Wholegrains and traditional bread making processes to enhance health benefits of baked goods

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Oliver Buddrick
April 2014
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Publications and presentations

Parts of the research reported in this thesis have been published and the details are as follows:

**Journal articles already published**


**Journal article accepted for publication**

Buddrick O, Cornell HJ, Small DM. (2014). Reduction of toxic gliadin content of wholegrain bread by the enzyme caricain. Food Chemistry

**Journal articles prepared for submission**


**Refereed conference proceeding papers**


**Other conference presentations**


Abstract

The processes of grain milling as well as bread making both have a very long history and the evolution of current practices has seen many changes in both processing and formulation of baked goods. White flour is preferred as the primary ingredient rather than wholemeal. In addition there has been a trend to abandon sourdough fermentation and adopt rapid bread making procedures. However, growing evidence indicates that traditional approaches offer significant health advantages to consumers.

Accordingly, the purpose of this project was to investigate the significance of various aspects of bread making in relation to the health implications of bread. For this purpose loaves was prepared by applying bulk and sourdough fermentation using freshly milled whole grains. Various components were investigated and the effects of fermentation time and temperature evaluated for selected blends of wholemeal grains. In order to study the stability and retention of vitamin E components, the possibility and advantages of replacing hexane with the less toxic (and greener) option of heptane for HPLC analysis was evaluated. Strategies to increase the quantity of vitamin E were found and the optimal fermentation conditions established. The use of palm oil as an ingredient in bread making was demonstrated as a means to increase the vitamin E content of wholemeal breads and a further advantage was the wider range of E vitamers retained during baking.

Two components for which there is increasing evidence of health implications are phytate and resistant starch (RS) formation. The effects of fermentation time and temperature on these during bread making for selected wholemeal flours from rye, oats and wheat were also investigated. Fermentation temperature was found to make only a small difference to the degree of phytate reduction and the formation of (RS). The presence of palm oil in formulations decreased the RS content in rye bread when added at 8 %. The rye sourdough fermentation gave the greatest formation of RS and temperature also influenced the levels to some extent. Rye sourdough fermentation because of the lowering of pH appeared to have a greater effect on RS formation than that observed for bulk fermented wholemeal breads.
Abstract

A further series of experiments were carried out to evaluate the detoxification of gliadin by a partially purified preparation of the papaya enzyme caricain. The incorporation of the enzyme directly into wholemeal wheat dough demonstrated that caricain is able to detoxify wheat to a level at where it is less of a problem for coeliac sufferers and this approach warrants further investigation in baking as well as other processes using cereal grains, particularly wheat and rye.

Accordingly, the results of the phases of this investigation show that some of the more traditional approaches in milling and baking do have potential as ways to enhance the nutritional profile of baked goods. Various ways to provide health benefits are therefore recommended and these impact upon the vitamin E and resistant starch contents of breads.
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List of abbreviations

µg  Microgram
µL  Microliter
α-T  α-tocopherol
β-T  β-tocopherol
γ-T  γ-tocopherol
δ-T  δ-tocopherol
α-T3  α-tocotrienol
β-T3  β-tocotrienol
γ-T3  γ-tocotrienol
δ-T3  δ-tocotrienol
AA  Ascorbic acid
AACC  American Association of Cereal Chemists
AI  Adequate Intake
AOAC  Association of Official Agricultural Chemists
APCI  Atmospheric-pressure ionization
ASE  Accelerated solvent extraction
DRI  Dietary reference intake
FDA  Food and Drug Administration
EPS  Exopolysaccharides
GRDC  Grains Research Development Corporation
HPIC  High performance ion-exchange chromatograph
HPLC  High performance liquid chromatography
HDL cholesterol  High density lipoprotein cholesterol
ICC  International Association for Cereal Science and Technology
LAB  Lactic acid bacteria
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>LDL cholesterol</td>
<td>Low density lipoprotein cholesterol</td>
</tr>
<tr>
<td>NP-HPLC</td>
<td>Normal-phase high performance liquid chromatography</td>
</tr>
<tr>
<td>PLE</td>
<td>Pressurized liquid extraction</td>
</tr>
<tr>
<td>RDA</td>
<td>Recommended dietary allowance</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reversed-phase high performance liquid chromatography</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>UL</td>
<td>Upper Intake</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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Explanatory notes

The purpose of these notes is to briefly describe the approaches adopted during the preparation of this thesis. They relate to the calculation and expression of enzyme activity data, spelling, as well as the referencing of literature sources:

1. Where alternative spellings are in common use the British rather than the American approach has been adopted in the text. Examples include words ending with –ise (rather than –ize) and some technical terms.

2. Typically, calculations relating to the concentration of phytate, vitamin E and gliadin toxins were expressed on a dry weight basis unless otherwise specified. This was done to ensure that a direct comparison could be performed between results obtained at different stages of processing for wholegrain bread.

3. Wholegrain and wholemeal: these two terms are used interchangeably. They describe the ground/milled product obtained when all the grain components (bran, germ and endosperm) are incorporated. The ground, powdered ingredient is then used in bread making and other food processes.

4. In the citation and listing of references and information sources, the current recommendations to authors for the relevant journal have been applied. This includes Plant Foods for Human Nutrition published by Springer (Chapter 7), RSC Advances by RSC Publishing (Chapter 8), Journal of Cereal Science published by Elsevier (Chapter 9), Journal of Agricultural and Food Chemistry (Chapter 10), Food Chemistry published by Elsevier (Chapter 11). For the remaining Chapters, the recommendations of the American Psychological Association (APA) have been used.
Chapter 1

Introduction

The purpose of this chapter is to outline the broad context within which this research program was developed. Among the significant issues considered were:

• Wholegrain foods have been the focus of significant scientific, governmental and commercial interest during the past 10 years since epidemiological studies have increasingly shown their protective role against the risk of many chronic diseases;

• Wheat is often regarded as an important dietary source of vitamin E as are cereals in general. During manufacture of wheat products for human consumption, a marked reduction in vitamin E content occurs. Accordingly one of the purposes of the current research is to investigate this component in breads prepared from freshly milled whole grains and the addition of palm oil to increase the vitamin E content. Research has increasingly highlighted the importance of vitamin E in human health and nutrition;

• Vitamin E is essential to human health and it is possible that coeliac disease patients which are deficient in vitamin E may be helped by dietary supplementation rich in vitamin E;

• The more traditional forms of fermentation, including sourdough, may have the potential to enhance the health benefits of cereal foods in a number of ways: enhancing texture and palatability of wholegrain, retarding of starch bioavailability (products with low glycaemic index) and enhancing mineral bioavailability (reduced phytate levels);

• Long-time fermentation process as well as sourdough production may also promote retrogradation of starch, hence increase the resistant starch formation in the finished products;
• Wholegrain products are known to have high phytate content and they have also recently been recognised for a variety of possible benefits to human health;

• Wholegrain contains more dietary fibre than refined flour;

• Increasingly the number of individuals being diagnosed with some form of sensitivity to the proteins in wheat grains represents a cause for concern. The most commonly reported condition has been the sensitivity to gliadins which is known as coeliac disease although other sensitivities as well as allergic responses to wheat have also been identified. Whilst the formulation of baked goods without the use of wheat proteins is possible there are potentially other strategies which will effectively detoxify the wheat based ingredients during processing and provide solutions for those who suffer from these conditions.

In the context of the many changes that have occurred in milling and bread making over the centuries, along with increasing evidence of the potential health benefits of some of the more traditional approaches to processing, the broad aim of this project has been to investigate the significance of various aspects of bread making in relation to the health implications of breads.
Chapter 2

Background and literature review: from grain to the science of bread making

The purpose of this chapter is to provide the background of the research and to review current knowledge on the significance and utilisation of cereal grains, particularly for the production of wholegrain bread. A brief explanation of the various cereal grains used for bakery products is given, along with a description of the primary ingredients used to produce bread.

2.1 Introduction

The cereals are members of the grass family known botanically as Poaceae and previously referred to as Gramineae. Wheat is the most widely cultivated cereal grain and is grown in more than 120 countries (Shewry, 1998). Commonly used in the production of bread, wheat is also the primary ingredient of a variety of other food products including cakes, crackers as well as pasta and a wide range of Asian noodle products (Bushuk and Rasper 1994).

The first cultivation of wheat probably occurred around 10,000 years ago, as a part of the ‘Neolithic revolution’, which saw the transition from hunting and gathering of food to settled agriculture. These earliest cultivated forms of wheat were diploid (genome AA) (einkorn) and tetraploid (genome AABB) (emmer) wheats and their genetic relationship indicates that they originate from the south-eastern part of Turkey (Dubcovsky & Dvorak, 2007). European researchers analysed the composition of einkorn and found that it contains high levels of carotenoids and Vitamin E vitamers. Significant levels of lipophilic antioxidants were also present, providing further evidence that wheat, especially older varieties, enjoys significant nutritional advantages compared to other cereal grains (Hejtmánková, Lachman, Hejtmánková, Pivec, & Janovská, 2010; Hidalgo, Brandolini, Pompei, & Piscozzi, 2006).

Cultivation of wheat spread to the near east approximately 9,000 years ago when varieties with hexaploid genotypes were being used to produce bread (Shewry, 1998). Spelt wheat (Triticum aestivum ssp. spelta) originated from Poland and is an ancient crop that is still
grown in several countries of central Europe. It demonstrates a higher resistance to environmental factors than common wheat and can be cultivated in harsher conditions, sometimes without the use of pesticides (Bonafaccia et al., 2000). However, Spelt may not be as nutritious as other varieties. For example, the levels of vitamin E compounds in spelt bread were half of those found in common wheat rolls (Zielinski, Ceglinska, & Michalska, 2008).

Rye (*Secale cereale*) is a cereal crop grown extensively as a source of grain and also as a forage crop. It is a member of the wheat tribe (Triticeae) and is closely related to barley (*Hordeum sp*) and wheat (*Triticum sp*). Rye grain is used for rye bread, rye beer, some whiskeys, some vodkas, and animal fodder. It can also be eaten whole, either as boiled rye berries, or by being rolled, similar to rolled oats (Slavin, Jacobs, & Marquart, 2001). Rye bread is still very widely consumed in central and eastern Europe.

The common oat (*Avena sativa*) is another temperate species of cereal grown for its grain, which is known by the same name (unlike other grains usually in the plural viz oats). While oats are suitable for human consumption as oatmeal and rolled oats, one of the most common uses is as livestock feed. Oats contain more soluble fibre than any other grain, resulting in slower digestion and an extended sensation of fullness (Paralkar, & Sengupta, 2013). One particular form of soluble fibre, beta-glucans, helps lower cholesterol (Jensen et al., 2004). Oat bran is the outer casing of the oat and its consumption is believed to lower particular cholesterol components which have adverse health associations and possibly to reduce the risk of heart disease (Jensen et al., 2004).

Australia is a major agricultural producer and exporter of cereal grains (Table 2.1). Agriculture and its closely related sectors earn $155 billion-a-year for a 12% share of GDP (Food and Agriculture Organisation, 2011). Land under cereal production (hectares) in Australia was last measured at 19,424,894 hectares in 2012 (World Bank, Trading Economics, 2013). In this context it is worth noting that land under cereal production refers to the harvested area in this case but some other countries report only sown or cultivated area. The importance of cereal grains in Australian agriculture is further emphasized in Figure 2.1.
Table 2.1  The annual production for Australia and the world of cereal grains 2011, 1,2

<table>
<thead>
<tr>
<th>Cereal crop</th>
<th>Australia</th>
<th>World</th>
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<tbody>
<tr>
<td>Barley</td>
<td>7,994,720</td>
<td>134,279,415</td>
</tr>
<tr>
<td>Oats</td>
<td>1,127,680</td>
<td>22,504,708</td>
</tr>
<tr>
<td>Rye</td>
<td>39,750</td>
<td>12,948,840</td>
</tr>
<tr>
<td>Triticale</td>
<td>355,078</td>
<td>13,384,848</td>
</tr>
<tr>
<td>Wheat</td>
<td>27,410,100</td>
<td>704,080,283</td>
</tr>
</tbody>
</table>

Notes 1  Data are expressed in units of tonnes per annum  
2  Source of data: (Food and Agriculture Organisation, 2011)

![Figure 2.1](image_url)  
A comparison of current production of Australian crops in 2011  
(Food and Agriculture Organisation, 2013)

2.2 Wholegrain

The terms wholegrain and wholemeal are now widely used to refer those flours and products involving the incorporation of each part of the original grain including bran, germ and endosperm. Wholegrain foods have been the focus of significant scientific, governmental and commercial interest during the past 10 years since epidemiological studies have increasingly shown their protective role against the risk of many chronic diseases (Shewry, 2009; Winter,
2009). Greater knowledge on the part of consumers of the association between a nutritious diet and health and wellbeing has been the main driver for the increase in popularity of wholegrain foods. The nearly ubiquitous consumption of wholegrain all over the world gives it an important position in international nutrition. Besides the high starch content, as an energy source, cereals provide dietary fibre, nutritious protein and lipids rich in essential fatty acids. Important micronutrients present in cereals include vitamins, especially many of the B vitamins, minerals, antioxidants such as vitamin E as well as phytochemicals (Dewettinck et al., 2008).

2.3 From grain to meal

Globally, the most common starting material for bread is wheat flour but a wide variety of other cereal grains, including rye, barley, oats and corn may be used. The flour milling process as it is typically known and practiced today evolved considerably during the years 1830 - 1870 (Cutler, 1953). Prior to that time milling equated to grinding and the resultant meal was a wholemeal material. Today almost all grain products have been refined in some way or another (Nagyová, Rovný, Stávková, Uličná, & Maďarova, 2009). The white flour popular today is made by processing natural whole grain, removing the outer layers which contain the majority of the micronutrients (Kristensen et al., 2010).

In the context of increasing global interest in utilising wholemeal ingredients, there has been renewed focus on the nutritional significance of the bran components. As an example, a group of Swedish researchers recently studied vitamin E vitamers. They reported that the steaming and flaking of dehulled oat groats (hulled kernels) resulted in moderate losses of tocotrienols but tocopherols were not affected. Drum drying of steamed rolled oats also resulted in an almost complete loss of all of the components studied. However, autoclaving of grains including the hulls caused increased levels of all tocopherols and tocotrienols analysed except β-tocotrienol, which was not affected (Bryngelsson, Dimberg, & Kamal-Eldin, 2002).
2.4 Wholegrain bread in health and disease

In a recent review in most epidemiological studies, wholegrain ingredients are associated with reduced risk of many chronic diseases (Jones, 2011). This confirms the conclusion of an extensive meta-analysis (Mellen et al., 2007). From the study, it has been found that whole-grain food intake was inversely associated with common carotid intimal medial thickness in the middle-aged, multiethnic cohort. This was not attributable to individual risk intermediates, single nutrient constituents or larger dietary patterns. Foods and food patterns of consumption act synergistically to influence the risk of a variety of chronic diseases (Jacobs & Steffen, 2003). Whole grain consumption and the risk of disease are presented as a model of food synergy which has been defined as having additive or indeed greater than additive, influences of foods and food constituents on health.

There is accumulating evidence that overall health appears to be better with increased consumption of wholegrain foods rather than those involving refined grain ingredients (De Munter, Hu, Spiegelman, Franz, & Van Dam, 2007; Esmaillzadeh, Mirmiran, & Azizi, 2005); that is, benefit accrues when all edible parts of the grain such as bran, germ and endosperm, are consumed (Mellen, Walsh, & Herrington, 2008). In addition, risk is further reduced if wholegrain foods are consumed in a diet that is otherwise high in plant foods. These researchers have suggested that phytochemicals located within the fibre matrix, in addition to, or instead of the fibre itself, may be responsible for the reduced risk (Mellen, Walsh, & Herrington, 2008).

2.5 Functional ingredients in bread making

An alternative term for the functional ingredients in common usage as ingredients for bread formulations is bread improver. These have been common ingredients in bread since the early 1950s and may be incorporated singly, or as a combined ingredient. The primary role is to facilitate and speed up bread production. Every ingredient used in bread-making has a function and in the alternative sense, enhances the quality such as freshness, softness and mouth-feel of the resultant products. While the ingredients of improvers can vary widely depending on their use and the manufacturer, a few important ingredients are found in many improvers around the world: ascorbic acid is used to strengthen the gluten; cysteine hydrochloric acid and sodium metabisulfite are added for gluten softening and clearing,
ammonium chloride and phosphates are used as food for yeast, amylases are used to hydrolyse starch into simple sugars, thereby facilitating rapid proliferation of yeast to enhance fermentation and protease enzymes may be used to improve extensibility of the dough. These ingredients are usually distributed in soy flour (filler), as the amounts of some of the different ingredients can be as little as 120 mg per kg.

2.5.1 Yeast

Bakers yeast (Saccharomyces cerevisiae) is available in a number of different forms. Cream yeast is the closest form to the yeast slurries of the 19th century, being essentially a suspension of yeast cells in liquid, siphoned off from the growth medium used in their production. Its primary use is in industrial bakeries with special high volume dispensing and mixing equipment and it is not readily available to small bakeries or home cooks. Compressed yeast is essentially cream yeast with most of the liquid removed whereas active dry yeast is the form of yeast most commonly available to non-commercial bakers.

2.5.2 Salt

Salt (sodium chloride, NaCl), is a white crystalline material, being essentially soluble in both hot and cold water and having a characteristic taste. Generally, the amount of salt used in a bread dough formulation is 1.8 to 2% based on flour weight. Salt is used in the making of bread for various reasons. Firstly, to give the necessary flavour; in addition to its own, saline flavour, the presence of salt stimulates the capacity of the palate for recognising flavours of other substances (Beck, Jekle, & Becker, 2012). Thus, minute quantities of sugar are recognised in the presence of salt which in its absence would be unnoticed (Burseg, Brattinga, de Kok, & Bult, 2010). This is doubtless is one of the reasons for the importance of salt as a flavouring agent in cooking in general.

Secondly, salt actively controls some of the chemical changes which proceed during fermentation; thus, salt, in the quantities employed in bread making, produces a binding effect on gluten of the dough (Miller & Hoseney, 2008). It retards the conversion of the starch of the flour into dextrin and maltose. Salt also controls alcoholic fermentation (Lynch, Dal Bello, Sheehan, Cashman, & Arendt, 2009). The retarding influence of salt extends to other reactions such as the development of lactic acid so tends to prevent undesirable fermentation occurring in the dough.
A third aspect of salt in bread relates to its significant role in the development of the gluten matrix (McCann & Day, 2013; Tuhumury, Small, & Day, 2014). Numerous studies have indicated that salt increases the mixing tolerance of wheat flour dough, extends the dough development time and increases the dough resistance, elasticity and extensibility (Uthayakumaran, Batey, Day, & Wrigley, 2011).

One of the compelling reasons for seeking a greater understanding of the function of salt is the increasing evidence of adverse health effects of current levels of salt consumption (Dotsch et al., 2009; Miller & Hoseney, 2008).

2.5.3 Fat and oil

Fats and oils are the primary enriching ingredients which are used in bread recipes to change the character of the resulting dough. For example, they are used to enhance gas retention and thereby increase both volume and softness. The levels used will vary according to the type of flour, with wholemeal flours typically requiring higher levels of fat or oil addition than white, often two to three times more. A fat content of approximately 3% by weight is the concentration that will produce the greatest leavening action. In addition to their effects on leavening, fats also serve to tenderise breads and preserve freshness (Cauvain, 2003). Interestingly, canola oil is increasingly used in a wide range of food applications and this crop is the leading oilseed produced in Australia currently (Figure 2.1). Over a million tonnes of this commodity is exported annually.

2.5.4 Water

The properties of the dough will vary according to the level of added water. In particular in the processing of wholemeal dough, if there is, too little water and the dough will be firm and difficult to mould, producing breads that have small volume and poor external appearance. On the other hand, with too much water, the dough will be soft and also difficult to mould; it will tend to flow in the prover and give poor quality bread (Cauvian, & Young, 2008). The optimum level of water is really the maximum quantity that can be incorporated into a dough and still be able to mould the pieces and give bread of acceptable quality.
2.6 Bread making today

Bread produced from cereal grains has accompanied humanity for many centuries, wherever it has been possible to grow or import grain. There is no record of when or where bread first originated but baked products have evolved to take many forms, each having its own particular characteristics. In very early times, whole grain was pounded and eaten as a watery paste, without further processing. Later the grain was ground in primitive mills and the resultant products were sieved prior to mixing into dough. This was then used to form flat bread that was baked on hot stones or directly in a fireplace (Piponnier, 1999).

Bread making is a centuries old traditional craft, practiced in any country that is able to either grow or import wheat. Today bread making in most western and in many eastern countries is mechanized (Belderok, 2000). The process of bread manufacture can be divided into a few major parts, each of which is of equal significance in producing an acceptable end product: dough making and baking. Dough making includes dough mixing of wheat flour, water, yeast and other functional ingredients and the expansion of the dough mass through the generation of carbon dioxide gas development and leavening. Recently, domestic bread makers that automate the process of making bread have become popular in the home.

2.6.1 Wheat bread

Whole wheat bread is a type of bread made using flour that is partly or entirely made from whole or almost whole wheat grains. Other varieties are made of refined flour, bran and germ removed and lacks thousands of healthy phytochemicals including vitamins and minerals. Nutritionally, whole grain bread is superior to refined wheat bread (Shewry, 2009).

2.6.2 Rye bread

In countries where rye bread is a staple, it often is claimed to be nutritionally superior to wheat bread. In the production of rye bread consisting either entirely or partly of rye flour, the so called sourdough fermentation is used. A sourdough is one that is acidified and leavened by its micro-organisms. Sourdough performs two functions in rye bread: acidification and flavour development. Acidification of the dough results from the action of lactic acid bacteria. Such acidification is essential for best results in baking rye bread; a high ratio of lactic acid to acetic acid is desirable (Lindhauer & Dreisoerner, 2003).
2.6.3 Oat bread

There are varieties of oat meal breads which claim to be suitable for those with celiac disease on the market in 2014. However, the doughs produced are heavy and not easily moulded. It is not possible to produce a loaf of bread with 100% oat meal/flour without using other functional ingredients such as wheat gluten (toxic to coeliac) or hydrocolloids. The addition of oat meal (up to 30%) to wheat bread may contribute to the reduction of gliadin toxicity (Pulido et al., 2009).

2.7 Summary of current utilization of cereals in bread making

While several cereals can be used in producing leavened baked goods, most of our daily bread is made from milled wheat products. However, in different parts around the world including Australia, other cereal grains including oat, rye and barley are used in bread making formulations. The basic functional ingredients to produce bread are flour, salt, yeast and water. Recently, the inclusion of wholegrain/wholemeal in bakery products has the protective roles against the risk of many chronic diseases (Belobrajdic & Bird, 2013). To increase the health benefit of wholegrain bakery product, several processes can be applied to further enhance health benefits of baked goods. These health beneficial bread making processes will be discussed in the following chapter.
References


Chapter 2


Chapter 3

Background and literature review: potential ways to enhance health benefits of wholegrain baked goods

The purpose of this chapter is to provide background and review current knowledge on various aspects of wholegrain cereals having potential health implications. The areas reviewed encompass a brief description of the fermentation processes, particularly sourdoughs and their impact on phytates as well as the toxicity of gliadin in wholegrain bakery products. The possibilities for detoxification of gliadin using an enzyme treatment will also be discussed along with the potential nutritional benefits of reduced starch digestibility.

3.1 Introduction to sourdough fermentation

Cereal grains are usually eaten after grinding and mixing with water. Such a mixture, resulting in the formation of a dough and characterised by sour aroma when left on its own for a while, may have been the first example of fermented food employed by mankind. The earliest evidence of baking of leavened dough has been dated at around 1,500 BC from mural Egyptian paintings, although sourdough bread was probably part of the European diet 5,000 years ago (Corsetti & Settanni, 2007).

Sourdough is useful in enhancing the texture and palatability of wholegrain (Vieno, 2011) and fibre rich products and it may stabilise or increase the level of bioactive compounds (Katina et al., 2005). In order to generate sufficient amounts of gaseous compounds, the fermentation process requires multiple steps of approximately 12-24h. By comparison, when bakers yeast is used in modern bakeries the fermentation is completed within a few hours. Bacterial proteolysis during sourdough fermentation contributed much more to the development of typical sourdough flavours of baked breads when compared to bread produced from chemically acidified or yeast doughs (Gänzle, Loponen, & Gobbetti, 2008; Wieser, Vermeulen, Gaertner, & Vogel, 2008).
Mathematical modelling of sourdough fermentation design can be used to define process conditions which enable the combination of health benefits with good sensory quality. In addition, enzymes present during bread making originate from different sources including those that already existing in the flour, those associated with the metabolic activity of yeast or lactic acid bacteria (LAB) and those intentionally added to the formulation. For example, amylase is added to increase the degree of saccharification (the process of breaking a complex carbohydrate (as starch or cellulose) into its monosaccharide components) (Juodeikiene et al., 2012). Microbial lipase is used to modify the lipid components in order to produce emulsifying compounds which influence texture and volume of baked loaves (Martínez-Anaya, 1996).

3.2 **Bacterial sourdough fermentation**

During sourdough fermentation, LAB produce a number of metabolites which have positive effects on the texture as well as retarding the staling of bread (Flander, Suortti, Katina, & Poutanen, 2011), e.g. organic acids, exopolysaccharides (EPS) and/or enzymes. EPS produced by LAB have the potential to replace more expensive hydrocolloids used as bread improvers (Arendt, Ryan, & Dal Bello, 2007). In contrast to the use of predominantly homofermentative species of LAB in the majority of fermented food applications, heterofermentative species play a major role in sourdough fermentation, especially when sourdoughs are prepared in a traditional manner (Gobbetti, De Angelis, Corsetti, & Di Cagno, 2005). As many as 50 different species of LAB have been isolated from sourdough. Some of these, including Lactobacillus reueri and L acidophilus, may have originated from intestinal sources by cross-contamination.

3.3 **Lactobacilli and coeliac disease**

Coeliac disease (CD) is a life-long intolerance to gluten proteins, with a prevalence of 1-2 % worldwide and having health consequences if not treated (Mendoza & McGough, 2005). Currently the treatment is dietary gluten withdrawal, but commercial gluten free foodstuffs present undesirable textural properties. LAB have been shown to possess a strong potential in decreasing coeliac disease and the effects of gliadin toxicity (Calasso et al., 2012; Gobbetti, Giuseppe Rizzello, Di Cagno, & De Angelis, 2007). Di Cagno et al. (2004) demonstrated active hydrolysis of various proline-rich peptides, including a particular peptide chain that induces sensitivity reactions, by some *Lactobacillus*
species. Moreover long-time fermentation of dough by selected LAB could be considered as a potential tool to decrease the risk of rye contamination of gluten-free products for coeliac patients (Angelis et al., 2006; De Angelis et al., 2006). Long-time fermentation by sourdough and enzymic modification have been attempted to modify the immunogenic sequence of gluten in order to avoid recognition by the immune system (Cabrera-Chávez & Calderón de la Barca, 2010). Baked goods made from hydrolysed wheat flour are not toxic to coeliac diseased patients (Greco et al., 2011). Wheat flour was fermented with sourdough lactobacilli and fungal proteases were also incorporated into the formulation. While the study was limited in scope, this process decreased the concentration of gluten and patients who ate the fully hydrolysed baked goods had no clinical symptoms, indicating the potential of this approach.

3.4 Enzyme treatment to reduce gliadin toxins

In a recent study by Cornell et al. (Cornell, Doherty, & Stelmasiak, 2010) another enzyme source was utilised in order to hydrolyse gluten components during breadmaking. Using papaya latex as the starting material, an enzyme preparation rich in the caricain (EC 3.4.22.30) was obtained by a series of steps involving ion exchange chromatography on CM Sephadex-C50, size exclusion chromatography with Sephareryl S-300 and size exclusion HPLC. Assays of the resultant partially purified enzyme provided an insight into the relative contribution of the enzymes present in detoxifying gluten. It was demonstrated that the benefits were due largely to caricain and to a lesser extent, chymopapain and glutamin cyclotranferase. These studies have provided a basis for further evaluation of caricain as a means of reducing gluten in foods for those sensitive to such components (Cornell, Doherty, & Stelmasiak, 2010).

3.5 Phytate

Another aspect of grain, particularly wholemeal products, is the presence of phytate as a molecule having significance in human health and life. In relation to nomenclature phytate is one of various salt forms of phytic acid. In this thesis, the terms phytae and phytic acid are used interchangeably. Phytic acid (inositol hexaphosphate, InsP₆) represents the major storage form of phosphorus (P) and inositol in plants and has also been suggested as a storage of trace elements in plants (Lewis, 1981). In cereals, phytate is concentrated in the aleurone layer and the scutellum cells of the germ, while the
starchy endosperm is almost devoid of phytate. In cereals, this component is typically constitutes 0.5-2 % of the grain dry weight (Reddy, Sathe, & Salunkhe, 1982).

The phytate molecule consists of inositol esterified with six phosphoric acid groups. There are nine possible molecular stereoisomers of inositol: cis-, epi-, alio-, neo-, myo-, muco-, D-chiro-, L-chiro- and scyllo-inositol, depending whether the various phosphate groups are in axial or equatorial positions. Figure 3.1 shows the structure of the naturally occurring form which is myo-inositolhexaphosphoric acid.

![Fig. 3.1 The structure of myo-inositolhexaphosphoric acid (phytic acid)](image)

3.5.1 The discovery of phytate

Phytate was discovered as long ago as 1855 when Hartig isolated small particles of non-starch grains from several plant seeds (Reddy & Sathe, 2002). Later researchers (Pfeffer, 1872) further characterised the grains isolated by Hartig into three groups: crystals of calcium oxalate, a protein substance and “globoids”, which was a nitrogen free organic substance containing calcium, magnesium and phosphorus. The “globoids” were later named as “inosite-phosphoric acid” as they could be hydrolysed into inosite and phosphorus (Winterstein, 1897). Phytate is now more fully referred to as myo-inositolhexakisphosphate, commonly abbreviated as IP6, which is a naturally occurring
organic compound found in all plant life. It is a mineral-binding compound which prevents monogastric animals and humans from absorbing nutrients contained within the plant cells. In its native state it also complexes with proteins as well as mono and divalent cations and is consumed by humans and animals chiefly in cereal grains and legumes or foods derived from them (Harland & Morris, 1995).

3.5.2 Phytate in wholegrain and bakery products

Worldwide, cereals and legumes are important sources of total calories, carbohydrates, proteins and various other nutrients in the diet of many populations. Especially wholegrain and legumes are the major source of dietary phytate intake. The milling of grain to give white flours reduces the levels of many nutrients as well as phytate. The phytate content of bread and cereals are presented in Table 3.1.

Table 3.1. The phytate content of bread and cereals

<table>
<thead>
<tr>
<th>Cereal/cereal products</th>
<th>Phytate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>0.39-1.35</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>0.25-1.37</td>
</tr>
<tr>
<td>Rye</td>
<td>0.54-1.46</td>
</tr>
<tr>
<td>Rye flour</td>
<td>0.33-1.08</td>
</tr>
<tr>
<td>Oats groats</td>
<td>1.37</td>
</tr>
<tr>
<td>Whole wheat bread</td>
<td>0.43-1.05</td>
</tr>
<tr>
<td>Rye bread</td>
<td>0.03-0.41</td>
</tr>
<tr>
<td>Sour rye bread</td>
<td>0.03</td>
</tr>
<tr>
<td>Pumpernickel bread</td>
<td>0.16</td>
</tr>
<tr>
<td>White bread</td>
<td>0.03-0.23</td>
</tr>
</tbody>
</table>


Although whole grain products are known to have high phytate content, this component is now being recognised for a variety of possible benefits to human health and the previous emphasis on its role as an anti-nutrient needs to be re-evaluated (Wu, Zhao, & Tian, 2010).
In western countries, most wheat based food products are made with refined endosperm from which the germ and peripheral layers are excluded, although these tissues have considerable nutritional potential and contain most of the micronutrients, phytochemicals and fibre of the grain (Alan, Ofelia, Patricia, & Rosario Maribel, 2012). As most of the phytate in cereals is located in the aleurone layers (bran), milling of cereals and subsequent separation of bran result in a significant reduction of phytate in flour.

### 3.5.3 Antioxidative properties of phytate

Phytic acid forms an iron chelate that inhibits iron-catalysed hydroxyl radical formation and lipid peroxidation (Empson, Labaza, & Graf, 1991). In order to further characterise its antioxidant properties in a model food system, Empson et al. investigated the effects of phytic acid on ascorbic acid degradation in aqueous solution. They found that iron-induced oxidative damage in foods can be inhibited with the use of small amounts of phytic acid. This compound reduced the degradation rate of ascorbic acid and slowed lipid peroxidation in oil-in-water emulsions and in cooked refrigerated food items. Many other potential antioxidant uses of phytic acid exist, including various biomedical applications, corrosion inhibition and other food preservation possibilities (Empson et al., 1991).

A study by (Lee et al., 2007) in relation to phytate antioxidant capability has been carried out on the interaction of lipid metabolism that induces the hypertriglycemia, hypercholesterolaemia, and cardiovascular disease that occur during aging. They have focused on the intervention strategies to control serum lipid levels (Lee et al., 2007). Dietary phytate may have health benefits for diabetes patients because it lowers the blood glucose response by reducing the rate of starch digestion and slowing gastric emptying (Kumar, Sinha, Makkar, & Becker, 2010; Thompson, 1993). This study was carried out with the intention to investigate the effect of phytic acid on serum and hepatic lipid levels in aged mice. A total of 40 aged ICR male mice were fed purified diets supplemented with 0% (P0), 0.5% (P5), 1.0% (P10), and 1.5% (P15) sodium phytate for 12 weeks. As for the result, there were no differences in food intake, body weight, and organ weight among the experimental groups. The concentrations of the
serum low-density lipoprotein cholesterol, hepatic triacylglycerol, and total cholesterol and the apparent absorption rates of total lipid and cholesterol were lower in the P15 group than in the P0 group. Serum high density lipoprotein cholesterol levels of all groups fed phytate-containing diets were higher than that of the P0 group. The severity of fatty liver decreased as phytate percentage in the diet increased. Fatty liver, also known as fatty liver disease, is a reversible condition wherein large vacuoles of triglyceride fat accumulate in liver cells via the process of steatosis (i.e., abnormal retention of lipids within a cell). The amounts of faecal total lipid, triacylglycerol, and total cholesterol were higher in the P10 and the P15 groups. These results suggested that phytate affected the serum and hepatic lipid levels in aged mice by increasing their faecal lipid content: consuming phytate-rich foods may reduce serum and hepatic lipid levels in the aged population (Lee et al., 2007).

During a 36 week study, F344 rats were injected with azoxymethane to induce colon cancer. Beginning stages appeared as aberrant crypts in unembedded segments of colon tissue. When it is left untreated, this could progress to tumors in a dose-response fashion. The higher the incidence of aberrant crypts (crypts are clusters of abnormal tube-like glands in the lining of the colon and rectum), the greater the risk a tumor will form. The addition of phytate to the drinking water reduced the number and depth of the colonic crypts when rats were examined at 12 and 36 weeks. The incidence of tumors in rats denied phytate water was 83 % compared with 25 % in rats drinking phytate water. This study also showed that phytate in drinking water administered after azoxymethane injection could not prevent the formation of aberrant crypts but it could prevent the next progressive step of tumor formation (Harland & Morris, 1995; Pretlow, 1992).

Another pilot study was conducted to determine whether IP6 could reverse or suppress initiating events. Eight months after four injections of azoxymethane, only 10 % of rats drinking 2 % phytate water developed lower intestinal cancer compared with a 43 % incidence in the controls. There was also a beneficial residual effect after discontinuing the phytate water regimen. Adjusting the phytate water toward a neutral pH had greater therapeutic effects: commercially available phytate contains a small amount of the lower inositol phosphate and intestinal phosphatases dephosphorylate IP6 to lower forms. A ready availability of Ins in the vicinity of dephosphorylation reactions might enhance the formation of lower inositol phosphates. These compounds were postulated
as possible the mediators of the antineoplastic effect. Animals drinking the mixture of 1 % Ins and 1 % IP6 fared better. This mixture was more effective in suppressing cell proliferation in both rats and mice (Harland & Morris, 1995; Shamsuddin, 1992).

### 3.5.4 Antinutritional effects of phytate

Among all the antinutritional components, phytic acid has long been one of the prime concerns for human nutrition and health management. The unique structure of phytic acid offers it the ability to strongly chelate with cations including calcium, magnesium, zinc, copper, iron and potassium to form insoluble salts. Therefore it is believed to adversely affect the absorption of these minerals by the consumer (Kumar et al., 2010; Victor, 2001).

Phytate accumulates in the seeds during the ripening period and is the main storage form of both phosphate and inositol in plant seeds and grains (Kumar et al., 2010; Loewus, 2002). Phosphorus in this form is not utilised by human beings, dogs, pigs, birds or agastic animals because they lack the intestinal digestive enzyme phytase (Kumar et al., 2010). Phytate forms complexes with proteins at both low and high pH values (Cheryan, 1980). These complexes alter protein structure, which may result in decreased protein solubility, enzymatic activity and proteolytic digestibility. Hitherto, extensive investigations have been carried out on the negative aspects of phytate overwhelming evidence that dietary phytate is an antinutrient. As a solution, the phytate degrading enzyme, phytase, is popular for degrading phytate during food processing and in the gastrointestinal tract. Major efforts have been made to reduce the amount of phytate in foods by different processes and/or the addition of exogenous enzymes (Kumar et al., 2010; Shamsuddin, 2002).

An overview of negative interactions of phytate with nutrients in food is the formation of insoluble phytate mineral complexes leads to decrease in mineral availability particularly zinc, iron, calcium, magnesium, manganese and copper (Brune, 1992; Iqbal, 1994; Konietzny, 2003; Kumar et al., 2010; Lopez, 2002). In addition there is the formation of non-specific phytate protein complexes, not readily hydrolysed by proteolytic enzymes (Kumar et al., 2010; O'Dell, 1976; Ravindran, 1995). Other reported effects are the formation of carbohydrate complexes making carbohydrates less
degradable, inhibition of amylase activity by complexing with calcium ions and a
decrease of carbohydrate degradation (Kumar et al., 2010; Rickard, 1997; Selle, 2000).
Formation of ‘lipophytin’ complexes, may lead to metallic soaps in gut lumen, resulting
in lower lipid availability (Kumar et al., 2010; Leeson, 1993; Matyka, 1990).

Accordingly it has long be considered that degradation of phytate is a prerequisite for
improving nutritional value because removal of phosphate groups from the inositol ring
decreases the mineral binding strength of phytate. This results in increased
bioavailability of essential dietary minerals (Kumar et al., 2010; Sandberg, 1999).
Various food processing and preparation techniques, along with the addition of
exogenous enzymes are the major efforts made to reduce the amount of phytate in
foods.

3.5.5 Methods for analysis of phytate

During the early and mid 1900s, quantitative analysis of phytate (InsP₆) was based on
precipitation with ferric chloride (Heubner & Stadler, 1914). This method was improved
by Young in 1935 (Young, 1935) and then developed by Haug and Lantzsch in a much
quicker assay by modifying an indirect method (Haug & Lantzsch, 1983). Another
method which is called purification using anion exchange chromatography (Smith &
Clark, 1952) was employed in the separation and identification of phytate and inositol
phosphates. A drawback of these methods is the lack of specificity in distinguishing
between InsP₆ and its degradation products. There are also difficulties in determining
low InsP₆ levels using the precipitation and anion-exchange methods.

A quantitative HPLC method for the analysis of phytic acid in food was developed
based on the precipitation of phytic acid with ferric chloride followed by conversion to
sodium phytate before injection onto a C₁₈ reversed-phase column. In that study,
standard food grade wheat bran samples were analysed by the method of standard
addition and recovery of phytic acid ranged from 99-103%. 3% H₂SO₄ was found to be
as effective as 3% trichloroacetic acid in the extraction of phytic acid. Atomic
absorption spectrometry was potentially valuable as a metal specific detector for the
HPLC of phytate-metal complexes (Camire & Clydesdale, 1982).
With the development of ion-pair HPLC procedures (Sandberg & Ahderinne, 1986) and capillary electromigration methods (Blatny, Kvasnicka, & Kenndler, 1994; Buscher, van der Hoeven, Tjaden, Andersson, & van der Greef, 1995), it became possible to study \( \text{InsP}_6 \) and some of its hydrolysis products during food processing and digestion. These methods are relatively easier to handle with a short and simple procedure but do not differentiate isomeric forms of inositol phosphates. Furthermore, a number of isomer-specific high performance ion-exchange chromatograph (HPIC) methods have been developed using gradient elution to separate inositol phosphates in biological samples (Mayr, 1988).

### 3.5.6 Influence of food processing on phytate

Cereals and legumes are the major source of dietary phytate intake (Anjum, Butt, Ahmad, & Ahmad, 2002). Because phytates chelate minerals which result in insoluble complexes, the removal of phytates may be desirable because such reduction in mineral solubility may lead to a decrease in mineral absorption and bioavailability. The most common food processing methods include heat treatments, germination, soaking, fermentation and bread making.

During boiling and heat treatment, phytate in food shows a high stability up to 100 °C for a boiling time of 1 h (Schlemmer, Muller, & Jany, 1995). It can not be easily removed by conventional heat treatments, such as cooking or baking. Enzymatic degradation of phytate either by phytases occurring naturally during food processing or by adding phytases effectively degrades phytate in food (Sandberg & Andlid, 2002).

During the course of food processing or preparation, phytate is not fully hydrolysed by endogenous phytase. Phytate must be reduced to very low levels to increase mineral bioavailability, especially of iron (Hurrell, 2003; Kumar et al., 2010). For this reason, the addition of exogenous phytase is desired. So far, commercial phytase products have been mainly used as animal feed additives in diets, largely for swine and poultry, and to some extent for fish. In spite of its immense potential in processing and manufacturing of food application, no phytase has found its way to the market. Many researchers have reported a convincing improvement of food products by adding microbial based phytase.
during food processing for bread making, plant protein isolates, corn wet milling and the fractionation of cereal bran (Antrim, 1993; Fredrikson, 2001; Haros, 2001; Kumar et al., 2010; Kvist, 2005). For example, addition of phytase (from *Aspergillus niger*) to a level not considered to affect iron absorption, into wholemeal bread, has resulted in a lowered pH and increased degradation of phytate (Türk & Sandberg, 1992). Furthermore, by adding phytase enzyme and citric acid, bioavailability of minerals may increased in high dietary fibre cereal products (Ekholm, Virkki, Ylinen, & Johansson, 2003).

In Sweden (Türk, Carlsson, & Sandberg, 1996), bakers traditionally incorporate lingonberry into wholemeal bread formulation, which was found to lower the pH of the dough and resulted in degradation of phytate. (Türk, Carlsson, & Sandberg, 1996) found that yeast and wheat contributed some phytase activity under the conditions of bread making. Wheat phytases could not improve phytate degradation compared to microbial phytases (Brejnholt, Dionisio, Glitsoe, Skov, & Brinch-Pedersen, 2010). A high phytase activity in beer-related strains of *Saccharomyces pastorianus* (yeast) was a valuable source of phytase for the production of wholegrain bread (Nuobariene, Hansen, Jespersen, & Arneborg, 2011).

### 3.5.7 Effect of soaking and germination on phytate

Phytic acid and its sodium and potassium salts are hydrophilic and hygroscopic. Although the salts take time to dissolve in water, they are readily soluble. Free phytic acid is not found in plants because it is too strong an acid and phytate complexes with various minerals at neutral or slightly acidic conditions. Yet, for accuracy in any qualitative technique, assurance of complete extraction is the imperative first step in the analytical process (Barbara & Donald, 2001). Previous studies have been designed to develop ways to eliminate phytate from foods by soaking or extracting the food in acid solution as phytate is soluble in an acidic solution, or by enhancing fermentation, thus creating phytate hydrolysis products which have weak mineral binding properties.

Germination is also used to enhance the eating quality of cereals and legumes. During germination, reserve nutrients are mobilised to provide for the growth of roots and
shoots. Phytate, being a major source of phosphorus, is hydrolysed by endogenous enzymes to release inorganic phosphorus.

3.5.8 Fermentation and bread making

During fermentation, cereals and legumes undergo overall changes in composition, textural and flavour properties. Wholegrain bread is an important source of minerals but it also contains considerable amounts of phytic acid, which is also known to impair their absorption. Leenhardt, Levrat-Verny, Chanliaud, & Rémésy, (2005), trial assessed the changes in vitro of dough pH by sourdough fermentation or exogenous organic acid addition on phytate hydrolysis. A slight acidification of the dough with sourdough or lactic acid addition allowed a significant phytate breakdown (70% of the initial flour content compared to 40% without any leavening agent or acidification) (Nielsen, Damstrup, Thomsen, Rasmussen, & Hansen, 2007).

Fermentation can be considered as one the most successful methods to decrease the amount of phytate in wholegrain cereals. Palacios and co-workers selected strains of high phytate-degrading activity from different parts of the gastrointestinal tract of chickens followed by a phytate-rich diet and to test their suitability for the bread making of whole wheat bread (Palacios, Haros, Rosell, & Sanz, 2008; Palacios, Haros, Sanz, & Rosell, 2008a, 2008b). Different lactobacilli and bifidobacteria strains were isolated and individually assayed for phosphatase and phytase activities, since both enzymes could contribute to the degradation of phytate. The isolates with the highest phytate-degrading activity belonged to the species Bifidobacterium dentium, Lactobacillus reuteri (L-M15) and Lactobacillus salivarius (L-ID15). The fermentative ability of the L-M15 and L-ID15 in whole wheat bread making were selected and tested. The whole wheat extended freshness. Moreover, their presence resulted in bread crumbs with lower levels of inositol phosphates. Overall, the two intestinal lactobacilli strains with high phytate degrading activity had good properties as starters in wholewheat bread making process.

3.6 Resistant starch and dietary fibre in baking
A further aspect of the more traditional aspects of breadmaking has been the higher level of dietary fibre associated with wholegrain products. Polysaccharides in foods consist of starch and plant cell wall constituents. More recent research (Fuentes-Zaragoza, Riquelme-Navarrete, Sánchez-Zapata, & Pérez-Álvarez, 2010) has confirmed the positive health effects of dietary fibre. Wholegrain is known to contain more dietary fibre than refined flour. Englyst and Cummings, (1987) classified starch into three groups based on its digestibility, namely readily digestible starch, partially resistant starch and resistant starch. According to this classification, starch that is not accessible to digestive enzymes, such as that protected by “hard-to-digest” coatings in whole and coarsely-milled grains, starch in green banana and potato is considered to be partially resistant starch. Retrograded amylose that can be solubilised in potassium hydroxide and which subsequently becomes susceptible to hydrolysis by amyloglucosidase is referred to as resistant starch (RS). The definition of RS was then modified to include ‘the sum of starch and starch-degradation products that on average reach the human large intestine’ (Englyst, Kingman, Hudson, & Cummings, 1996).

It is hypothesised that the long time fermentation process as well as the sourdough production may also promote retrogradation of starch, thereby increasing resistant starch formation in the finished products. Resistant starch has been identified as a new bioactive component, which may also be formed in cereal fermentations (Lappi et al., 2010; Poutanen, Flander, & Katina, 2009).

3.6.1 Methods of resistant starch determination

Methods of analysis of RS can be grouped into two broad categories: these are based on either in vivo or in vitro approaches. Some of those reported in the literature are summarised in Tables 3.2 and 3.3. As a result of the challenges associated with in vivo analysis for routine purposes, the modified in vitro procedures summarised in Table 3.3 have been validated and an approved procedure is available in the methods of AACC International and AOAC. This approach has been selected for the current investigation. Further details of the procedure are provided in Chapters 6 and 10.
Table 3.2  Tabulated summary of *in vivo* resistant starch analyses

<table>
<thead>
<tr>
<th>Method</th>
<th>Description</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂ breath test</td>
<td>Determination of the increase in hydrogen in the breath after the consumption of malabsorbed carbohydrates</td>
<td>• Simple and non-invasive&lt;br&gt;• Healthy subjects</td>
<td>• Semi-quantitative&lt;br&gt;• Strict standardisation necessary&lt;br&gt;• Large intra- and inter-individual variation in H₂ excretion</td>
</tr>
<tr>
<td>Ileostomy model</td>
<td>Patients who have had a colectomy for ulcerative colitis and Crohn’s disease</td>
<td>• Direct collection of the ileal effluent =&gt; quantitative&lt;br&gt;• Easy to perform</td>
<td>• Cannot be considered as healthy&lt;br&gt;• Physiological adaptation&lt;br&gt;• Water and electrolytes absorption&lt;br&gt;• Bacterial overgrowth&lt;br&gt;• Transit time (≠ from normal)</td>
</tr>
<tr>
<td>Intubation of healthy subjects</td>
<td>Collection of the ileal content in healthy subjects after intubation using a constant perfusion technique of solution containing an unabsorbable marker</td>
<td>• Healthy subjects&lt;br&gt;• Direct collection of the ileal effluent</td>
<td>• Disturbance of the normal physiology by the long triple lumen tube&lt;br&gt;• Quantification of the flow rate using a liquid phase marker&lt;br&gt;• Risk of selectivity of the tube in case of heterogeneous food&lt;br&gt;• Expensive and time-consuming</td>
</tr>
</tbody>
</table>

Source Champ, Kozlowski, & Lecannu, (2008)
Table 3.3  Tabulated summary of in vitro resistant starch analyses

<table>
<thead>
<tr>
<th>References</th>
<th>Description</th>
<th>Advantages, drawbacks and characteristics</th>
</tr>
</thead>
</table>
| Björck et al. (1986) | This method quantifies part of the starch present in the dietary fibre residue obtained after enzymatic solubilisation according to Asp et al. (1983), then final digestion of protein and starch (using Termamyl), then pancreatin. The soluble dietary fibre components are precipitated with 95% ethanol and the total dietary fibre components are then recovered by filtration. Resistant starch is calculated as total starch remaining in the fibre residue after solubilisation in KOH minus the value obtained without prior KOH treatment. | • Can be performed together with total dietary fibre analysis  
• The main component measured is retrograded starch  
• Has been validated with a rat model (antibiotic-treated rat) (Björck and Asp 1991). |
| Englyst et al. (1992) | The various types of starch are determined by controlled enzymatic hydrolysis with measurement of the released glucose using glucose oxidase. Resistant starch is defined as the starch which is not hydrolysed after incubation with pancreatin (Pancrex V, Paines and Byrne, Greenford, UK) and amyloglucosidase (Novo Nordisk, Bioindustries, Copenhagen, Denmark), after 120·min at 37°C. Due to contamination of amyloglucosidase with an invertase activity, invertase is also added; sucrose has thus to be determined separately. RS is calculated by deducting the Rapidly Digestible Starch (RDS) plus the Slowly Digestible Starch (SDS) contents (i.e. after 20 and 120·min incubation, respectively) of the sample hydrolysed, from Total Starch content. | • The conditions of hydrolysis have been optimised  
• Has been adapted to a large variety of substrates  
• A laborious method giving poor reproducibility without extensive training  
• Need for very specific equipment (mincer, shaking water-baths)  
• Enzymes seem to be no more available from Novo Nordisk Bioindustries, but must be purchased from Englyst Carbohydrate Services Ltd.  
• RS is not measured directly, but calculated by difference between total starch and RDS + SDS  
• No pepsin step in this version of the method. |
Table 3.3  Tabulated summary of *in vitro* resistant starch analyses (continued)

<table>
<thead>
<tr>
<th>Authors</th>
<th>Procedure</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muir and O’Dea</td>
<td>The chewed food sample is incubated with pepsin and then hydrolysed by α-amylase (Speedase PNA-8, Halcyon Chemicals, Sandrigam, Australia) and amyloglucosidase (Sigma, St Louis, MO, USA). The insoluble residue, collected by centrifugation, contains RS.</td>
<td>This method has been validated with in-vivo studies on ileostomates (Muir and O’Dea 1993; Muir et al. 1995). From the seven comparisons available, it seems satisfactory. However, no comparisons with other in-vitro methods have been published. Until now, it has been used mainly by the authors; thus difficulties and reproducibility cannot be evaluated. No alcoholic precipitation to isolate RS after α-amylase digestion.</td>
</tr>
<tr>
<td>Goñi et al.</td>
<td>The food sample is milled (dry samples) or homogenised (wet samples), defatted when fat content is ≥5%, incubated with pepsin, then hydrolysed by pancreatic α-amylase. The insoluble residue, collected by centrifugation, contains RS.</td>
<td>This method has not been validated on human subjects. No use of amyloglucosidase. No alcoholic precipitation to isolate RS after α-amylase digestion.</td>
</tr>
<tr>
<td>Åkerberg et al.</td>
<td>The analytical procedure was chosen to mimic physiological conditions, and included chewing as a pre-step before incubation with pepsin, pancreatin (Sigma) and amyloglucosidase (Boehringer, Mannheim, Germany). The non-digestible polysaccharide, including RS, is recovered by ethanol precipitation and subsequent filtration. RS is analysed as total starch in the filter residue.</td>
<td>RS values obtained from in-vivo and/or in-vitro analyses have been compared for identical products. The pooled standard deviation for the method is 2.9%. The correlation obtained with in-vivo values from the literature (19 foods) was: $r = 0.97; y = 0.77x + 0.45$. According to the authors, the method allows parallel determination of the potentially available starch fraction and dietary fibre.</td>
</tr>
</tbody>
</table>
Champ et al. (1999a) defined RS as the starch not hydrolysed by incubation with \(\alpha\)-amylase and amylglucosidase. Amyloglucosidase (Novo Nordisk Bioindustries) is added to the pancreatic \(\alpha\)-amylase (Sigma) to avoid inhibition of the amylase by the products of the digestion. Hydrolysis products are extracted with 80% ethanol and discarded. RS is then solubilised with 2 N KOH and hydrolysed with amylglucosidase.

- Simple and relatively rapid. Ten samples can easily be analysed (in duplicate) in a normal day of work. No particular training is needed.
- The procedure has been validated in collaboration with Dr. Langkilde and Prof. H. Andersson using in-vivo values obtained from ileostomates and with intubation studies (Faisant et al. 1995a; Champ et al. 1998; Noah et al. 1998). However, more comparisons should be performed between in-vitro and in-vivo values to check the validity of the method on a large range of starches and foods.
- Amyloglucosidase is no longer available from Novo Nordisk Bioindustries
- No proteolysis.
3.7 **Summary of current knowledge**

The fermentation that occurs during bread making is different from those utilised in most other food processes in that the purpose is not to extend shelf life of the raw materials, itself, but rather is a means of the converting grain into a more functional and consumable form. Various fermentation techniques applied may be beneficial to increase bioavailability when decreasing the phytate content and the formation of health beneficial prebiotics (in the form of resistant starch) in wholemeal bakery products. A further aspect of incorporating wholemeal cereal grains is the possibility of using enzymatic treatments to detoxify gliadin in order to develop bread suitable for coeliacs and gluten intolerant individuals. This may represent an additional means to enhance the health benefits of wholegrain baked goods.
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Chapter 4

Background and literature review: wholegrains dietary source of vitamin E

The purpose of this chapter is to provide background and to review current knowledge on vitamin E in the context of the potential health benefits of consuming wholegrains and their utilization for the manufacture of bakery products. The areas that will be discussed include the nutritional significance, chemical stability and fortification strategies. In addition, various methods of vitamin E analysis will also be described.

4.1 Introduction

Tocopherols and tocotrienols, collectively known as tocochromanols, are lipid soluble antioxidants with a chromanol ring and a hydrophobic side chain. These food components are better known as the vitamin E compounds which play an essential role in human nutrition and health. The term ‘vitamin E’ was first introduced by Evans and Bishop (1923) to describe an important dietary factor for animal reproduction. The same research group isolated α-tocopherol from wheat germ in 1936 for the first time. They named the vitamin E “tocopherol” from the Greek term pherein “carry” and tococs “to birth” (Evans, Emerson, & Emerson, 1936).

More than 40 years passed before vitamin E was first associated with antioxidant properties (Epstein, Forsyth, Saporoschetz, & Mantel, 1966). Thereafter, the role of this vitamin was extensively studied in humans and animals and later the function of tocochromanols was studied in plants, including cereals (Falk & Munne-Bosch, 2010; Žilić et al., 2011).

Wheat is often regarded as an important dietary source of vitamin E (Hussain et al., 2012; Sen, Khanna, & Roy, 2007) as are cereals in general. However, during manufacture of wheat products for human consumption, a marked reduction in vitamin E content occurs. It is well known, for instance, that in the production of white flour from the whole grain wheat and rye as well as oats, the vitamin E content declines by
approximately 50% due to the removal of bran and germ as well as the effects of heat processing (Edge, Jones, & Marquart, 2005; Zhou, Su, & Yu, 2004).

The level of vitamin E in cereals is also influenced by various factors including genotypic environmental and agronomic factors (Lampi, Nurmi, Ollilainen, & Piironen, 2008; Lampi, Nurmi, & Piironen, 2010). These factors along with growing conditions may have either a positive or negative impact on the vitamin E levels. Cereal processing including milling (Ko et al., 2003), baking (Piironen, Varo, & Koivistoinen, 1987), malting, extrusion and cooking have a major influence on the vitamin E content (Tiwari & Cummins, 2009).

4.2 The molecular structures of vitamin E

Vitamin E is the generic term used for all of the compounds in this group. The vitamin can exist as two types of structures: the tocopherol and tocotrienol. Both structures are similar except for the tocotrienol structure having double bonds on the isoprenoid units (e.g. Figure 4.1-4.5). There are many derivatives of these structures due to the different substituents possible on the aromatic ring at positions 5, 6, 7, and 8 as shown in Figures 4.2-4.5.

Tocopherols are equipped to perform an almost unique function. They can interrupt free radical chain reactions by capturing the free radical and this gives them their antioxidant properties. The hydrogen from the free hydroxyl group on the aromatic ring is donated to the free radical, resulting in a relatively stable free radical form of the vitamin. This mechanisms imparts antioxidant properties to the vitamin.

![Fig. 4.1 The chemical structure of α-tocotrienol](image-url)
Fig. 4.2 The structure of α-tocopherol

Fig. 4.3 The structure of β-tocopherol

Fig. 4.4 The structure of γ-tocopherol

Fig. 4.5 The structure of δ-tocopherol
4.3 Vitamin E and human health

Until now, only eight substances have been found to have vitamin E activity: $\alpha$-, $\beta$-, $\gamma$- and $\delta$-tocopherol and $\alpha$-, $\beta$-, $\gamma$- and $\delta$-tocotrienol. These tocol derivatives, are responsible for vitamin E activity in cereal grains and inhibit cholesterol biosynthesis (Qureshi, Qureshi, Hasler-Rapacz, et al., 1991; Qureshi, Qureshi, Wright, et al., 1991). Yet, of all the publications on vitamin E appearing in the scientific databases, less than 1% are related to the tocotrienols. According to Sen et al., (2006) “The abundance of $\alpha$-tocopherol in the human body and the comparable efficiency of all vitamin E molecules as antioxidants, led biologists to neglect the non-tocopherol vitamin E molecules as topics for basic and clinical research”.

The benefits of the tocotrienol isomers of vitamin E in red palm oil to possess potential blood cholesterol lowering and cardioprotective effects and more is postulated antioxidant activity in biological systems (Das et al., 2008; Das et al., 2005; Nesaretnam, 2008; Nesaretnam, Yew, & Wahid, 2007). Aggarwal, Sundaram, Prasad, & Kannappan, (2010) mentioned that whereas there is a substantial body of knowledge regarding the tocopherols, very little is known about tocotrienols. Nevertheless, these workers investigated the role of tocotrienols and hypothesised that these may have superior biological properties. Additionally, the anti-inflammatory and antioxidant activities might contribute to prevention of cancer, diabetes as well as cardiovascular and neurodegenerative disease (Ryan, Galvin, O'Connor, Maguire, & O'Brien, 2007).

Research with humans given a tocotrienol-rich palm oil, indicated that supplementation decreased (12%) total cholesterol levels in hypercholesterolemic subjects (Yong et al., 1997). Further studies with animals substantiated that vitamin E supplementation could enhance the blood lipid profile (Fau - Chen et al., 2006).

Earlier studies completed in model systems, including edible oils and other food matrices showed that tocopherols are pro-oxidative at levels above those concentrations that provide antioxidant protection (Huang, Hopia, Schwarz, Frankel, & German, 1996; Jung & Min, 1992). More recently, studies completed with pure triacylglycerols have indicated that the tocopherols are not pro-oxidants but may act synergistically with pro-oxidants already in the system (Kulás & Ackman, 2001; Lampi, Kataja, Kamal-Eldin, &
Vieno, 1999). One particular study has indicated that the ratio of vitamers is important: a preparation of mixed tocopherols containing γ-, δ- and α-tocopherol (5:2:1) has better antioxidant and anti-inflammatory effects than α-tocopherol alone in animal models and in a limited number of preliminary clinical investigations (Saldeen & Saldeen, 2005).

4.3.1 Vitamin E deficiency in human health

Vitamin E deficiency occurs infrequently in humans and is almost always due to factors other than dietary insufficiency. Vitamin E deficiency can occur through various conditions that affect absorption of fat or vitamin E (Franke, Murphy, Lacey, & Custer, 2007). Malabsorption of vitamin E results from pancreatic and liver abnormalities that lower fat absorption and cause abnormalities of intestinal cells, length of the intestine and defects in the synthesis or assembly of the chylomicrons (Diet Health Council, 1989). Genetic abnormalities in lipoprotein metabolism that produce low to non-detectable circulating chylomicrons, very low density lipoprotein (VLDL) and low density lipoprotein (LDL) levels affect both absorption and plasma transport of vitamin E (Di Filippo et al., 2012).

4.3.2 Vitamin E deficiency and coeliac disease

Aslam, Misbah, Talbot, & Chapel (2004) reported vitamin E deficiency causes a neurological disorder characterized by sensory loss, ataxia and retinitis pigmentosa due to free radical mediated neuronal damage. Symptomatic vitamin E deficiency is a genetic defect of the vitamin E transport protein and in malabsorption complicating cholestasis, abetalipoproteinaemia, coeliac disease, cystic fibrosis and small bowel resection (Barton, Kelly, & Murray, 2007). Hallert et al., (2002) indicated that coeliac patients showed a higher total plasma homocysteine level than the general population, indicative of a poor vitamin status. It has been suggested oxidative stress is an important factor in the pathogenesis of coeliac disease (Szafarska-Poplawska et al., 2010). The vitamin E antioxidant capacity of coeliac patients was reduced, primarily by a depletion of glutathione. It was concluded that natural antioxidants, including vitamin E along with appropriate dietary supplements could be important complements to the classic therapy of coeliac disease (Hozyasz, Chelchowska, & Laskowska-Klita, 2003; Odetti et al., 1998; Stojilković et al., 2009). The inflammation and vacuolar myopathy was
reversed by a gluten free diet and vitamin E supplementation (Kleopa, Kyriacou, Zamba-Papanicolaou, & Kyriakides, 2005).

4.3.3 Required levels of vitamin E intake

Intake recommendations for vitamin E and other nutrients are provided in the Dietary Reference Intakes (DRIs) developed by the Food and Nutrition Board (FNB) at the Institute of Medicine in the USA. DRI is the general term for a set of reference values used to plan and assess nutrient intakes of healthy people. These values, which vary by age and gender can be categorised into: Recommended Dietary Allowance (RDA) which is the average daily level of intake sufficient to meet the nutrient requirements of nearly all (97%–98%) healthy people, Adequate Intake (AI) which is established when evidence is insufficient to develop an RDA and is set at a level assumed to ensure nutritional adequacy and Tolerable Upper Intake Level (UL) which is the maximum daily intake unlikely to cause adverse health effects. The recommended dietary allowance for vitamin E is presented in Table 4.1.
Table 4.1 US recommended dietary allowance and tolerable upper intake levels for vitamin E

<table>
<thead>
<tr>
<th>Life stage</th>
<th>RDA (mg/d⁻¹)</th>
<th>UL (mg/d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Infants month</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-6</td>
<td>4</td>
<td>b</td>
</tr>
<tr>
<td>7-12</td>
<td>6</td>
<td>b</td>
</tr>
<tr>
<td><strong>Children (years )</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-3</td>
<td>6</td>
<td>200 (465 µmol)</td>
</tr>
<tr>
<td>4-8</td>
<td>7</td>
<td>300 (698 µmol)</td>
</tr>
<tr>
<td><strong>Males (years)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-13</td>
<td>11</td>
<td>600 (1395 µmol)</td>
</tr>
<tr>
<td>14-18</td>
<td>15</td>
<td>800 (1860 µmol)</td>
</tr>
<tr>
<td>19-30</td>
<td>15</td>
<td>1000 (2326 µmol)</td>
</tr>
<tr>
<td>32-50</td>
<td>15</td>
<td>1000 (2326 µmol)</td>
</tr>
<tr>
<td>51-70</td>
<td>15</td>
<td>1000 (2326 µmol)</td>
</tr>
<tr>
<td>&gt;70</td>
<td>15</td>
<td>1000 (2326 µmol)</td>
</tr>
<tr>
<td><strong>Female (years)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-13</td>
<td>15</td>
<td>600 (1395 µmol)</td>
</tr>
<tr>
<td>14-18</td>
<td>15</td>
<td>800 (1860 µmol)</td>
</tr>
<tr>
<td>19-30</td>
<td>15</td>
<td>1000 (2326 µmol)</td>
</tr>
<tr>
<td>32-50</td>
<td>15</td>
<td>1000 (2326 µmol)</td>
</tr>
<tr>
<td>51-70</td>
<td>15</td>
<td>1000 (2326 µmol)</td>
</tr>
<tr>
<td>&gt;70</td>
<td>15</td>
<td>1000 (2326 µmol)</td>
</tr>
<tr>
<td><strong>Pregnancy (years)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤18</td>
<td>15</td>
<td>800 (1860 µmol)</td>
</tr>
<tr>
<td>19-30</td>
<td>15</td>
<td>1000 (2326 µmol)</td>
</tr>
<tr>
<td>31-50</td>
<td>15</td>
<td>1000 (2326 µmol)</td>
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<tr>
<td><strong>Lactation (years)</strong></td>
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<tr>
<td>≤18</td>
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<td>800 (1860 µmol)</td>
</tr>
<tr>
<td>19-30</td>
<td>15</td>
<td>1000 (2326 µmol)</td>
</tr>
<tr>
<td>31-50</td>
<td>15</td>
<td>1000 (2326 µmol)</td>
</tr>
</tbody>
</table>

- **Bold type**: Recommended dietary allowance; **ordinary type**: adequate intake (AI).
- **b Not possible to establish; source of intake should be formula and food only.**

4.4 Stability of vitamin E

The vitamin E content of food is greatly affected by the processing conditions. These include light, heat, pH, reactions catalysed by the enzyme lipoxidase (also known as lipoxygenase) and various metals, primarily iron and copper, as well as the presence of free radicals in the oil that are able to initiate autoxidation. In the absence of oxygen, tocopherols and tocotrienols are stable to heat and alkaline conditions including those used to saponify samples that contain lipids during extraction for laboratory analysis.

Vitamin E loss during the baking of French and wheat/rye bread was studied (Wennermark & Jagerstad, 1992). No vitamin E loss occurred in either the scalding and fermentation stages. However, the sourdough preparation and dough-making resulted in 20-60% reduction in the content of tocopherols and tocotrienols. In addition vitamin E yield in the wheat/rye and French bread when using solvent and saponification extraction methods were compared. The two methods were associated with analytical differences after the baking stage. In addition, the storage stability of the vitamin in wholemeal flour, white flour, bran and germ of wheat were also investigated. Storage at 20 °C for 1 year decreased vitamin E activity of the various wheat fractions by 28-40%.

Similarly, dough making caused a substantial loss of vitamin E (up to 30%) (Nanditha & Prabhasankar, 2009). The Chorleywood rapid dough technique resulted in a substantial incorporation of oxygen into the dough, facilitating the lipoxidase catalysed oxidation of the unesterified polyunsaturated fatty acids. Lipid oxidation and vitamin E destruction were more pronounced in stored than in fresh flour. While in the preparation of wheat and rye bread, the scalding procedure had no effect on vitamin E retention. Sourdough preparation from rye wholemeal decreased the vitamin E content by about 60%. This excessive loss of vitamin E activity was apparent after 10 min of mixing, indicating a rapid degradation of vitamin E when in contact with water. Low speed mixing used to prepare the dough of wheat and rye bread caused lesser losses of vitamin E activity.

Leenhardt et al., (2006) provided a solution to optimise the unsaponifiable antioxidants content of bread. A thorough analysis of carotenoids and vitamin E contents from grain to bread and highlighted the most important processing steps affecting their level in
wheat bread. It was concluded that a reduction in kneading time and intensity associated with a longer period of dough fermentation may enhance the retention of carotenoids and vitamin E by limiting oxygen incorporation.

### 4.5 Vitamin E fortification

Food fortification is the process of adding of nutrients to foods and beverages. Some food experts prefer to characterise enrichments as ingredients that restore vitamins and minerals that are lost during processing. It can be purely a commercial choice to provide extra nutrients in a food, or sometimes it is a public health policy which aims to reduce the numbers of people with dietary deficiencies in a population.

In 1941, bread fortification was commenced in the US: niacin, riboflavin, thiamin and iron were required to be added to help reduce diseases related to nutritional deficiency (American Dietetic Association, 2005). Subsequently, a substantial amount of research has been carried out in relation to the incorporation of vitamins and minerals into bakery products. A decade ago, Al-Saqer and co-workers (2004) prepared two functional foods, pan bread and sugar snap cookies, using red palm olein and red palm shortening (RPS) a yellow-orange vegetable fat. Their objective was to provide higher amounts of antioxidant vitamin E in the diet. That study found that tocotrienols were the predominant fraction in cookies made either with RPS (58 to 68%) or with red palm olein (corresponding to approximately 69 to 83 % of total vitamin E). Their results also indicated that the cookies recipe containing higher amounts of fat would provide higher amounts of those desirable phytochemicals and antioxidant vitamin than typical breads.

#### 4.5.1 Palm oil and carotenoids

Palm oil is derived from the mesocarp of the oil palm fruit *Elaeis guineensis*. Its physical and chemical properties are quite distinct from those of oil obtained from the kernel inside the nut. The oil palm originated in West Africa and the cultivar grown widely in Malaysia is a cross of the *dura and pisifera* varieties known as *tenera*.

The carotenoids, the name of which is derived from the fact that they constitute the major pigment in the carrot root, *Daucus carota*, where they were first identified, are undoubtedly among the most widespread and important pigments in living organisms.
They are present in numerous vegetable oils and palm oil contains the highest known concentration of agriculturally derived carotenoids. In fact, crude palm oil is to be the world's richest natural plant source of carotenes in terms of retinol (provitamin A) equivalent. It is these carotenoids that impart the orangey-red colour typical of this oil (May, 1994).

Palm oil olein which is rich in carotenoids may act as an antioxidant by quenching singlet oxygen and scavenging free radicals (Song, Lu, & Chen, 2003; Young & Lowe, 2001). Carotenoids synergistically act with vitamin E and ascorbic acid (Stahl & Sies, 2003). A similar mechanism to that thought to be functioning with vitamin E and ascorbic acid, has been postulated for regeneration of tocopheroxyl radicals by interaction with carotenoids. In food manufacture, carotenoids may be added to enhance vitamin E content as well as colour in the finished product (Böhm, Edge, Land, McGarvey, & Truscott, 1997).

### 4.6 Extraction of vitamin E

In the context of food analysis procedures, the purpose of this section is to introduce issues regarding extraction of Vitamin E components. These components are lipid-soluble compounds which are readily soluble and can be extracted with relatively non-polar organic solvents. In cereals, tocopherols and tocotrienols are in the grain matrix with complexes of fibre, starch and protein and it is therefore very important that the samples are finely ground to optimise extractability (Ge et al., 2002). It is also important to store the samples at low temperature and in the dark to avoid degradation of the tocols due to oxidation, otherwise, analyses need to be performed on meals freshly after grinding.

Many workers have used direct solvent extraction methods to isolate tocopherol and tocotrienols from cereal samples. A classic practice includes extraction of sample in methanol (Michalska et al., 2007; Peterson & Qureshi, 1993). Until recently, the saponification method of extracting tocols from ground oats offered advantages over the previously used direct methanol extraction method (Peterson, Jensen, Hoffman, & Mannerstedt-Fogelfors, 2007). The saponification method increased yield by almost equal 25% and was less time consuming. An HPLC method was developed and
validated for the simultaneous determination of vitamin E and carotenoids in cereals after solid-phase extraction (SPE) (Irakli, Samanidou, & Papadoyannis, 2011).

### 4.6.1 Pressurized extraction of vitamin E

A rapid analytical method including pressurized liquid extraction (PLE) and liquid chromatography-electrospray ionisation-mass spectrometry (LC-ESI-MS) has been developed for the determination of tocopherols and tocotrienols in cereals (Bustamante-Rangel, Delgado-Zamarreno, Sanchez-Perez, & Carabias-Martinez, 2007). These workers also optimized the pressurized liquid extraction parameters using methanol as an extraction solvent at a temperature of 50 °C and a pressure of 110 psi, using one cycle of extraction with a static time of 5 min. The method was reliable for the determination of tocopherols and tocotrienols in cereals. Delgado et al. (2009) used PLE prior to LC determination of tocotrienols and tocopherols in wheat, rye, barley, maize and oat as well as palm oil samples.

Accelerated solvent extraction (ASE) was used by Moreau and co-workers et al. (2003) to extract tocols from freshly ground corn kernels and ground rolled oats using four different organic solvents: hexane, methylene chloride (also known as dichloromethane), isopropanol, and ethanol. They investigated two different temperatures (40 and 100°C) indicating that more lipid and hence tocols were extracted at 100°C and using the solvent methylene chloride. Freitas, Jacques, Richter, da Silva, & Caramao, (2008) used a similar extraction approach to extract vitamin E from grape seed, however they used 100% hexane as the solvent.
4.7 HPLC separation of tocopherols and tocotrienols

Currently, high performance liquid chromatograph (HPLC) methods are widely used in the analysis of tocopherols and tocotrienols for food and nutritional purposes (Nielsen & Hansen, 2008). Each form of tocopherols and tocotrienol can be separated and quantified individually using HPLC with either a UV or fluorescence detector (Panfili, Fratianni, & Irano, 2003). The analytical interferences are largely reduced after separation by HPLC. Therefore, the sensitivity and specificity of HPLC methods are much higher than those obtained with colorimetric, polarimetric or even gas chromatography (GC) methods (Durant, Dumont, & Narine, 2006). Furthermore, coupling of HPLC systems to a mass spectrometer by an atmospheric-pressure ionization (APCI) interface allowed the most selective and sensitive detectability that enabled elucidation and quantitation of tocopherols with a limit of detection in the femtomole range (Stoggl, Huck, Scherz, Popp, & Bonn, 2001). In addition, sample preparation for the HPLC methods is simpler and more efficiently duplicated than in older approaches to analysis.

A variety of HPLC methods for the quantification of tocopherols and tocotrienols in a range of foods have been reported. Normal as well as reversed-phase HPLC methods are used in the separation and quantification of tocopherols and tocotrienols preparations for various foods. Because plant tissues possess most forms of tocopherol and tocotrienol, it has long been suggested that normal phase HPLC method can be applied to food samples from plant sources (Rupérez, Martín, Herrera, & Barbas, 2001). In the reversed-phase HPLC methods, β- and γ-tocopherols and β- and γ-tocotrienols are not usually completely separated. On the other hand such methods can be applied for animal tissues, which either lack or have reduced levels of β- and γ-tocopherols and β and γ-tocotrienol. The resolution of the normal phase HPLC method is higher than for reversed phase method; however, the reversed HPLC column is more long lasting than normal phase HPLC column. By using reversed-phase HPLC with fluorescence detection for the separation of tocopherol and tocotrienol isomers was successfully applied to cereals, including as durum wheat, bread wheat, rice oat and corn. The column used was filled with a novel sorbent material of ultrapure silica gel (Irakli, Samanidou, & Papadoyannis, 2012).
4.8 Summary of current knowledge

Research has consistently demonstrated the health benefits of wholemeal products rich in vitamin E over those made from refined flour which has relatively low levels of vitamin E. The tocol derivatives, consisting of the tocopherols and tocotrienols, are responsible for vitamin E activity in cereal grains. These compounds play an essential role in human nutrition and health. Nevertheless, all types of baking and processing cause are associated with losses of various nutrients including vitamin E. Food fortification is the process of adding nutrients to foods and beverages. Recent evidence indicates that the incorporation of palm oil may have potential for increasing the vitamin E content in baked goods. Vitamin E as lipid-soluble compounds, are readily soluble and can be extracted with organic solvents. Normal phase HPLC methods are now routinely used in the separation and quantification of tocopherols and tocotrienols preparations for various foods.
References


Chapter 4


Chapter 5

Summary of background and description of project aims

The purpose of this chapter is to briefly summarise the context in which this project has been developed and to describe the aims of the research program.

5.1 Summary of the current situation and significance of the project

Currently, bread is an important part of the diet for millions of people around the world. Grain milling and bread manufacture has evolved over a long period of human history the resultant products continue to provide energy, protein, minerals and a variety of beneficial dietary components. However, the changes that have occurred as commercial milling and baking have developed have resulted in moves away from traditional approaches. The earlier sourdough and wholemeal products have been largely replaced by rapid processing procedures based upon white flour.

Research in recent decades has increasing demonstrated that the changes in processing have significant implications for health and wellbeing. Furthermore various studies shown that the consumption of wholegrains cereals and wholegrain foods may reduce the risks of developing certain diseases which are increasingly prevalent in western countries. These include coronary heart disease, colon cancer, diabetes and diverticular disease. Another trend that has occurred has been to utilise only wheat with reduced emphasis on rye by bakers around the world. Accordingly the potential of temperate cereal grains in bread making formulations warrants re-evaluation. In addition, the significance of some of the more traditional approaches in baking may be useful ways to enhance the availability of natural vitamin E components as antioxidants in human diets.

5.2 Hypothesis

The research reported in this thesis has been based upon the hypothesis that wholegrain cereals used for bread production and traditional fermentation methods may contribute to enhanced health benefits of bakery products.
5.3 Project aims

The broad aim of this PhD project has been to investigate the significance of various aspects of bread making, particularly traditional approaches, in relation to the health implications of breads.

The specific objectives have been:

1. To develop and validate enhanced procedures for extraction and analysis of the eight vitamer forms vitamin E with particular emphasis on reducing exposure to toxic solvents and to reduce the toxicity of the vitamin E separation method by replacing hexane with heptane;

2. To investigate the stability vitamin E at various stages of breadmaking using from freshly milled wholegrain meals and evaluate the potential to increase vitamin E content in baked products through palm oil fortification and changed dough processing conditions;

3. To compare the effect of fermentation on phytate contents at each stage of processing and in the final baked loaves thereby establishing suitable fermentation time and temperature combinations to modify the phytate levels of wholemeal breads prepared from selected combinations of cereal grains;

4. To study the formation of resistant starch as a means of varying the starch digestibility of wholemeal breads; and

5. To evaluate the ability of the enzyme caricain (from papaya latex) to detoxify gliadin in whole wheat flour and develop bread suitable for coeliacs and gluten intolerant individuals.
Chapter 6

Materials and methods

The purpose of this chapter is to provide selected information to that provided in subsequent chapters regarding equipment and methods used during this study. This includes procedures applied in the preparation of standards and enzyme solutions, sampling and production of whole grain breads, extraction procedures and analytical methods for the measurement of vitamin E and phytate as well as identification of lactobacillus. Furthermore the analysis of resistant starch using a Megazyme test kit and gliadin toxin using an ELISA test kit are also discussed.

6.1 Apparatus and auxiliary equipment

The items of equipment used, together with details of manufacturers and model numbers are presented in Table 6.1.
Table 6.1 Description of equipment and instrumentation used in the project

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer/supplier</th>
<th>Model no</th>
</tr>
</thead>
<tbody>
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<td>CS76083V</td>
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<td>pH meter</td>
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<td>Boeco Germany</td>
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<td>Shimadzu Spectrofluormetric detector</td>
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<td></td>
</tr>
<tr>
<td>Spectrophotometer</td>
<td>Agilent Technologies</td>
<td>Cary 60</td>
</tr>
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</table>
6.2 Preparation of wholegrain breads

A number of different varieties of wholegrain breads were made on a pilot scale and the samples of these products were analysed in triplicate. The averages of the results from the data were calculated and are presented in this thesis, representing the value of each variety of bread prepared. The rye bread variety was prepared using procedures similar described elsewhere (Zielinski, Michalska, Ceglinska, & Lamparski, 2008). The wheat and oat mix varieties were prepared using a method developed for the study with varied mixing speeds to minimize oxidation of vitamin E. It is specifically noted here that all steps in the preparation of bread for the vitamin E analysis were carried out subdued lighting conditions, where possible otherwise amber glassware or glassware covered with aluminium foil in order to minimize the potential impact of light on vitamin stability and non specific loss due to sample handling.

6.2.1 Milling of grain samples

For all bread varieties, grains were milled to provide wholemeal flour on the day of bread making was commenced. The mill used was a bench top unit (Grain Master Whisper Mill, Korea) which uses upright blades spinning at high speed (10,000 rpm) to produce a relatively fine meal with increased surface area.

6.2.2 Rye sourdough fermentation

All percentages values are relative to flour weight. For doughs prepared using wheat (10%) and rye (90%), 35 % of the rye flour was first fermented for 24 h. For the sourdough formulation a flour water ratio 1:1 was used along with 10% of starter culture (produced by back slashing). The final dough was prepared by mixing the sourdough and the remaining flours 65%, salt 2%, instant dry yeast 1%, red palm oil 4% and water 65%. The ingredients used to make rye bread were: 20g wheat meal, 120g rye meal, 120g sourdough, 140g water, 3.67g palm oil, 4g common table salt, 2g instant yeast. The procedure for making rye bread involved placing all ingredients in mixer 4 min slow and 6 min fast.
6.2.3 Wheat and oat bulk fermentation

The wheat as well as blends of oats and wheat were mixed and bulk fermented (4.5-6.5 h) at temperatures of either 23.5 or 37.0 °C. For the dough preparation the meals (ratio range 100, 90/10, 80/20, 70/30), water 70%, red palm oil 4%, salt 2%, instant dry yeast 0.2% was firstly mixed at slow speed (4min) and followed by fast speed (6min) until full dough development was achieved.

6.2.4 Proving and baking

All doughs were scaled (180g), shaped and placed into bread tins prior to the final proof at 37 °C for 45min. Baking was at 230 °C for 10 min followed by a further 15min at 200 °C. Representative sub-samples were taken at various stages during processing and these were freeze dried, ground and stored at -18°C until analysed.

6.3 Determination of moisture content

The moisture content of samples (grain samples, freeze-dried dough bread) was measured according to the AACC international (2000) air oven method. Empty aluminium moisture dishes were placed into a pre-heated oven set at 130 ± 3 °C. After 1 hour, the empty dishes were taken from the oven and cooled in a desiccator containing active silica gel desiccant for a period of 30 min and then weighed. Sub-samples (approximately 5 g) were accurately weighed into pre-weighed dishes. Then the dishes containing the samples were placed into the oven and dried at 130 ± 3 °C for 1 hour. The process of the drying, cooling and weighing was repeated after 1 hour until a constant weight was attained. The loss in weight was used to calculate the moisture content of the sample using the following equation:

\[
\text{Moisture content (\%) } = \frac{\text{Loss in weight of dish and sample upon drying}}{\text{Initial weight of sample}} \times 100
\]

6.4 Dry weight basis calculations

The results obtained for contents of the vitamins in the bread samples were routinely adjusted by calculation to a dry weight basis. The purpose was to facilitate the direct comparison of the results particularly for different sample types. All samples analysed for vitamins were also tested for moisture content. The following general equation was applied:
**Vitamin content (adjusted to a constant moisture basis) =**

\[
\text{Vitamin content (as is basis)} \times \frac{100 - \text{constant moisture figure}}{100 - \text{actual moisture of sample}}
\]

In all cases the data were recalculated to a dry weight basis (where the constant moisture figure is zero) so the equation was used in the form:

**Vitamin content (adjusted to a dry basis)**

\[
= \text{Vitamin content (as is basis)} \times \frac{100}{100 - \text{actual moisture of sample}}
\]

### 6.5 Procedures and calculation applied generally in the analysis of vitamin E

Due to the potential sensitivity of the vitamin E to light, all procedures were performed in the absence of direct light. In addition, samples and sample extracts were covered with aluminium foil to exclude light and brown glassware was used wherever possible. Except that otherwise indicated, all steps in analytical methods were performed without delay.

### 6.6 Procedures used in the validation of vitamin analysis methods

In all cases, a variety of approaches were used to ensure the validity of the methods and the resulting analytical data. During the development and establishment of methods the initial approach was to measure standard solutions of either individual vitamins or mixtures of these. Secondly, the procedure involved recovery studies in which bread samples were spiked with appropriate amounts of the standard vitamin compound prior to extraction.

### 6.7 Duplication and presentation of analytical results for vitamin content

In the analysis of samples for vitamin content, at least duplicate sub-samples of each sample was extracted on different days. In addition, multiple analyses were performed on each extract obtained. The results of replicate analyses of each sample have been calculated and presented as mean values with details of the Standard deviation (SD). These calculations were carried out using Microsoft Excel 2003 software. In the evaluation of results obtained when reference materials were repeatedly analysed, the relative standard deviation of a series of values was also calculated.
6.8 Calculation of results for vitamin content using HPLC

The vitamin content of the bread was calculated using the external standards, unless otherwise specified. At the start of a series of NP-HPLC measurements, at least 2 replicated analyses of the standard test solutions were performed and the average peak areas were calculated. These were then compared with those of the sample test peak areas. For this comparison, the weighed portion, the amount of aliquot used, the volume of solution needed for pH adjustment and the dilutions were taken into account. A spreadsheet prepared in Microsoft Excel, was used to calculate the average peak areas obtained for the individual standards and these were used as described.

6.9 Preparation of calibration curve

Firstly, the peak areas of individual standard was used directly and plotted using the scatter option in the software with concentration of individual vitamin (mg/L) on the x axis and the corresponding peak areas on the y axis. A linear regression equation of the form \([y = mx + c]\) typically gave the best statistical fit and the equation as well as the \(R^2\) value were recorded. The latter value was then considered and the analyses were repeated if the value was lower than 0.95.

6.10 Calculation of vitamin content

The average peak areas for each sample tested were then used in the calculation of vitamin concentrations of the sample solutions using the linear equations. The appropriate dilution factor was applied and allowance made for the original sample weight to express the result per 100 g of sample. After determination of individual vitamin contents, the data were expressed on a dry weight basis, following the calculation described in Section 6.4.
6.11 Accelerated solvent extraction (ASE)

The following extraction procedure was applied to all the grain samples and all the 4 stages of processed whole grain bakery products (after milling, dough mixing, fermentation and baking). The overall approach in the preparation of the samples extracts involved weighing 2.0 g of grain sample mixed with 0.05 g of ascorbic acid, 1.9 g drying agent (hydromatrix celite) and placed into a 22 mL stainless steel extraction cell. This was then closed and mounted in the carousel of a Dionex ASE 200 Accelerated Solvent Extractor equipped with solvent controller and then extracted. The extraction program consisted of a sample preheat for 5 min, heated for 5 min, 2 static cycles 10 min each, Flush time 60 sec, purge 120 sec, temperature 70 - 90°C, pressure 1600psi, 100% hexane. The extract was collected in a 250 mL glass vial and evaporated under nitrogen flow. The sample was redissolved in 1 mL mobile phase (the mobile phase was ethyl acetate acetic acid n-hexane 1:1:198 (v/v/v) vortexed, transferred to an HPLC sample vial and inject into NP-HPLC system.

6.12 Determination of phytate

The phytic acid contents of samples were determined by a rapid method as described by Haug and Lantzsh (1983), in which the phytic acid is precipitated with an iron-III-solution of known iron content and the decrease in iron in the supernatant is a measure of phytic acid content. Phytate reference solution containing phytic acid sodium salt hydrate from rice, type V 94% purity and about 6% water, was obtained from Sigma-Aldrich. Stock solutions were prepared with 1.3 mg mL⁻¹ phytic acid. Ferric solution was prepared by dissolving 0.2 g NH₄Fe (SO₄)₂ 12H₂O in 100 mL 2 moL⁻¹ HCl and the volume was made to 1000 mL with distilled water. 2, 2-Bipyridine solution was prepared by dissolving 1.0 g 2, 2 bipyridine and 1.0 mL thioglycollic acid in distilled water and the volume was made to 100 mL.

For the analysis of different cereal grains and bread doughs as well as bread, 0.06 g of meal, dough as well as bread was extracted with 10 mL of 0.2 mol L⁻¹ HCl at 4°C overnight and 0.5 mL of this extract was pipetted out into a test tube fitted with a ground-glass stopper. 1 mL of ammonium iron (III) sulphate solution was added into it. The test tube was then covered with a stopper and incubated in a boiling water bath for 30 min. After cooling to room temperature, 2 mL of (1% v/v) 2`, 2-bipyridine was added. The absorbance was immediately measured using a spectrophotometry set at 519 nm against distilled water.
The test method was calibrated with the reference solutions prepared by diluting the stock solutions with 0.2 mol L\(^{-1}\) HCl in a range of 0.1-1 mL (3.12-31.2 µg mL\(^{-1}\) phytate phosphorus).

6.13 **Analysis of gliadin residues using detection by ELISA kit**

The kit used for the analysis of gliadin residues was supplied by ELISA Systems (Table 6.2). The immunoassays typically utilise antibodies for the detection of specific allergenic proteins which serve as markers for allergenic food. Different classes of antibodies can be raised against allergenic proteins, including monoclonal and polyclonal antibodies. Monoclonal antibodies are well suited for the recognition of the specific antigen due to the recognition of one epitope. As polyclonal antibodies recognize multiple epitopes spread on the proteins (being more tolerant to small changes of the antigen) and have lower production costs, they are typically utilised by industry for manufacture of ELISA kits. However, this assay utilises monoclonal antibodies to omega gliadin protein in a non-competitive, sandwich type ELISA. The gliadin standards supplied in this kit (6 in total) allow measurement of gliadin levels in the samples and can be readily converted to indicate quantification within a range of 2.5-25 parts per million (ppm) in the sample.

6.14 **Analysis of resistant starch**

The procedure used was based on the analysis kit supplied by Megazyme International, following the method recommended by the manufacturer. Samples were incubated in a shaking water bath with pancreatic α-amylase and amyloglucosidase (AMG) for 16 h at 37°C, during which time non-resistant starch is solubilised and hydrolyzed to D-glucose by the combined action of the two enzymes. The reaction was terminated by the addition of an equal volume of ethanol or industrial methylated spirits (IMS, denatured ethanol) and the RS is recovered as a pellet on centrifugation. This was washed twice by suspension in aqueous IMS or ethanol (50% v/v), followed by centrifugation. Free liquid was removed by decantation. RS in the pellet was dissolved in 2 M KOH by vigorously stirring in an ice-water bath over a magnetic stirrer. This solution was neutralized with acetate buffer and the starch is quantitatively hydrolyzed to glucose with AMG. D-glucose is measured with glucose oxidase/peroxidase reagent (GOPOD) and this was a measure of the RS content of the sample. Non resistant starch (solubilised starch) was determined by pooling the orinal
supernatant and the washing, adjusting the volume to 100mL and measuring D-glucose content with GOPOD.

**Table 6.2  Sources and details of reagents used for gliadin residue detection**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micro-wells containing anti-Gliadin antibodies</td>
<td>48 wells 1 test strip holder ESGLI-48</td>
<td>ELISA Systems¹</td>
</tr>
<tr>
<td>Prepared concentrated gliadin standards</td>
<td>6 Gliadin standards 0 ppm, 2.5 ppm, 5.0 ppm, 10.0 ppm, 20.0 ppm, 25.0 ppm ESGLI-48</td>
<td>ELISA Systems¹</td>
</tr>
<tr>
<td>Enzyme Conjugate</td>
<td>HRP-Conjugated anti-Gliadin ESGLI-48</td>
<td>ELISA Systems¹</td>
</tr>
<tr>
<td>Substrate</td>
<td>Stabilized Tetramethylbenzidine (TMB) ESGLI-48</td>
<td>ELISA Systems¹</td>
</tr>
<tr>
<td>Extraction solution concentrate</td>
<td>Bottle was diluted 20 times in 40% ethanol prior to use ESGLI-48</td>
<td>ELISA Systems¹</td>
</tr>
<tr>
<td>Ethanol</td>
<td></td>
<td>AJAX²</td>
</tr>
<tr>
<td>Wash buffer solution concentrate</td>
<td>ESGLI-48</td>
<td>ELISA Systems¹</td>
</tr>
<tr>
<td>Dilution solution</td>
<td>ESGLI-48</td>
<td>ELISA Systems¹</td>
</tr>
<tr>
<td>Stop solution</td>
<td>1M Phosphoric acid ESGLI-48</td>
<td>ELISA Systems¹</td>
</tr>
</tbody>
</table>

Notes ¹ELISA Systems Pty Ltd, Unit 10, 121 Newmarket Road, Windsor, Queensland 4030 Australia  ²Ajax Chemicals, Melbourne, Australia
6.15 Statistical analysis

Where specified, Minitab (Minitab Inc, USA) Version 15, was used to develop a central composite design for the optimisation of the amount of red palm oil, fermentation temperature and time whilst a two level factorial was employed for the optimisation of the vitamin E content in wholegrain bread. A 3-Dimensional contour surface plots were created using Design Expert (Version 7.1, Stat-Ease Inc, USA).

In experimental designs, variables are coded with values of “+1” and “-1” given to “high” and “low levels respectively in the variable range. The centre points in experimental design are represented by “0”. This gives equal weight to all variables in the regression equation and removes bias, which may arise due to differences in magnitude of variables. Centre point replicates are used to estimate reproducibility of the method and are also included to check whether the response surface has curvature (Goupy, 2005).

Preliminary stages of optimisation often involve the use of a two- level factorial design which one or several centre points, as shown in Figure 7.1. Such design can facilitate the establishment of the main effects as well as the interactions between factors but cannot determine the second order curvature effects. The factorial design can be extended by adding axial points to create a central composite design (CCD). The experimental design can determine the main effects, the interaction between factors and second order effects.
Fig. 6.1 Two level factorial design for three factors. The levels -1, 0 and 1 are the coded levels for the low, centre and high values, respectively. Each vertex of the cube represents one set of experimental values. The point (0, 0, 0) is the centre point.

A CCD consists of a two-level factorial design with centre points and a star design forming a five level design where each factor is converted to the coded values $-\alpha$, -1, 0, +1, $+\alpha$. Coding enables factors with different units to be compared on a more equal basis. The cube is the factorial component of the design while the other points forming a star are the axial points. The corners of the cube and the axial points each represent one experimental run. The distance of the axial points, $\alpha$, varies depending on the number of factors, $k$, to be optimised. $\alpha$ Can be determined using the equation $\sqrt[4]{2^k}$ and if the design is rotatable, then there is a uniform distribution of information. For two or three factor designs to ensure rotatability $\alpha$ is equal to 1.414 and 1.682, respectively.

A multi-variate design such as CCD offers information on the relationship between the factors and the response. A three-dimensional response surface is typically created from the
response surface designs, providing a visual representation of the effects of two factors on the response. The response surface design is a polynomial model where the “surface” is represented mathematically. These plots can supply a graphical illustration of the data over the ranges observed and can be used to forecast areas of most favourable performance. From the experimental models the conditions for an optimum separation can then be found.

A common variation of the CCD used is the face-centered CCD as shown in (Figure 6.2). In this design $\alpha$ is set as 1. This means the design is not rotatable and the high and low levels for axial and cube points are the same. The CCD, along with multi-linear regression, can estimate the main effects, $b_i$, two way interactions, $b_ib_j$, and the second order effects, $b_i^2$. Coefficients of the polynomial equation can be determined by regression analysis. The polynomial equation communicates the response to the factors, forming the Empirical Model (Daali, Cherkaoui, Christen, & Veuthey, 1999). The polynomial equation can be used to assess the linear, quadratic and interactive effects of independent variables on the selected responses.

\[ Y = b_0 + b_1X_1 + \ldots + b_nX_n + b_{12}X_1X_2 + \ldots b_{n-1,n}X_{n-1}X_n + b_{11}X_1^2 + \ldots + b_{nn}X_n^2 \quad \text{Eqn} \]

Where:

- $b_0$: the intercept
- $b_1$ to $b_n$: the main effect coefficients
- $b_{12}$ to $b_{m,n}$: the two way interaction coefficients
- $b_{11}$ to $b_{nn}$: the second order coefficient
In the wholegrain bread production, factors influencing the quantity of vitamin E include fermentation time, temperature and amount of oil. The extent to which these factors affect the quantity and response varies with each component. Thus, in this study two face-centred experimental designs are employed (a) for the optimisation of fermentation time, temperature and amount of oil and (b) for the optimisation of the running buffer. A running buffer is used in gel (or any) electrophoresis to help keep a specific pH range for a certain process. In each case the response variable is the area ratio of red palm oil to NA. Minitab for Windows version 15 (Minitab Inc., State College, PA, USA) was used to generate and analyse the experimental designs.
References


Chapter 7

Results and discussion

Heptane as a less toxic option than hexane for the separation of vitamin E from food products using normal phase HPLC

Journal article published in RSC Advances by RSC Publishing presented as Chapter 7
Heptane as a less toxic option than hexane for the separation of vitamin E from food products using normal phase HPLC

Oliver Buddrick, Oliver A. H. Jones,* Paul D. Morrison and Darryl M. Small

The term ‘vitamin E’ refers to a group of eight vitamers (alpha-, beta-, gamma-, delta-tocopherols and tocotrienols). Its primary role is thought to be as an antioxidant commonly added to a variety of foods, e.g. bakery products. High-Performance Liquid Chromatography (HPLC) procedures are used for the separation and analysis of these tocopherols and tocotrienols in foods. The use of a normal phase column is the preferred approach in such methods, with hexane almost universally utilised as the mobile phase. However there is increasing concern regarding the toxicity of hexane. Here we evaluate the use of heptane as a replacement for hexane in HPLC based vitamin E analysis. The two solvents were compared using samples of bread fortified with palm oil (as a source of vitamin E). Accelerated solvent extraction procedure followed by HPLC showed the effective separation of the E vitamers in a variety of bread samples using both solvents. It is concluded that heptane provides effective separation and quantification of the E vitamers found in cereals and cereal products while also reducing operator risk.

Introduction

High Performance Liquid Chromatography (HPLC) was first applied to the resolution of vitamin E and other fat soluble vitamins in the early 1970s and proved to be a very effective method. Vitamin E occurs in eight vitamer forms, (α-, β-, γ- and δ-tocopherol and α-, β-, γ- and δ-tocotrienol) (see Fig. 1). Collectively these are referred to as tocols or tocochromanols. These have been successfully baseline separated on HPLC using silica columns. The polarity of tocols is primarily affected by the number of methyl groups attached to the chromanol ring and to a lesser extent by the slightly increased polarity of the unsaturated side chains of tocotrienols compared to those of tocopherols. It has been reported that the most difficult compounds to separate are the β- and γ-tocols (which are positional isomers) because they each have two methyl groups on their ring structure.

Vitamin E can currently be separated by both normal-phase HPLC (NP-HPLC) and reversed-phase HPLC (RP-HPLC). In NP-HPLC the vitamers are dissolved in relatively non-polar organic solvents and separated by absorption. This method is considered to be more effective for separating vitamin E vitamers present in cereal grains than reversed-phase HPLC. A review of the literature indicated that hexane has been the solvent of choice for the separation and extraction of vitamin E from cereal for over 20 years (see Table 1). However, hexane is very volatile and is metabolized in humans to 2,5-hexanedione, which is neurotoxic. There has therefore been increasing concern over operator exposure to this solvent in recent years.

It has been suggested that continued exposure to hexane or 2,5-hexanedione results in loss of sensorial and motor function and alterations in axonal neurofilament proteins. It should be stated that both hexane and heptane are toxic. However, hexane is more volatile, can cause peripheral neuropathy and is more neurotoxic than heptane. Animal studies have clearly shown that heptane is significantly less toxic than hexane. This has prompted the development of alternative solvents for use in HPLC.

Fig. 1 Diagram of the general chemical structure of tocopherols (a) and tocotrienols (b). Details of the different R groups in each of the alpha, beta, gamma and delta forms of each group apply to both structures.
demonstrated that n-hexane is far more toxic to the peripheral nerve of the rat than n-pentane or n-heptane.6,27–29

Human exposure studies also give causes for concern. For example, after a 6 month period of occupational exposure to n-hexane it was reported that a 27 year-old suffered a sequential optic neuropathy with the hallmarks of Leber Hereditary Optic Neuropathy (LHON). LHON is a maternally inherited loss of central vision related to pathogenic mutations in the mitochondrial genome, which are a necessary, but not sufficient condition to develop the disease.6,29–31 The toxic effects of hexane on the human body have been highlighted elsewhere in the literature and are well established.31–33

The vitamin E content of bakery products is often increased by the addition of vitamins in oil since, in the breadmaking process, vegetable oils are used to enhance the gas retention of dough thereby increasing volume and softness. The level used will vary according to the type of flour, with wholemeal requiring higher levels of oil addition than white flour (often two or three times more).24 However vitamin content in bakery products may be affected by the heat of the baking process with some oils being better suited to the process.25 For example red palm oil is known to have a higher heat resistance compared to other plant based oils (such as canola and sunflower) used in the food industry.25

In our study wholemeal dough was fortified with red palm oil to increase the vitamin E content in the final product. A validation method using Accelerated Solvent Extraction (ASE) and separation by NP-HPLC was applied to quantify the recovery and verify the fortification process of vitamin E vitamers after the breadmaking process. The primary aim was to reduce the toxicity of the method for separation of various forms of vitamin E by replacing hexane with heptane. The theory under test was that the use of heptane would be as effective as hexane in separating vitamin E vitamers while also being a less toxic option for HPLC based analysis of vitamin E in a variety of bread products.

Results and discussion

Since pure tocotrienols are not readily commercial available and tocotrienols are known to exhibit similar fluorescent responses to their respective tocopherols, tocotrienols are

<table>
<thead>
<tr>
<th>Mobile phase used</th>
<th>Extraction/purification</th>
<th>Sample matrix</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane/dioxane (96 : 4 v/v)</td>
<td>Methanol</td>
<td>Oats</td>
<td>9</td>
</tr>
<tr>
<td>Hexane/2-propanol (99 : 1 v/v)</td>
<td>Hexane</td>
<td>Barley, corn, wheat</td>
<td>10</td>
</tr>
<tr>
<td>Hexane/ethyl acetate/acetic acid (97.3 : 1.8 : 0.9 v/v/v)</td>
<td>Saponification</td>
<td>Oats, spelt, durum wheat, soft wheat, maize, barley, triticale</td>
<td>11</td>
</tr>
<tr>
<td>Hexane/tetrahydrofuran/isopropanol (93.7 : 6 : 0.3 v/v/v)</td>
<td>Chloroform and water</td>
<td>Pan bread and sugar snap cookies</td>
<td>12</td>
</tr>
<tr>
<td>Same as Panilli et al. (2003)</td>
<td>Hot saponification</td>
<td>Einkorn (wheat)</td>
<td>13</td>
</tr>
<tr>
<td>Described by14</td>
<td>Methanol</td>
<td>Rye/rye bread</td>
<td>15</td>
</tr>
<tr>
<td>Hexane/2-propanol (99.5 : 0.5 v/v)</td>
<td>Methanol</td>
<td>Oats</td>
<td>16</td>
</tr>
<tr>
<td>Hexane/dioxane (95 : 5 v/v)</td>
<td>Extraction with hexane</td>
<td>Wheat</td>
<td>17</td>
</tr>
<tr>
<td>Hexane/ethyl acetate/acetic acid (94.6 : 3.6 : 1.8 v/v/v)</td>
<td>Extracted with hexane</td>
<td>Wheat, barley, spelt, rye</td>
<td>18</td>
</tr>
<tr>
<td>Described by10</td>
<td>Methanol</td>
<td>Rye/rye bread</td>
<td>19</td>
</tr>
<tr>
<td>Hexane/ethyl acetate/acetic acid (94.6 : 3.6 : 1.8 v/v/v)</td>
<td>Methanol</td>
<td>Spelt (wheat)</td>
<td>20</td>
</tr>
<tr>
<td>Hexane/2-propanol (99 : 1 v/v)</td>
<td>Hexane</td>
<td>Wheat</td>
<td>21</td>
</tr>
<tr>
<td>Hexane/dioxane (96 : 4 v/v)</td>
<td>Methanol</td>
<td>Oats</td>
<td>22</td>
</tr>
<tr>
<td>Previously used by Lampi et al. (2008)</td>
<td>Saponification</td>
<td>Bread, water biscuit, pasta</td>
<td>23</td>
</tr>
<tr>
<td>Previously used by Lampi et al. (2010)</td>
<td>Hot saponification</td>
<td>Wheat</td>
<td>24</td>
</tr>
<tr>
<td>Hexane/ethyl acetate/acetic acid (97.3 : 1.8 : 0.9 v/v/v)</td>
<td>Saponification</td>
<td>Wheat</td>
<td>25</td>
</tr>
</tbody>
</table>

Fig. 2 Chromatogram of tocopherol standards (eluted in the following order: α, β, γ and δ) separated using hexane (upper panel) and heptane (lower panel) as the main solvent. The mobile phase was ethyl acetate, acetic acid, n-hexane 1 : 1 : 198 (v/v/v) at a flow rate 1.5 mL min⁻¹ for the upper panel and ethyl acetate, acetic acid, n-heptane 1 : 1 : 198 (v/v/v) at a flow rate 1.5 mL min⁻¹ for the lower panel.
commonly quantified using tocopherol standards.\textsuperscript{5,6} Previously published data on vitamin E HPLC separation was used in order to match retention times and thus aid in the identification of tocotrienols which have been shown to be eluted in the following order: $\alpha$-, $\alpha$-T, $\beta$-, $\gamma$-, $\gamma$-T3, $\delta$-T and $\delta$-T3.\textsuperscript{7} The chromatographic conditions were as detailed in Section 2.5 (below). In the case of tocopherols, we used standards and data from the literature to confirm that the elution order was the same in both hexane and heptane. In the case of the tocotrienols, previous studies in the literature indicate that the elution order would be the same in both solvents.\textsuperscript{3,8} In addition the peak size and shape did not vary between solvents (see Fig. 2 and 3) indicating the elution order did not vary.

Fig. 2 shows a comparison of the chromatograms of $\alpha$-, $\beta$-, $\gamma$- and $\delta$-tocopherols standards separated using both hexane and heptane as the main solvent. Chromatograms for vitamin E extracted from bread dough (after final proving) made using 100% wheat meal fortified with 5% red palm oil, and fermented at 30 °C are shown in Fig. 3. Using the mobile phase, ethyl acetate, acetic acid, and $n$-hexane in the ratio of 1 : 1 : 198 (v/v/v) with a silica column (Supelcosil LC-Si, Supelco, Sigma-Aldrich, Australia), the tocopherol standards were eluted in the order: $\alpha$, $\beta$, $\gamma$ and $\delta$. This order was the same when heptane was used as a main solvent. The only difference observed was the retention times for each vitamer were increased by up to 2 minutes when heptane was used.

Similar results were observed in the sample separation (Fig. 3) using heptane as a main separation solvent. However it is of note that compared to tocopherol standard, the bread samples show several peaks other than those in the standard chromatogram. Those peaks were identified as tocotrienols by reference to data from analytical standards previously published in the literature.\textsuperscript{4} All the samples in our study have been identified and quantified using the same method.\textsuperscript{4}

To enable a comprehensive analysis a variety of palm oil fortified bread samples (wheat, wheat/oat mix and rye flour based) were analysed for all eight forms of vitamin E using both heptane and hexane and the results are presented in Tables 2 and 3. The total tocot content recovered using heptane is higher than those recovered using hexane. However, the range of vitamers is greater when separated using hexane. It should also be noted here that the formulation for the rye bread necessitated the addition of palm oil after the fermentation stage rather than before as in the other bread types, this resulted in the lower values of total tocols observed prior to the mixing process. It was observed that with hexane all 4 tocopherols were not detected.

Table 2 Variation in different stages of wholemeal bread production regarding tocochromanol compounds and total tococromanols using heptane as a main solvent in normal phase HPLC separation.* ND = not detected.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\alpha$-T</th>
<th>$\alpha$-T3</th>
<th>$\beta$-T</th>
<th>$\beta$-T3</th>
<th>$\gamma$-T</th>
<th>$\gamma$-T3</th>
<th>Total tococromanols</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat 100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixing</td>
<td>4.40 ± 0.27</td>
<td>4.87 ± 0.17</td>
<td>4.30 ± 0.03</td>
<td>12.68 ± 1.26</td>
<td>0.36 ± 0.06</td>
<td>13.55 ± 1.45</td>
<td>40.70 ± 2.43</td>
</tr>
<tr>
<td>Fermentation</td>
<td>6.79 ± 0.16</td>
<td>2.98 ± 0.07</td>
<td>5.45 ± 0.48</td>
<td>14.45 ± 0.63</td>
<td>0.24 ± 0.41</td>
<td>13.63 ± 2.04</td>
<td>43.54 ± 2.52</td>
</tr>
<tr>
<td>Final proof</td>
<td>6.74 ± 0.47</td>
<td>2.96 ± 0.07</td>
<td>5.21 ± 0.07</td>
<td>13.49 ± 0.48</td>
<td>ND</td>
<td>15.78 ± 0.32</td>
<td>44.18 ± 1.41</td>
</tr>
<tr>
<td>Bake</td>
<td>7.13 ± 0.54</td>
<td>2.53 ± 0.47</td>
<td>4.32 ± 0.12</td>
<td>11.03 ± 0.23</td>
<td>0.14 ± 0.07</td>
<td>12.61 ± 0.04</td>
<td>37.76 ± 1.13</td>
</tr>
<tr>
<td>Wheat/Oat (70/30)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixing</td>
<td>6.37 ± 0.13</td>
<td>6.42 ± 0.53</td>
<td>3.78 ± 0.12</td>
<td>10.87 ± 0.47</td>
<td>0.74 ± 0.01</td>
<td>13.81 ± 0.90</td>
<td>42.00 ± 0.82</td>
</tr>
<tr>
<td>Fermentation</td>
<td>6.14 ± 0.29</td>
<td>5.43 ± 0.11</td>
<td>3.70 ± 0.43</td>
<td>10.57 ± 0.61</td>
<td>0.17 ± 0.30</td>
<td>15.08 ± 0.74</td>
<td>41.10 ± 1.92</td>
</tr>
<tr>
<td>Final proof</td>
<td>5.55 ± 0.33</td>
<td>4.38 ± 0.17</td>
<td>3.54 ± 0.37</td>
<td>10.09 ± 0.68</td>
<td>ND</td>
<td>13.93 ± 0.77</td>
<td>37.48 ± 1.96</td>
</tr>
<tr>
<td>Bake</td>
<td>5.09 ± 0.26</td>
<td>4.05 ± 0.18</td>
<td>3.02 ± 0.19</td>
<td>8.41 ± 0.27</td>
<td>ND</td>
<td>12.37 ± 0.88</td>
<td>32.95 ± 1.41</td>
</tr>
<tr>
<td>Rye</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inoculation</td>
<td>6.61 ± 0.56</td>
<td>6.13 ± 0.20</td>
<td>3.96 ± 0.33</td>
<td>5.88 ± 0.39</td>
<td>ND</td>
<td>ND</td>
<td>22.58 ± 0.51</td>
</tr>
<tr>
<td>Fermentation</td>
<td>6.03 ± 0.07</td>
<td>3.97 ± 0.13</td>
<td>2.63 ± 0.25</td>
<td>4.54 ± 0.86</td>
<td>ND</td>
<td>ND</td>
<td>17.17 ± 1.07</td>
</tr>
<tr>
<td>Mixing</td>
<td>9.76 ± 0.89</td>
<td>8.12 ± 0.47</td>
<td>3.55 ± 0.55</td>
<td>6.67 ± 0.78</td>
<td>0.94 ± 0.02</td>
<td>16.40 ± 0.83</td>
<td>45.45 ± 2.11</td>
</tr>
<tr>
<td>Final proof</td>
<td>8.91 ± 0.90</td>
<td>ND</td>
<td>3.85 ± 0.09</td>
<td>6.79 ± 0.12</td>
<td>0.98 ± 0.11</td>
<td>16.00 ± 0.19</td>
<td>36.52 ± 0.82</td>
</tr>
<tr>
<td>Bake</td>
<td>7.16 ± 0.37</td>
<td>ND</td>
<td>2.86 ± 0.02</td>
<td>5.63 ± 0.26</td>
<td>0.90 ± 0.10</td>
<td>11.92 ± 0.36</td>
<td>28.47 ± 0.60</td>
</tr>
</tbody>
</table>

* Recoveries were adjusted to a dry weight basis. All figures refer to the mean of 3 analytical runs.
Table 3: Variation in different stages of wholemeal bread production regarding tocochromanol compounds and total tocochromans using hexane as a main solvent in normal phase HPLC separation (data expressed as μg g⁻¹). ND = not detected.

<table>
<thead>
<tr>
<th>Sample</th>
<th>α-T</th>
<th>α-T3</th>
<th>β-T</th>
<th>β-T3</th>
<th>γ-T</th>
<th>γ-T3</th>
<th>δ-T</th>
<th>δ-T3</th>
<th>Total tocochromans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat 100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixing</td>
<td>3.98 ± 0.17</td>
<td>2.51 ± 0.23</td>
<td>3.27 ± 0.01</td>
<td>2.98 ± 0.11</td>
<td>0.34 ± 0.01</td>
<td>4.40 ± 0.17</td>
<td>0.61 ± 0.04</td>
<td>1.84 ± 0.09</td>
<td>19.94 ± 0.57</td>
</tr>
<tr>
<td>Fermentation</td>
<td>4.55 ± 0.48</td>
<td>2.67 ± 0.27</td>
<td>3.30 ± 0.17</td>
<td>3.13 ± 0.34</td>
<td>0.44 ± 0.11</td>
<td>5.82 ± 1.26</td>
<td>0.42 ± 0.01</td>
<td>1.61 ± 0.32</td>
<td>21.93 ± 1.86</td>
</tr>
<tr>
<td>Final proof</td>
<td>4.57 ± 0.73</td>
<td>2.85 ± 0.43</td>
<td>3.07 ± 0.24</td>
<td>3.32 ± 0.02</td>
<td>ND</td>
<td>6.07 ± 1.46</td>
<td>ND</td>
<td>ND</td>
<td>19.88 ± 2.88</td>
</tr>
<tr>
<td>Bake</td>
<td>3.22 ± 0.03</td>
<td>1.89 ± 0.07</td>
<td>2.53 ± 0.04</td>
<td>2.65 ± 0.05</td>
<td>0.58 ± 0.01</td>
<td>3.57 ± 0.18</td>
<td>ND</td>
<td>1.12 ± 0.08</td>
<td>15.57 ± 0.35</td>
</tr>
<tr>
<td>Wheat/Oat (70/30) mix</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixing</td>
<td>4.24 ± 0.04</td>
<td>3.77 ± 0.01</td>
<td>2.61 ± 0.03</td>
<td>2.87 ± 0.06</td>
<td>0.51 ± 0.01</td>
<td>4.67 ± 0.18</td>
<td>ND</td>
<td>1.68 ± 0.18</td>
<td>20.34 ± 0.39</td>
</tr>
<tr>
<td>Fermentation</td>
<td>3.93 ± 0.04</td>
<td>3.59 ± 0.10</td>
<td>2.13 ± 0.23</td>
<td>2.95 ± 0.23</td>
<td>0.40 ± 0.03</td>
<td>4.29 ± 0.20</td>
<td>ND</td>
<td>1.50 ± 0.20</td>
<td>18.77 ± 0.98</td>
</tr>
<tr>
<td>Final proof</td>
<td>3.63 ± 0.05</td>
<td>3.36 ± 0.07</td>
<td>2.39 ± 0.04</td>
<td>2.81 ± 0.07</td>
<td>0.56 ± 0.16</td>
<td>4.41 ± 0.01</td>
<td>ND</td>
<td>1.49 ± 0.14</td>
<td>18.66 ± 0.20</td>
</tr>
<tr>
<td>Bake</td>
<td>3.88 ± 0.11</td>
<td>3.40 ± 0.06</td>
<td>2.60 ± 0.07</td>
<td>3.05 ± 0.14</td>
<td>0.49 ± 0.08</td>
<td>4.81 ± 0.01</td>
<td>ND</td>
<td>ND</td>
<td>18.24 ± 0.10</td>
</tr>
<tr>
<td>Rye</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inoculation</td>
<td>5.01 ± 0.04</td>
<td>4.41 ± 0.07</td>
<td>3.31 ± 0.06</td>
<td>ND</td>
<td>ND</td>
<td>2.00 ± 0.02</td>
<td>ND</td>
<td>ND</td>
<td>14.73 ± 0.08</td>
</tr>
<tr>
<td>Fermentation</td>
<td>3.95 ± 0.24</td>
<td>3.41 ± 0.13</td>
<td>2.63 ± 0.01</td>
<td>3.63 ± 0.13</td>
<td>0.41 ± 0.03</td>
<td>1.67 ± 0.03</td>
<td>ND</td>
<td>ND</td>
<td>15.84 ± 0.39</td>
</tr>
<tr>
<td>Final proof</td>
<td>7.10 ± 0.13</td>
<td>6.11 ± 0.11</td>
<td>3.09 ± 0.03</td>
<td>2.39 ± 0.08</td>
<td>0.62 ± 0.10</td>
<td>6.01 ± 0.22</td>
<td>ND</td>
<td>ND</td>
<td>25.32 ± 0.55</td>
</tr>
<tr>
<td>Bake</td>
<td>6.57 ± 0.21</td>
<td>5.57 ± 0.07</td>
<td>2.67 ± 0.08</td>
<td>2.21 ± 0.04</td>
<td>0.68 ± 0.05</td>
<td>5.64 ± 0.05</td>
<td>ND</td>
<td>23.34 ± 0.28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.11 ± 0.10</td>
<td>4.09 ± 0.06</td>
<td>2.16 ± 0.02</td>
<td>1.82 ± 0.01</td>
<td>0.45 ± 0.09</td>
<td>4.53 ± 0.03</td>
<td>ND</td>
<td>ND</td>
<td>18.16 ± 0.17</td>
</tr>
</tbody>
</table>

* Recoveries were adjusted to a dry weight basis. All figures refer to the mean of 3 analytical runs.

seen as well as in some cases 4 tocotrienols. With heptane, all 4 tocopherols were again seen but only 3 tocotrienols were observed. However the response of the tocotrienols which were present were greater with heptane. Thus, the range of vitamers was greater using hexane but that the amount of each vitamer was greater when heptane was used.

**Experimental**

2.1 Standards, reagents and solutions

Standards of α-tocopherol (>98%), β-tocopherol (90%), γ-tocopherol and δ-tocopherol were supplied by Sigma-Aldrich (Australia). HPLC grade hexane and heptane were obtained from Merck (Castle Hill, New South Wales, Australia). HPLC grade ethyl acetate and acetic acid were supplied from Honeywell Burdick & Jackson (Taren Point, New South Wales, Australia).

2.2 Bread preparation

2.2.1 Milling process. For all bread varieties, grains were milled to provide wholemeal flour on the day of breadcrumbing. The mill used was a bench top unit (Grain Master Whisper Mill, Retsel Dandenong, Victoria, Australia) which uses upright blades spinning at high speed (10 000 rpm) to produce a relatively fine meal with increased surface area.

The wheat meal dough was mixed and bulk fermented for 5 h at 30 °C. For the dough preparation, the 100% wheat meal, 70% water, 5% red palm oil, 2% salt, 0.2% instant dry yeast were first mixed using a bench mixer with 10 different speeds (Kitchen Aid Heavy Duty, Model 5KPM50, Benton Harbor, USA) at slow speed (speed setting 2) for 4 min and followed by fast speed (speed setting 4) for 6 min until full dough development was achieved where the dough clears from dough hook and mixing bowl.37 The dough was weighed and 180 g of each type was placed into bread tins prior to the final proving stage of 37 °C for 45 min. Baking was at 230 °C for 10 min followed by a further 15 min at 200 °C in order to bake the bread evenly without causing an increase in crust colour. Both the wheat and the oat flour based breads were prepared using the procedure described above.

Rye breadmaking is different from the wheat meal bread process. The first step involves sour dough fermentation where 35% of the total rye flour weight was fermented with 10% starter culture. The production of the starter culture itself involved a 24 h incubation of rye meal slurry with the ratio of rye meal to water 1 : 1, inoculum enrichment with 1% of the ripe sour dough. This was repeated every 24 h until the microbiota were established. The rye bread formulation consists of 90% rye meal, 10% wheat meal, 100% water, 5% red palm oil, 2% salt and 1% instant dry yeast. The rye dough preparation consists of 24 h incubated sour dough, the remaining 65% flour and ingredient listed above. The dough was mixed for 10 min at slow speed with a paddle. The dough was scaled (250 g) and placed into bread tins prior to the final proof at 37 °C for 45 min. Baking was at 230 °C for 10 min followed by a further 15 min at 200 °C.

2.2.2 Freeze drying process. All samples were immediately frozen at −40 °C and placed in a controlled freeze dryer (VirTis SP Industries Company, Gardiner, USA) to obtain low-moisture-content samples for further analysis. Freeze dried samples were ground in a mortar to form powder and samples were stored in an air-sealed container at −18 °C pending further use.

2.2.3 Sampling. During the making of the wheat meal and wheat blend bread, samples were taken from the dough after mixing, then after fermentation and after final proof (just before
entering the oven) and finally, after baking. In the rye bread-making procedure, the samples were taken after inoculation, fermentation, mixing, final proof and baking.

2.3 Accelerated solvent extraction

The following extraction procedure was applied to all the bread samples and all four stages of processed whole grain bakery products (dough mixing, fermentation, final proofing and baking). The overall approach in the preparation of the sample extracts involved weighing 2.0 g of grain sample mixed with 0.05 g of ascorbic acid and 1.9 g drying agent (Hydromatrix celite, Sigma-Aldrich) which were placed in a 22 mL stainless steel extraction cell. The cell was closed and mounted in the carousel of extraction of a Dionex ASE 200 Accelerated Solvent Extractor (Thermo Scientific, Scoresby, Victoria, Australia) equipped with solvent controller and then extracted using an in-house extraction program. The cell was filled with 90% of hexane and 10% of ethyl acetate and heated to a temperature of 80 °C and pressurized at 1600 psi. These conditions were maintained for 5 min followed by two static cycles for 10 min. After that the cell was flushed for 60 s and purged for 120 s. The extract was collected in a 60 mL glass vial and evaporated under nitrogen. Each sample extraction was redissolved in 1 mL mobile phase, vortex-mixed then transferred to an HPLC sample vial.

2.4 Characterisation of samples

The moisture content of samples was measured according to the American Association of Cereal Chemists, (AACC) International air oven method.\(^5,6,8,12\) Empty aluminium moisture dishes were placed into a pre-heated oven set at 130 ± 3 °C. After 1 h, the empty dishes were taken from the oven and cooled in a desiccator containing active silica gel desiccant for a period of 30 min and then weighed. Sub-samples (~5 g) were accurately weighed into pre-weighed dishes. The dishes containing the samples were placed then into the oven and dried at 130 ± 3 °C for 1 h. The process of the drying, cooling and weighing was repeated three times until a constant weight was attained.

2.5 Separation and detection of tocopherols and tocotrienols by HPLC

Chromatography was performed using a Shimadzu HPLC system fitted with a Waters (Milford, MA, USA) solvent delivery system (510 Model). The data were stored and processed by a Shimadzu interface CBM-10A Communicator Bus module connected to an auto injector (SIL-10A) the injection volume was 25 μL and a fluorescence detector (RF-10A). The analytical column was a 4.6 mm × 25 cm, 5 μm particle size: silica Supelcosil LC-Si, Supelco normal phase. The mobile phase was ethyl acetate, acetic acid, \(n\)-hexane 1 : 1 : 198 (v/v/v) at a flow rate 1.5 mL min\(^{-1}\). Fluorometric detection of all peaks was performed using excitation 290 nm and emission of 330 nm respectively, the typical run lasted 30 min, the gain was ×1, the speed was fast and sensitivity high. Separation conditions were identical in all cases except for the replacement of hexane with an equal amount of heptane.

Conclusion

This study illustrated the possibility and advantages of replacing hexane used as a solvent to separate \(\varepsilon\) vitamers with the less toxic option of heptane. Although the retention time was increased by up to 2 min this did not extend the overall run time per sample significantly. The increase in quantity of vitamers recovered was another positive outcome. The results show that a simple direct replacement (without eluotropic strength adjustments) of \(n\)-hexane with \(n\)-heptane led to a compatible performance. This suggests that large and complicated efforts are not always necessary in order to replace harmful solvents with less damaging ones. This in turn should encourage beginners and experienced analysts alike to experiment with solvent systems for extractions, rather than just following traditional recipes which may have been developed many years ago and do not take into account the capabilities of modern, analytical instruments.

Acknowledgements

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References

Effect of Fermentation and Oil Incorporation on the Retention of E Vitamers during Breadmaking

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ABSTRACT

All forms of baking and processing cause a loss of nutrients, including vitamin E but little is known about these occurrences or if they could be avoided. The objective of this research was to study the incorporation of palm oil and the stability of vitamin E in palm oil during breadmaking. Wheatmeal and rye breads were baked with and without the addition of 0, 2, 5 or 8 % palm oil. The eight E group vitamers (tocopherols and tocotrienols) were extracted using accelerated solvent extraction, freeze dried then analysed using normal phase HPLC. Compared to the controls, the inclusion of palm oil was found to increase the quantity of all forms of vitamin E in the final baked products. It is concluded that palm oil is very effective in increasing the vitamin E content of wholegrain bread.

Keywords: dough fermentation; palm oil; vitamin E; wholegrain;
1. INTRODUCTION

Vitamin E is a term encompassing eight separate compounds (4 tocopherols and 4 tocotrienols) collectively known as tocochromanols or tocols. These are lipid-soluble antioxidants with a chromanol ring and a hydrophobic side chain, which play an essential role in human nutrition and health. The history and chemical structures of the eight vitamin E vitamers are well described and details can be found in Eitenmiller and Ye (2004). Vitamin E has many biological functions in humans, with the most important and best known being its antioxidant role. Other functions include regulating enzymatic activities and cell signalling (Azzi 2007; Zingg and Azzi 2004).

Wheat is often regarded as an important dietary source of vitamin E, as are cereals in general (Sen et al 2007). However, the total tocol content decreases during the mixing, fermentation and baking stages of the breadmaking process (Dewettinch et al 2008). Owing to the importance of vitamin E in the human diet, fats and oils are used as primary enriching ingredients. Vegetable oils with high vitamin E content are widely used to fortify bakery products, when used in bread formulations they improve the characteristics of the resulting dough by enhancing gas retention and thereby increasing the volume and softness of the final product (Cauvain 2003). The proportion of oil required varies according to the type of flour being used, with wholemeal flours requiring higher levels of oil in comparison to white, often as high as two to three times more (Williams and Pullen 1998).

Palm oil is an excellent oil to use in baked products. It contains similar amounts of both unsaturated and saturated fatty acids making the oil semi-solid at room temperature and is heat stable with a high smoke point (232 °C). These properties, permit its use as a major
component in baking and cooking without the need for hydrogenation and removes the problem of trans fatty acids, generated by the latter process, which are detrimental to human health (Cauvain 2003). In contrast to many other vegetable oils, palm oil also contains up to all eight vitamin E vitamers and recent research has also demonstrated the benefits of the tocotrienols isomers of vitamin E as possessing potential blood cholesterol lowering and cardioprotective effects and a more efficient antioxidant activity in biological systems (Das et al 2005; Das et al 2008; Nesaretnam 2008; Nesaretnam et al 2007). Tocotrienols have been shown to have greater anti-inflammatory and antioxidant properties that could reduce incidence of cancer, diabetes as well as cardiovascular and neurodegenerative diseases compared to tocopherols (Ryan et al 2007; Lau et al 2007; Akhtar and Ashgar 2011).

Despite this, there are few studies which investigate vitamin enrichment of bakery products using palm oil (Akhtar and Ashgar 2011; Agnoletti et al 2011). One of the more detailed examples of that is Al-Saquer et al. (2004), who prepared pan bread and sugar snap cookies, using red palm olein (RPO) and red palm shortening (RPS). The results showed that tocotrienols were the predominant form of vitamin E in cookies made either with RPO or RPS. The same authors also demonstrated that a cookie recipe containing higher quantities of fat would provide larger amounts of vitamin E and other desirable phytochemicals (including β-carotene) than various bread formulations (Al-Saquer et al 2004; Lenfant and Thyrion 1996).

Research has consistently demonstrated the health benefits of wholemeal or wholegrain products over those made from refined flour. There is also increased interest in rye flours for health benefits. Within the context of a broader study of the influence of fermentation conditions during baking, the aim of the current research has been to investigate the retention of E vitamers, for wholemeal rye and wheat formulations. Specific objectives were the
comparison of different temperature and fermentation times, along with a comparative
evaluation of varying levels of incorporation of a palm oil as a potential source of the various
E group vitamers to the final baked product.

2. MATERIALS AND METHODS

2.1 Materials

We have recently reported on procedures for the analysis of vitamin E by normal phase
HPLC using heptane rather than hexane for the separation of the eight vitamer forms in wheat
bread (Buddrick et al 2013). The standards and other chemicals used in the analysis of the E
vitamers, along with the breadmaking materials are the same as in that study (Buddrick et al
2013). Standards, reagents and solutions, α-Tocopherol (>98 %), β-tocopherol (90 %), γ-
tocopherol and δ- tocopherol were supplied by Sigma-Aldrich (Australia). HPLC grade
hexane and heptane were obtained from Merck (Castle Hill, New South Wales, Australia).
HPLC grade ethyl acetate and acetic acid were supplied from Honeywell Burdick & Jackson
(Taren Point, New South Wales, Australia).

2.2 Bread sample preparation

2.2.1 Flour milling process

Wholemeal flours were freshly milled on the day of baking. Organically grown wheat grain
of mixed variety (11.7% protein) and rye (11.5% Protein) from the Laucky Flour Mill
(Bridgewater, Victoria, Australia). The mill used was a bench top unit (Grain Master Whisper
Mill, Retsel Dandenong, Victoria, Australia) which uses upright blades spinning at high
speed (10,000 rpm) to produce a relatively fine meal with increased surface area.
2.2.2 Bread samples preparation

The wheat fermentation involved yeast and a bulk fermentation for periods of 3, 5 and 7 h and temperatures of 23, 30 and 37 °C. For the dough preparation, the 100 % wheat meal, 70 % water, 5 % red palm oil, 2 % salt, 0.2 % instant dry yeast were first mixed using a bench mixer with 10 different speeds (Kitchen Aid Heavy Duty, Model 5KPM50, Benton Harbor, USA) at slow speed (speed setting 2) for 4 min and followed by fast speed (speed setting 4) for 6 min until full dough development was achieved where the dough clears from dough hook and mixing bowl. The dough was weighed and 180 g of each type was placed into bread tins prior to the final proving stage of 37 °C for 45 min. Baking was at 230 °C for 10 min followed by a further 15 min at 200 °C in order to bake the bread evenly without causing an increase in crust colour.

Rye breadmaking is different from the wheat meal bread process. The first step involves sour dough fermentation where 35 % of the total rye flour weight was fermented with 10% starter culture. The production of the starter culture itself involved a 24 h incubation of rye meal slurry with the ratio of rye meal to water 1:1, inoculum enrichment with 1 % of the ripe sour dough. This was repeated every 24 h until microbiota was established. The rye bread formulation consists of 90 % rye meal, 10 % wheat meal, 100 % water, 5 % red palm oil, 2 % salt and 1 % instant dry yeast. The rye dough preparation consists of 24 h incubated sour dough, the remaining 65 % flour and ingredient listed above. The dough was mixed for 10 min at slow speed with a pedal. The dough was scaled (250 g) and placed into bread tins prior to the final proof at 37 °C for 45 min. Baking was at 230 °C for 10 min followed by a further 15 min at 200 °C. Wheat and rye breadmaking as previously described (Buddrick et al 2013).
2.2.3 Freeze Drying Process

All samples were immediately frozen at -40 °C and placed in a controlled freeze dryer (VirTis SP Industries Company, Gardiner, USA) to obtain low-moisture-content samples for further analysis previously described (Buddrick et al 2013). Freeze dried samples were ground in a mortar to form powder and samples were stored in an air-sealed container at -18 °C pending further use.

2.3 Accelerated Solvent Extraction

The following extraction procedure was applied to all the bread samples and all four stages of processed whole grain bakery products (dough mixing, fermentation, final proofing and baking). The overall approach in the preparation of the samples extracts involved weighing 2.0 g of grain sample mixed with 0.05 g of ascorbic acid and 1.9 g drying agent (Hydromatrix celite, Sigma Aldrich) which were placed in a 22 mL stainless steel extraction cell. The cell was closed and mounted in the carousel of extraction of an Dionex ASE 200 Accelerated Solvent Extractor (Thermo Scientific, Scoresby, Victoria, Australia) equipped with solvent controller and then extracted using an in house extraction program. The cell was filled with 90 % of hexane and 10 % of ethyl acetate and heated to a temperature of 80 °C and pressurized at 1600 psi. These conditions were maintained for 5 min followed by two static cycles for 10 min. After that the cell was flushed for 60 s and purged for 120 s. The extract was collected in a 60 mL glass vial and evaporated under nitrogen. Each sample extraction was redissolved in 1 mL mobile phase, vortex-mixed then transferred to an HPLC sample vial.
2.4 Method for moisture analysis

The moisture content of samples was measured according to the American Association of Cereal Chemists, (AACC) International air oven method \(^1\). Empty aluminium moisture dishes were placed into a pre-heated oven set at 130 ± 3 °C. After 1 hour, the empty dishes were taken from the oven and cooled in a desiccator containing active silica gel desiccant for a period of 30 min and then weighed. Sub-samples (~5 g) were accurately weighed into pre-weighed dishes. The dishes containing the samples were placed then into the oven and dried at 130 ± 3 °C for 1 hour. The process of the drying, cooling and weighing was repeated three times until a constant weight was attained.

2.5 Separation and Detection of Tocopherols and Tocotrienols by HPLC

Chromatography was performed using a HPLC analytical water system (Milford, MA, USA) comprising a Model 510 solvent delivery system. The data were stored and processed by a Shimadzu interface CBM-10A Communicator Bus module connected to an auto injector (SIL-10A) the injection volume was 25 µL and a fluorescence detector (RF-10A). Analytical column, 4.6 mm x 25 cm, 5µm particle size: silica Supelcosil LC-Si, Supelco normal phase. The mobile phase was ethyl acetate acetic acid \(n\)-hexane 1:1:198 (v/v/v/) at a flow rate 1.5 mL/min. Fluorometric detection of all peaks was performed using excitation 290 nm and emission of 330 nm respectively. The typical run lasted 30 min. The gain was \(\times 1\) and the speed was fast and sensitivity high. Analysis of the tocopherols and tocotrienols by HPLC were carried out using the procedures previously described (Buddrick et al 2013).
RESULTS

In order to facilitate comparisons and evaluation of retention of the vitamers, samples of freshly milled wheat and rye as well as palm oil were analysed for all eight forms of vitamin E and the results are presented in Table 1.

Table 1 Tocopherol and tocotrienol contents in freshly milled cereal grains, (wheat and rye) as well as the red palm oil used in this study.

<table>
<thead>
<tr>
<th>Sample</th>
<th>α-T (µg/g)</th>
<th>α-T3 (µg/g)</th>
<th>β-T (µg/g)</th>
<th>β-T3 (µg/g)</th>
<th>γ-T (µg/g)</th>
<th>γ-T3 (µg/g)</th>
<th>δ-T (µg/g)</th>
<th>δ-T3 (µg/g)</th>
<th>Total (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat*</td>
<td>5.43</td>
<td>2.16</td>
<td>6.71</td>
<td>21.05</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>35.35</td>
</tr>
<tr>
<td>Rye*</td>
<td>9.36</td>
<td>8.85</td>
<td>4.76</td>
<td>7.31</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>30.28</td>
</tr>
<tr>
<td>Palm oil</td>
<td>177.4</td>
<td>149.1</td>
<td>19.53</td>
<td>34.62</td>
<td>ND</td>
<td>491.5</td>
<td>ND</td>
<td>ND</td>
<td>872.2</td>
</tr>
</tbody>
</table>

Note: *Recoveries were adjusted to a dry matter basis (dmb).

T= tocopherol; T3= tocotrienol; ND= not detected.

The total vitamin E content in rye grain was 16 % lower than the freshly milled wheat grain. The quantity of vitamin E extracted from freshly milled rye and wheat kernels shown in Table 1 are similar to those reported by other researchers (Hidalgo et al 2006; Nystrom et al 2008). As shown in Table 1, palm oil has a significant amount of γ-tocotrienol. In a bread formulation containing 2 % oil (density of palm oil 0.9165 g/mL), the addition of 1.83 g of oil to 100 g of meal approximates to 1596 µg vitamin E.
The content of tocopherols and tocotrienols in 100 % wheat bread prepared without and with the addition of oil is shown in Table 2. In this experiment the purpose of producing bread without the addition of oil was to verify the effect of incorporating palm oil on the vitamin E content of wholegrain products. The tocopherols present were α-T and β-T and γ-T while δ-T3 was not detected. The tocotrienol content found in the wheat meal bread samples consisted of only β-T3 and ranged from 1.55 to 3.76 µg/g. No α-T3, γ-T3 or δ-T3 was detected. The highest level of total tocols was found in the bread fermented at 37 °C for 3 h.

The content of tocopherols (α-T, β-T, γ-T) in wheat breads with the addition of oil was significantly increased compared to those without oil, ranging from 1.06 to 7.89 µg/g. The principal tocopherol in wheat is α-T and no δ-T was detected. The total tocotrienol content ranged from 0.98 to 23.17 µg/g. The primary wheat bread tocotrienols fraction consisted of β-T3 and γ-T3. As expected, the highest overall levels of tocopherol and tocotrienols was found in bread with the addition of 8 % palm oil.
Table 2 Content of tocopherols and tocotrienols in loaves baked from 100 % wheat without (control experiment) and with the addition of oil.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tocols concentration, µg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-T</td>
</tr>
<tr>
<td>23 °C 3 h</td>
<td>1.42</td>
</tr>
<tr>
<td>23 °C 7 h</td>
<td>2.41</td>
</tr>
<tr>
<td>30 °C 5 h (1)</td>
<td>1.50</td>
</tr>
<tr>
<td>30 °C 5 h (2)</td>
<td>1.35</td>
</tr>
<tr>
<td>37 °C 3 h</td>
<td>2.42</td>
</tr>
<tr>
<td>37 °C 7 h</td>
<td>1.56</td>
</tr>
<tr>
<td>2 % 23 °C 3 h</td>
<td>2.49</td>
</tr>
<tr>
<td>2 % 23 °C 7 h</td>
<td>2.72</td>
</tr>
<tr>
<td>2 % 37 °C 3 h</td>
<td>2.94</td>
</tr>
<tr>
<td>2 % 37 °C 7 h</td>
<td>2.63</td>
</tr>
<tr>
<td>5 % 30 °C 5 h (1)</td>
<td>4.83</td>
</tr>
<tr>
<td>5 % 30 °C 5 h (2)</td>
<td>4.14</td>
</tr>
<tr>
<td>8 % 23 °C 3 h</td>
<td>6.72</td>
</tr>
<tr>
<td>8 % 23 °C 7 h</td>
<td>7.11</td>
</tr>
<tr>
<td>8 % 37 °C 3 h</td>
<td>6.80</td>
</tr>
<tr>
<td>8 % 37 °C 7 h</td>
<td>7.89</td>
</tr>
</tbody>
</table>

The contents of tocopherols and tocotrienols in rye bread, with and without the addition of oil, are presented in Table 3. In this series of experiments, when no oil was incorporated the content of tocopherols (α-T and β-T) reflected the naturally occurring vitamin E in the whole-rye kernel particularly the germ. The main tocopherol was α-T and no γ-T and δ-T were detected. The tocotrienol content in the rye bread was formed by β-T3, with lesser amounts of α-T3 and no γ-T3 or δ-T3 were found. The highest total level of tocopherol and tocotrienols were found in the bread fermented at 23 °C.

In the wholemeal rye bread with the addition of oil tocopherol was α-T. As observed for the wheat breads, the final bread had no detectable δ-T. The main rye bread tocotrienol fraction was formed by β-T3 and γ-T3, although a small quantity of δ-T3 was detected. The addition of 8 % palm oil again resulted in the highest levels of the E vitamers in the final product.
Table 3 Content of tocopherols and tocotrienols in rye bread without (control experiment) and with the addition of oil.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tocols concentration, µg/g</th>
<th>α-T</th>
<th>α-T3</th>
<th>β-T</th>
<th>β-T3</th>
<th>γ-T</th>
<th>γ-T3</th>
<th>δ-T</th>
<th>δ-T3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without the addition of oil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23 °C</td>
<td></td>
<td>1.42</td>
<td>0.53</td>
<td>0.38</td>
<td>1.15</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>3.48</td>
</tr>
<tr>
<td>23 °C</td>
<td></td>
<td>1.38</td>
<td>0.39</td>
<td>0.26</td>
<td>1.49</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>3.52</td>
</tr>
<tr>
<td>30 °C (1)</td>
<td></td>
<td>1.50</td>
<td>0.46</td>
<td>0.18</td>
<td>1.29</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>3.43</td>
</tr>
<tr>
<td>30 °C (2)</td>
<td></td>
<td>1.36</td>
<td>0.54</td>
<td>0.21</td>
<td>1.15</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>3.26</td>
</tr>
<tr>
<td>37 °C</td>
<td></td>
<td>1.06</td>
<td>0.44</td>
<td>0.21</td>
<td>1.20</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2.90</td>
</tr>
<tr>
<td>37 °C</td>
<td></td>
<td>1.45</td>
<td>0.40</td>
<td>0.25</td>
<td>1.34</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>3.44</td>
</tr>
<tr>
<td>With the addition of oil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 % 23 °C</td>
<td></td>
<td>6.20</td>
<td>0.42</td>
<td>1.51</td>
<td>2.12</td>
<td>0.77</td>
<td>2.49</td>
<td>ND</td>
<td>0.40</td>
<td>13.92</td>
</tr>
<tr>
<td>2 % 37 °C</td>
<td></td>
<td>5.70</td>
<td>ND</td>
<td>1.32</td>
<td>1.88</td>
<td>0.73</td>
<td>2.04</td>
<td>ND</td>
<td>ND</td>
<td>11.66</td>
</tr>
<tr>
<td>5 % 30 °C (1)</td>
<td></td>
<td>7.55</td>
<td>ND</td>
<td>1.37</td>
<td>2.39</td>
<td>1.32</td>
<td>5.91</td>
<td>ND</td>
<td>ND</td>
<td>18.53</td>
</tr>
<tr>
<td>5 % 30 °C (2)</td>
<td></td>
<td>7.70</td>
<td>ND</td>
<td>1.56</td>
<td>3.12</td>
<td>1.56</td>
<td>6.27</td>
<td>ND</td>
<td>ND</td>
<td>20.19</td>
</tr>
<tr>
<td>8 % 23 °C</td>
<td></td>
<td>8.39</td>
<td>0.45</td>
<td>1.37</td>
<td>2.59</td>
<td>0.96</td>
<td>9.34</td>
<td>ND</td>
<td>0.49</td>
<td>23.60</td>
</tr>
<tr>
<td>8 % 37 °C</td>
<td></td>
<td>9.29</td>
<td>ND</td>
<td>1.50</td>
<td>2.78</td>
<td>0.96</td>
<td>9.93</td>
<td>ND</td>
<td>ND</td>
<td>24.46</td>
</tr>
</tbody>
</table>

The losses of vitamin E content in bread occur in the dough-making and fermentation stage. The reduction of vitamin E at various stages in this study are shown in figure 1 and 2.
Figure 1. Vitamin E content in wheat bread sample (5% palm oil fermented at 30°C for 5 h) production including after mixing, fermentation, final proof and baking.

Figure 2. Vitamin E content in rye bread production, including inoculation, fermentation (at 30°C, 5% palm oil), mixing final proof and baking.
US recommended dietary allowance and tolerable upper intake levels for vitamin E RDA (mg/d-1) is 15mg. In Table 4. We will demonstrate the approximate quantity of bread that one has to consume of the final breads produced to follow dietary allowance and intake levels. Assuming a loaf of bread weighs 680 g and contains approximately 20 slices weighing around 34 g each.

<table>
<thead>
<tr>
<th>Sample</th>
<th>µg/g</th>
<th>µg in 100 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat breads</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>4.88</td>
<td>488</td>
</tr>
<tr>
<td>2%</td>
<td>19.47</td>
<td>1947</td>
</tr>
<tr>
<td>5%</td>
<td>30.93</td>
<td>3093</td>
</tr>
<tr>
<td>8%</td>
<td>47.40</td>
<td>4740</td>
</tr>
<tr>
<td>Rye breads</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>3.34</td>
<td>334</td>
</tr>
<tr>
<td>2%</td>
<td>12.79</td>
<td>1279</td>
</tr>
<tr>
<td>5%</td>
<td>19.36</td>
<td>1936</td>
</tr>
<tr>
<td>8%</td>
<td>24.03</td>
<td>2403</td>
</tr>
</tbody>
</table>
The sensory characteristics of the loaves of bread produced as a result of the inclusion of palm oil were evaluated on the basis of a comparison of cross-sectional slices and examples are presented in Figure 3.

Figure 3. Images of bread produced with (a) 0%, (b) 2% (c) 5% and (d) 8% of red palm oil

The control breads (a) had the appearance of a normal baked wholemeal bread. The introduction of palm oil into the formulation had a noticeable effect on the crumb colour (image c and d). The bread shown in Figure (c) appears to be the best loaf of bread made with the inclusion of 5% palm oil based upon the greater uniformity of crumb structure and volume compared with other oil untreated and treated loaves. Although a full sensory evaluation of the product was beyond the scope of this study all breads produced were tested by the authors and colleagues at RMIT and bakery teaching staff at the William Angliss
Institute of TAFE (Melbourne, Australia), apart from a slight orange colour at high % of oil but no negative comments on taste were received and the bread looked and felt like a normal loaf.

3. DISCUSSION

The quantity of vitamin E extracted from freshly milled rye and wheat kernels confirm that wholegrain wheat (containing the wheat germ and thus the wheat germ oil) has a considerable amount of vitamin E. However, because of its high levels of polyunsaturated fatty acids, it is also prone to rancidity/oxidation depending on storage and handling conditions (Enig 2000). Here the wholegrain wheat was milled and immediately combined with the other ingredients (water, salt, yeast) and mixed into a dough. This was found to result in a substantial loss of vitamin E. This may be due to the fact that the dough making process (without the addition of palm oil) resulted in a substantial incorporation of oxygen in the dough which may have facilitated lipoxygenase catalysed oxidation of unesterified, polyunsaturated fatty acids, hence the destruction of vitamin E as has been reported previously (Wennermark and Jägerstad 1992). Grains when milled including the germ have a very high enzyme activity, which are naturally occurring in cereal grain. These enzymes may have a negative effect on bread making qualities. A reduced enzyme activity in refined flours has been reported in literature and the ageing for refined flour is recommended to enhance baking performance. (Personal communication chief scientist David A.I. Suter (2013).

It was found in this work that both temperature and fermentation time had minimal effect on total tocol quantity in the final wheatmeal bread and the increase of vitamin E content in the bread is mainly attributed to the addition of palm oil. In the wheat bread variety without oil,
the decline of vitamin E from flour to bread ranged from 76 to 89 % while the wheat bread with an addition of 2 % palm oil had a reduction of 48 %. The total reduction of vitamin E content decreases with the content of palm oil, where at 8 % palm oil the total of vitamin E content is increased by 52 % when compared to wheat meal with no oil.

Most typical rye bread made from wholemeal is prepared using the sourdough process in which the main ingredients: flour, water and starter culture are mixed and fermented for about 24 h and the red palm oil in this study was added after the fermentation stage. The first step involved 35 % of the total rye meal without the addition of yeast. Yeast when added to a wheat dough formulation consumes oxygen (which may account for the increased vitamin E in the final baked bread). However, the absence of yeast in the rye bread formulation, gave a reduction of vitamin E when compared to the wholemeal wheat bulk fermentation and previous studies have reported a reduction of up to 60 % of vitamin E activity in rye sourdough process (Wennermark and Jägerstad 1992). The content of vitamin E in rye bread without the addition of red palm oil was also diminished by 88 to 90 % when compared to the raw meal. However, with the addition of 2 % palm oil, the loss was reduced to 61 %. Furthermore, reduction was even lower at 8 % of palm oil with only a deterioration of only 18 % of the vitamin E content.

Palm oil contains similar amounts of unsaturated and saturated fatty acids, making it a very stable ingredient that is: less prone to oxidise, well known to have high levels of β-carotene (the precursor of vitamin A), including other carotenes and vitamin E. Some of the commercial grade palm oil appears a reddish colour due to high carotene content (Enig 2000). β-carotene is insoluble in water and soluble in fats and oils as a result of its conjugated double-bond structure of the molecule. However, once exposed to air it will usually not only
oxidise but decompose. For example, Heinonen et al. (1997) reported a synergistic effect of α-tocopherol and β-carotene on oxidation of 10 % oil in water emulsion of rapeseed oil. Natural secondary antioxidants such as carotenoids often possess excellent singlet oxygen-quenching properties (Decker 1998). This synergistic effect of α-tocopherol and β-carotene could possibly be the reason for the increasing the amount of vitamin E recovered in final baked loaves. However, this is beyond the scope of the current study. Nevertheless the results demonstrate that, environmental considerations aside, palm oil could be a very useful ingredient in wholemeal and rye flour based baked goods as it will increase the health benefits of the final products.

4. CONCLUSION

Our results show that compared to the control loaves baked in this study, the inclusion of palm increased the quantity of tocopherols and trienols found in both wholemeal wheat and rye. It is concluded that palm oil was effective in increasing the vitamin E content of wholegrain breads. Palm oil appeared to provide further advantages as there was a wider range of E vitamers retained during breadmaking.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.
REFERENCES


Chapter 9

Results and discussion

The influence of fermentation processes and cereal grains in wholegrain bread on reducing phytate content

Journal article published in Journal of Cereal Science published by Elsevier presented as Chapter 9
The influence of fermentation processes and cereal grains in wholegrain bread on reducing phytate content

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Abstract
Wholegrain bread is generally thought of as being more healthy than white bread due to it having a higher content of dietary fibre, vitamins (especially vitamin B and E) and many important minerals. However, wholegrain bread also contains high levels of phytate (myo-inositolhexakisphosphate, InsP-6) which may bind desirable nutrients, preventing their absorption in the gut and thereby reducing the nutritional value of the end product. In order to evaluate factors influencing phytate levels, the effects of fermentation and selected wholemeal flours from rye, oats and wheat were investigated. Phytate levels were assessed using a spectrophotometric assay based on the measurement of iron with 2,2'-bipyridine. Phytate decreased in freshly ground wholegrain flour dough during the fermentation process with time of fermentation being the most important factor. Fermentation temperature was found to make only a small difference to the process of phytate reduction. Since the potential benefits of wholemeal breads incorporating various grains (e.g. oats and rye) are increasingly evident, this research has important implications for human health.

1. Introduction
The milling of grain to give white flours reduces the levels of many nutrients as well as phytate (myo-inositolhexakisphosphate, InsP-6). In contrast, wholegrain products are known to have both high phytate levels and high nutrient content (Fardet, 2010; Stevenson et al., 2012). Wholegrain products also have a wide range of benefits to human health, for example they are associated with reduced risk of many chronic diseases including heart disease and diabetes (Jones, 2011; Mellen et al., 2007). Wholegrain cereals and legumes are the major sources of dietary phytate intake. The interaction of phytate and dietary minerals and beneficial health effects of phytate have been the subject of a review (Kumar et al., 2010). Phytates can chelate and bind minerals, resulting in insoluble complexes that may lead to a decrease in mineral absorption and bioavailability, and therefore the removal of phytates from baked goods has long been considered desirable. Evidence demonstrating a diverse range of benefits to health and wellbeing is now accumulating (Kumar et al., 2010).

Fermentation has been shown to decrease the amount of phytate in wholegrain cereals (Liukkonen et al., 2003; Sanz-Penella et al., 2012). For example, Leenhardt et al. (2005) assessed changes in phytate hydrolysis brought about by sourdough fermentation or exogenous organic acid addition using an in vitro trial. They found that a slight acidification (pH ~ 5.5) of the dough with either sourdough or via the addition of lactic acid caused a significant phytate reduction (~ 35%).

A direct comparison of the reduction in phytate using wholegrain for the bulk fermentation of wheat doughs and rye sourdough fermentation has not previously been reported. Therefore, the objective of the present study has been to thoroughly investigate the effect of fermentation of doughs prepared with selected wholegrain cereal meals on the levels of phytate during breadmaking. This includes the optimisation of suitable fermentation conditions, including time and temperature in relation to various blends of cereal grains. The effect of different amounts of palm oil on phytate levels was also investigated as fats and oils are commonly used to enhance volume and softness of wholegrain bread.

2. Materials and methods
2.1. Preparation of bread samples
2.1.1. Milling process
For all bread varieties, grains were milled to provide wholemeal flour on the day of the experiment. The mill was used was a bench top unit (Grain Master Whisper Mill, Retsel Dandenong, Victoria, Australia), which uses upright blades spinning at high speed
(10,000 rpm) to produce a relatively fine meal with small particle size and a large surface area. This gives a greater area that microflora can interact with and thus increases the fermentability of the meal.

2.1.2. Preparation of bread

For all baking trials, a central composite design was developed using Minitab software (version 16). The centre points of each were used to estimate reproducibility of the method and to check whether the response surface had curvature.

For the dough preparation, 100% wheat meal, 70% water, 2, 5, 8% red palm oil, 2% salt, 0.2% instant dry yeast (NB: All percentage values are relative to the total flour weight) were first mixed using a bench mixer with 10 different speeds (Kitchen Aid Heavy Duty, Model 5KPM50, Benton Harbor, USA) at slow speed (setting 2) for 4 min and followed by fast speed (setting 4) for 6 min until full dough development was achieved — corresponding to the time when the dough could be readily removed from the dough hook and the mixing bowl (Suas, 2008). The wheatmeal dough was mixed and then bulk fermented for 5 h at a temperature of 30 °C.

The dough was weighed (180 g) and placed into bread tins prior to baking. The rye bread making procedure differed from that for wheatmeal bread since the two meals with small particle size were also prepared using the procedure described above to facilitate direct comparisons.

The rye bread making procedure differed from that for wheatmeal bread since the first step involved sourdough fermentation where 35% of the total rye meal weight was fermented with 10% starter culture. The production of starter culture involved a 24 h incubation of rye meal dough with the ratio of rye meal to water 1:1, activation with 1% of the ripe sourdough and this was repeated every 24 h over three days after which time the microbial community had developed fully, assessed by the presence of a characteristic sourdough aroma. The rye bread formulation consisted of 90% rye meal, 10% wheatmeal, 100% water, 2, 5 and 8% red palm oil, 2% salt and 1% instant dry yeast; again all percentage values are relative to the total flour weight. Following the 24 h incubation of the sourdough, the remaining 65% flour and the other ingredients were incorporated. The dough was mixed for 10 min at slow speed (speed setting 2), and then dough was scaled (250 g) and placed into bread tins prior to the final proofing stage which was at 37 °C for 45 min. Baking was then carried out at 230 °C for 10 min followed by a further 15 min at 200 °C.

2.1.3. Sampling

During the making of the wheatmeal bread and wheat blends, samples were taken from the dough at four stages; after mixing, then after fermentation, after the final proof (just before entering the oven) and finally, after baking. In the rye bread making procedure, the samples were taken after inoculation, fermentation, mixing, final proof and baking.

2.1.4. Freeze drying

All samples were immediately frozen at −40 °C in a blast freezer and placed in a controlled freeze dryer (VirTis SP Industries Company, Gardiner, USA) to obtain low-moisture-content samples for further analysis. Freeze dried samples were ground in a mortar to form powder and samples were stored in air-sealed containers at −18 °C prior to analysis.

2.2. Characterisation of samples

2.2.1. Moisture content

The moisture content of samples was measured according to the AACC International air oven method (AACC International, 2010b).

Empty aluminium moisture dishes were placed into a pre-heated oven set at 130 ± 3 °C. After 1 h, the empty dishes were taken from the oven and cooled in a desiccator containing active silica gel desiccant for a period of 30 min and then weighed. Sub-samples (~5 g) were accurately weighed into pre-weighed dishes. The dishes containing the samples were then placed into the oven and dried at 130 ± 3 °C for 1 h. The process of the drying, cooling and weighing was repeated three times, until a constant weight was attained.

2.2.2. Determination of phytate

The phytic acid was determined by the method described by Haug and Lantzsch (1983), in which the phytic acid is precipitated with an iron-III solution of known iron content and the decrease in iron in the supernatant is taken as a measure of phytic acid content. Phytate reference solution containing phytic acid sodium salt hydrate from rice (type V 94% purity and ~6% water) was obtained from Sigma–Aldrich, Sydney, Australia. Stock solutions were prepared with 1.3 mg/mL phytic acid. Ferric ammonium sulphate solution was prepared by dissolving 0.2 g NH₄Fe(SO₄)₂·12H₂O in 100 mL 2 mol/L HCl and the volume was made to 1000 mL with distilled water. The 2.2′-bipyridyl solution was prepared by dissolving 1.0 g 2,2′-bipyridine and 1.0 mL thiglycollic acid in distilled water and making up the volume to 1000 mL.

For the analysis of the cereal grains (wheat, rye and oat) and bread doughs as well as bread, 0.5 g of sample was extracted with 50 mL of 0.5 mol/L HCl for 3 h followed by centrifugation for 30 min at 3000 rpm. The extract (0.5 mL) was pipetted into a 15 mL centrifuge tube. Then 1 mL of ammonium iron (III) sulphate solution was added. The tubes were incubated in a boiling water bath for 30 min then cooled in ice water to adjust to room temperature. Once the tubes had reached room temperature, 1.5 mL of 1% v/v 2,2′-bipyridyl solution was added. The absorbance was immediately measured at 519 nm against distilled water and the test method was calibrated with the reference solutions prepared by diluting the stock solution with 0.2 mol/L HCl in a range of 0.1–1.0 mL (3.12–31.2 μg/mL phytate phosphorus).

2.2.3. Determination of pH and total titratable acidity (TTA)

The pH values of all samples were measured according to the AACC International hydrogen ion activity (pH)-electrometric method (AACC International, 2010a). For this, 15 g of samples were weighed and agitated with 100 mL distilled water using a bath mixer until an even suspension, free of lumps, was obtained. The suspension was rested (25 °C) for 30 min, then agitated continuously to keep particles in suspension, then rested for a further 10 min. The supernatant liquid was placed into an electrode vessel and the pH value was immediately determined using a potentiometer and electrode that had been calibrated against known buffer solutions. After the pH value was obtained and recorded, 0.1 mol/L NaOH for rye bread varieties and 0.01 mol/L for the wheat bread varieties was slowly added from a burette and stirred constantly until a constant pH of 6.6 was obtained. The volume of NaOH (mL) used is reported as the TTA.

3. Results

3.1. pH profile and TTA in sourdough

The acidity of the sample during various processing stages affects the phytate levels. The pH and phytate content for 100% wholemeal, a blend of wholemeal 70% wheat and 30% oat and rye bread during various processing stages are summarized in Tables 1–3, respectively.
The pH of bulk fermented wheat dough, as well as that prepared from the wheat and oat blend, decreased from around 6.5 following mixing to 5.3 after fermentation. The losses in phytate for these two doughs during mixing were considerable (approx 45% of the initial levels) with further reductions upon fermentation to 52 and 62% respectively (Tables 1 and 2). There was no significant change in pH after final proofing in both samples; however the reduction of phytate was greater in the wheat bread after final proofing (63%) than for the wheat and oat blend (53%). There was no decrease in pH nor a reduction in phytate during the baking process in either of the bread samples. A similar pattern of pH changes in rye sourdough fermentation were different from that of the bulk fermentation of the wheat and wheat and oat blend. After inoculation and a 24 h fermentation period at various temperatures (23, 30 and 37 °C), the pH of rye sourdough dropped from 6.53 to as low as 3.95 (Table 3). At this stage we also observed a high reduction of phytate, which may also be due to the higher TTA value 4.3–10.4, reflecting an increase in acidity. This confirms observations reported in a previous study (Fretzdorff and Brummer, 1992).

3.2. Phytate content in sourdough

The results of phytate degradation during the wheat bread process using 100% wholemeal are displayed in Fig. 1 and those for the blend of wholemeal 70% wheat and 30% oat are in Fig. 2. Phytate levels are lower at the low pH brought about by fermentation, than after mixing in both wholemeal and wholemeal oat blends. In the case of rye bread (Fig. 3), a very large reduction in phytate occurs after inoculation followed by the expected rise after further mixing with rye meal and a further reduction after final proof.

3.3. Effect of processing conditions

The results of processing conditions tested in these experiments, namely the percentage of palm oil incorporated (2, 5 and 8%), temperature of fermentation and proofing (23, 30 and 37 °C) and time for fermentation and proofing (3, 5 and 7 h) are also shown in Figs. 1–3. Varying the content of palm oil appeared to have no detectable effect on the reduction in phytate in any of the breads prepared. Likewise the effect of temperature, for the range considered, was of relatively little consequence and was only revealed at short (3 h) fermentation times.

The data in Fig.1 show that for 100% wheat bread, fermentation even for 3 h, was sufficient to give noticeable reductions in phytate levels. However, greater reductions were obtained using 5 h fermentations and no further reduction was achieved by extending this to 7 h. For 70% wheat and 30% oat blends, (Fig. 2) the results were similar to the 100% wheat bread and showed again that longer fermentation times (5, 7 h) gave the highest reduction in phytate with temperature of lesser importance. In both cases, proofing and baking caused no significant reductions after fermentation. With regard to the rye bread (Fig. 3), large reductions in phytate were obtained after inoculation and fermentation, with marginally lower results at 37 compared to 30 °C. At a temperature of 23 °C, with incorporation of 2% palm oil, less phytate was lost in comparison with others in the series.

One important difference in the results for the rye bread experiment was that the reductions in phytate were greater (up to 85.5% reduction) compared with wheat (63%) and wheat oat blends (53%). Furthermore reductions during the final proof were observed, but there was no further reduction upon baking (Table 3).

4. Discussion

4.1. TTA and pH profile in sourdough

Many studies have indicated that phytate hydrolysis during dough fermentation significantly enhances the bioavailability of minerals including calcium, copper, magnesium, zinc and iron (Frontela et al., 2011; Haros et al., 2008; Pozrl et al., 2009; Reale levels. However, greater reductions were obtained using 5 h fermentations and no further reduction was achieved by extending this to 7 h. For 70% wheat and 30% oat blends, (Fig. 2) the results were similar to the 100% wheat bread and showed again that longer fermentation times (5, 7 h) gave the highest reduction in phytate with temperature of lesser importance. In both cases, proofing and baking caused no significant reductions after fermentation. With regard to the rye bread (Fig. 3), large reductions in phytate were obtained after inoculation and fermentation, with marginally lower results at 37 compared to 30 °C. At a temperature of 23 °C, with incorporation of 2% palm oil, less phytate was lost in comparison with others in the series.

One important difference in the results for the rye bread experiment was that the reductions in phytate were greater (up to 85.5% reduction) compared with wheat (63%) and wheat oat blends (53%). Furthermore reductions during the final proof were observed, but there was no further reduction upon baking (Table 3).

Table 1

<table>
<thead>
<tr>
<th>Condition</th>
<th>pH</th>
<th>TTA</th>
<th>Phytate reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>After mixing</td>
<td>6.51</td>
<td>11.5</td>
<td>45.6</td>
</tr>
<tr>
<td>5% (1)^a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% (2)^a</td>
<td>6.53</td>
<td>11.5</td>
<td>45.6</td>
</tr>
<tr>
<td>After fermentation</td>
<td>5% 30 °C 5 h (1)^a</td>
<td>5.36</td>
<td>19.1</td>
</tr>
<tr>
<td>5% 30 °C 5 h (2)^a</td>
<td>5.36</td>
<td>19.1</td>
<td>60.8</td>
</tr>
<tr>
<td>After final proof</td>
<td>5% 30 °C 5 h (1)^b</td>
<td>5.29</td>
<td>21.5</td>
</tr>
<tr>
<td>5% 30 °C 5 h (2)^b</td>
<td>5.27</td>
<td>21.4</td>
<td>63.20</td>
</tr>
<tr>
<td>After baking</td>
<td>5% 30 °C 5 h (1)^b</td>
<td>5.43</td>
<td>14.0</td>
</tr>
<tr>
<td>5% 30 °C 5 h (2)^b</td>
<td>5.43</td>
<td>14.0</td>
<td>63.2</td>
</tr>
</tbody>
</table>

Note: 5% (w/w) indicate oil content in bread formulation.  
^a mL 0.01 mol/L NaOH/15 g sample. Raw ingredient wheatmeal contained 1.25 μg/g of phytate.  
^b Centre point.

Table 2

<table>
<thead>
<tr>
<th>Condition</th>
<th>pH</th>
<th>TTA</th>
<th>Phytate reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>After mixing</td>
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<td></td>
<td></td>
</tr>
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<td>6.45</td>
<td>12.7</td>
<td>44.0</td>
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<tr>
<td>5% (2)^a</td>
<td>6.44</td>
<td>12.6</td>
<td>43.2</td>
</tr>
<tr>
<td>After fermentation</td>
<td>5% 30 °C 5 h (1)^a</td>
<td>5.32</td>
<td>20.8</td>
</tr>
<tr>
<td>5% 30 °C 5 h (2)^a</td>
<td>5.31</td>
<td>20.7</td>
<td>52.7</td>
</tr>
<tr>
<td>After final proof</td>
<td>5% 30 °C 5 h (1)^b</td>
<td>5.28</td>
<td>22.2</td>
</tr>
<tr>
<td>5% 30 °C 5 h (2)^b</td>
<td>5.29</td>
<td>22.2</td>
<td>53.5</td>
</tr>
<tr>
<td>After baking</td>
<td>5% 30 °C 5 h (1)^b</td>
<td>5.35</td>
<td>14.8</td>
</tr>
<tr>
<td>5% 30 °C 5 h (2)^b</td>
<td>5.37</td>
<td>14.8</td>
<td>53.5</td>
</tr>
</tbody>
</table>

Note: 5% (w/w) indicate oil content in bread formulation.  
^a mL 0.01 mol/L NaOH/15 g sample. Raw ingredient rye meal contains 0.69 μg/g.  
^b Centre point.

Table 3

<table>
<thead>
<tr>
<th>Condition</th>
<th>pH</th>
<th>TTA</th>
<th>Phytate reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>After inoculation</td>
<td>6.53</td>
<td>4.3</td>
<td>31.8</td>
</tr>
<tr>
<td>After fermentation</td>
<td>5% 30 °C 24 h (1)^a</td>
<td>3.94</td>
<td>29.9</td>
</tr>
<tr>
<td>5% 30 °C 24 h (2)^a</td>
<td>3.95</td>
<td>28.0</td>
<td>81.1</td>
</tr>
<tr>
<td>After mixing</td>
<td>5% 30 °C 5 h (1)^b</td>
<td>5.21</td>
<td>10.4</td>
</tr>
<tr>
<td>5% 30 °C 5 h (2)^b</td>
<td>5.21</td>
<td>11.1</td>
<td>68.1</td>
</tr>
<tr>
<td>After final proof</td>
<td>5% 30 °C 12 h (1)^b</td>
<td>4.82</td>
<td>10.3</td>
</tr>
<tr>
<td>5% 30 °C 12 h (2)^b</td>
<td>4.86</td>
<td>10.3</td>
<td>85.5</td>
</tr>
<tr>
<td>After baking</td>
<td>5% 30 °C 12 h (1)^b</td>
<td>5.12</td>
<td>10.4</td>
</tr>
<tr>
<td>5% 30 °C 12 h (2)^b</td>
<td>5.13</td>
<td>10.4</td>
<td>85.5</td>
</tr>
</tbody>
</table>

Note: 5% (w/w) indicate oil content in bread formulation.  
^a mL 0.1 mol/L NaOH/15 g sample. Raw ingredient rye meal contains 0.69 μg/g.  
^b Centre point.
et al., 2007; Saleh et al., 2013). Phytate hydrolysis occurs throughout the different stages of bread making and obviously depends on the type of bread being prepared. In our study, the results indicate that the proving time had the most significant impact on the phytate reduction which might enhance mineral bioavailability of the final products. Breadmaking with sourdough fermentation may result in more suitable pH conditions for the degradation of phytate by endogenous phytases and the sourdough fermentation may also be a source of microbial phytases.

Lactic fermentation is a traditional method for food processing and preservation developed over a long period of time. Due to the production of lactic acid and other organic acids, the pH is lower and the phytase activity is likely to be increased. A correlation between pH and phytate content can be obtained showing that samples with higher pH have higher phytate content. It can be concluded that phytate content depends on the pH of the sample. In our samples with lower pH, phytate content was also lower. The influence of lower pH could be observed especially during the rye sourdough fermentation. When the remaining flour content was added to the sourdough, the phytate levels increased as expected. However, the levels later decreased again to levels lower than those observed after the initial fermentation process. A slight increase in pH was also observed after the baking process.

4.2. Sourdough phytate content

The phytate content is higher in whole grain and in wholemeal flour compared to flour with low extraction rate (refined flour) (García-Estepa et al., 1999). The content of phytic acid in rye grain/rye meal has been reported to vary between 0.54 and 1.46% (Reddy, 2001). Freshly ground whole grain was incorporated into the bread formulation in the current study. The barrier to destruction of phytate in whole wheat dough above pH 6 is the insolubility of its magnesium and calcium salts whereas at pH 5, the limiting factor appears to be the level of phytase activity (Tangkongchitr et al., 1982). Other workers (Fretzdorff and Brummer, 1992) found that the pH was the most important factor influencing the reduction in content of phytic acid during breadmaking as phytic acid in dough of pH 4.3–4.6 lowered more than in dough with higher pH. This is also indicated in our rye bread data (Table 3).

Our results show some interesting changes in phytate level for the bread formulation with long term sourdough fermentation (3–7 h) which allows the study of suitable fermentation conditions, including time and temperature. The phytate levels in wheat, oat and rye after milling were 1.25, 1.31 and 0.62 g/100 g, respectively and each of these was lowered considerably during breadmaking. For the wheat variety, this ranged between 44 and
phytase action which in turn is in consequence of the activity of naturally occurring phytase. The reduction of phytate content during breadmaking depends on the activity of phytase during normal wholemeal breadmaking has previously been investigated (Fretzdorff and Brummer, 1992; Türk et al., 1996). It is known that during the transformation of wholemeal into dough and finally into bread, the phytate content decreases as a consequence of the activity of naturally occurring phytase. The reduction of phytate content during breadmaking depends on the phytase action which in turn is influenced by several other factors, particularly proving time, temperature and acidity of the dough (Türk et al., 1996). If one is considering eating bread containing phytate for health reasons, wholemeal bread made from wheat and oat blends is the best option as the maximum phytate reduction was only 44%. On the contrary, it is suggested that those who wish to enhance bioavailability of minerals consume sour-dough rye breads.

5. Conclusion

In this study, phytate degradation was investigated to study its dependence on the flour meal type and different breadmaking procedures (bulk fermentation and rye sourdough fermentation). Both fermentation methods were found to be useful techniques to influence phytate level and hence mineral bioavailability. During breadmaking, significant degradation of phytate occurred, being higher for those formulations having a higher initial phytate content. The highest content was found in the bulk fermented sample while the lowest phytate content was detected after the rye sourdough fermentation and final proof. The rye sourdough fermentation gave the greatest reduction in phytate levels and temperature also influenced these levels but to a lesser extent. Rye sourdough fermentation because of the lowering of pH had a greater effect on phytate reduction than the bulk fermented wholemeal bread. Significant reductions in phytate of wheat and wheat-containing bread can be brought about by fermentation.

Acknowledgements

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References


Chapter 10

Results and discussion

The Effect of Fermentation and Addition of Vegetable Oil on Resistant Starch Formation in Wholegrain Breads
Chapter 10

The Effect of Fermentation and Addition of Vegetable Oil on Resistant Starch Formation in Wholegrain Breads

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Abstract

Dietary starches are significant sources of energy for human populations and they can also make a specific input to overall health. In this study, the formation of resistant starch in wholemeal bread products was evaluated in relation to the processing conditions including fermentation time, temperature and the inclusion of palm oil as a vitamin source. In addition, the impact on final starch content of traditional sourdough fermentation of wholemeal rye bread, as well as the bulk fermentation process of wheat and wheat/oat blends of wholemeal bread, was assessed by enzyme assay. Varying the content of palm oil was found to have a significant effect on the formation of resistant starch in all of the breads prepared. In contrast, both fermentation time and temperature made only a small impact on resistant starch formation. Sourdough fermentation of rye bread was found to have a greater impact on resistant starch formation than the bulk fermentation wheat and wheat blend breads, possible due to an increase in organic acid content in the sourdough process.

Keywords: Dietary fibre; Dough fermentation; Prebiotics; Resistant starch; Wholegrain
1. Introduction

Until relatively recently, starch was considered to be a completely digestible carbohydrate. However, this assumption was incorrect when starch remnants were detected in faeces after consumption of a high starch meal \( (1) \). Significant amounts of starch escape digestion and absorption in the small intestine of healthy individuals but can be fermented in the large intestine where they encourage the growth of health-promoting bacteria, reduce pH and increase the production of \( \text{t butanoic (butyric) acid through fermentation. This type of starch is known as resistant starch (RS)} \) \( (2) \).

RS has now been identified as a bioactive component of food, which can be formed in cereal fermentations \( (3) \). It involves the conversion of carbohydrates to alcohol and carbon dioxide and/or organic acids using yeast, bacteria or a combination of both, under anaerobic conditions. It is hypothesised that the time-intensive bulk fermentation and sourdough production processes promote retrogradation of starch via realignment of amylose and amylopectin chains, thereby increasing RS formation in the finished products. The fermentation process and its potential nutritional benefits may contribute to an enhanced health status, as well as reduction of starch digestibility \( (3, 4) \).

The increase in consumer demand for healthy, high quality foods in recent years has led to a growth in the use of new technologies as well as novel ingredients including RS. Wholegrain flour contains more RS than white (refined) flour and the health benefits of consumption of RS in the form of wholegrain products include its role as a prebiotic \( (5, 6) \), a reduction in the incidence of chronic diseases, particular diabetes and colon cancer \( (7) \) along with weight loss \( (8) \).
Previous studies have indicated that processing conditions can affect the formation of RS by influencing the gelatinisation and retrogradation of normal starch (9, 10). It has also been reported that it is possible to make a physically functional RS ingredient by the application of physical processes to a starch suspension (11). Furthermore it is also possible to increase the RS content in foods by modifying processing conditions including pH, heating temperature and time, number of heating and cooling cycles as well as by freezing and drying (12).

To the best of our knowledge, an investigation of the formation of RS using either wholegrain flours for the bulk fermentation of wheat doughs or rye sourdough fermentation has not been reported. Therefore, the objective of the present study was to investigate the effect of fermentation of doughs prepared with selected wholegrain cereal meals on the levels of RS during breadmaking. This includes the optimisation of suitable fermentation conditions, including time and temperature, in relation to various blends of cereal grains. Since fats and oils are commonly used to enhance volume and softness of wholegrain bread the effect of incorporating different amounts of palm oil on RS levels was also investigated.

2. Materials and Methods

2.1 Preparation of Bread Samples

2.1.1 Milling process

For all bread varieties, grains were milled to provide wholemeal flour on the day of the experiment. The mill used was a Grain Master Whisper Mill bench top unit (Retsel
Dandenong, Victoria, Australia), which uses upright blades spinning at high speed (10,000 rpm) to produce a relatively fine meal with small particle size and a large surface area for microbiota to interact with, thus increasing the fermentability of the meal.

2.1.2 Optimisation of Fermentation Time and Temperature

In a preliminary experiment to establish the fermentation time for the bulk fermented bread freshly prepared wheat and wheat/oat blends doughs were placed in glass beakers, plastic wrapped and placed in an incubator at 37°C (the proving temperature used in commercial bakeries) or at room temperature (23°C). Three fermentation times for further study were selected as those when the prepared dough started to increase in size, after ~3 h (minimum time) and when it started to collapse, which was after ~7 h. The time and temperature variables were then entered into Minitab software (version 16, Minitab Pty, Sydney, Australia) and the centre points of each data set were used to estimate reproducibility of the method and to check whether the response surface had curvature, calculated at 5h and 30°C respectively. The temperatures but not the fermentation times were applied to the rye sour dough fermentation. The fermentation time for rye sour dough was set at 24h (which is the minimum time needed for traditional rye sourdough fermentation).

2.1.3 Preparation of Bread

The dough ingredients for the wheat and wheat-/oat blend flours were the same: 100% wheatmeal, 70% water, 2, 5, or 8% red palm oil, 2% salt, 0.2% instant dry yeast (note: all percentage values are relative to the total flour weight). These were first mixed using a Kitchen Aid Heavy Duty bench mixer with 10 different speeds (Model 5KPM50, Benton
Harbor, USA). Mixing was carried out initially at slow speed (setting 2) for 4 min and followed by fast speed (setting 4) for 6 min until full dough development was achieved – defined as the time when the dough could be readily removed from the dough hook and the mixing bowl (13). The wheatmeal dough was mixed and then bulk fermented for 5 h at a temperature of 30 °C. The dough was weighed out into 180 g sections and placed into bread tins prior to the final proof at 37 °C for 45 min. Baking was at 230 °C for 10 min followed by 15 min at 200 °C.

The bread making procedure for rye differed from that for wheatmeal since the first step involved sourdough fermentation. For this, 35% of the total rye meal weight was fermented with 10% starter culture. This is due to their different biochemistries. Wheat amylases are generally not heat-stable and break down at temperatures over 40 °C, so can not affect the wheat glutens that give wheat bread its structure. In contrast, rye amylase remains active up to 50 °C (14), and rye gluten is not particularly stable. In Sourdough fermentation the acidic *Lactobacillus* culture and an acid-tolerant yeast strain are added to the flour to lower the pH which inactivates the rye enzymes and helps gelatinize starches in the dough matrix. The production of starter culture involved a 24 h incubation of rye meal dough with the ratio of rye meal to water 1:1, activation with 1% of the ripe sourdough, and this was repeated every 24 h over 72 hours to allow the microbial community to develop fully; this was assessed by the presence of a characteristic sourdough aroma (15).

The rye bread formulation consisted of 90% rye meal, 10% wheatmeal, 100% water, 2% salt and 1% instant dry yeast. Again, all percentage values are relative to the total flour weight. Three treatment levels of red palm oil were used (2, 5 and 8%). Following the 24 h incubation of the sourdough, the remaining 65% flour and the other ingredients were
incorporated into the dough which was mixed for 10 min at slow speed (speed setting 2). This was then divided into 250 g portions and placed into bread tins prior to the final proofing stage which was at 37 °C for 45 min. Baking was then carried out at 230 °C for 10 min followed by a further 15 min at 200 °C.

2.1.4 Freeze Drying of samples

All samples of dough and baked breads were immediately frozen at -40 °C in a blast freezer and placed in a controlled freeze dryer (VirTis SP Industries, Gardiner, Montana, USA). Each freeze dried samples was ground in a mortar to form a powder and samples were stored in air-sealed containers at -18 °C prior to analysis.

2.2 Characterisation of Samples

2.2.1 Moisture Content

The moisture content of samples was measured according to the American Association of Cereal Chemists (AACC) International air oven method (AACC International, 2010a). Empty aluminium moisture dishes were placed into a pre-heated oven set at 130 ± 3 °C. After 1 hour, the empty dishes were taken from the oven and cooled in a desiccator containing active silica gel desiccant for a period of 30 min and then weighed. Sub-samples (~5 g) were weighed into the pre-weighed dishes. The dishes were placed into the oven and dried at 130 ± 3 °C for 1 h. The process of drying, cooling and weighing was repeated three times so that a constant weight was attained for each bread sample.
2.2.2 Determination of Resistant Starch

The analysis of RS was undertaken using an enzymatic assay (Megazyme International, Bray, Co. Wicklow, Eire) following procedure provided by the manufacturer. The samples were incubated in a shaking water bath with pancreatic α-amylase and amyloglucosidase (AMG) for 16 h at 37 °C. During this time non-RS was solubilised and hydrolyzed to D-glucose by the combined action of the two enzymes. The reaction was terminated by the addition of an equal volume of ethanol and the RS recovered as a pellet on centrifugation. Free liquid is removed by decantation. RS in the pellet was dissolved in 2 M KOH by vigorously stirring in an ice-water bath using a magnetic stirrer. This solution was neutralized with acetate buffer and the starch quantitatively hydrolysed to glucose with AMG. D-Glucose was measured with glucose oxidase/peroxidase reagent (GOPOD) and this measurement was used to calculate the total RS content of the sample. In order to validate the assay procedure a reference sample was analysed (regular maize starch 0.67% RS ; 14% moisture). The RS contents found for the reference sample are represented in Table 1 and the figures reported here were adjusted to reflect these recovery rates.

<table>
<thead>
<tr>
<th>Sample</th>
<th>RS (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set 1</td>
<td>0.69 ± 0.02</td>
</tr>
<tr>
<td>Set 2</td>
<td>0.70 ± 0.02</td>
</tr>
<tr>
<td>Set 3</td>
<td>0.67 ± 0.01</td>
</tr>
</tbody>
</table>

* Recoveries were adjusted to the dry matter basis (DMB).
2.2.3 Determination of pH and Total Titratable Acidity (TTA)

The pH values of all samples were measured according to the AACC International hydrogen ion activity (pH)-electrometric method (AACC International, 2010a). For this, 15 g of each sample was weighed and agitated with 100 mL distilled water using a bath mixer until an even suspension, free of lumps, was obtained. The suspension was rested at 25 °C for 30 min, then agitated continuously to keep particles in suspension, then rested for further 10 min. the supernatant liquid was placed into a beaker and the pH value was immediately determined using a potentiometer and electrode that had been calibrated against buffer solutions of known pH. After the pH value was obtained and recorded, 0.1 mol/L NaOH for rye bread varieties or 0.01 mol/L for the wheat bread varieties was slowly added from a burette and stirred constantly until a constant pH of 6.6 was obtained. The volume of NaOH (mL) used is reported as the TTA.

Table 2 levels of the factors used for the 2 level full factorial design

<table>
<thead>
<tr>
<th>Fermentation</th>
<th>time (h)</th>
<th>Temperature (°C)</th>
<th>Palm oil %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Actual values and coded values</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>3</td>
<td>-1</td>
<td>23</td>
</tr>
<tr>
<td>High</td>
<td>7</td>
<td>+1</td>
<td>37</td>
</tr>
<tr>
<td>Centre points</td>
<td>5</td>
<td>0</td>
<td>30</td>
</tr>
</tbody>
</table>
3. Results

Table 3 tabulates the regression coefficients of the variables.

Would avoid regression coefficients and use ‘main and interaction effects’; easier to explain

Table 3 Regression coefficients of second-order polynomial models for responses performed.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Resistant starch (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rye 100a 90/10b Wheat/Oat 80/20c 70/30d</td>
</tr>
<tr>
<td></td>
<td>Rye 100a 90/10b 80/20c 70/30d</td>
</tr>
<tr>
<td>Constant</td>
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<td>0.000</td>
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<td>Percent c</td>
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<td>0.0061</td>
</tr>
<tr>
<td>Time e</td>
<td>0.0007</td>
</tr>
<tr>
<td>Percent * temperature</td>
<td>-0.0037</td>
</tr>
<tr>
<td>Percent * time</td>
<td>-0.0022</td>
</tr>
<tr>
<td>Temperature * time</td>
<td>-0.0135</td>
</tr>
<tr>
<td>Percent * temperature * time</td>
<td>-0.0060</td>
</tr>
<tr>
<td>R² (%)</td>
<td>99.48</td>
</tr>
</tbody>
</table>

*Percent = percentage of oil in the bread; Temperature = fermentation temperature; Time = fermentation time; *Coef. = coefficient; 
* p is the probability that H₀ (null hypothesis) is true, i.e. coefficient is not significant
The comparison of RS content in wholemeal wheat, rye and wheat and oat blend breads (including literature references for comparison) is presented in figure 1. The amount of RS formation in sour dough fermented rye bread ranged from 3.91 to 2.86 %. This was higher than the amount recovered in bulk fermented wheat bread which was only 1.23% at the lowest and 2.06 % at the highest. In the wheat and oat blend varieties, the maximum quantity of RS recovered was 2.21 % and the minimum was 1.47 %.

A factorial design was adopted to evaluate the results of processing conditions including the percentage of palm oil incorporated (2, 5 and 8 %), temperature of fermentation and proofing (23, 30 and 37°C) and time for fermentation and proofing (3, 5 and 7h) results are shown in
Figure 2. The RS content decreases with the increase in oil content (2% palm oil in the bread formulation shows then highest formation of RS and 8% shows the lowest). A slightly higher RS formation in bread samples containing 30% of oats in the formulation was also observed (data note shown). Temperature had little effect on the formation of RS in this study.

Note: Recoveries were adjusted to the dry matter basis (dmb).  
Note: 2, 5 and 8% indicate oil content in bread formulation. * = Centre point

Figure 2. The comparison of RS content in wholemeal wheat bread with varies percentage addition of red palm oil and their fermentation temperatures.
Note: Recoveries were adjusted to the dry matter basis (dmb). Note: 2, 5 and 8% indicate oil content in bread formulation. * = Centre point

Figure 2 The comparison of RS content in wholemeal sourdough fermented rye breads including amounts of oil added to dough formulation and their fermentation temperatures. Error bars on the graph show standard deviation of the mean.

The effect of various processing stages of 100% wheat and 70% wheat 30% oat blend bread including processing stages of rye bread making process on pH and TTA in centre point experiment were carried out and results were previously reported (18). The pH of bulk fermented wheat dough, as well as that prepared from the wheat and oat blend, decreased from around 6.5 following mixing to 5.3 after fermentation. There was no significant change in pH after final proofing in both samples. The pattern of pH changes in rye sourdough fermentation was different from that in the bulk fermentation of the wheat and wheat and oat blend. After inoculation and a 24 h fermentation period at various temperatures (23, 30 and 37°C) the pH of rye sourdough dropped from 6.53 to as low as 3.95.
4. Discussion

4.1 The Effect of Varying Amounts of Palm Oil on RS Levels

The effect of palm oil addition on the formation of RS was investigated. As can be observed in Figure 1 and 2, the presence of the palm oil decreased the yields of RS. This confirms the observations of Escarpa, González, Morales, & Saura-Calixto, (19), who found that the addition of an excess of lipids to autoclaved potato starch reduced the RS yields. Amylose crystallization (RS formation) is competitively affected by amylose complexion with vegetable oil. Lipids mechanically cover part of the starch granules, thus reducing the capacity of the starch molecules to absorb water. This inhibits the swelling of starch granules and subsequent collapse or rupture, and hence ultimate the formation of RS which appears to takes place within hours of the gelatinisation of starch. It involves recrystallization of amylose fraction (linear-(1, 4)-α-D-glucose) whereas staling is believed to involve retrogradation of amylopectin. It is known that the certain lipid derivatives and surface active agents reduce the effective water concentration of starch and assist in increasing the moisture retention of gluten.

4.2 Effect of Sourdough Fermentation Methods on RS Content

In rye sourdough fermentation, after inoculation for 24 h fermentation period at various temperature (23, 30 and 37°C), the pH of the dough dropped from 6.53 to as low as 3.95. This may be the reason for the observed increase in RS. The presence of lactic acid may substantially increase the RS content in bakery products and indeed lactic fermentation is an more traditional method for food processing and preservation.
Other workers (3) have proposed the ability of sourdough processes to reduce starch digestibility, hence, increase in resistant starch. The chemical changes taking place during sourdough fermentation which may diminish the degree of starch gelatinisation, have been reported, hence increasing the formation of RS. It has previously been shown that rye sourdough fermentation increases the formation of RS in bakery products (20, 21). This was also confirmed in our study.

4.3 Effect of Bulk Fermentation Methods on RS Content

Bulk fermentation was used for the doughs made with wheat and wheat/oat blends with periods of 3, 5 and 7 h. This may increase the formation of lactic acid produced by the *Lactobacillus* naturally occurring in cereal grain (22). The acidification of the dough may have an impact on structure-forming components particularly gluten and starch. In bulk fermentation at various temperature (23, 30 and 37°C), the pH was significantly higher than in the sourdough fermentation method and had a negative effect on the formation of RS in those loaves. Bulk fermented breads moreover have shown a decline in TTA when compared to sourdough fermentation. The influence on TTA and pH during fermentation in breadmaking process has been investigated in a recent study (23).

**Conclusion**

In this study, RS was investigated to study its dependence on the fermentation technique and different breadmaking conditions (fermentation time and temperature) as well as the addition of palm oil into bread formulation. Both fermentation methods were found to be useful techniques to influence RS formation and hence potentially increase the known health
benefits. The highest content was found in rye sourdough fermentation sample while the
lowest RS content was detected after the bulk fermentation of wheat and wheat/oat blend
varieties with addition of 8 % palm oil. Palm oil also decreased the RS content in rye bread
when added at 8 % attributed to the amylose complexion with vegetable oil. The rye
sourdough fermentation gave the greatest formation in RS levels and temperature also
influenced these levels but to a lesser extent. Rye sourdough fermentation because of the
lowering of pH had a greater effect on RS formation than the bulk fermented wholemeal
breads.

Acknowledgements

The authors thank David Hogan of Laucke Flour Mill of Bridgewater, Victoria for supplying
the wheat and rye grain samples. One of the authors (OB) also thanks the Grains Research
and Development Corporation for the award of a PhD Scholarship.

(In the conclusion you need to focus on what the FFD design tells us – formalise conclusions
by discussing (a) whether each effect or variable significantly affects RS and (b) their relative
importance. What recommendations would you make about the ideal settings? While this is
not a true optimisation study we can still draw conclusions)
References


Chapter 11

Results and discussion

Reduction of toxic gliadin content of wholegrain bread by the enzyme caricain

Manuscript accepted for publication in Food Chemistry presented as Chapter 11
Increasingly the number of individuals being diagnosed with some form of sensitivity to the proteins in wheat grains represents a cause for concern. Currently, the treatment is dietary withdrawal of gluten, but commercial gluten-free bread presents some undesirable properties. The objective of this study has been to assess the ability of the enzyme caricain (from papaya latex) to detoxify gliadin in whole wheat flour and develop bread suitable for coeliacs and gluten intolerant individuals. Ion exchange chromatography was used to enrich the caricain in papaya latex and an enzyme-linked immunosorbent assay test kit was used for the analysis of gliadin residues in the baked bread. The partially purified enzyme was found to be more effective in reducing gliadin content than the crude papain and the resultant loaves had acceptable crumb and crust characteristics. Caricain appears to be capable of detoxifying gliadin and has the potential to mitigate the problems confronting coeliacs.

1. Introduction

Coeliac disease (CD) is a sensitivity to certain cereals, including wheat, rye, barley, triticale and oats (Mäki et al., 2003). The particular fractions in these cereals, identified as those responsible for CD, are the prolamin group of proteins. These components, notably gliadin in wheat, are a group of storage proteins found in the kernels of cereal grains and these are high in proline content. When ingested by a person with CD, these proteins cause an inappropriate immune response, the consequences of which are damage to the mucosa of the small intestine and malabsorption of nutrients (Mendoza & McCough, 2005; Thompson, 1997). Patients may present with a variety of symptoms and the typical signs including weight loss, diarrhoea, fatigue and flatulence. Other indications of malabsorption may occur in the presence or absence of gastrointestinal symptoms, and particularly include iron deficiency anaemia, fat deficiency and oestrogenic bone disease (Frick & Olsen, 1994; Trier, 1991). Left unchecked, the problems associated with CD may have life-threatening consequences (Frick & Olsen, 1994). It has been reported that CD affects 1% of children and adults both in the United States (Fasano et al., 2003) and Europe (Mäki et al., 2003) with similar prevalence rates in many other countries worldwide, particularly those in which high amounts of wheat bread are consumed.

In most cases the primary symptoms of CD develop during early childhood. These typically include diarrhoea as well as a failure to grow and thrive. However in recent times it has become clear that CD may also arise during adulthood (Grodzinsky, Franzen, Hed, & Strom, 1992). In such cases, symptoms are typically bloating, diarrhoea, abdominal pain, skin rash, anaemia and thinning of the bones (osteoporosis) although these might be a result of many causes and therefore may be associated with conditions other than CD. Such non-specific symptoms can occur for several years before being correctly diagnosed and treated. The aetiology of CD has been the subject of much study and a unified hypothesis (Cornell & Stelmasiak, 2007) recognises the involvement of immunological factors as well as an enzyme deficiency in this disease.

CD is not classified as an allergic reaction, but is an immune-mediated condition initiated in genetically susceptible individuals following the ingestion of foods made from grains containing gluten. Whilst it is a genetic condition, there are reports that CD may be triggered by other factors and these have been investigated in some detail (Anderson, Van Heel, Tye-Din, Jewell, & Hill, 2006). In an early study the toxic action of wheat in susceptible patients was eliminated by pre-digestion of the gluten with crude papain (Messer, Anderson, & Hubbard, 1964). In the form of dry papaya latex, papain contains a variety of proteolytic enzymes including thiolhydrisases such as chymopapain, as well as glutamine cyclotransferase and a number of other activities (Dubey,
Pande, Singh, & Jagannadham, 2007). In a more recent study (Cornell, Doherty, & Stelmasiak, 2010), papaya latex was used as the starting material for the preparation and partial purification of gliadin-detoxifying enzymes containing caricain (EC 3.4.22.30). Previous work has indicated that coeliacs are deficient in particular digestive enzymes (Cornell, 2005) justifying work on the use of an oral enzyme supplement to complete the digestion of small amounts of gluten proteins ingested inadvertently. The main requirement for the detoxification of gluten proteins is that any amounts of gluten proteins ingested inadvertently. The main requirement for the detoxification of gluten proteins is that any amounts of gluten proteins ingested inadvertently. The main requirement for the detoxification of gluten proteins is that any amounts of gluten proteins ingested inadvertently. The main requirement for the detoxification of gluten proteins is that any amounts of gluten proteins ingested inadvertently. The main requirement for the detoxification of gluten proteins is that any amounts of gluten proteins ingested inadvertently.

There is much conjecture as to the types of enzymes responsible in conjunction with studies of the types of peptides responsible for toxicity in CD. Stepniak et al. (2006) have identified a prolyl endopeptidase from Aspergillus niger that is able to degrade T-cell stimulatory peptides in vitro. It is also suspected that caricain, which contains endopeptidases, can achieve detoxification by digestion of immunoreactive fragments of gluten (Cornell & Stelmasiak, 2007, 2012).

In order to evaluate the effects of in vitro experimental treatments, the use of an ELISA analytical method is a valid way of detecting immunoreactive gliadin (Doña et al., 2010). In the development of enzyme supplements and the treatment of foods with enzymes, the use of an ELISA method may be useful as a way to evaluate the efficacy of this approach. Immunoassays typically utilise antibodies for the detection of specific proteins which serve as markers for allergenic food. Different classes of antibodies can be raised against allergenic proteins, including monoclonal and polyclonal antibodies with the former being well suited to the recognition of the specific antigen due to the recognition of one epitope. As polyclonal antibodies recognise multiple epitopes located in the protein chains (being more tolerant to small changes of the antigen) and are cheaper, they are typically utilised by industry for manufacture of ELISA kits. The detection of gliadins using the currently available commercial kit involves monoclonal antibodies to ø-gliadin protein in a non-competitive, sandwich-type ELISA. The gliadin standards supplied in such kits allow measurement of gliadin levels in samples, facilitating quantification across a range of 2.5–25 parts per million (ppm).

The aim of the current investigation has been to evaluate enzyme treatment during processing of a bread product as a means of potentially reducing toxicity for coeliacs.

2. Materials and methods

2.1. Preparation of enriched caricain

The procedure was based on that previously described (Cornell et al., 2010): a column (3.2 x 20 cm) of CM Sephadex C-50 (Pharmacia, Sweden) was equilibrated with 0.02 mol/L phosphate buffer adjusted to pH 4.6 in a cold room at 5°C. A sample (2.5 g) of dry papaya latex, MG 50.000 (Enzyme Solutions, Pty Ltd., Melbourne) dissolved in starting buffer (30 mL) was applied to the column and the eluate monitored for protein at 280 nm. After the unabsorbed material eluted, the pH was increased with a pH 6.5 phosphate buffer (0.02 mol/L). Further elution using the same buffer involved application of a gradient from 0.1 to 0.3 mol/L NaCl in the buffer, followed by 0.8 mol/L NaCl. Fractions corresponding to the peaks at 280 nm were monitored, but only those obtained with the latter eluant were collected and dialysed against distilled water (x3 changes) and freeze dried to obtain enriched caricain. The yield obtained was 16% of the applied crude papaya latex.

Assays of proteolytic activity were carried out using the method of Gravett, Viljoen, and Oosthuizen (1991), based on the use of the benzoylarginine p-nitroanilide (BAPNA) assay.

2.2. Bread dough preparation

Organically grown wheat grain of mixed cultivars (11.7% protein) from the Laucke Flour Mill (Bridgewater, Victoria, Australia) was freshly milled to provide wholemeal flour for breadmaking. The mill used was a bench top unit (Grain Master Whisper Mill, Korea) which uses upright blades spinning at high speed (10,000 rpm) to produce a relatively fine meal with increased surface area. For dough preparation, a bench mixer with 10 different speeds (Kitchen Aid Heavy Duty, Model 5KPM50, Benton Harbor, USA) was utilised. The meal (100%), water (70%), red palm oil (5%), salt (2%) and instant dry yeast (0.2%) were firstly mixed at slow speed (4 min), followed by fast speed (6 min) until a dough was achieved. Caricain in the form of either a crude (CE) or purified (PE) preparation was added (0.01% or 0.03% on a dough weight basis, respectively) after the completion of the slow speed mixing step. The wheat dough was bulk fermented (5–7 h) at a temperature of 30 or 37°C to evaluate the effect of temperature. All doughs were weighed (180 g) and placed into bread tins prior to the final proof at 37°C for 45 min. Baking was at 230°C for 10 min followed by a further 15 min at 200°C in order to bake the bread evenly.

2.3. Characterisation of bread samples

The moisture content of samples was measured according to the AACC International air oven method (AACC International, 2010). Empty aluminium moisture dishes were placed into a pre-equilibrated oven at 130 ± 3°C for 1 h. The dishes were taken from the oven and cooled in a desiccator containing active silica gel desiccant for a period of 30 min and then weighed. Sub-samples (approximately 5 g) were accurately weighed into pre-weighed dishes, prior to placing these into the oven and drying at 130 ± 3°C for 1 h. The process of drying, cooling and weighing was repeated until a constant weight was attained.

2.4. Immunoassay of gliadin content

The kit used for the analysis of gliadin residues was supplied by ELISA Systems (Windsor, Queensland, Australia) and the procedures described by the manufacturer were followed. Samples for gliadin residue detection were prepared following the approach recommended: bread samples (1.00 g) were extracted with a 40% ethanol solution (10 mL). The sample tubes were then vortexed and placed in a water bath at 60°C. After a 5 min interval, samples were mixed again; this procedure was repeated twice. After the incubation and mixing stage, the samples were rested for at least 30 min at room temperature (21°C). For those without enzyme and also the CE treatments further dilution was necessary (in the range of 500–2000-fold) beyond that described in the instructions supplied with the test kit, because of the high absorbance readings obtained. The gliadin content was determined from the calibration graph using a spectrophotometer (Agilent Technologies, Cary 60) at a wavelength of 450 nm.

2.5. Description of treatment of samples

For baking trials, samples enzyme (CE or PE) were incorporated and the conditions used are presented in Table 1.

3. Results

In the initial experiments, partially purified caricain was prepared and incorporated into a wholegrain bread formulation. The bread doughs were bulk fermented and the enzyme treated loaves containing either CE or PE evaluated against the control...
Fig. 1. The comparison of the gliadin content between untreated and treated wholegrain bread fermented at 37 °C for 5 h. Set 1 and set 2 refer to results of duplicate baking trials.

(no enzyme) by measuring the gliadin contents using the immunoassay. It is emphasised that samples with higher gliadin contents were diluted prior to analysis. The results are presented in Fig. 1. The untreated (control) bread sample, shows the highest gliadin content, as expected. After the addition of crude papain, a reduction in gliadin content was found and the effect was more pronounced with the higher amount of enzyme added. Furthermore, the purification of crude papain to give the enriched caricain has resulted in a reduction in the level of gliadin measured (0.03% PE, fermented at 30 °C for 5 h).

The results of the addition of PE for different fermentation times and temperatures are exhibited in Table 2. In is noted that all data were calculated from an average of 3 replicate samples from each set. Figures are in ppm on bread solids with their corresponding standard deviations. Statistical analysis of the results using a “t” test was carried out in order to test the significance of the difference between the means of treatment with PE and controls. The following equation was employed:

\[ t = \frac{x_1 - x_2}{s \sqrt{\left( \frac{1}{n_1} + \frac{1}{n_2} \right)}} \]

Where \( x_1 \) and \( x_2 \) are the means of \( n_1 \) and \( n_2 \) samples and \( s \) is the joint standard deviation. The degrees of freedom are \( n_1 + n_2 - 2 = 4 \). Probabilities (p) for both sets of results in Table 2 were \( p < 0.001 \) for Set 1 and \( p < 0.002 \) for Set 2.

Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Set 1</th>
<th>Set 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>831.85 + 2661</td>
<td>64.276 ± 2353</td>
</tr>
<tr>
<td>PE 37 °C for 5 h</td>
<td>1878 ± 95</td>
<td>2296 ± 119</td>
</tr>
<tr>
<td>PE 30 °C for 5 h</td>
<td>1581 ± 84</td>
<td>1972 ± 81</td>
</tr>
<tr>
<td>PE 37 °C for 7 h</td>
<td>1770 ± 104</td>
<td>1829 ± 78</td>
</tr>
</tbody>
</table>

The data indicate that even at a lower fermentation temperature (30 °C) there was a large effect on the gliadin reduction with the use of PE and there was no further reduction when the fermentation time was extended from 5 to 7 h.

The extent to which gliadin was detoxified in bread containing crude papain and the PE caricain was compared with untreated controls and the results are shown in Table 3. The higher purity enzyme has a greater effect on the gluten detoxification than the corresponding weight of crude papain (CE). A high extent of detoxification was obtained at 30 °C after 5 h with PE and the reduction in gliadin content was greater than in samples for which CE was incorporated.

Again, data were calculated from an average of 3 replicate samples in each set. The values are the relative reduction in gliadin content after enzyme treatment compared against no treatment, expressed as percentages.

The sensory characteristics of the loaves of bread produced as a result of the inclusion of PE (0.03% on dough weight) were evaluated on the basis of a comparison of cross-sectional slices and examples are presented in Fig. 2. The samples of control bread had the appearance expected for wholemeal bread. The introduction of PE to the formulation had a slight darkening effect on the crust colour and the crust structure appeared to be a little more open. At the lower fermentation temperature (30 °C), a separation of crust from crumb was noticeable, as shown in Fig. 2(c). In addition, all enzyme treated bread demonstrated lower oven spring. The bread shown in Fig. 2(d) appears to be the best loaf of bread made with the inclusion of enzyme based upon the greater uniformity of crumb structure compared with other enzyme treated loaves. This was prepared using PE 37 °C with a fermentation period of 7 h.

4. Discussion

The most important functional component in wheat flour is gluten. Therefore, anything that influences or modifies gluten or individual gluten proteins and, thus, the ability to form a network, is likely to have an influence on the dough and final bread quality. In this study wholemeal bread has been baked using freshly milled wholegrain wheat. Preliminary experiments carried out using CE (0.01% and 0.03%) and conditions of 37 °C and 5 h fermentation resulted in loaves with darker crusts and bitter taste (images not shown). This result indicates that extensive protein hydrolysis had occurred contributing to increased Maillard reactions, affecting taste as well as darkening of the crust. The protein hydrolysis is related to detoxification of gliadin (Cornell & Stelmasiak, 2011). In the current study the enriched form of caricain was able to detoxify wheat dough, at least to a level where it is likely to be less toxic to coeliacs. The small amounts of residual gliadin detected are sufficiently low to indicate that the product could be safely consumed by some individuals who are sensitive, without adverse effects, provided that enzyme therapy was also available as a safeguard (Cornell et al., 2005). The results of this trial indicate that the procedure of purification of the enzyme by ion exchange reported in previous studies was required in order to achieve high levels of detoxification.

### Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>No enzyme, fermented at 37 °C for 5 h</td>
</tr>
<tr>
<td>CE</td>
<td>0.01% Crude enzyme, fermented at 37 °C for 5 h</td>
</tr>
<tr>
<td>CE</td>
<td>0.03% Crude enzyme, fermented at 37 °C for 5 h</td>
</tr>
<tr>
<td>PE</td>
<td>0.03% Purified enzyme, fermented at 37 °C for 5 h</td>
</tr>
<tr>
<td>PE</td>
<td>0.03% Purified enzyme, fermented at 30 °C for 5 h</td>
</tr>
<tr>
<td>PE</td>
<td>0.03% Purified enzyme, fermented at 37 °C for 7 h</td>
</tr>
</tbody>
</table>

### Table 3

Relative reduction of gliadin in duplicate sets of bread samples treated with CE and PE, expressed as percentage values.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Set 1</th>
<th>Set 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE (0.01%) 37 °C for 5 h</td>
<td>37.7</td>
<td>60.1</td>
</tr>
<tr>
<td>CE (0.03%) 37 °C for 5 h</td>
<td>65.7</td>
<td>70.1</td>
</tr>
<tr>
<td>PE (0.03%) 37 °C for 5 h</td>
<td>97.8</td>
<td>96.4</td>
</tr>
<tr>
<td>PE (0.03%) 30 °C for 5 h</td>
<td>98.1</td>
<td>96.9</td>
</tr>
<tr>
<td>PE (0.03%) 37 °C for 7 h</td>
<td>97.9</td>
<td>97.2</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>No enzyme, fermented at 37 °C for 5 h</td>
</tr>
<tr>
<td>CE</td>
<td>0.01% Crude enzyme, fermented at 37 °C for 5 h</td>
</tr>
<tr>
<td>CE</td>
<td>0.03% Crude enzyme, fermented at 37 °C for 5 h</td>
</tr>
<tr>
<td>PE</td>
<td>0.03% Purified enzyme, fermented at 37 °C for 5 h</td>
</tr>
<tr>
<td>PE</td>
<td>0.03% Purified enzyme, fermented at 30 °C for 5 h</td>
</tr>
<tr>
<td>PE</td>
<td>0.03% Purified enzyme, fermented at 37 °C for 7 h</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Set 1</th>
<th>Set 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>83,851 ± 2,661</td>
<td>64,276 ± 2,353</td>
</tr>
<tr>
<td>PE 37 °C for 5 h</td>
<td>1,878 ± 95</td>
<td>2,296 ± 119</td>
</tr>
<tr>
<td>PE 30 °C for 5 h</td>
<td>1,581 ± 84</td>
<td>1,972 ± 81</td>
</tr>
<tr>
<td>PE 37 °C for 7 h</td>
<td>1,770 ± 104</td>
<td>1,829 ± 78</td>
</tr>
</tbody>
</table>
Ion exchange chromatography on a CM Sephadex column (Cornell et al., 2010) was used for a threefold enrichment. This step was essential to eliminate darker crust colour and bitter taste in the final product and also increased the degree of detoxification from 65.7% to 97.8% (Set 1) and from 70.1% to 96.4% (Set 2) when compared with the bread treated with the same amount of crude papain (Table 3). Statistical analysis demonstrated that the purified enzyme achieved a reduction in immunoactive gliadin even after 5 h at 30°C.

The ELISA assay adopted for the current study appears to be a rapid and reliable method for the measurement of changes in the gliadin brought about by the treatment with caricain. The data obtained generally had low standard deviation values with most being in the range of 3–6%. Furthermore, the results were independently confirmed (Australian Government National Measurement Institute, 2013) with a high degree of reproducibility using the same assay. The results extend previous reports describing the use of caricain for in vitro detoxification of gliadin based on the use of a supplement containing caricain (Cornell & Stelmasiak, 2011).

5. Conclusion

A series of trials was carried out to evaluate the detoxification of gliadin by caricain incorporated directly into a wholemeal wheat dough. The results extend the previous work on the effectiveness of the enzyme in detoxifying gliadin and applied the partially purified enzyme. This is the first report of the application of the enzyme in the formulation of baked products. The data from this study show that caricain is able to detoxify wheat gliadin to a level where it is likely to be less of a problem for coeliacs and could be safely consumed without adverse effects providing that enzyme therapy is available as a safeguard. This research demonstrates the potential of specific enzymes for detoxification as a useful way forward in patient management. This practical approach warrants further investigation, forming the basis of further studies designed to enhance the quality of life for coeliacs and others who are sensitive to wheat proteins.

Acknowledgements

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References


Fig. 2. Images of bread produced by treatment with PE, (b) PE 37°C for 5 h, (c) PE 30°C for 5 h and (d) PE 37°C for 7 h and without enzyme (a) Control.


Chapter 12

General discussion and conclusions

The purpose of this final chapter is to briefly summarise the results obtained during the current study, draw final conclusions and make recommendations for further research.

12.1 Discussion

In Chapter 1 of this thesis, the broad context of cereal processing was described in relation to the contribution of bread to health and wellbeing. As milling practices have changed to achieve more refined white flour, bakers around the world have adopted more rapid approaches to bread making. These developments have been accompanied by overall trends away from incorporating rye into bread formulations and the traditional use of sourdough cultures. Despite these changes, over the past fifty years, the role of wholemeal and some of the older practices in bread making have been promoted for their health benefits. This partly reflects the growing awareness and evidence on the importance of dietary fibre components, leading to recommendations that adults should consume at least 30g of these each day. During the past decade there has been increasing evidence from clinical trials that many of the older approaches in baking, including the use of wholemeal flours, can assist in reducing and delaying the challenges to health particularly those involving chronic conditions affecting older people.

Accordingly, the underlying aim of this research has been to re-evaluate traditional approaches to bread making. Aspects of ingredient formulation and processing variables have been investigated in order to provide a clearer understanding of the ways traditional methods contribute to the various health benefits. The results then have the potential to inform changes which can be adopted industrially. At the same time they can also provide information to enhance the knowledge of consumers and promote bread of a healthy, balanced diet.
The research reported in this thesis provides a systematic investigation encompassing:

1. The use of freshly prepared wholemeal flour for bread making;

2. The incorporation of flours prepared from oats and rye in conjunction with wheat flour;

3. The use of extended fermentation times as well as a sourdough culture to enhance the properties of the resultant products;

4. The development of enhanced approaches for analysis of the E vitamers and the application of these to studies on the effects of varying fermentation conditions on the retention of the vitamers during bread making;

5. The potential application of caricain for reducing the content of gliadins components which adversely affect coeliac individuals;

6. A study of phytate hydrolysis during processing, focussed upon the influence of fermentation conditions and the contribution of the various cereal flours to overall phytate levels;

7. Resistant starch was investigated to study its dependence on the fermentation technique and different bread making conditions (fermentation time and temperature) as well as the addition of palm oil into bread formulation.

As an overview, these individual areas of investigation all relate to important aspects of the health benefits of breads for consumers. Each part of the study provides a basis for understanding the potential of some of the more traditional practices in bread making, in conjunction with new innovations which offer additional benefits. The current study provides opportunities for the enhanced intakes of vitamin E regarding overall levels, as well as those of selected vitamers for which there is growing evidence of significant health benefits.
Baked bread into which wholemeal rye or oats have been incorporated have been proposed to offer benefits due to their contents of fibre, including soluble components. In addition they also appear to contribute to a low glycaemic index, so that there is a lower resultant spike in blood sugar in comparison with white bread and other breads. In addition, whole-grain rye flour has more soluble fibre and resistant starch than wholegrain wheat flour making wholemeal rye more effective for controlling the cholesterol level in the blood.

The times and temperatures during the fermentation stage of baking also decreased the phytate content in the final baked loves. This provides a basis for understanding the effects on phytate so that the extent of hydrolysis can be manipulated. The way this might be applied depends upon the balance of the evidence between regarding this component as an anti-nutrient or as a highly beneficial agent that offers protection against chronic diseases. It is also significant that the results of the current research demonstrate that fermentation processes, bulk and sourdough fermentation, also increased the resistant starch contents in the resultant products, thereby also providing health benefits.

12.2 Major conclusions

The final conclusions of this study are summarised here:

1. In vitamin E analysis, hexane can be replaced as a solvent for the separation of E vitamers, with the less toxic option of heptane. The results show that a simple direct replacement of n-hexane with n-heptane led to a compatible performance. This indicates that large and complicated efforts are not always necessary in order to replace harmful solvents with less damaging ones.

2. The inclusion of palm oil increased the quantity of tocopherols and trienols found in both wholemeal wheat and rye. It is concluded that palm oil was effective in increasing the vitamin E content of wholegrain breads. Palm oil appeared to provide further advantages as there was a wider range of E vitamers retained during bread making. The effects of varying fermentation conditions were also studied and
provide new knowledge that can be used to enhance the retention of the vitamin E components in baked products.

3. Phytate degradation was investigated to study its dependence on the flour meal type and different bread making procedures (bulk fermentation and rye sourdough fermentation). Both approaches were found to be useful techniques to influence phytate level and these provide a basis for understanding the factors that may influence mineral bioavailability as well as the beneficial effects of phytate for health and wellbeing.

4. Resistant starch was investigated to evaluate the dependence of formation on the fermentation technique and different bread making conditions (particularly fermentation time and temperature) as well as the addition of palm oil into bread formulation. Both fermentation methods led to the formation of this component and hence these have potential to contribute to the health benefits of baked products.

5. A series of experiments were carried out to evaluate the detoxification of gliadin by caricain incorporated directly into wholemeal wheat dough. The results demonstrate that the levels of the toxic gliadin fraction were reduced dramatically while the resultant products retained the overall characteristics of bread.

### 12.3 Possible areas for future research

In relation to the objective of the study regarding the stability of vitamin E during bread making, new approaches to analysis were developed. The vitamin E vitamers (tocopherols and tocotrienols) were extracted using accelerated solvent extraction, freeze dried then analyzed using normal phase HPLC. A further aspect that warrants further study would be that instead of using normal phase HPLC, reverse phase approaches are under consideration as these are even more environmentally friendly. Accordingly it recommended that the use of heptane in NP-HPLC reported here might be compared with procedures for RP-HPLC which are under development.
The results reported on enzyme detoxification in this thesis represent a relatively preliminary study. In order to confirm the effectiveness of the enzyme as a detoxifying agent it is necessary to carry out further studies.

Firstly, a limited number of analyses to measure the detoxification of gliadin have been studied here. It would be useful to digest the dough with a range of alternative proteolytic enzymes, possibly including the well known food enzymes pepsin and trypsin to make a water-soluble product for *in vitro* testing as reported in previous studies (Cornell, et al., 2010). The pepsin trypsin digest must be analysed for serine-containing peptides, activity of the peptide linked to the presence of PSQQ and also probably QQQP motifs. With the tyrosine-containing peptides, sequence such as QQPY and/or QPYP are associated with immunological activity and hence toxicity (Cornell & Wills-Johnson, 2001). If these approaches resulted in signs of toxicity it would then be necessary to modify conditions or assess alternative enzymes until negative signs of toxicity were achieved. Following such research this would form the basis of proceeding to incorporate the enzyme into a variety of food products. These might also be evaluated using *in vivo* experiments.

Further consideration could also be given to the application of the enzyme caricain and others identified as potentially useful, in the context of a broader range of foods and products where gluten is present, but for which the role of gluten is less significant as a determinant of product texture and quality in a wider context.

Secondly, use of mass spectrometry could be valuable as a tool to extend the information available on the specific amino acid sequences involved. This may facilitate the detection with high specificity of gliadins and related prolamins in flours and in wheat dough samples. This method might permit a more rapid and accurate detection of the toxic gliadin in food samples.

While further work is needed to confirm the results and to provide a fuller understanding of the detoxification process, the present study does give promising results which may lead to the availability of foods which contain wheat and can be
consumed by those who are sensitive to gliadin protein. There is also a need to confirm this study conducting clinical experiments to evaluate the enzyme-treated products.

Finally, and in conclusion, it is the sincere hope of the author that the research reported in this thesis will make a significant contribution to the enhanced nutrition and wellbeing of people around the world and may lead to greater enjoyment of cereal grain foods by those who are coeliacs or who otherwise are sensitive to cereal proteins in their diets. May the work described here also provide a strong basis and added stimulus to further research into the challenging areas of cereal grains and their utilisation as foods for the support of the world’s expanding population.