1993) Analysis and distribution of vitamin E in vegetable oils and foods. In 'Vitamin E in health and disease.' (Eds L Packer, J Fuchs.) pp. 9-31. (Marcel Dekker, Inc., New York:
- Shintani, D, DellaPenna, D (1998) Elevating the vitamin E content of plants through metabolic engineering. *Science* **282**, 2098-100.
- Skrbic, B, Milovac, S, Dodig, D, Filipcev, B (2009) Effects of hull-less barley flour and flakes on bread nutritional composition and sensory properties. *Food Chemistry* **115**, 982-988.
- Spano, C, Bottega, S, Lorenzi, R, Grilli, I (2011) Ageing in embryos from wheat grains stored at different temperatures: oxidative stress and antioxidant response. *Functional Plant Biology* **38**, 624-631.
- Su, WL, Kleinhanz, RR, Schadt, EE (2011) Characterizing the role of miRNAs within gene regulatory networks using integrative genomics techniques. *Molecular Systems Biology* **7**, 1-12.
- Sudha, ML, Vetrmani, R, Leelavathi, K (2007) Influence of fibre from different cereals on the rheological characteristics of wheat flour dough and on biscuit quality. *Food Chemistry* **100**, 1365-1370.
- Sumner, AK, Gebreegziabher, A, Tyler, RT, Rossnagel, BG (1985) Composition and properties of pearled and fines fractions from hulled and hull-less barley. *Cereal Chemistry* **62**, 112-116.

- Tang, S, Kerry, JP, Sheehan, D, Buckley, DJ, Morrissey, PA (2001) Antioxidative effect of added tea catechins on susceptibility of cooked red meat, poultry and fish patties to lipid oxidation. *Food Research International* **34**, 651-657.
- Tapsell, L, Williams, P, Droulez, V, Southee, D, Patch, C, Lethbridge, A (2005) Functional Foods for the Australian industry: definitions and opportunities. National Centre of Excellence in Functional Foods, 2005.
- Temple, NJ (2000) Antioxidants and disease: More questions than answers. *Nutrition Research* **20**, 449-459.
- Theriault, A, Chao, JT, Gapor, A (2002) Tocotrienol is the most effective vitamin E for reducing endothelial expression of adhesion molecules and adhesion to monocytes (vol 160, pg 21, 2002). *Atherosclerosis* **164**, 389-389.
- Topping, DL, Morell, MK, King, RA, Li, ZY, Bird, AR, Noakes, M (2003) Resistant starch and health - Himalaya 292, a novel barley cultivar to deliver benefits to consumers. *Starch-Starke* **55**, 539-545.
- Trogh, I, Courtin, CM, Goesaert, H, Delcour, JA, Andersson, AAM, Aman, P, Fredriksson, H, Pyle, DL, Sorensen, JF (2005) From hull-less barley and wheat to soluble dietary fiber-enriched bread. *Cereal Foods World* **50**, 253-260.
- Tyopponen, JT, Hakkarainen, RVJ (1985) Thermal stability of vitamin E in barley. *Acta Agriculturae Scandinavica* **35**, 136-138.
- U.S. Department of Agriculture (2011) 'Vitamin E content of selected food per common measure, sorted by nutrient content.' (U.S. Department of Agriculture, Agricultural Research Service, USA:
- Ullrich, SE, Baik, BK, Quinde-Axtell, Z, Nair, S (2008) Barley for food: traits and improvements. In 'International Barley Genetics Symposium. Egypt'. (Eds S Ceccarelli, S Grandi) (International Center for Agricultural Research in the Dry Areas:
- Varriamomaston, E, Ke, V, Huang, G, Ponte, J (1980) Comparison of methods to determine starch gelatinization in bakery foods. *Cereal Chemistry* **57**, 242-248.
- Viljoen, J (2001) Select grain stores carefully to control costs. *Farming Ahead* **119**, 44-45.
- Wanasundara, NU, Shahidi, F (1996) Stabilization of sea blubber and menhaden oils with green tea catechins. *Journal of American Oil Chemists' society* **42**, 1285-1290.
- Wang, JS, Rosell, CM, de Barber, CB (2002) Effect of the addition of different fibres on wheat dough performance and bread quality. *Food Chemistry* **79**, 221-226.
- Wang, L, Xue, Q, Newman, RK, Newman, CW (1993) Enrichment of tocopherols, tocotrienols, and oil in barley fractions by milling and pearling. *Cereal Chemistry* **70**, 499-501.
- Wang, M, Zheng, QS, Shen, QR, Guo, SW (2013) The critical role of potassium in plant stress response. *International Journal of Molecular Sciences* **14**, 7370-7390.
- Wheeler, R (2015) Barley variety sowing guide 2015. Primary Industries and Regions. South Australia.
- Willet, W (1994) Coffee and coronary heart disease. A new problem with old brew? *Annals of epidemiology* **4**, 497-8.



- Wong JC, Lambert RJ, Y, T (2003) QTL associated with accumulation of tocopherols in maize. *Crop Science* **43**, 2257–2266.
- Yang, B (2003) Natural vitamin E: activities and sources. *Lipid Technology* **125-130**,
- Yang, F, Basu, TK, Ooraikul, B (2001) Studies on germination conditions and antioxidant contents of wheat grain. *International Journal of Food Sciences and Nutrition* **52**, 319-330.
- Young, IS, Woodside, JV (2001) Antioxidants in health and disease. *Journal of Clinical Pathology* **54**, 176-186.
- Zeev, BA (1997) The chemistry of free radicals: peroxy radicals. In 'Radical Chemistry.' Vol. 5 pp. 546. (Wiley, New York:
- Zhao, H, Fan, W, Dong, J, Lu, J, Chen, J, Shan, L, Lin, Y, Kong, W (2008) Evaluation of antioxidant activities and total phenolic contents of typical malting barley varieties. *Food Chemistry* **107**, 296-304.
- Zielinski, H, Ciska, E, Kozłowska, H (2001) The cereal grains: focus on vitamin E. *Czech Journal of Food Sciences* **19**, 182-188.

# Appendices

## **Appendix A**

Statement of Authorship

Manuscript 1 Chapter 3

Title of paper	Antioxidant capacity and vitamin E in barley: genotypic dependence and effect of storage.
Publication status	Accepted for publication
Publication details	DO, T. T. D., COZZOLINO, D., MUHLHAUSLER, B., BOX, A. & ABLE, A. J. 2015. Antioxidant capacity and vitamin E in barley: effect of genotype and storage. <i>Food Chemistry</i> , 187, 65-74.

### Author contribution

By signing the statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of principal author (Candidate)	Thi Thu Dung Do		
Contribution to the paper	Performed the majority of experimental work, data interpretation and wrote manuscript.		
Signature		Date	24/9/2015

Name of Co-author	Daniel Cozzolino		
Contribution to the paper	Contributed to MIR work and helped to write the relevant section of the manuscript.		
Signature		Date	24/9/2015

Name of Co-author	Beverly Muhlhausler		
Contribution to the paper	Supervised work development and critical assessment of the manuscript.		
Signature		Date	24/9/2015

Name of Co-author	Amanda Box		
Contribution to the paper	Supervised work development, provided material and critical assessment of the manuscript.		
Signature		Date	24/9/2015

Name of Co-author	Amanda J. Able		
Contribution to the paper	Principal supervisor of the project, helped in data interpretation, critical assessment and editing of the manuscript. Acted as corresponding author.		
Signature		Date	25/9/2015

**Appendix B**

Statement of Authorship

Manuscript 2 Chapter 4

Title of paper	Effect of malting on antioxidant capacity and vitamin E in different barley genotypes.
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### Author contribution

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Name of principal author (Candidate)	Thi Thu Dung Do		
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Contribution to the paper	Principal supervisor of the project, helped in data interpretation, critical assessment and editing of the manuscript. Acted as corresponding author.		
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### Appendix C

Statement of Authorship

Manuscript 3 Chapter 5

Title of paper	Enrichment of antioxidant capacity and vitamin E in pita made from barley
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Contribution to the paper	Principal supervisor of the project, helped in data interpretation, critical assessment and editing of the manuscript. Will acted as corresponding author.		
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**Appendix D**

Statement of Authorship

Manuscript 4 Chapter 6

Title of paper	Quantitative trait loci for antioxidant capacity and vitamin E content in barley.
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**Author contribution**

By signing the statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of principal author (Candidate)	Thi Thu Dung Do		
Contribution to the paper	Performed the majority of experimental work, data interpretation and wrote manuscript.		
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