PHENOLIC AND POLYPHENOLIC COMPOUNDS OF WHEAT (*Triticum* spp.): EXTRACTION AND ANTIOXIDATIVE PROPERTIES

CHANDRIKA M. LIYANAPATHIRANA

Phenolic and Polyphenolic Compounds of Wheat (*Triticum* spp.): Extraction and Antioxidative Properties

By

© **Chandrika M. Liyanapathirana**

A thesis submitted to the

School of Graduate Studies

in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

Department of Biology

Memorial University of Newfoundland

August 2005

St. John's Newfoundland and Labrador Canada

Your file Votre référence
ISBN: 978-0-494-15660-5
Our file Notre référence
ISBN: 978-0-494-15660-5

I dedicate this thesis

to my loving husband Janaka and

to my little daughter Rashmi,

with love

ABSTRACT

Phenolic and polyphenolic compounds were extracted from wheat (*Triticum aestivum* L.) in order to assess their biological activity under different *in vitro* conditions. The effects of primary processing such as milling and pearling were also examined. The antioxidant capacity of wheat was evaluated using a number of *in vitro* assays based on scavenging of several free radicals and reactive oxygen species (ROS) and control of lipid and DNA oxidation.

The optimum conditions for the extraction of crude phenolics from whole grain and bran of soft and hard wheats were determined using response surface methodology (RSM). A face-centred cubic design (FCD) was used to investigate the effects of three independent variables, namely solvent composition (%), extraction temperature (^{o}C) and time (min) on the response, that is the total antioxidant capacity (TAC). Although the optimum extraction conditions were established, in the interest of operational costs related to energy consumption, phenolics were generally extracted using 80% aqueous ethanol for prolonged periods at low temperatures. Crude phenolics extracted with 80% aqueous ethanol yielded 75-80% antioxidant activity as compared to that under optimum conditions. The extractability of phenolics from wheat under simulated gastric pH conditions revealed that pH treatment facilitated the extraction of phenolics. In the latter study wheat phenolics of non-treated and treated samples were extracted into water. The antioxidant activity of treated samples was much higher than that of non-treated samples. Phenolics extracted belonged to the free, soluble esters and insoluble-bound fractions. Soluble esters of phenolics and insoluble-bound phenolics were extracted after alkaline hydrolysis of samples. The contribution of bound phenolics to the total phenolic content was significantly higher than that of free and esterified fractions. In the *in vitro* antioxidant assays, the bound phenolic fraction demonstrated a significantly higher antioxidant capacity than free and esterified phenolics. In all experiments detailed above, two commercial samples of soft (70% Canadian Eastern soft red spring and 30% Canadian Eastern soft white winter) and hard (90% Canadian Western hard red spring and 10% Canadian Eastern hard red winter) wheat mixtures were used. The antioxidant potential of the milling fractions examined decreased in the order of bran >

whole grain > flour. Most of the phenolics were concentrated in the bran and these were not available for analysis under normal extraction conditions.

The effects of primary processing, namely pearling and milling, on the phenolic content and antioxidant capacity were determined using two wheat cultivars, namely CWAD (Canadian Western Amber Durum; *Triticum turgidum* L. var. durum) and CWHRS (Canadian Western hard red spring; *Triticum aestivum* L.). After pearling the phenolic content and antioxidant capacity in the grains were significantly decreased. As the degree of pearling was increased there was a gradual decrease in the phenolic content and hence the corresponding antioxidant capacity in the resultant pearled product. The by-products resulting from 10-20% pearling possessed the greatest antioxidant capacity. Among different milling fractions the bran had the highest phenolic contents while the endosperm possessed the lowest and this was also reflected in antioxidant capacity of different milling fractions in the two cultivars.

The HPLC analysis of commercial wheat mixtures revealed that ferulic acid was the predominant phenolic acid while in the pure cultivars examined sinapic acid was the major phenolic acid. This study demonstrated the importance of bran in the antioxidant activity of wheat, hence consumption of wheat as whole grains may render beneficial health effects.

ACKNOWLEDGEMENTS

This thesis could not have been accomplished without the help of numerous people and it is a great pleasure to thank all of them. However, I specifically note my appreciation to those people who stand out most notably in my mind as contributing to my thesis. Moreover, I truly believe that all people whom I have not personally mentioned here are aware of my deep appreciation.

Most naturally, I would like to express my sincere gratitude to Dr. Fereidoon Shahidi, my doctoral thesis advisor, without whom this thesis would not exist. I cannot overstate my gratitude to Dr. Shahidi for providing an environment in which it has been possible to freely pursue independent avenues of thought. Throughout my program Dr. Shahidi provided sound advice, excellent teaching, good company and lots of useful ideas. The discussions, encouragements and critiques made by him were essential to the progress of this work and my thesis certainly benefited from his insight and innumerable suggestions. He has contributed enormously to my development over the past six years and I hope we will have the opportunity to continue to work together in the future as well.

My appreciation goes to my other members of the doctoral committee, Dr. Arya K. Bal and Dr. Peter J. Scott. I respectfully thank both not only for being on my committee but also for providing lucid and valuable insights into my work and contributing to its development in numerous ways.

I thank the heads, Department of Biology for allowing me to carry out my research in her/his department.

I thank the Natural Sciences and Engineering Research Council (NSERC) of Canada for funding me through a Postgraduate Scholarship B and for providing me paid maternity leave during my study. I am also grateful to the Dean of the School of Graduate Studies for funding me through a graduate fellowship. Financial support through Dr. Shahidi's research grants is also gratefully acknowledged.

I would also like to express my sincere gratitude to Dr. Ryszard Amarowicz for helping me with chromatographic analyses of ethanolic extracts of the wheat samples. I am thankful to Dr. Jim Dexter for supplying me wheat samples and Mr. Rollie Desjardins for preparing pearled wheat samples. I am grateful to Shirley Kenny, Sheena Quinton,

Christine Everson, Wendy Roach, Roy Ficken and Gary Collins in the Department of Biology for helping me in many different ways. I also express my gratitude to Peter Earle, Department of Biology for helping me solve problems with computer analysis. I am also very grateful to Craig Skinner of Department of Biochemistry for his help during my stay at Memorial. I would like to thank my friends at Memorial for all the emotional support, camaraderie, entertainment and caring they provided throughout.

I also want to thank everyone in my family, because a good family is the basis for a good life. Most importantly, my warmest thanks belong to my parents who bore, raised, supported, taught and loved me. My parents, not only gave me life, but also filled it with all the love and affection one can wish for. I am forever indebted to my mother for her understanding, endless support, patience and encouragement given when they were most required. My mother has been a motivation throughout my life. I am deeply indebted to her for all she is and all she has done for me. Unfortunately, my father is no longer with me to celebrate the completion of my thesis. However, this thesis is a little tribute to him and the immense loss I feel.

My dearest thanks are also extended to my three brothers who have always been supportive, are generous with love, have good advice and help me find my way. Their wide-ranging interests and their integrity as academics provided a wonderful environment in which to grow. I gratefully acknowledge their dedication to my academic success.

Lastly, my deepest gratitude must extend to two people dearest to my heart; my husband and my little daughter. Words cannot express my gratitude to my wonderful husband, Janaka, who frequently knows me better than I know myself and who has unfailing faith in me. His patience and love have been a great source of strength all through this work. Without his devoted self sacrifice for me this work would likely have never been completed. Life blessed me with the divine gift of my daughter, Rashmi. I am short of my vocabulary to thank her for being the sunshine of my life. Her cherubic smiles and words have lightened the burden of my workload, making it all seem worthwhile. She is my inspiration.

May you all have a long life – AYUBOWAN !!!

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AAPH	2,2'-Azobis-(2-methylpropionamidine) dihydrochloride
$ABTS^{\bullet-}$	2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) radical anion
$ABTS^{\bullet+}$	2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) radical cation
$ABTS^{2-}$	2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate)
ACL	Antioxidative capacity of lipid-soluble
ACS	American Chemical Society
ACW	Antioxidative capacity of water-soluble
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
C.V.	Coefficient of variance
CCl_4	Carbon tetrachloride
CHD	Coronary heart disease
CWAD	Canadian western amber durum
CWHRS	Canadian western hard red spring
DF	Degree of freedom
DNA	Deoxyribose nucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EC	(-)-Epicatechin
ECG	(-)-Epicatechin-3-O-gallate
EDTA	Ethylene diamine tetraacetic acid
EGC	(-)-Epigallocatechin
EGCG	(-)-Epigallocatechin-3-O-gallate
EPR	Electron paramagnetic resonance
FAE	Ferulic acid equivalents
FCD	Face-centered cube design
GC/MS	Gas chromatography/ mass spectrometry
HBA	Hydroxybenzoic acids
HCA	Hydroxycinnamic acids

$HO^{\bullet}$	Hydroxyl radical
HOCl	Hypochlorous acid
HPLC	High performance liquid chromatography
HWB	Hard wheat bran
HWW	Hard whole wheat
LDL	Low density lipoprotein
$^1O_2$	Singlet oxygen
$O_2^{\bullet-}$	Superoxide radical anion
O_3	Ozone
$ONOO^-$	Peroxynitrite
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffered saline
PCL	Photochemiluminescence
PF	Protection factor
PG	Propyl gallate
ppm	Part per million
PUFA	Polyunsaturated fatty acids
R^2	Coefficient of multiple determination
r^2	Correlation coefficient
RMCD	Randomly methylated β-cyclodextrin
$ROO^{\bullet}$	Peroxyl radical
ROS	Reactive oxygen species
R-PE	R-phycoerhthrin
RSM	Response surface methodology
RSREG	Response surface regression
RTE	Ready-to-eat
SAS	Statistical analysis software
SBO	Seal blubber oil
SCO	Stripped corn oil
SWB	Soft wheat bran
SWW	Soft whole wheat
TAC	Total antioxidant capacity

TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBHQ	Tertiary butyl hydroquinone
TE	Trolox equivalents
TEAC	Trolox equivalent antioxidant capacity
TPC	Total phenolic content
TRAP	Total radical-trapping antioxidant parameter
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
USDA	United States Department of Agriculture
UV	Ultraviolet
VCEAC	Vitamin C equivalent antioxidant capacity

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

CHAPTER 1

1.1 Introduction

There is a growing interest in phytonutrients or plant-derived bioactives that occur naturally in the foods and possess biological activities associated with curative and preventive properties and/or nutritive value (Balentine et al., 1999). In general, fruits and vegetables have been shown to contain compounds that contribute to human health (Kurulich et al., 1999). However, little attention has been paid to cereals with respect to their contribution in enhancing human health, despite the fact that they serve as staple foods for most of the world's population (Belderok, 2000). Among different cereals wheat is the most important in the temperate climate (Belderok, 2000). Durum wheat or pasta wheat and common or bread wheat are the two important commercially grown wheats (Dick and Matsuo, 1988).

Antioxidant activity is an important biological property of many phytochemicals that protects living organisms from oxidative damage thereby preventing various diseases such as cancer and cardiovascular diseases (Wang et al., 1999). Phenolic compounds possess antioxidant activity and are aromatic secondary metabolites that constitute one of the diverse classes of compounds found in plants (Cuppett, 1998). Simple phenols, phenylpropanoids, flavonoids, tannins and lignins are among numerous categories of phenolic compounds that exist in plants (Brielmann, 1999). Cereals have been known to contain phenolic acids, phytoestrogens and small quantities of flavonoids (Zielinski and Koslowska, 2000). The phenolic acids in cereals belong to benzoic and cinnamic acid series; the latter being most common in cereals (Natella et al., 1999). Cereals are also a major source of lignans, that function as potent antioxidants, in the human diet (Cassidi, 1996).

Phenolic acids are concentrated in the cell walls of outer layers of cereal grain (Maillard and Berset, 1995). In general, ferulic acid is the main phenolic acid in many cereals that exists predominantly in the seed coat (Watanabe et al., 1997). The health benefits of cereal grains may possibly be attributed to the nature of their cell wall polymers and chemical architecture (Bunzel et al., 2001 & 2003). Phenolic acids, that are covalently bound to the insoluble wheat bran matrix, have been shown to possess different antioxidative functions (Kroon et al., 1997). However, little is known about distribution of phenolics in any detailed and systematic manner in wheat grain. Thus, this research was carried out to fill an important gap in the existing knowledge in the field and to shed light on phenolics of wheat and their associated potential.

1.2. Objectives

The objectives of the study were categorized into two main parts.

Part I

Investigating the effects of extraction conditions on the biological activity of wheat to achieve the following.

1. To optimize the extraction of phenolics from soft and hard wheat
2. To study the effects of long-term (16 h) extraction conditions at 4 °C on antioxidant activity of wheat phenolics
3. To study the effects of simulated gastric pH condition on the antioxidant activity of soft and hard wheat
4. To determine the contribution of free, esterified and bound phenolics to the antioxidant activity of whole wheat and milling fractions upon alkaline hydrolysis

Part II

Effects of primary processing on biological activity of wheat

1. To study the effects of different levels of pearling on the antioxidant activity
2. To investigate the antioxidant activity of whole grain and milling fractions, namely bran, flour, shorts and feed flour.

The experiments carried out have been described under two main headings. In Part I, the effects of various extraction conditions on the antioxidant activity of wheat and its different milling fractions were determined. The experiments in Part I were carried out using two commercial hard and soft wheat mixtures obtained from milling suppliers of Robin Hood Multifoods Inc. (Markham, ON) in Saskatchewan. Firstly, the optimum conditions for extraction of wheat phenolics were determined by assessing the total antioxidant capacity (TAC) of their extracts. The optimum conditions were derived statistically using response surface methodology (Chapter 4). Although the optimum conditions for extraction of phenolics from wheat were determined, consideration of the economy of the process and excessive energy costs for water removal from the extracts, dictated the use of 80% aqueous ethanol over a prolonged period of time at low temperatures (Chapter 5). Wheat phenolics were also extracted using water alone. In this latter experiment, the effects of simulated gastric conditions on the extractability of phenolic compounds from wheat were determined (Chapter 6). Therefore, harsh experimental procedures such as extreme pH conditions and high temperature were avoided. The last chapter of Part I (Chapter 7) describes the antioxidant capacity of free, esterified and bound phenolics of the wheat samples examined. Since under normal extraction conditions only free and esterified phenolics are extracted, the

samples were subjected to alkaline hydrolysis in order to release the bound phenolics (Chapter 7) for subsequent evaluation.

In Part II, the effects of primary processing, namely pearling and milling, on the antioxidant activity of wheat phenolics were examined. These experiments were carried out using two wheat cultivars obtained from the Canadian Grain Commission (Winnipeg, MN). Thus, the effects of pearling (Chapter 8) and milling (Chapter 9) on the antioxidant activity of wheat phenolics were investigated.

This thesis is presented in the manuscript format that includes individual research papers (Chapters 4–9). Chapter 2 of the thesis provides a general literature review and Chapter 3 summarizes the general Materials and Methods used in reporting results in Chapters 4-9. However, Chapters 4-9 begin with a short introduction relevant to the work presented therein. Moreover, references are given as a separate section at the end of the thesis.

CHAPTER 2

Literature Review

2.1. Biology, production and classes of wheat

Cereals, in general, play an important role in human nutrition. Botanically, cereals are classified in Poaceae, the grass family (Belderok, 2000) and include wheat, rice, barley, oats, rye, maize, sorghum and millets. Wheat, barley, rye and oats can readily be grown in temperate zones while rice, sorghum and various kinds of millet are grown in tropical zones (Belderok, 2000). Wheat is the most important cereal for the population of temperate areas while rice, sorghum and millet are most important in the tropics (Belderok, 2000). The importance of wheat has mainly been attributed to its ability to be ground into flour and semolina that form the basic ingredients of bread and other bakery products and pasta (Beldrok, 2000). Wheat can be grown over a wide range of elevations, climatic conditions and soil fertility. Further, wheat that can be consumed by humans with minimal processing, can also be transported and safely stored over long periods (Bushuk, 1998). The main constituents of wheat kernel are bran, germ and endosperm as depicted in Fig. 2.1.

Wheat belongs to the genus *Triticum* and is classified into three groups, namely diploid, tetraploid and hexaploid (Dick and Matsuo, 1988). Durum wheat and common or bread wheat are the commercial wheats and are products of natural hybridization of ancestral types. The best known ancestor of today's commercial wheats is *T. monococcum* or einkorn wheat.

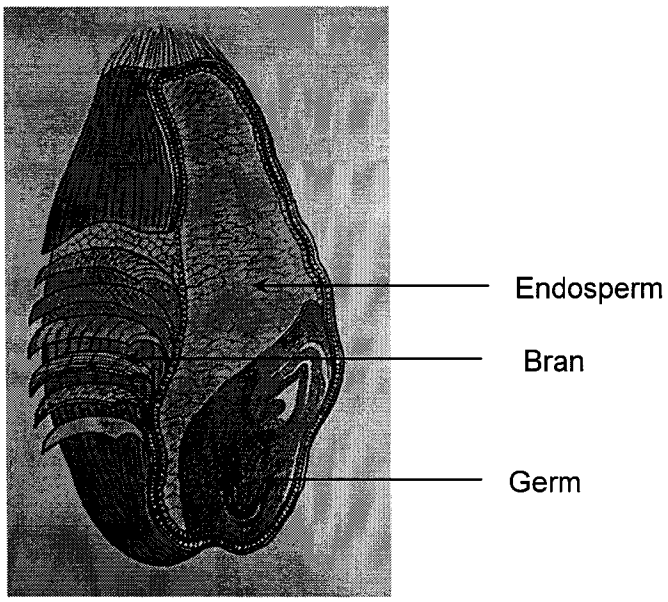


Fig. 2.1. The structure of the wheat kernel

T. monococcum is a diploid wheat and its genome is called the A-genome (Belderok, 2000). Among tetraploids there are the wild *T. dicoccoides* and cultivated *T. dicoccum*, which are often referred to as emmer wheats, and *T. durum*, another cultivated variety. Presently *T. durum* is grown widely as durum or pasta wheat. The genome of the tetraploid species is called the AB genome (Belderok, 2000). *Triticum aestivum*, the most remarkable species among the common or bread wheats, is hexaploid and a hybrid of the tetraploid *T. dicoccoides* and the diploid *Aegilops squarrosa* or *T. tauscii*, a species without any economic value. The genome of *T. aestivum* is called the ABD-genome. The unique milling and baking properties associated with common or bread wheat have been attributed to the presence of the D-genome derived from *A. squarrosa* (Belderok, 2000). Among different wheats, *T. aestivum* and *T. turgidum* are by far the most widely grown (Orth and Shellenberger, 1988). Approximately 95% of wheat grown today is of the common or bread wheat type while the remaining 5% is durum wheat.

Common or bread wheat is mainly used for bread and other bakery products while durum wheat is used primarily for production of pasta and couscous (Bushuk, 1998).

Wheat is one of the most widely grown cereal crops in the world. Nutritive value and storage qualities have made wheat a staple food for more than one-third of the world's population (Poehlman and Sleper, 1995). Wheat provides over 20% of calories and protein in the human diet (Bushuk, 1998). Wheat is known for its unique protein, especially the gluten proteins, that have the ability to form a strong cohesive mass that retains gas evolved during fermentation of the dough, one of the common ways used in leavening doughs. This leads to the formation of a light, well-aerated baked product (Tipples et al., 1994).

In 2002/03, the world production of wheat was approximately 571 million tonnes (Koo and Taylor, 2003). According to 2002 production statistics the European Union was the largest producer of wheat followed by China and the United States of America; the amounts produced were 103, 90 and 59 million tonnes, respectively. Other major wheat-producing countries include the former Soviet Union, Canada, Australia, Turkey, India and Argentina which together produced about 74% of the total world production (Koo and Taylor, 2003). Saskatchewan, Manitoba and southern Alberta are the major Canadian wheat-producing areas. Canadian wheats primarily include hard red spring wheat, Canadian Western red spring and durum wheat. Average Candian wheat production for 1998–2002 period is shown in Table 2.1.

There are different wheat grading and classifying systems. According to Dexter and Marchylo (2001), the effective grading and classification systems assign and preserve the commercial value of wheat on the basis of processing potential. The class

and physical condition of wheat are among the most important factors that determine wheat milling potential and end-product qulity (Dexter and Edwards, 1998 a & b).

Table 2.1. Average Canadian wheat production from 1998 to 2002 by class

Wheat class	Production (Million tonnes)						Percent (%)
	1998	1999	2000	2001	2002	Average	Share[a]
Common/bread	16.6	20.9	19.4	16.7	16.4	18.0	3.1
Durum	6.0	4.3	5.6	3.1	3.3	4.5	0.8
All	22.6	25.2	25.0	19.8	19.7	22.5	3.9

[a]Cobtribution to the world production

Source: Koo and Taylor, 2003

Intended end-use of wheat is a function of the class of wheat. There are different wheat classes, namely hard or soft, high protein or low protein, strong gluten or weak gluten, among others (Dexter, 1993). Moreover, wheats can be classified as winter or spring wheat depending on their growth habitat (Orth and Shellenberger, 1988). Spring wheats change from vegetative to reproductive growth without exposure to cold winter temperature. In contrast, winter wheats require a period of cold winter temperature for heading to occur. Winter wheats planted in late summer or fall may germinate using fall moisture and the young plants remain in a dormant phase in winter. They resume growth in early spring (Orth and Shellenberger, 1988). Wheats may also be divided into different classes on the basis of colour of their outer layer of the kernel (Koo and Taylor, 2003). The colour of the wheat kernels varies from light yellow to red brown due to the presence of pigments in the seed coat and is influenced by growing conditions. However, in a true-breeding cultivar colour does not vary and hence may be classified as red or white consistently (Evars and Bechtel, 1988).

In general, milling, baking and end-use quality of wheat is primarily influenced by the grain hardness. Hardness is determined by both genotype and growing conditions (Pomeranz and Afework, 1984). Wheat has been classified on the basis of kernel hardness that dictates the end-use quality. The different categories of wheat kernel hardness are listed in Table 2.2 (Williams, 1993).

Table 2.2. Different wheat classes based on the kernel hardness

Hardness category	Wheat class
Extra hard	Durum, Polish
Very hard	Some hard white spring
Hard	Hard red spring, hard white spring
Medium hard	Hard red winter, hard white winter, Canada prairie red spring
Medium soft	Canada prairie white spring
Soft	Soft white spring, soft red spring
Very soft	Soft white winter, soft red winter
Extra soft	Soft red winter, Club

Source: Williams, 1993

The cultivated wheats i.e. *T. aestivum* L. and *T. durum* Desf., have been separated into three distinct classes of grain hardness, namely soft hexaploid, hard hexaploid and durum (Anjum and Walker, 1991; Pomeranz and Williams, 1990). Hardness of wheat is defined as the resistance to deformation or fracture properties, the partical size distribution and the level of starch damage after milling or grinding (Anjum and Walker, 1991; Pomeranz and Williams, 1990). Hardness, is the strength by which the starch granules are adhered to the protein matrix (Stenvert and Kingswood, 1977). Anjum and Walker (1991) reported that kernel hardness in wheat is controlled by several major and minor genes. According to Giroux and Morris (1997) soft wheat kernels

fracture more easily, release numerous intact starch granules and produce fine-textured flour with less starch damage. Hard wheats result in coarse-textured flours with broken starch granules and hence damage to starch. Thus, wheat is classified into two classes, namely hard and soft, on the basis of endosperm texture which determines the milling process and end-use properties (Pomeranz and Williams, 1990). Moreover, durum wheat represents an additional increase in grain hardness. Hence, durum wheats may require greater force to fracture and result in coarser-textured flours with greater starch damage compared to hard wheats (Giroux and Morris, 1997). In general, harder wheats tend to possess a higher protein content than softer wheats (Appendix 1.1, Bushuk, 1998).

Different wheat types have diverse end-uses and quality requirements. Thus, the main quality requirement for hard spring wheat in bread-making include flour extraction or milling yield, flour protein concentration and composition of rheological properties such as dough handling characteristics (Finney et al., 1987). On the other hand, soft wheat quality is primarily determined by starch characteristics, pentosan concentration, and protein concentration and composition (Kaldy et al., 1991). According to Peterson et al. (1992) these characteristics are greatly influenced by the cultivar and interactions of cultivar and environment. However, the magnitude of interactions was much less than the main effects of the cultivar genotype. In general, the content of protein and its composition are primary determinants of flour functionality (Graybosch et al., 1996). Temperature and fertilization are among environmental factors that contribute to end-use quality of wheat. High temperatures during grain filling elevate the protein content while lowering its functionality. Consequently, rheological properties of flour may be affected (Corbellini et al., 1997). Durum (*T. durum*) wheat has been shown to be the most

appropriate cereal for production of high quality pasta products, where proteins have received much attention as factors determining pasta quality (Feillet, 1988; Fortini, 1988). However, Dexter and Matsuo (1979) reported on the role of starch fraction in the determination of quality of durum wheat. In general durum wheat starch gelatinizes at a slightly lower temperature than starches from other wheat classes. Moreover, the percentage of amylase is slightly higher in durum wheat starches than in bread wheat starches (Lintas, 1988).

2.2. Plant secondary metabolites

Plants have developed a mechanism of chemical defense which plays a major role in their survival. The process by which a plant produces these chemicals is referred to as secondary metabolism, while the compounds produced are referenced as secondary metabolites (Cuppett, 1998). Secondary metabolites have been used by mankind for thousands of years as dyes, flavours, fragrances, stimulants, hallucinogens, insecticides, poisons for vertebrates and humans as well as therapeutic agents. Their putative functions in plants have often been a matter of controversy (Roberts and Wink, 1999).

In general, secondary plant metabolites include all plant chemicals excluding primary constituents such as carbohydrates, lipids and proteins, among others. They may vary in their distribution from plant to plant and consist of a large variety of compounds (Harborne, 1999a). The production of a high diversity of secondary metabolites is a typical trait of plants. These compounds include both nitrogen-free and nitrogen-containing metabolites. Some of the nitrogen-free constituents include phenolics, terpenes, polyketides and polyacetylenes while alkaloids, amines, cyanogenic

glycosides, non-protein amino acids and glucosinolates belong to the nitrogen-containing class of secondary metabolites (Wink, 1999a).

In general, several major secondary metabolites commonly occur in plants along with many other minor components. Consequently, these complex mixtures may differ from organ to organ, sometimes between individual plants but more often between different species (Wink and Schimmer, 1999). Secondary metabolites are synthesized in plants in a tissue-, organ-, and developmental-specific manner by means of specific biosynthetic enzymes (Cseke and Kaufman, 1999). One of the characteristic features of secondary metabolites is that they are accumulated and stored in high concentration in plants producing them; hence, levels of 1-3% of dry weight are often encountered (Wink, 1999a). Hydrophilic secondary metabolites are generally stored in the vacuole while lipophilic substances are deposited in organs such as resin ducts, laticifers, trichomes or even in the cuticle (Harborne, 1999a). On the other hand, sites of synthesis of secondary metabolites may be different from the sites of storage, and long-distance transport of them has often been demonstrated in the xylem, phloem or via the apoplast (Wink and Schimmer, 1999).

Secondary metabolites have evolved as compounds that are important for fitness of the organisms producing them. Many of these compounds are known to interfere with numerous pharmacological targets (Wink, 1999a). There is considerable interest in the role that plants and, more specifically, plant-based foods play in human nutrition and health. Much of these health-promoting properties have been attributed to secondary plant metabolites (Kochian and Garvin, 1999). All these discoveries have made secondary metabolites interesting for several biotechnological applications. One of the main areas of interest is phytomedicine, where thousands of plants are in use worldwide

to treat human ailments and diseases (Wink, 1999a). Moreover, secondary metabolites have been used in agriculture as natural plant protectants due to their insecticidal, fungicidal and phytotoxic properties (Wink, 1993).

One of the earliest hypotheses argued that secondary metabolites are just waste products with no function at all, however, it was later shown that some secondary metabolites serve as signaling compounds to attract animals for pollination and seed dispersal (Cipollini and Levey, 1997). Thus, plants synthesize thousands of metabolites that are used for their growth and development, reproduction, defense against attack by many different kinds of organisms.

A characteristic feature of plants is their capacity to synthesize an enormous variety of secondary metabolites. Only 20-30% of plant species have been investigated for their secondary metabolites so far. However, several thousand secondary metabolites have already been isolated and their structures determined (Wink, 1999b). Despite the enormous variety of plant secondary metabolites, the number of distinct basic biosynthetic pathways involved in their synthesis is relatively few. Precursors of secondary metabolites usually originate in basic metabolic pathways such as glycolysis, Krebs cycle or shikimic acid pathway (Wink, 1999b).

Among different secondary metabolites the terpenoids or isoprenoids are the largest and most diverse class of organic compounds found in plants (Brielmann, 1999). The terpenoids may be distinguished from other classes of secondary metabolites by their biosynthetic origin and their broadly lipophilic properties (Harborne, 1999a). Phenolic compounds are aromatic secondary metabolites bearing one or more hydroxyl groups. The majority are polyphenols having several hydroxyl groups where one or more are further substituted by methyl or glycosyl groups (Cuppett, 1998). In general,

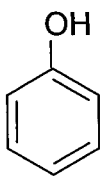
most phenolics are potentially toxic to life and hence they are frequently stored in plants conjugated with sugars or sulphates or both. Such water-soluble forms are stored in plant vacuoles (Harborne, 1999 a & b). Most classes of phenolic compounds occur in nature as glycosides. Nevertheless, many phenolics may also occur naturally in plants in the free state where they concentrate in bud exudates or in leaf surface waxes (Harborne, 1999a). Flavonoids may be present in any part of the plant while this is also true for many other phenolics such as phenylpropanoids and hydroxybenzoic acids (HBA; Harborne, 1994). By contrast, distribution of tannins is mainly restricted to bark and leaves in woody plants (Haslam, 1989).

2.3. Biosynthesis of phenolic compounds in plants

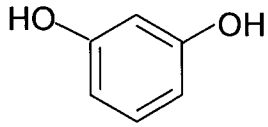
Phenolic compounds share a common biosynthetic origin, mainly from phenylalanine and, in some plants, from tyrosine, which are amino acids generated via shikimic acid pathway (Harborne, 1999a). Numerous categories of phenolic compounds exist; some of them being simple phenols, phenylpropanoids, flavonoids, tannins, lignins and quinines (Brielmann, 1999). The different classes of phenolic compounds range from simple phenols such as catechol, resorcinol and salicylic acid to complex anthocyanin pigments with molecular weights of 2,000 Da or more to the highly polymeric condensed tannins where molecular weights may exceed 20,000 Da (Fig. 2.2).

The biosynthesis of hydroxycinnamic acids (HCA), namely caffeic, ferulic and sinapic acids occurs via hydroxylation and methylation of *p*-coumaric acid (Fig. 2.3). Caffeic acid synthesis is catalyzed by the enzyme, monophenol mono-oxygenase, that results in the addition of a second hydroxyl group into *p*-coumaric acid while methylation

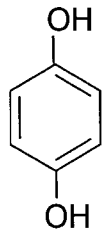
of caffeic acid catalyzed by *O*-methyltransferase leads to the formation of ferulic acid (Strack, 1997; Macheix et al., 1990).



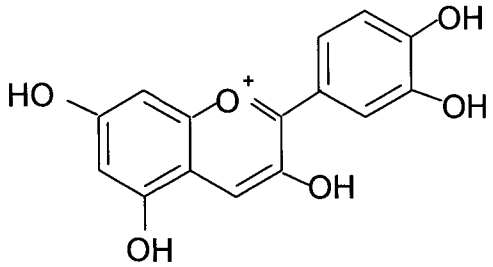
Phenol



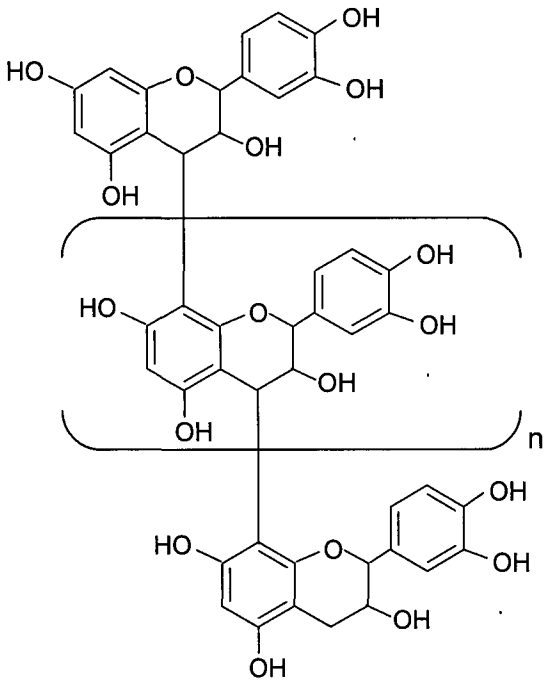
Resorcinol



Hydroquinone



Cyanidin (An anthocyanidin)



Condensed tannin

Fig. 2.2. Chemical structures of some simple phenols, anthocyanin and condensed tannin

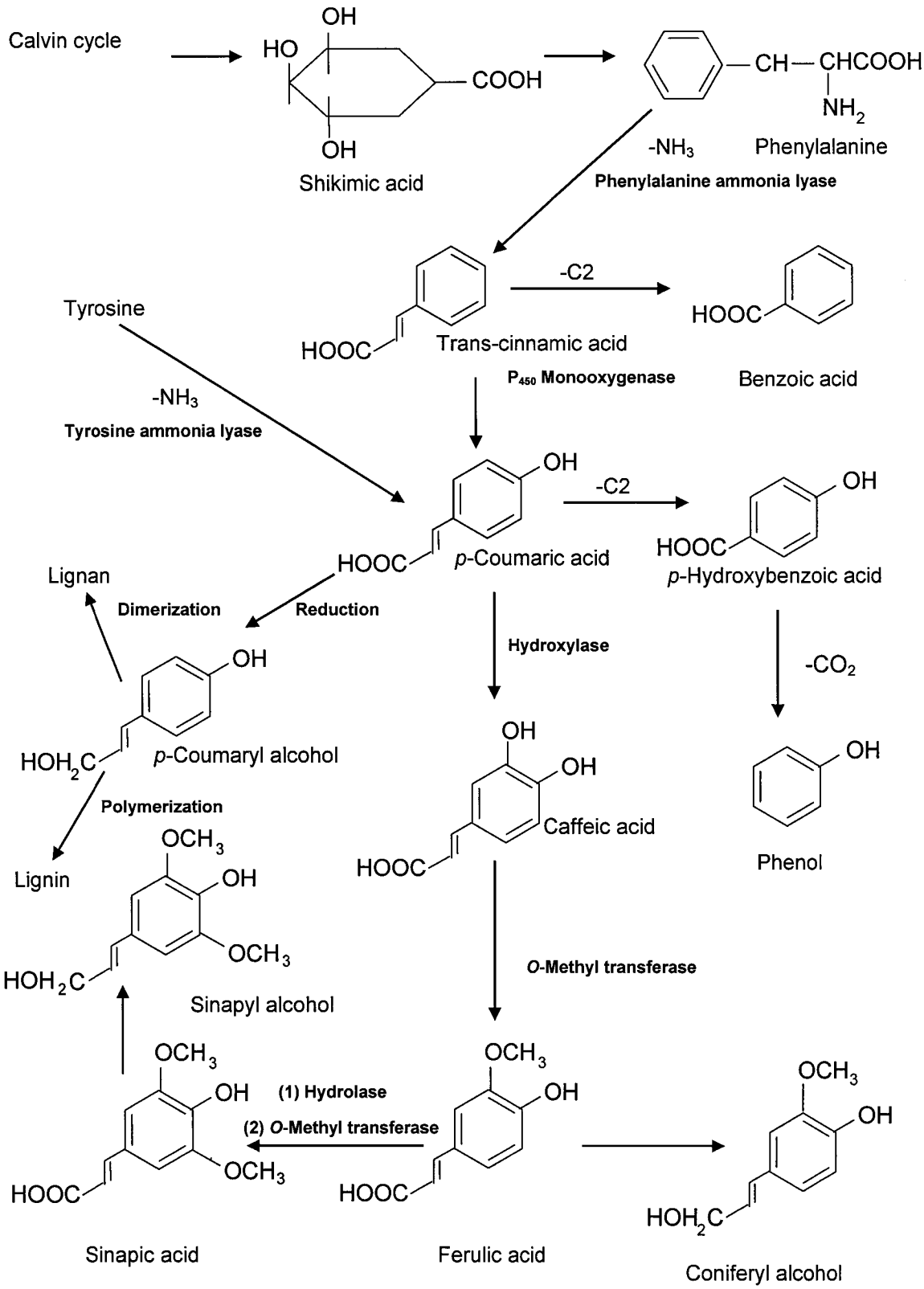


Fig. 2.3. Biosynthesis of phenylpropanoids

Source: Shahidi and Naczk, 2004

Sinapic acid, on the other hand, is synthesized by *O*-methylation of 5-hydroxyferulic acid that is formed from caffeic acid. Synthesis of HCA derivatives also takes place; formation of *p*-coumaroyl-CoA catalyzed by *p*-coumaroyl-CoA ligases or *O*-glycosyl transferases being significant. *p*-Coumaroyl-CoA undergoes condensation reactions with three malonyl-CoA to give rise to flavonoids (Fig. 2.4, Strack, 1997; Macheix et al., 1990). Biosynthesis of HBA may occur via several pathways depending on the plant; they may be derived directly from shikimic acid pathway that synthesizes gallic acid (Strack, 1997) or alternatively HBA may be produced by the degradation of HCA via cinnamoyl-CoA esters (Strack, 1997; Machiex et al., 1990). Moreover HBA may seldom be produced by the degradation of flavonoids (Strack, 1997). Further hydroxylations and methoxylations, catalyzed by various enzymes, may lead to numerous derivatives of HBA similar to HCA synthesis (Strack, 1997). Some commonly encountered HBA and HCA are depicted in Fig. 2.5 and Fig. 2.6, respectively.

Among various phenolic/polyphenolic categories, flavonoids are the best known group of polyphenols and comprise some 4000 compounds. In fact, all flavonoids share a common skeleton of flavone or 2-phenylbenzopyrone shown in Fig. 2.7 (Harborne, 1999b). There is a large number of naturally occurring flavonoids based on the C15 skeleton, which is formed by the same pathway from three malonate units condensing with phenylalanine-derived C6-C3 precursor (Harborne, 1991a). Thus, condensation of three malonyl-CoA molecules with *p*-coumaroyl-CoA, catalyzed by chalcone synthase, to form the C6-C3-C6 intermediate 4,2',4'6'-tetrahydroxychalcone is the key to the synthesis of flavonoids (Figure 2.4, Strack, 1997; Harborne, 1988). *p*-Coumaric (4-hydroxycinnamic) acid, which is derived from phenylalanine may be considered as a universal component in higher plant tissues. Since 4-hydroxycinnamic acid provides the

substrate for the synthesis of the A ring of the flavonoid group of compounds this remains highly significant compared to other phenolic compounds (Miller and Rice-Evans, 1997).

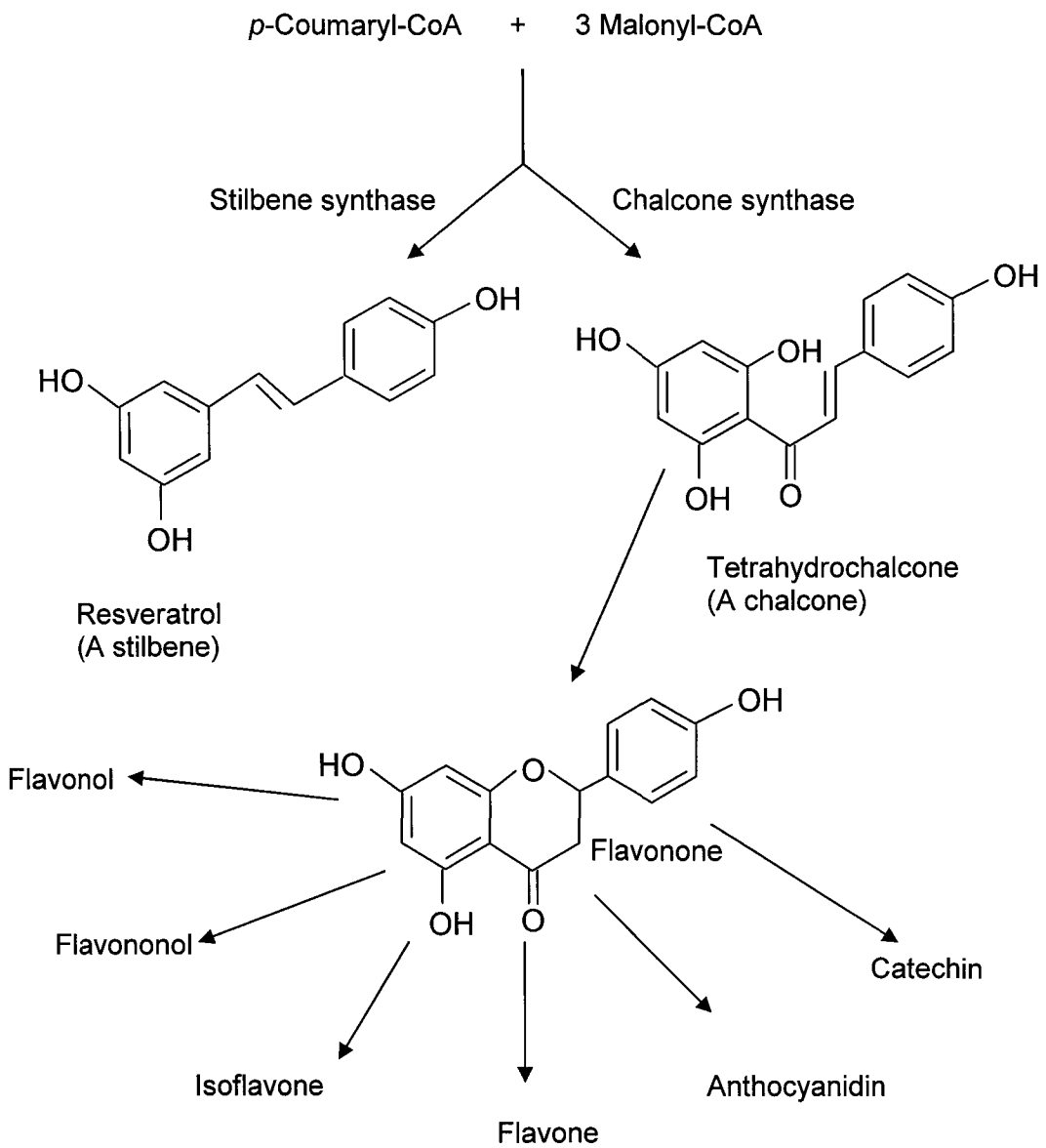
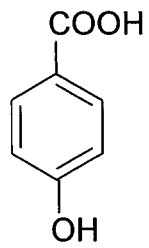
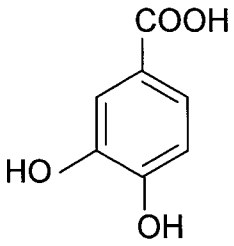


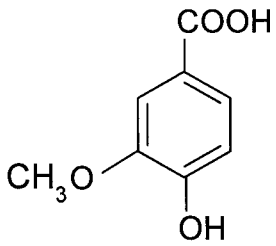
Fig. 2.4. Biosynthesis of flavonoids from phenylpropanoid derivative *p*-coumaryl-CoA
Source: Shahidi and Naczk, 2004



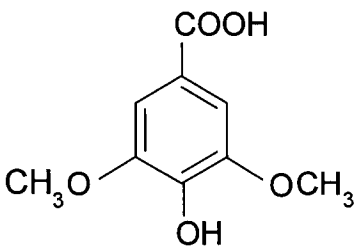
p-Hydroxybenzoic acid
(4-Hydroxybenzoic acid)



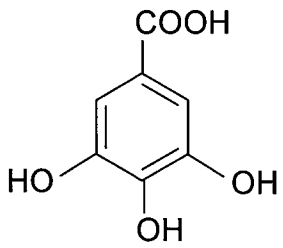
Protocatechuic acid
(3,4-Dihydroxybenzoic acid)



Vanillic acid
(4-Hydroxy-3-methoxybenzoic acid)

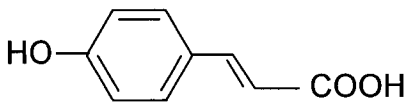


Syringic acid
(3,5-Dimethoxybenzoic acid)

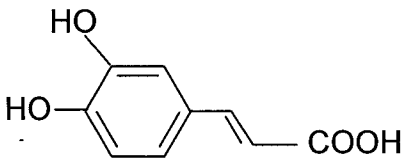


Gallic acid
(3,4,5-Trihydroxybenzoic acid)

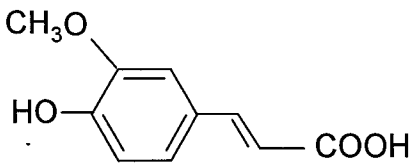
Fig. 2.5. Chemical structures of common hydroxybenzoic acids



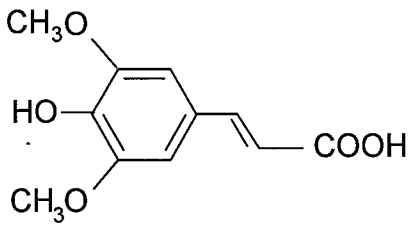
p-Coumaric acid
(4-Hydroxycinnamic acid)



Caffeic acid
(3,4-Dihydroxycinnamic acid)



Ferulic acid
(4-Hydroxy-3-methoxycinnamic acid)



Sinapic acid
4-Hydroxy-3,5-dimethoxycinnamic acid

Fig. 2.6. Chemical structures of common hydroxycinnamic acids

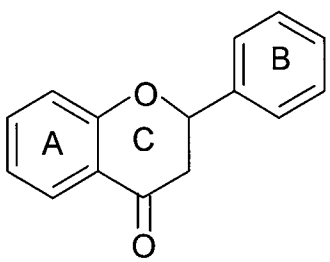


Fig. 2.7. Chemical structure of the flavone nucleus

Hydroxycinnamic acids such as *p*-coumaric, ferulic and caffeic acids are the most widely distributed phenolic acid constituents in plant tissues. The HCA, synthesized via shikimic acid pathway, are present in many plant-derived diets at higher concentrations than flavonoids and anthocyanins (Rice-Evans et al., 1997). These phenolic acids, in general, occur as various conjugates resulting from enzymatic hydroxylation, *O*-glycosylation, *O*-methylation or esterification (Rice-Evans et al., 1997). Caffeic acid is the most abundant phenylpropanoid in fruits (Rice-Evans et al., 1997) while ferulic acid is frequently the most abundant in cereal grains (Watanabe et al., 1997). Flavonoids occur often as glycosides in many plant tissues. For instance, the common flavonol, quercetin, may exist in over 135 different glycosidic forms (Harborne, 1994). Glucose, galactose, rhamnose, xylose and arabinose are the most frequently encountered sugar moieties in various flavonoid glycosides where glycosylation is catalyzed by glycosyl transferase enzyme (Strack, 1997). There are two major types of linkages observed in glycosides namely, *O*- or *C*-glycosidic linkages (Harborne, 1994). Flavonoids may also occur as acylated sugars where the most common acyl groups include HCA or aliphatic acids such as malonate (Strack, 1997).

2.4. Role of phenolic compounds in plants

In general, several secondary plant metabolites have evolved for protection against viruses, bacteria, fungi, competing plants and, more importantly, against herbivores (Harborne, 1994). Such chemical defenses have obvious selective and survival value for the plants as plants are generally attached to their substrates making them easily attacked by herbivores (Cseke and Kaufman, 1999). There are two major strategies by which plants may defend themselves against attack by herbivores,

pathogenic fungi, bacteria and viruses, namely structural and chemical defense strategies (Cseke and Kaufman, 1999). Structural defenses include lignification, silicification, callose formation and wax deposition while chemical defenses include almost all chemical compounds that deter attack based on their chemical nature (Cseke and Kaufman, 1999). Almost all classes of secondary plant metabolites have been suggested as antiherbivore agents (Roberts and Strack, 1999). However, within a class some molecules may be more effective than others. Nevertheless, almost every secondary metabolite that accumulates in plant tissues is potentially hostile to herbivores (Harborne, 1991b).

Phenolic compounds have numerous defense functions in plants. Environmental factors such as light, temperature, humidity and genetic factors may influence the synthesis of phenolic compounds (Strack, 1997). In general, light stimulates the synthesis of flavonoids especially anthocyanins and flavones by inducing the enzyme phenylalanine ammonia lyase (Fig. 2.3, Macheix et al., 1990). Biosynthesis of phenolic compounds has been shown to be induced under stress conditions of excessive UV light, wound or infection, among others. Hence, there may be either the rapid deposition of lignin barriers or exertion of direct toxic effects by the free radicals formed from lignin precursors as defensive mechanisms (Strack, 1997). Induction of phytoalexins against a microbial attack is another defensive mechanism existing in plants. Phytoalexins are present in healthy tissues at low concentrations and upon microbial attack they rapidly accumulate in the tissues. Phenolics such as hydroxycoumarins and HCA are among important constituents that form low-molecular-weight, inducible phytoalexins (Strack, 1997).

Phenolic compounds play a major role as cell wall support materials (Strack, 1997). Hydroxycinnamic acids, especially ferulic acid and *p*-coumaric acid, occur widely in cell wall of graminaceous plants (Sun et al., 1998) and are principal components that dictate cell wall integrity, shape and defense against pests and insects (Sun et al., 2000). Cross-linking of cell wall material is known to have a profound influence on the growth of plant cell walls and its mechanical properties and biodegradability (Hartley et al., 1990). Phenolics such as lignin, the second most abundant organic material on earth after cellulose, provides mechanical support to the plants. In addition, lignin forms a structural barrier against microbial invasion (Strack, 1997).

Many species of flowering plants have evolved the ability to produce various compounds that appeal to the visual, olfactory and taste senses of insects that impart an adaptive advantage to the plant (Cseke and Kaufman, 1999). Attraction of pollinators is achieved by several mechanisms, such as flower colour and odour (Roberts and Strack, 1999). Flavonoids, particularly anthocyanins together with co-pigments, namely flavones and flavonols, are functionally important as floral and fruit pigments (Strack, 1997; Harborne, 1994).

Allelopathy refers to the control of competition among higher plants by the diffusion of toxic, soluble substances (Butt and Lamb, 1981). Thus, plants give off chemical substances that are injurious to other plants or prevent other plants from becoming established in their vicinity, which are referred to as allelopathic chemicals or allomones. Such chemicals are beneficial to the plant that produces them by preventing the growth of other plant species that may compete for soil nutrients, carbon dioxide or sunlight (Cseke and Kaufman, 1999). Among numerous natural products tannins are common phenolic compounds that are toxic or may act as a deterrent to insects and

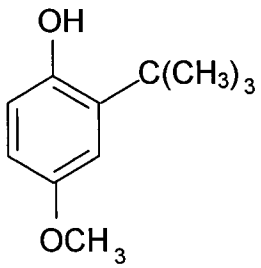
vertebrates (Roberts and Strack, 1999). Moreover, toxic water-soluble phenolics such as simple phenols, HCA and HBA may also serve as allelopathic chemicals (Strack, 1997).

Another important function of secondary metabolites in plants is to establish symbiotic associations between plants and other organisms such as bacteria and fungi (Cseke and Kaufman, 1999). Thus, some phenolics especially flavonoids, are known to act as signal molecules for host recognition in the interaction between certain leguminous plants and nitrogen-fixing bacteria (Strack, 1997).

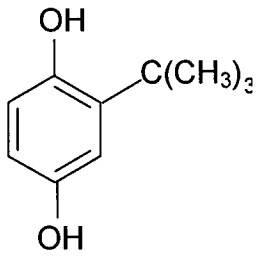
2.5. Grain phytochemicals

Common foods of plant origin contain a variety of flavonoids and phenolic acids in amounts ranging from traces to several grams per kilogram of fresh weight (Bravo, 1998). Since synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary-butylhydroquinone (TBHQ) and propyl gallate (PG) (Fig. 2.8) have been shown to cause or promote negative health effects there is a strong trend towards development of naturally occurring antioxidants (Nunez-Delicado et al., 1997). Well known antioxidants have been derived from tea (Cao et al., 1996a), red wine (Fogliano et al., 1999; Kanner et al., 1994), spices (Madsen and Bertelsen, 1995), fruits and vegetables (Velioglu et al., 1998; Vinson et al., 1998 & 2001; Wang et al., 1996). Moreover, many other plant species have been investigated in search for novel antioxidants (Madhujith et al., 2004; Siriwardhana and Shahidi, 2002; Wettasinghe and Shahidi, 1999; Kim et al., 1994; Yen and Duh, 1994; Chevolleau et al., 1992).

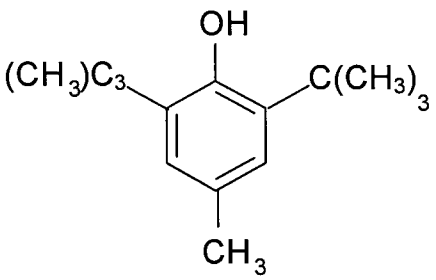
Cereal grains have also been known to contain phenolic acids, saponins and phytoestrogens. They are also known to contain small quantities of flavonoids (Zielinski



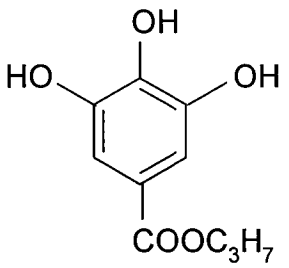
BHT
(3-Tertiary-butyl-4-hydroxyanisole)
(2-Tertiary-butyl-4-methoxyphenol)



TBHQ
Tertiary-butylhydroquinone



BHT
3,5-Di-tertiary-butyl-4-hydroxytoluene
2,6-Di-tertiary-butyl-4-methylphenol



PG
Propyl gallate
3,4,5-Trihydroxypropylbenzoate

Fig. 2.8. Chemical structures of common synthetic antioxidants

and Koslowska, 2000). According to Cassidy (1996) cereals are a major source of lignans in the human diet. Lignans may function as potent antioxidants by decreasing the production of reactive oxygen species (ROS) thereby exerting anticancer effects. Cereals are known to contain a wide range of phenolic acids that belong to benzoic and cinnamic acid series in particular (Yu et al., 2001). In general, HCA derivatives are the most common phenolic acids, strengthening their potential role as nutritional antioxidants (Natella et al., 1999). Recently, phenolic acids have gained attention due to

their antioxidative, antiinflammatory, antimutagenic and anticancer properties as well as their ability to modulate some key enzymatic functions in the cell (Ho et al., 1992).

In plants, phenolic acids may be esterified with other small molecules of aliphatic alcohols, phenols, phenolic acids and alkaloids, among others, which may be hydrolyzed by acids releasing them (Yu et al., 2001). Phenolic acids are, in general, capable of binding with starch and other polysaccharides through hydrogen bonds, chelation and covalent bonds via their carboxyl and hydroxyl groups thereby forming bridges or crosslinks (Ho et al., 1992). In cereal grains phenolic acids are concentrated in the cell walls of the outer layers of the grain mainly esterified to the arabinose side groups of arabinoxylans (Maillard and Berset, 1995). On the other hand, Goupy et al. (1999) have reported that phenolic acids are mainly found in the aleurone layer and endosperm of cereals. In general, ferulic acid is the major phenolic acid in many cereals predominantly existing in the seed coat (Watanabe et al., 1997) while traces of ferulic acid may be present in the starchy endosperm (Pussayanawin et al., 1988). Sosulski et al. (1982) reported the presence of trans-ferulic, syringic and vanillic acids in wheat while Hatcher and Kruger (1997) reported six phenolic acids, namely sinapic, ferulic, vanillic, syringic, caffeic and coumaric acids in wheat. Oat and corn also contain several different phenolic compounds including *p*-hydroxybenzoic, vanillic, protocatechuic, syringic, ferulic, caffeic and synapic acids (Sosulski et al., 1982). Presence of trans-ferulic, syringic, *p*-hydroxybenzoic and protocatechuic acids was also reported in rice (Sosulski et al., 1982). In addition, oat (*Avena sativa* L.) has been considered as a good source of anioxidants. According to Gary et al. (2000) oat also contains a range of functional ingredients that are concentrated in different parts of the kernel. The main antioxidative constituents of oat include vitamin E (tocols), phytic acid, phenolic compounds and

avenantheramides. In addition, flavonoids and sterols are also present to a lesser extent. These antioxidative constituents are concentrated in the outer layers of the oat kernel (Peterson, 2001). Emmons and Peterson (1999) identified ten phenolic compounds from oat extracts; avenantheramides and caffeic acid being the major constituents. The oat pearling fractions have particularly been rich in phenolics and tocols (Emmons et al., 1999; Bryngelsson et al., 2002). The most common phenolic acids in oat flour are *p*-hydroxybenzoic, protocatechuic, vanillic, trans-*p*-coumaric, syringic, trans-sinapic, caffeic and ferulic acids; ferulic acid being the most abundant (Xing and White, 1997). According to Terao et al. (1993) ferulic acid is the dominant phenolic acid in rye, wheat and barley. Barley (*Hordeum vulgare* L.) is one of the world's most important cereal crops used as a source of starch and energy. Thus, barley has been used extensively in fermentation products. According to Goupy et al. (1999) flavan-3-ols, flavonols and phenolic acids have been identified as major phenolic compounds in nine varieties of barley and their corresponding malts. Besides phenolic compounds, barley and malt extracts have also been reported to have carotenoids and tocopherols. Seven phenolic acids have been separated by HPLC after alkaline hydrolysis of barley or malt; trans- ferulic acid, trans-sinapic acid and cis-ferulic acid being the major ones (Maillard and Berset, 1995). Yu et al. (2001) have identified benzoic acid (*p*-hydroxybenzoic, vanillic and protocatechuic acids) and cinnamic acid (*p*-coumaric, caffeic, ferulic and chlorogenic acids) derivatives in 30 barley varieties. Ferulic acid is the major low-molecular-weight phenolic acid constituent in barley (Zupfer et al., 1998).

According to Andreasen et al. (2000 & 2001) ferulic acid, its dehydrodimers and sinapic acid occur in significant quantities in rye grain where they are concentrated in the

bran. These authors have isolated four ferulic acid dehydrodimers from rye bran, namely 8-*O*-4-diferulic acid, 8-5-diferulic acid, 5-5-diferulic acid and 8-5-benzofuran-diferulic acid; the former being the most abundant. The steryl ferulate contents of rye and wheat grains and their milling fractions were analyzed using a reversed-phase HPLC method and identified using HPLC-MS (Hakala et al., 2002). Uneven distribution of steryl ferulates in the grains led to differences in milling products; steryl ferulate contents were higher in the bran fraction than that of the flour.

2.6. Bioavailability of antioxidants

The antioxidant activity of phenolic compounds depends largely on their bioavailability which is influenced by several factors. Thus, release of phenolics from the food matrix in the gut, stability of phenolics in the gut flora, their modification on the intestine and absorption into circulatory system through intestinal wall, and stability in the liver and accessibility to the tissue at the target site are some of the important factors affecting bioavailability of antioxidative components (Donovan et al., 1999; Nielsen et al., 1999). Cereals and cereal-based foods may be an important vehicle to deliver nutraceutical components to the consumer as such foods are consumed widely and consistently as staples in many parts of the world (Awika et al., 2003).

Hydroxycinnamates have been shown to be absorbed in humans (Kroon and Williamson, 1999; Ghesson et al., 1999). Therefore, a diet rich in cereal bran may render beneficial health effects. However, there are no reports on the potential absorption and metabolic fate and effects of ferulic acid dehydrodimers on human health (Andreasen et al., 2000). The most abundant phenolic acids in rye include ferulic acid, sinapic acid and the dimer 8-*O*-4-diferulic acid that exert antioxidant activity against

oxidation of low density lipoprotein (LDL) cholesterol under *in vitro* conditions. However, the effects of these compounds *in vivo* are still uncertain since the mechanisms of their absorption and bioavailability remain unknown (Andreasen et al., 2001). Olthof et al. (2001) have found that chlorogenic acid is partially absorbed in humans and may have biological effects in blood circulation while the fraction not absorbed may bring about biological effects in the colon. Caffeic acid has been shown to be a promising natural antioxidant that contributes to the antioxidant defense system under *in vivo* conditions using a rat model. Hence, caffeic acid may either contribute directly to the antioxidant system or it may impart a sparing effect on α-tocopherol in LDL cholesterol (Nardini et al., 1997). In the gut the action of microbial esterases may release monomeric HCA and their absorption into the circulatory system has been demonstrated in both rats (Wende et al., 1997) and humans (Kroon et al., 1997). Ohta et al. (1997) reported that some of the absorbed HCA may be conjugated to form the more soluble glucuronide and/or sulphate conjugates. However, the structures of these conjugated forms have not been determined yet and their *in vivo* effect uncertain (Andreasen et al., 2001). Since absorption of dietary phenolics in human has been demonstrated, phenolic compounds of fruits, vegetables and whole grains may result in increased antioxidant capacity of plasma after ingestion. Hence, phytochemicals may constitute an important source of dietary antioxidants responsible for the health benefits (Prior and Cao, 2000).

The absorption of phenolics *in vivo* primarily depends on their basic structure, degree of glycosylation, size of the molecule, and their degree of conjugation with other compounds (Schneider et al., 1999; Bravo et al., 1994). Thus, the proportion of phenolics available in the circulatory system may be limited.

2.7. Measurement of antioxidant activity

In general, the total antioxidant capacity of plant-derived materials cannot be evaluated by any single method due to the complex nature of phytochemicals (Chu et al., 2000). Therefore, two or more methods should always be employed in order to evaluate total antioxidant activity. Moreover, despite the use of several methods in testing antioxidant properties, these methods cannot be used as such to measure the antioxidant activity of plant extracts due to their often complex composition (Yamaguchi et al., 1998). Measurement of antioxidant activity in a biological system is of paramount importance since antioxidant properties allow the prevention of oxidative stress and related diseases. Hence, it is possible to reach an opinion about the intake of dietary antioxidants and to evaluate their contribution in the antioxidant status of humans (Halliwell et al., 1995). In the case of a food system, measurement of antioxidant activity allows one to evaluate the total antioxidant capacity of the system and its significance in enhancing the health of an individual (Fogliano et al., 1999).

Numerous *in vitro* assays have been used in the measurement of the antioxidant potential of food and biological materials. Many chemical assays have been based on the ability to scavenge different free radicals while others may eliminate any source of oxidative initiation such as inhibition of an enzyme, chelation of metal ions and reducing power, among others. There are some chemical assays that evaluate lipid peroxidation, in which a lipid or lipoprotein substrate is used and the degree of inhibition of oxidation of the substrate is measured (Sanchez-Moreno and Larrauri, 1998).

2.7.1. Free radical and reactive oxygen species (ROS) scavenging assays

The antioxidant potential of a compound may be expressed by measuring its capacity to scavenge numerous ROS such as superoxide radical anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), hypochlorous acid (HOCl), peroxynitrite ($ONOO^-$) and peroxyl radical ($ROO^{\bullet}$), among others (Sanchez-Moreno, 2002). All these ROS are produced in the body during regular aerobic metabolism and they may also be generated in foods and other biological systems. Halliwell et al. (1995) reported that since all of these ROS participate in free radical chain reactions the ability of a compound to scavenge them may be a relevant way to determine antioxidant capacity of that compound.

The antioxidant potential of plant extracts can be evaluated using the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. In this assay the antioxidant compound may react with the stable DPPH radical and convert it to 2,2-diphenyl-1-picrylhydrazine; the degree of discoloration measured at 519 nm, using a spectrophotometer, indicates the antioxidative potential of the extract (Singh et al., 2002). Alternatively, scavenging of DPPH radical may be evaluated using electron spin resonance (EPR) spectroscopy on the basis that the signal intensity of the radical is inversely related to the concentration of antioxidant and to the reaction time (Wettasinghe and Shahidi, 1999). The DPPH radical scavenging assay is considered as a valid and easy assay for evaluating antioxidant potential due to its stability. Moreover, the radical does not have to be generated as in other radical scavenging assays (Sanchez-Moreno, 2002). Free radical scavenging property, in general, is attributed to the hydrogen-donating ability of the extracts. Thus, phenolic compounds may donate a hydrogen atom from their hydroxyl groups and form a stable intermediate, which is incapable of initiating or propagating free radical chain

reactions (Sherwin, 1978). Since DPPH radical scavenging is performed in alcohol this method is not useful for measuring the antioxidant activity of plasma as protein is precipitated in the medium (Sanchez-Moreno, 2002).

One of the most commonly-used methods to measure total antioxidant activity of plant extracts is the Trolox equivalent antioxidant capacity (TEAC) assay (van den Berg et al., 1999). Miller et al. (1993) introduced the TEAC assay for the clinical investigation of premature infants' low serum antioxidant capacity caused by vitamin E deficiency. As Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is used as the standard, results are expressed as Trolox equivalents. The ABTS (2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate)) radical, used in this assay, has been procured in different ways. In the original assay $ABTS^{\bullet+}$ are prepared using metmyoglobin/ABTS in the presence of H_2O_2 (Miller et al., 1993). In this assay the radical is generated by activation of metmyoglobin with H_2O_2 via the formation of ferrylmyoglobin radical, which then oxidizes ABTS to $ABTS^{\bullet+}$. Moreover, Rice-Evans and Miller (1997) found that $ABTS^{\bullet+}$ can be generated directly by chemical reduction of manganese dioxide in the absence of heme protein and H_2O_2. These authors established that antioxidants acted by scavenging the $ABTS^{\bullet+}$ and not by inhibition of its formation through reduction of ferrylmyoglobin or reaction with H_2O_2. Later Re et al. (1999) modified the method by introducing a decolorization technique where a stable radical is formed directly by using potassium persulphate. Recently, van den Berg et al. (1999) proposed a modification to the TEAC assay by pregeneration of the $ABTS^{\bullet-}$ that prevents the interference of radical formation by the presence of plant extract or biological sample thereby making the assay less susceptible to artifacts and prevents overestimation of antioxidant capacity. These authors described a convenient way to generate the $ABTS^{\bullet-}$ by heating $ABTS^{2-}$ with a

thermo-labile azo compound ABAP {2'2'-azobis(2-amidinopropane) dihydrochloride} or AAPH {2'2'-azobis (2-methyl-propionamidine) dihydrochloride}.

Benzie and Strain (1996) developed a method to determine the reducing ability of plasma as a measure of its antioxidant power based on a redox reaction. Hence, this method assesses the reduction of a ferric-tripyridyltriazine complex to its ferrous, coloured form in the presence of plama antioxidants. This method is referred to as ferric reducing ability of plasma or FRAP assay. However, the method was later used to determine antioxidant activity of tea and wine (Benzie and Strain, 1999; Benzie and Szeto, 1999). Subsequently, the method has been renamed as the ferric reducing/antioxidant power assay (Benzie and Strain, 1999).

Another well known method for measuring antioxidant capacity of biological material is the total radical-trapping antioxidant parameter (TRAP) that is quite time consuming (Serafini et al., 1998; Ghiselli et al., 1995). Total radical-trapping antioxidant parameter has also been introduced to determine antioxidant status of human plasma (Wayner et al., 1985). In TRAP assay peroxyl radicals are generated at a controlled rate by the thermal decomposition of ABAP. In order to evaluate antioxidant power, ABAP is mixed with the plasma and the oxidation is monitored by measuring oxygen consumption during the reaction. The induction period of plasma is compared with that of the reference antioxidant, Trolox, and hence the antioxidant activity of plasma is expressed in terms of Trolox equivalents. Subsequently several modifications have been introduced in this method (Ghiselli et al., 1995; Delange and Glazer, 1989; Wayner et al., 1987). In the modified assay the rate of peroxidation induced by ABAP is measured through the loss of fluorescence of the protein, R-phycoerythrin (R-PE), and the lag phase induced by the antioxidant is compared to that induced by Trolox.

Oxygen radical absorbance capacity (ORAC) has also been commonly used to measure the antioxidant capacity of biological material. In fact, ORAC has been described as superior to other antioxidant assays for two reasons; first, it combines both inhibition time and degree of free radical action by an antioxidant into a single quantity (Cao et al., 1995) and secondly numerous free radical generators or oxidants may be used since antioxidant activity of a biological sample depends on the free radical employed in the assay (Cao et al., 1996 a & b). According to Cao et al. (1996a) and Wang et al. (1996) the ORAC procedure allows the accurate measurement of total antioxidant capacity of plant extracts. In the ORAC assay the antioxidant capacity is obtained by determining the net protection area under the florescence decay curve, where R-PE is the florescent probe. The antioxidant activity determined using ORAC is also expressed as Trolox equivalents (Cao et al., 1995). Ghiselli et al. (1995) reported on the interference in the scavenging of peroxyl radicals by protein thiol groups present in plasma or other protein-containing samples. However, Prior and Cao (1999) modified the ORAC assay by eliminating proteins using trichloroacetic acid. The principal drawback of the original ORAC assay is that R-PE can be photobleached after exposure to excitation light as it is not photostable. Moreover, R-PE can interact with polyphenols and result in erroneous ORAC values (Ou et al., 2001). Recently, these authors improved the ORAC assay by introducing a new florescence probe, fluorescein, that has been shown to be quite stable over time. Fluorescein, as compared to R-PE, has demonstrated excellent photostability. In addition, fluoroscein may not interact with antioxidants in the assay medium (Ou et al., 2001). The ORAC procedure has become a widely used method for assessing antioxidant capacity in biological samples and foods. However, one of the limitations associated with ORAC method has been the

inability to determine both hydrophilic and lipophilic antioxidants in a sample (Prior et al., 2003). Therefore, Huang et al. (2002) have improved the ORAC assay to assess lipophilic antioxidants by using randomly methylated β-cyclodextrin (RMCD) that enhances the water solubility of lipophilic antioxidants. Thus, 7% RMCD (w/v) in a 50% acetone-water mixture has found to sufficiently solubilize vitamin E and other lipophilic antioxidants, which play a critical role in biological defense, in a phosphate buffer system (Huang et al., 2002).

In TEAC, TRAP and ORAC, antioxidant capacity is expressed in terms of Trolox equivalents. Trolox, a water-soluble analogue of vitamin E, is not naturally existing in foods (Kim et al., 2002). Kim et al. (2002), therefore, have developed the vitamin C equivalent antioxidant capacity (VCEAC) assay to measure antioxidant capacity of plant extracts. Since vitamin C is a naturally-occurring compound VCEAC is more desirable for measuring antioxidant capacity than TEAC, TRAP and ORAC (Kim et al., 2002).

2.7.2. DNA scission assays

The hydroxyl radical is an extremely reactive free radical encountered in biological systems. It has the ability to damage every molecule found in living cells and, hence, is implicated as a highly-damaging free radical species (Hochstein and Atallah, 1998). The hydroxyl radical is capable of joining nucleotides in DNA and causes strand breakage leading to mutagenesis, carcinogenesis and cytotoxicity (Hochstein and Atallah, 1998). Oxidative damage of DNA is associated with a wide range of base damaged products, structural identification and measurement of which may be performed using gas chromatography-mass spectrometry (GC-MS) (Aruoma, 1999). Base damage is useful in the identification of oxidative species that attack DNA

(Aruoma, 1999). DNA must be hydrolyzed using enzymes or acids and subsequently subjected to derivatization prior to GC-MS analysis. Hydrolysis releases nucleosides or free bases while derivatization leads to conversion of polar nucleosides/bases and internal standards to volatile, thermally-stable products which possess characteristic mass spectra (Aruoma, 1999). The $HO^{\bullet}$-mediated damage to DNA may be diminished or induced in the presence of a compound hence forming an essential tool to assess the antioxidant or prooxidant potentials, respectively, of such compounds (Aruoma, 1999). The attack of $HO^{\bullet}$ on DNA is very characteristic as these radicals can cause changes in all four of the purine and pyrimidine bases (Aruoma and Halliwell, 1998). Other ROS such as $O_2^{\bullet-}$ and H_2O_2 either do not attack DNA or they target guanine base preferentially. Consequently, 8-hydroxyguanine is the major product of DNA damage (Dizdaroglu, 1991). DNA damage is often measured as single strand breaks and double strand breaks or chromosomal aberrations (Aruoma, 1999). The level of DNA damaged, in general, reflects the rate of DNA damage in comparison to rate of repair. Moreover, oxidative damage to DNA is a useful biomarker of overall oxidative stress and provides evidence of an early stage of carcinogenesis (Aruoma et al., 1999).

2.7.3. Lipid oxidation assays

Free radicals are capable of initiating lipid peroxidation by abstracting hydrogen atoms from unsaturated fatty acids, and these oxidation products can be measured using several *in vitro* assays. Miller (1971) developed the β-carotene linoleate assay which determines the coupled oxidation of β-carotene and linoleic acid which is a simple, reproducible and quick method for evaluation of antioxidant capacity of a substance. The formation of conjugated dienes may be detected by measuring the absorbance of a

sample at 234 nm. During oxidation of a polyunsaturated fatty acid (PUFA) the double bonds are converted to conjugated double bonds. Thus, measuring the conjugated dienes formed in the presence of a substance allows one to evaluate its antioxidant capacity (Esterbauer et al., 1989). El-Saadani et al. (1989) described an iodometric method to quantify lipid oxidation by measuring lipid peroxides, the primary lipid oxidation products. It has been shown that there is an accumulation of primary lipid oxidation products followed by a decline which indicates the formation of aldehydes, upon decomposition of unstable hydroperoxides. Thus, decomposition of lipid hydroperoxides may lead to the formation of aldehydes such as hexanal, malondialdehyde and 4-hydroxynonenal (Buege and Aust, 1978). The thiobarbituric acid reactive substances (TBARS) assay was used to detect the secondary oxidation products; the assay is based on the formation of a stable pink coloured chromergen between aldehydes and thiobarbituric acid (TBA) in the aqueous phase.

2.7.4. Food model systems

Antioxidant activity of plant extracts has been evaluated using a variety of model systems that involve various analytical techniques. Meat, bulk oil and oil-in-water emulsion systems have been commonly employed in evaluating antioxidant activity.

2.7.4.1. Meat model system

Different meat types tested include beef (Murphy et al., 1998), chicken (El-Alim et al., 1999), turkey (Lee et al., 1996), pork (Wettasinghe and Shahidi, 1996 & 1997; Shahidi et al., 1987) and mutton (Mendiratta et al., 2000). Efficacy of antioxidants has also been evaluated using fish meat model systems (He and Shahidi, 1997). In general,

lipids in muscle tissues can undergo oxidative deterioration resulting off-flavours and off-odours. Consequently, palatability, nutritional quality and functionality of meat constituents may be affected(Tim and Watts, 1958). This problem is even greater in cooked meat that undergoes rapid deterioration leading to off-flavour development known as "warmed-over" flavours (Tim and Watts, 1958). A mixture of aldehydes, ketones, alcohols and hydrocarbons resulting from lipid oxidation causes the warmed-over flavour in meat (Tim and Watts, 1958).

One of the most frequently used methods to analyse lipid oxidation products is the 2-thiobarbituric acid (TBA) test (Lai et al., 1995). Malonaldehyde, which is an end product of lipid peroxide decomposition, reacts with TBA resulting a pink chromergen that has an absorption maximum at 532 nm (Shahidi and Hong, 1991). Thus, the thiobarbituric acid reactive substances (TBARS) formed are measured and expressed as mg malonaldehyde equivalents/kg sample (Esterbauer et al., 1991). According to Kansci et al. (1997) malonaldehyde can be measured using gas chromatography. Moreover, malonaldehyde can also be determined using high performance liquid chromatography (HPLC).

Lipid oxidation can be monitored by measuring the carbonyl compounds formed upon decomposition of lipid hydroperoxides. Thus, hexanal serves as an important index of meat oxidation and flavour quality deterioration (Shahidi and Pegg, 1994 a & b). It has been reported that headspace gas chromatography may be used to monitor hexanal and other volatiles resulting from lipid oxidation (Shahidi and Pegg, 1994 a & b). On the other hand, upon oxidative deterioration of marine lipids, that are rich in omega-3 fatty acids such as eicosapentaenoic and docosahexaenoic acids, result in propanal (Frankel et al., 1993).

2.7.4.2. Bulk oil and oil-in-water emulsion systems

Bulk oil and oil-in-water emulsion systems have commonly been used to investigate antioxidant properties of different compounds. The efficiency of an antioxidant in different systems is dependent upon concentration of antioxidative substance, nature of the lipid substrate, duration of storage period, method employed to measure lipid oxidation and presence of other interacting compounds in the system (Frankel and Meyer, 2000; Hopia et al., 1996; Huang et al., 1996 & 1994). Hydrophilic antioxidants have shown to be less active in emulsion systems compared to bulk oil systems while lipophilic compounds have performed better in emulsion systems than in bulk oils (Huang and Frankel, 1997; Hopia et al., 1996; Huang et al., 1996 & 1994). Thus, polar antioxidants such as propyl gallate, TBHQ and Trolox have shown to be more effective in bulk oils than non-polar antioxidants such as BHT, BHA and tocopherols (Coupland and McClements, 1996).

Various oil systems, as bulk or emulsions, have been used for evaluation of the antioxidant activities of different compounds. Thus, corn (Hopia et al., 1996; Huang et al., 1996 & 1994), flax (Chen and Ahn, 1998), sunflower (Abdalla and Roozen, 1999; Yanishlieva and Marinova, 1995), olive (Marinova and Yanishlieva, 1997), soybean, peanut and fish (Frankel et al., 1996) oils have been employed in bulk oil and emulsion systems.

A variety of analytical techniques has been used to measure oxidative status in bulk oil and oil emulsion systems. These analytical techniques measure factors such as loss of reactants (oxygen or lipid), formation of intermediates (hydroperoxides and conjugated dienes) and degradation products (alcohols, aldehydes, ketones and hydrocarbons) (Coupland and McClements, 1996). Formation of intermediates such as

hydroperoxides can be detected as a peroxide value (Marinova and Yanishlieva, 1997; Yanishlieva and Marinova, 1995) or conjugated dienes (Abdalla and Roozen, 1999; Huang and Frankel, 1997; Frankel et al., 1996). Peroxides are the main initial product of lipid oxidation and peroxide value is expressed as milliequivalents of oxygen/kg of sample (AOCS, 1990). According to Shahidi and Wanasundara (1998) unsaturated fatty acids upon reaction with molecular oxygen produce conjugated dienes and trienes with absorption maxima at 234 nm and 268 nm, respectively.

Weight gain of a sample of oil due to the uptake of oxygen and formation of hydroperoxides provides another method to measure lipid oxidation (Wanasundara and Shahidi, 1996). The active-oxygen method (AOM) is a commonly used method for assessing oxidative stability of fats and oils, which determine the peroxide value (deMan et al., 1987). Moreover, the Rancimat method is a rapid alternative that evaluates volatile oxidation products by measuring conductivity (Nawar, 1996). Formic acid is the main compound that causes changes in the conductivity (deMan et al., 1987).

The oxidative stability of oil treated with different antioxidants has been assessed using proton nuclear magnetic resonance (NMR) spectroscopy (Shahidi 1992; Wanasundara and Shahidi, 1993; Senanayake and Shahidi, 1999). According to Shahidi et al. (1994) NMR is a useful means for measuring the oxidative deterioration of lipids. Thus, this method can be used to measure the relative changes in NMR absorption pattern of lipid fatty acids and formation of both primary and secondary oxidation products.

2.8. Physiological and biological activities of phytochemicals and their health effects

The growing concern for general health, chronic disease prevention and prevention of the effects of aging has intensified consumers' interests in phytonutrients which are plant-derived, naturally-occurring compounds with curative, preventive or nutritive value (Balentine et al., 1999). In general, fruits and vegetables are known to contain compounds that contribute to health and wellness both by their traditional nutritive value and through enhancing the body's defense against chronic diseases (Kurulich et al., 1999). Thus, physiological functionality of plant chemicals has received much attention due to the increasing interest in human health (Azuma et al., 1999). For instance, antioxidative action, one of the important physiological functions of plant chemicals, is supposed to protect living organisms from oxidative damage and thus prevent various diseases such as cancer, cardiovascular diseases and diabetes (Wang et al., 1999).

In general, free radicals may play a major role in the progression of a wide range of pathological disturbances such as brain dysfunction, cancer, cardiovascular disease and inflammation (Haraguchi et al., 1997). In the food industry, free radicals are responsible for lipid oxidation that may lead to the deterioration of foods during processing and storage, and, hence, considerable attention has been paid to adding antioxidants to food and biological systems for scavenging free radicals (Larson, 1988). A number of natural compounds from plants including vitamin E (tocols), flavonoids, phenolic acids, chlorophyll derivatives and carotenoids have been found to be effective antioxidants (Mazza et al., 1999). Phenolic compounds, especially anthocyanins, flavonols, catechins and other flavonoids have been demonstrated to have a wide range

of biochemical and pharmacological effects including anticarcinogenic, antiatherogenic, anti-inflammatory, antimicrobial and antioxidant activities (Mazza et al., 1999).

Physiologically, the formation and elimination of free radicals are balanced by the antioxidant system in the body; a disturbance in this balance may result in increased production of free radicals and a decrease in antioxidant capacity which may eventually lead to cell and tissue damage (Sies, 1991). Free radicals are able to attack a variety of targets such as lipids, proteins and nucleic acids and hence are implicated in a number of important degenerative diseases including the process of aging (Weisburger, 1999; Beckman and Ames, 1998; Ozawa, 1997). Therefore, the function of antioxidants is to intercept and react with free radicals at a faster rate compared to the substrate thereby neutralizing the free radicals. Oxidative damage to DNA, protein and other macromolecules accumulates with age and has been postulated to be a major type of endogenous damage leading to aging (Fraga et al., 1990).

Free radicals attack unsaturated fatty acid moieties of biomembranes leading to membrane lipid peroxidation that has been shown to be strongly associated with aging, carcinogenesis and atherosclerosis (Soler-Rivas et al., 2000; Halliwell et al., 1992). Lipid oxidation products are responsible for protein cross-linking, denaturation, polypeptide chain scission, enzyme inactivation and amino acid destruction (Erikkson, 1982). Moreover, free radicals may attack DNA and cause mutation thereby leading to cancer (Ames et al., 1995).

A wide range of plant-derived compounds has attracted medical and pharmacological interest during the last decade. Among numerous secondary metabolites, many compounds have been identified and characterized with anticancer, antiviral, antimalarial, anti-inflammatory, antidepressant, anti-ischemic and

immunostimulatory properties (Heilmann et al., 1999). These plant-derived secondary metabolites have been used directly as drugs, while many pharmacologically-active compounds have served as leading models for semi-synthetic and synthetic drugs. Thus plants, may continue to serve as a rich and valuable source of new compounds with potent biological activity. Plants accumulate efficient antioxidative compounds such as polyphenols in the body and their isolation and the identification of their chemical structures are of much importance for their application as novel drugs and food additives (Masuda et al., 2003).

Antioxidant defenses in the organisms against ROS produced during normal cell aerobic respiration may be of endogenous or dietary origin (Harman, 1995). Dietary antioxidants such as polyphenolic compounds have been shown to be a major dietary factor responsible for numerous protective effects in the body (Hertog, 1996; Hertog et al., 1994, 1995, 1997). Epidemiological studies suggest that increased consumption of phenolic antioxidants correlates with reduced coronary heart disease (CHD, Hertog et al., 1993). Despite high saturated fat intake, CHD mortality has been low in certain parts of France. People in these areas consume wine, a dietary factor that has been suggested as an explanation for their low mortality from CHD. Thus, the evidence suggests that the intake of wine may counteract the effects of a high fat diet by reducing the incidence of CHD; a phenomenon known as the "French paradox" (Frankel et al., 1995). The phenolic components in red wine have been shown to possess potent antioxidant activity in inhibiting the oxidation of human LDL cholesterol *in vitro* (Frankel et al., 1993).

Dietary intake of antioxidants is associated with a reduced risk of atherosclerosis and, in turn, cardiovascular diseases, which is possibly due to their ability to prevent

oxidation of LDL cholesterol (Dittrich et al., 2003). Animals, including humans, have developed a system of antioxidants, including antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase and catalase, and non-enzymatic defenses such as high-molecular-weight (albumin, ceruloplasmin, ferritin) and low-molecular-weight (ascorbic acid, α-tocopherol, β-carotene, reduced glutathione, uric acid, bilirubin and flavonoids) compounds that may protect the organism from detrimental effects of reactive species (Prior and Cao, 1999). When free radicals and other reactive species are generated in the living system a wide variety of antioxidants may start to function against them to prevent possible damage (Cao et al., 1997).

Giovannelli et al. (2000) have demonstrated the ability of dietary polyphenols to modulate *in vivo* oxidative damage in the gastrointestinal tract of rodents thereby suggesting their protective and therapeutic potential in oxidative damage-related pathologies. According to Kerry and Abbey (1997) phenolic antioxidants effectively inhibit LDL cholesterol oxidation under *in vitro* conditions; hence, exhibiting a protective effect against experimentally-induced oxidative stress. Polyphenolic compounds have also been shown to exert a protective action in experimental colon carcinogenesis using animal models (Weisburger et al., 1998). Halder and Bhaduri (1998) have demonstrated the protective function of polyphenols against oxidative damage of human red blood cells using an *in vitro* model. Phenolic antioxidants have demonstrated *in vitro* antioxidant activity by scavenging free radicals in the aqueous phase and their chain-breaking properties against lipid peroxidation (Salah et al., 1995).

The antioxidant activity of simple phenolic acids has been studied in different model systems. The simple phenolic acids include the derivatives of benzoic and cinnamic acids (Andreasen et al., 2001). Graf (1992) reported that ferulic acid may

function as a potent antioxidant with potential applications in pharmaceutical and food industries. Garcia-Conesa et al. (1997 a&b, 1999) reported that some ferulic acid dimers exerted antioxidant activity in two *in vitro* assays; one measuring the inhibition of peroxidation of phospholipid liposomes and the other measuring the scavenging of $ABTS^{\bullet-}$. Ferulic acid may serve an important function in preserving the physiological integrity of cells exposed to oxidative degradation and protect against various inflammatory diseases by effectively scavenging deleterious free radicals (Graf, 1992).

Kanski et al. (2002) suggested that naturally-occurring antioxidants such as ferulic acid are important as therapeutics against neurodegenerative disorders such as Alzheimer's disease where oxidative stress plays a critical role. It has also been shown that ferulic acid is a potent inhibitor against hydroxyl and peroxyl radicals and, thereby, may act against oxidative stress.

According to Satué-Garcia et al. (1997 a&b) several antioxidant mechanisms such as hydrogen donation, metal chelation and protein binding may explain the antioxidant activity against *in-vitro* human LDL cholesterol oxidation. Moreover, there are several factors that may govern the oxidizability of LDL cholesterol including lipid composition and size of the particle, the concentration of chain-breaking antioxidants and the level of endogenous lipid hydroperoxides (De Graaf et al., 1991; Esterbauer et al., 1992; Chait et al., 1993; Frei and Gaziano, 1993; Hodis et al., 1994; Lass et al., 1996;). Protein binding of phenolic compounds may provide another mechanism that explains their antioxidant activity. Thus, the apolipoprotein B moiety of LDL has been found to have specific copper-binding sites (Gieseg and Esterbauer, 1994). Binding of Cu^{2+} to LDL cholesterol is essential for initiating its copper-mediated oxidation.

Lipophilic antioxidants such as tocopherols and carotenoids may accumulate in the plasma lipoproteins such as LDL cholesterol (Esterbauer et al., 1991). Thus, lipophilic antioxidants may act as highly efficient scavengers of the lipid peroxyl radical formed within lipoprotein particles upon peroxidation (Esterbauer and Ramos, 1995). In general, hydrophilic antioxidants are less efficient at scavenging lipophilic radicals. However, they tend to act synergistically by regenerating lipophilic antioxidants. Hence, regeneration of the α-tocopheroxyl radical by phenolic compounds to α-tocopherol may increase the resistance of LDL cholesterol against oxidation (Kagan et al., 1992). According to Abuja et al. (1998), consumption of tocopherol in the LDL particle is much slower in the presence of an antioxidant than in the absence of an antioxidant, indicating a protective effect of the antioxidant. The ability of an antioxidant to decrease copper-promoted LDL cholesterol oxidation may also be attributed to the efficient removal of Cu^{2+} from the surface of LDL by the antioxidant (Decker et al., 2001; Retsky et al., 1999). According to Chen and Frei (1997) direct binding of Cu^{2+} to LDL is crucial for the reactivity of copper. It has been suggested that HCA mainly act as peroxyl radical scavengers and hence increase the resistance of LDL cholesterol to oxidation (Castelluccio et al., 1995). Thus, ferulic acid may function as a chain-breaking antioxidant exerting its antioxidant properties against LDL cholesterol oxidation from the aqueous phase (Castelluccio et al., 1996).

According to Giessauf et al. (1992), copper-mediated oxidation of tryptophan residues in LDL-apolipoprotein B may be a major cause of initiating lipid oxidation in LDL cholesterol. A unique antioxidant mechanism that involves the blockage of copper binding sites on apolipoprotein B tryptophan has been proposed (Meyer et al., 1998). This may be achieved by the binding of antioxidants to apolipoprotein B on the LDL

molecule. Hence, structural features that confer differences in protein binding may affect the antioxidant activity of phenolics in inhibiting oxidation of LDL cholesterol (Meyer et al., 1998). It is very unlikely that ferulic acid exhibits antioxidant activity against copper-mediated LDL cholesterol oxidation by chelating copper since ferulic acid may not chelate metal ions to any large extent (Graf, 1992). Under certain circumstances a phenolic compound may not exhibit any metal chelation ability, but still could show high lipid peroxidation inhibition activity. This could be explained by their lipophilicity that renders better antioxidant activity (van Acker et al., 1996). Moreover, HCA such as ferulic acid and *p*-coumaric acid bind to apolipoprotein B of LDL and then block copper access to the LDL particle (van Acker et al., 1996). Natella et al. (1999) reported that inhibition of copper-catalyzed oxidation represents the association of both chelation of metal ions and scavenging of free radical species in the LDL system.

Cereal grains are one of the most important food groups and cereal fibres are known to impart some health benefits, possibly attributed to the nature of their cell wall polymers and chemical architecture (Bunzel et al., 2001 & 2003). Although HCA are known to be good antioxidants the significance of their substantial intake is poorly understood (Scott et al., 1993). In general, ferulic acid that is covalently bound to the insoluble wheat bran matrix may have a different antioxidative function from the soluble forms of esterified ferulic acid and free ferulic acid (Kroon et al., 1997). It has been found that only a small portion of esterified feruloyl groups may be released from wheat bran prior to fermentation in the colon. This is important since anticarcinogenic properties of wheat bran may be related to their insolubility. Moreover, a significant quantity of ferulic acid is released from insoluble cereal fibre into the soluble phase by fermentation in the human colon (Kroon et al., 1997). The protective effects, modified by

the process of fermentation, has been shown to be greatest for the insoluble fibres against carcinogenesis (Harris and Ferguson, 1993). The insoluble whole grain components may dilute colonic contents by increasing fecal weight, accelerate intestinal transit time, and increase defecation frequency thereby decreasing the opportunity for fecal mutagens to interact with intestinal epithelial cells; reducing the likelihood of cellular mutation (Pins et al., 2001). Thus, potential anticarcinogenic activity of wheat bran may be partially related to its low fermentability in the large intestine (Kroon et al., 1997). In general, bound phenolics cannot be digested by human enzymes and pass through digestion in the stomach and small intestine, thereby reaching the colon where they exert site-specific health benefits (BeMiller and Whistler, 1996). Thus, grain consumption has been associated with a reduced risk of colon cancer (Slavin et al., 1997). It has been reported that in 15 out of 18 epidemiological studies grain intake provided protection against colorectal and gastric cancers (Slavin et al., 1997. Since phenolic compounds are bioactive substances occurring widely in food plants their presence in the human plasma in a diet-dependent concentration has been suggested (Nardini et al., 1995).

Ferulic acid in its free form has been shown to render astringency and hence may act as an antifeedant to insects and animals (Arnason et al., 1992). The increased concentration of ferulates in the outer layers of the cereal grains may be implicated in resistance to both insect and fungal pathogens. Thus, cross-linking of phenolic compounds may provide a physical barrier to invasive disease development and consumption by insects (Zupfer et al., 1998). Cross-linking of plant cell walls via ferulate dehydrodimerization is a well-established phenomenon where ferulates acylate various polysaccharides, notably arabinoxylans in grasses (Ishii, 1997). Therefore,

ferulate dehydrodimerization is a mechanism for linking two polysaccharide chains providing structural integrity to the cell wall and, thereby, inhibiting fibre degradability (Grabber et al., 1998 a&b). Ferulic acid is also known for its antioxidant properties (Marinova and Yanishlieva, 1992). In general, phenolic acids have been recognized as potent antioxidants (Graf, 1992; Natella et al., 1999). Recently, several ferulic acid dehydrodimers have been shown to possess antioxidant activity in different *in vivo* systems (Garcia-Cornesa et al., 1997 a & b, 1999). Ferulic acid possesses useful antioxidant properties and may serve as a good candidate for a natural replacement for synthetic antioxidant food additives (Scott et al., 1993). The liver protective and antioxidative effects of certain plant extracts, possibly due to free radical scavenging, have been demonstrated for carbon tetrachloride-induced liver injury (Chidambara Murthy et al., 2002; Singh et al., 1999). Baranowski et al. (1980) have reported on the antifungal activity of phenolic acids such as caffeic, *p*-coumaric, ferulic and protocatechuic acid against the fungus, *Saccharomyces cerevisiae*.

The consumption of plant foods provides protection against various diseases such as cancer, cardio- and cerebrovascular diseases (Weisburger, 1999). The capacity of antioxidants present in plant-derived foods to scavenge free radicals is responsible for the above protection. Phenolics have been recognized as a major group of non-essential dietary constituents that have been associated with the inhibition of atherosclerosis and cancer (Teissedre et al., 1996).

Free radical oxidative attack is a major cause of disruption and deteriorative changes in aged wheat seeds and the endogenous antioxidative mechanisms may remove potentially damaging molecular species (Pinzino et al., 1999). These authors have further reported that carotenoids, particularly lutein, which are present in wheat

may act as radical scavengers. Hence, the content of lutein has shown a rapid decrease during seed aging. According to Krinsky (1994) carotenoids act as radical scavengers thereby providing protection against oxidative damage. It has been found that free radical content in wheat glutens obtained from flours of wheat seeds after long term storage is high and antioxidative components may become particularly important in scavenging the free radicals formed (Pinzino et al., 1999). Aged wheat seeds show reduced viability, abnormal seedling development, reduced rate of germination and disruption of respiration, ATP production and protein synthesis, among others (Bewley and Black, 1994). The protective effects of phenolics against oxidative stress may also be suggested. Wilson and MacDonald (1986) reported that free radical-mediated lipid peroxidation is responsible for plasma membrane deterioration during seed aging.

2.9. Structure-activity relationship and mechanisms of action of phenolic acids

Hydroxycinnamic acids and their derivatives are widely distributed in plants and are known as antioxidants in various biological and food systems. The antioxidant activity of HCA and its derivatives has been studied extensively and structure-activity relationship has mainly been related to the type and number of characteristic groups on the aromatic ring (Rice-Evans et al., 1996). The ability to donate hydrogen or electrons and to form stable radical intermediates has been attributed to the antioxidant properties associated with phenolic acids (Maillard et al., 1996). The antioxidative activity of phenolic compounds may be attributed to the presence of hydroxyl groups although this is not the only factor that determines their potency as antioxidants (Kuo et al., 2002). The presence of electron-donating groups such as $-CH_3$ and -OH in the aromatic ring increases the ease of hydrogen atom abstraction while groups with electron-withdrawing

properties such as –COOH, -CHO, and –COOR have the opposite effect. The presence of methoxy groups on the structure confers a higher negative charge compared to OH groups and hence phenolics that also contain methoxy group(s) function as strong hydrogen donors (Satue-Garcia et al., 1997). The effects of such substituents may be transmitted to the aromatic centre through extended conjugation in the side chain (Nenadis et al., 2003).

The role of caffeic acid *in vivo* in antioxidant defense has been demonstrated in a rat model (Nardidni et al., 1997). Further antioxidant activity of few other phenolic acids has been studied using different models (Natella et al., 1999; Nardini et al., 1997; Rice-Evans et al., 1996; Laranjinha et al., 1995 a & b, 1994; Marinova and Yanishlieva, 1992). All these studies consistently reported that antioxidant activity of phenolic acids is to some extent related to their chemical structures. Thus, antioxidant activity of phenolic acids depends on substitutions on the aromatic ring and the nature of the side chains present (Shahidi and Wanasundara, 1992). However, different model systems used to determine the antioxidant activity make comparison of the results difficult (Natella et al., 1999).

In general, HCA have a higher antioxidant activity than their corresponding HBA. Moreover, antioxidant activity improves as the number of hydroxy and methoxy groups increases, and specifically in the presence of a *O*-dihydroxy group in the phenolic ring. Thus, caffeic acid consistently exhibits a greater antioxidant activity than other HCA (Natella et al., 1999; Meyer et al., 1998). The high antioxidant capacity of HCA may be linked to the presence of the propenoic side chain that may bring about a stabilizing effect by resonance of the phenoxyl radical. Thus, the presence of a conjugated double bond may enhance the antioxidant activity of aromatic ring unlike the carboxylic side

chain in benzoic acid derivatives (Natella et al., 1999). Ferulic acid readily forms a resonance-stabilized phenoxyl radical which accounts for its potent antioxidant potential due to its phenolic nucleus and the extended side chain conjugation (Graf, 1992). Moreover, ortho substitution with an electron donor methoxy group increases the stability of the phenoxyl radical and therefore increases the antioxidative efficiency (Terao et al., 1993). The para substitution of a phenolic compound leads to the stabilization of the phenoxyl radical to be stabilized due to delocalization of electrons across the entire molecule as in the case of sinapic acid (Heilmann et al., 2000). Caffeic acid may act as a potent inhibitor against LDL cholesterol oxidation in various *in vitro* model systems (Chen and Ho, 1997; Abu-Amsha et al., 1996; Nardini et al., 1995; Laranjinha et al., 1994). The lower antioxidant capacity of ferulic acid compared to caffeic acid is due to the methylation of one of the *O*-dihydroxy groups in the former while substitution of 3-methoxy group of ferulic acid by hydrogen further lowers the antioxidant activity as in the case of *p*-coumaric acid (Meyer et al., 1998). Moreover, *O*-dihydroxy groups in caffeic acid may enhance the metal chelating properties of caffeic acid (Nardini et al., 1995). This is the same structure-activity relationship that had been recognized for flavonoids as one of the antioxidant mechanisms (van Acker et al., 1996).

Monophenols are, in general, less efficient than polyphenols in inhibiting LDL cholesterol oxidation. In gallic acid the inductive effect of the three hydroxyl groups enhances the inhibitory activity of the molecule against LDL cholesterol oxidation (Sanchez-Moreno et al., 1998; Salah et al., 1995). Another important factor is the methoxy substitution that substantially increases the antioxidant power of monophenols as seen in the synthetic antioxidant BHA. Methoxy substitution may, on one hand, be far from equivalent to the addition of a hydroxyl group; ferulic acid is much less efficient than

caffeic acid (Sanchez-Moreno et al., 1998; Salah et al., 1995). The presence of two hydroxyl groups in the ortho position leads to the formation of copper-phenolic acid complex, hence caffeic acid may serve as an efficient metal ion chelator in the cooper-catalyzed oxidation of LDL (Natella et al., 1999; Nardini et al., 1995). Phenolic compounds bearing a substituent in the ortho position to the hydroxyl group form an intramolecular hydrogen bond which is energetically favourable (Zhang et al., 1999).

CHAPTER 3

Materials and Methods

3.1. Materials

3.1.1. Wheat samples

Whole grain, flour, germ and bran of commercial soft (70% Canadian Eastern soft red spring and 30% Canadian Eastern soft white winter) and hard (90% Canadian western hard red spring and 10% Canadian Eastern hard red winter) wheat mixtures were obtained from milling suppliers of Robin Hood Multifoods Inc. (Markham, ON) in Saskatchewan. Whole grains and their milling fractions, namely bran, flour, shorts and feed flour of two wheat cultivars namely CWAD (Canadian Western Amber Durum; *Triticum turgidum* L. var. durum) and CWHRS (Canadian Western hard red spring; *Triticum aestivum* L.) that were grown in Manitoba (crop year 2002) and Saskatchewan (crop year 2001), respectively, (personal communication) were obtained from Canadian Grain Commission, Winnipeg, Manitoba. Pearled grains and their corresponding by-products were also obtained for CWAD and CWHRS cultivars.

Composition of wheat milling fractions are as follows:

Whole grain – unprocessed wheat grain

Bran – the outermost layers of the wheat kernel including aleurone layer

Flour – endosperm of wheat kernel

Germ – wheat embryo

Shorts – a mixture of bran, endosperm and germ

Feed flour – a mixture of bran and low grade endosperm

Semolina – coarse flour prepared from durum wheat

3.1.2. Chemicals

The compounds 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-di[3-ethylbenzthiazoline sulphonate (ABTS), 2,2'-azobis-(2-methylpropionamidine) dihydro-chloride (AAPH) 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ferulic acid and Folin-Ciocalteu phenol reagent, sodium carbonate, monobasic potassium phosphate, dibasic potassium phosphate, butylated hydroxytoluene (BHT), ferulic acid, α-tocopherol, β-carotene, human low density lipoprotein (LDL) cholesterol, hydrogen peroxide, ethylenediaminetetraacetic acid (EDTA), L-ascorbic acid, 2-deoxyribose, ferric chloride, fluorescein, ferrozine, ferrous chloride, sodium bicarbonate, deoxyribonucleic acid (DNA, pBR 322 Plasmid, *E. coli* strain RRI), Trizma base, boric acid, ethdium bromide, agarose, ferrous sulphate, copper sulphate, bromophenol blue, xylene cyanol, glycerol, were purchased from Sigma-Aldrich Chemical Co. (Oakville, ON). All other chemicals and solvents were purchased from Fisher Scientific (Nepean, ON) and were of ACS grade or better.

3.1.3. Preparation of samples

Whole grains and their milling fractions, where necessary, were ground in a coffee bean grinder (Model CBG5 series, Black and Decker Canada Inc., Brockville, ON) and passed through a mesh size 16 sieve (Tylor Test Sieve, Mentor, OH). Wheat flour was used as such for the extraction of crude phenolics. All samples were defatted by blending the ground material with hexane (1:5, w/v, 5 min x 3) in a Waring blender (Model 33BL73, Waring Products Dividion, Dynamics Corp. of America, New Hartford, CT) at ambient temperature. Defatted wheat samples were air-dried for 12 h and stored in vacuum packaged polyethylene pouches at -20 °C until used for analysis.

3.2. Experimental

3.2.1. Determination of total phenolic content (TPC)

The content of total phenolics was determined according to a modified version of the procedure described by Singleton and Rossi (1965). Extracts were dissolved in methanol to obtain a 5 mg/mL concentration solution. Folin-Ciocalteu's reagent (0.5 mL) was added to a centrifuge tube (50 mL) containing 0.5 mL of the extract. Contents were mixed and 1 mL of saturated sodium carbonate solution was added to each tube, followed by adjusting the volume to 10 mL with distilled water. The contents in the tubes were thoroughly mixed by vortexing. Tubes were allowed to stand at ambient temperature for 45 min until the characteristic blue colour developed; centrifugation was then carried out at 4000 X g for 5 min (ICE Centra M5, International Equipment Co., Needham Heights, MA). Absorbance of the clear supernatants was measured at 725 nm using a diode array spectrophotometer (Model 8452A, Agilent Technologies Canada Inc., Mississauga, ON). The content of total phenolics in each extract was determined using a standard curve prepared using ferulic acid and expressed as micrograms of ferulic acid equivalents (FAE) per gram of defatted material.

3.2.2. Measurement of total antioxidant capacity

Total antioxidant activity was determined according to the Trolox equivalent antioxidant capacity (TEAC) assay described by van den Berg et al. (1999) with slight modifications. The extracts and reagents were prepared in a 0.1 M phosphate buffer (pH 7.4) containing 0.15 M sodium chloride (PBS). A solution of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) radical anion ($ABTS^{\bullet-}$) was prepared by mixing 2.5 mM 2,2'-azobis-(2-methylpropionamidine) dihydrochloride (AAPH) with 2.0 mM 2,2'-

azinobis-(3-ethylbenzthiazoline-6-sulphonate) ($ABTS^{2-}$) at a 1:1 (v/v) ratio, and heating at 60 °C for 12 min. The absorbance of the freshly prepared radical solution at 734 nm was about 0.4. The radical solution protected from light was stored at room temperature. A standard curve was prepared using different concentrations of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). Thus, the reduction in the absorbance (ΔA) of the $ABTS^{\bullet-}$ solution (1960 μL) at different concentrations of Trolox (40 μL) over a period of 6 min was measured and plotted. The TEAC values of wheat extracts (5 mg/mL) were determined in the same manner and expressed as μmol Trolox equivalents/g of defatted material. A blank measurement was recorded for each measurement that corresponded to decrease in absorbance without any compound added. The TEAC of an unknown compound represents the concentration of a Trolox solution that has the same antioxidant capcity as the compound.

TEAC values were determined as follows:

$$\Delta A_{Trolox} = (A_{t=0\ min\ Trolox} - A_{t=6\ min\ Trolox}) - \Delta A_{Radical(0\text{-}6\ min)}$$

$$\Delta A_{Trolox} = m \times [Trolox]$$

$$TEAC_{Extract} = (\Delta A_{Extract} / m) \times d$$

where, ΔA = reduction of absorbance, A = Absorbance at a given time, m = slope of the standard curve, [Trolox] = concentration of Trolox, d = dilution factor.

3.2.3. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The method described by Kitts et al. (2000) was used with slight modifications in order to assess the DPPH radical-scavenging capacity of wheat extracts. A 0.075 mM (final concentration) DPPH solution in ethanol was mixed with wheat extracts and vortexed thoroughly. The absorbance of the mixtures at ambient temperature was

recorded for 60 min at 10 min intervals. Ferulic acid, BHT and tocopherol were used as reference antioxidants. The absorbance of the remaining DPPH radicals was measured at 519 nm using a diode array spectrophotometer (Agilent Co., Mississauga, ON). The scavenging of DPPH was calculated according to the following equation.

% scavenging = $\{(Abs_{contol} - Abs_{sample})/Abs_{control}\} * 100$

where, $Abs_{control}$ = absorbance of DPPH radical + methanol

Abs_{sample} = absorbance of DPPH radical + wheat extract/ standard

The scavenging activity was expressed as μmol DPPH radical scavenged/g of defatted material.

3.2.4. β-Carotene-linoleate model system

The antioxidant activity of the wheat samples or standards was evaluated using the β-carotene-linoleate model system (Miller, 1971). A solution of β-carotene was prepared by dissolving 25 mg of β-carotene in 5 mL of chloroform. Three millilitres of β-carotene solution were pipetted into a 100 mL round bottom flask and chloroform was removed under vacuum using a rotary evaporator at 40 °C. Forty milligrams of linoleic acid, 400 mg of Tween 40 emulsifier and 100 mL of aerated distilled water were added to the flask. Contents were mixed thoroughly with vigorous shaking. Aliquots (3.0 mL) of the emulsion were transferred into a series of tubes containing 2.0 mL of the wheat extracts in methanol (final concentrations of phenolics in the assay media were 100 ppm as ferulic acid equivalents). Ferulic acid, BHT and tocopherol were used as reference antioxidants. Absorbance values were recorded over a 2 h period at 20 min intervals while keeping the samples in a water bath at 50 °C. Blank samples devoid of β-carotene

were prepared for background subtraction. Antioxidant index (AI) was calculated using the following equation.

AI = (β-carotene content after 2 h of assay/Initial β-carotene content) * 100

The antioxidant capacity was expressed as nmol β-carotene retained/g of defatted material.

3.2.5. Inhibition of oxidation of human low density lipoprotein (LDL) cholesterol

The procedure described by Hu and Kitts (2000, 2001) was employed in this study. LDL was dialyzed in 10 mM PBS (pH 7.4) at 4 °C in the dark for 24 h. LDL (0.2 mg LDL/mL) was mixed with different amounts of wheat extracts dissolved in 10 mM PBS. Ferulic acid was used as the reference antioxidant. Reaction was initiated by adding a solution of cuprous sulphate (10 μM) and samples were incubated for 22 h at 37 °C. The formation of conjugated dienes was measured at 234 nm using a diode array spectrophotometer (Agilent Technologies Canada Inc., Mississauga, ON). The inhibitory effect of wheat extracts on the formation of conjugated dienes (% $Inhibition_{CD}$) was calculated using the following equation. A separate blank, containing all reagents except LDL, was used for each extract.

% $Inhibition_{CD}$ = ($Abs_{oxidative}$ - Abs_{sample}/$Abs_{oxidative}$ – Abs_{native})/ * 100

where, Abs_{sample} = absorbance of LDL + $CuSO_4$ + wheat extract or standard

Abs_{native} = absorbance of LDL + PBS

$Abs_{oxidative}$ = absorbance of LDL + $CuSO_4$ + PBS

Using percentage values, the amount of protein (μg) that can be protected against copper-mediated oxidation by 1 g of defatted wheat samples was obtained.

3.2.6. Determination of hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging assay was carried out following the procedure of Ruch et al. (1989). Wheat samples were dissolved in 3.4 mL of 0.1 M phosphate buffer (pH 7.4) and mixed with 0.6 mL of a 43 mM solution of hydrogen peroxide prepared in the same buffer. The absorbance of the reaction mixture was recorded over a 40-min period at 10 min intervals. A blank sample devoid of hydrogen peroxide was used for background subtraction. The concentration of hydrogen peroxide in the assay medium was determined using a standard curve and hydrogen peroxide scavenging activity of samples was calculated using the following equation.

Hydrogen Peroxide Scavenging Activity (%) = 100 – [(hydrogen peroxide concentration of medium containing the extract)/(hydrogen peroxide concentration of the control medium)] x100

Antioxidant activity was expressed as µmol hydrogen peroxide scavenged/g of defatted material.

3.2.7. Determination of superoxide radical scavenging activity

The superoxide radical was generated with an enzymatic reaction according to a modified version of the method explained by Nishikimi et al. (1972). The reaction mixture contained 1 mL of each of 3 mM hypoxanthine, xanthine oxidase (100 mIU), 12 mM diethylenetriaminepentaacetic acid, 178 mM nitro blue tetrazolium and the sample. The absorbance of the medium was read at 560 nm over a 60 min period at 10 min intervals. The absorbance values were corrected by subtracting 0 min readings from those obtained subsequently. Superoxide radical scavenging activity (at 10 min) of additives was calculated using the following equation.

Superoxide Radical Scavenging Activity (%) = 100 – [(absorbance of medium containing the additive of concern) / (absorbance of the control medium)] x 100

Antioxidant activity of wheat samples was expressed as nmol superoxide radical anion scavenged/g of defatted material.

3.2.8. Determination of reducing power

The reducing power of wheat samples was determined following the method of Oyaizu (1986). The assay medium contained 2.5 mL of sample in a 0.2 M phosphate buffer (pH 6.6) and 2.5 mL 1% potassium ferricyanide. After incubation at 50 °C for 20 min, 2.5 mL of 10% trichloroacetic acid were added to the mixture followed by centrifugation at 1750*g* for 10 min. One millilitre of the supernatant was mixed with 2.5 mL HPLC-grade water and 0.5 mL of 0.1% ferric chloride, and the absorbance of the resultant solution was read at 700 nm. A standard curve was prepared using various concentrations of ascorbic acid and the reducing power was expressed as µmol ascorbic acid equivalents/g of defatted material.

3.2.9. Measurement of hydroxyl radical scavenging capacity

The effect of hydroxyl radical was assayed using the deoxyribose method as described by Halliwell et al. (1987). One millilitre of final reaction mixture contained 500 µL of extracts and 100 µL of each of 100 µM $FeCl_3$, 100 µM EDTA, 20 mM H_2O_2, 100 µM L-ascorbic acid and 30 mM deoxyribose in 0.2 M phosphate buffer (pH 7.4). The reaction mixture was incubated at 37 °C for 1 h followed by heating in a boiling water bath for 15 min after addition of 1 mL of trichloroacetic acid (2.8%, w/v) and 1 mL of a 1% (w/v) solution of 2-thiobarbituric acid. The absorbance of the solution was

measured at 532 nm against a phosphate buffer blank. Antioxidant capacity was expressed as μmol hydroxyl radical scavenged/g of defatted material.

3.2.10. Determination of oxygen radical absorbance capacity (ORAC)

Oxygen radical absorbance capacity of wheat extracts was determined according to the method of Dávalos et al. (2004) using 75 mM phosphate buffer (pH 7.4) at 37 °C. A FLUOstar OPTIMA microplate reader (BMG LABTECHNOLOGIES GmbH, Offenberg, Germany) equipped with FLUOstar OPTIMA evaluation software version (1.30-0) and black, polystyrene, non-treated 96-well microplates (Costar® Corning Inc., Corning, NY) were used. The outer wells of the microplate were not used in the analysis. The excitation and emission filters were 485-P and 520-P, respectively. The final reaction mixture (200 μL) consisted of antioxidant (20 μL), florescein (120 μL, 70 nM final concentration) and AAPH (60 μL, 12 mM final concentration). The samples were loaded into designated wells in the microplate. A gain adjustment was performed by pipetting 200 μL of florescein into a designated well before starting the program in order to optimize signal amplification. In cycle one, pump 1 was programmed to inject florescein after which the samples and solutions were incubated at 37 °C for 15 min followed by the addition of AAPH using the second pump in cycle 2. The instrument read the florescence of each well after addition of AAPH over 100 cycles. All the measurements were expressed relative to the initial reading. The results were calculated using the differences in area under the florescein decay curve between the blank and the sample and expressed as μmol Trolox equivalents/g of defatted material. The standard curve was prepared using 1-25 μM Trolox (final concentration).

3.2.11. Measurement of iron (II) chelating activity

The Fe (II) chelating activity of wheat extracts was measured as reported by Carter (1971). The reaction was performed in an aqueous medium. The wheat extracts (3 or 6 mg/mL, 2.0 mL) were mixed thoroughly with 2 mM $FeCl_2$ (0.2 mL) and 5 mM ferrozine (0.4 mL). The mixtures were left at room temperature for 10 min. The absorbance of the resultant solution was read at 562 nm. The Fe (II) chelating activity of wheat extracts was calculated as follows:

Iron (II) chelating activity (%) = {1 - (absorbance of sample at 562 nm/ absorbance of control at 562 nm)} x 100

The iron (II) chelating capacity of samples was expressed as µg EDTA equivalents/g of defatted material using a standard curve prepared with EDTA.

3.2.12. Evaluation of antioxidant activity using photochemiluminescence (PCL)

The antioxidant capacity of water- (ACW) and lipid-soluble (ACL) compounds was assessed using a PHOTOCHEM® (Analytik Jena USA, Delaware, OH). The extracts were dissolved in distilled water and methanol, respectively, for ACW and ACL systems followed by centrifugation. The supernatants were used in the determination of antioxidant activity with further dilution using respective solvents, if necessary. Both ACW and ACL analysis were carried out in 0.1 M carbonate buffer (pH 10.5) (Popov and Lewin, 1994, 1996, 1999). The photosensitizer luminol (Reagent 3) was procured from Analytik Jena USA (Delaware, OH). The compositions of reaction mixtures for ACW and ACL are shown in Table 3.1. The antioxidant activity of water-soluble compounds is expressed as µmol ascorbic acid equivalents/g of defatted material while that of lipid-

soluble compounds is expressed as μmol α-tocopherol equivalents/g of defatted material.

Table 3.1. Composition of assay mixtures in ACW and ACL systems

Assay kit	Buffer (μL)	Water (μL)	Methanol (μL)	Luminol (μL)	Sample (μL)
ACW	1000	1500 - X	0	25	X[a]
ACL	200	0	2300-X	25	X

[a]X = Volume of sample (μL)

3.2.13. Measurement of antioxidant activity using Rancimat

The oxidative stability of fats and oils in the presence or absence of wheat extracts was determined using an automated Metrohm Rancimat apparatus (Model 743, Herisen, Switzerland) capable of operating over a temperature range of 50-200 °C. The oxidative stability of seal blubber oil (SBO) and stripped corn oil (SCO) was determined at 100 °C while that of shortening was determined at 120 °C. The glassware was thoroughly cleaned and dried prior to each determination. Samples of oil or fat were weighed directly into the reaction vessels. Wheat extracts were added at 25 mg/g oil or fat concentration. The air flow rate through the sample was adjusted to 20 L/h. The volatile reaction products released during oxidation of oil or fat sample were collected in 60 mL of distilled water in the collection vessel. The change in the conductivity was plotted automatically until the end point was reached. With each oil or fat a control test (with no additives) was included and subjected to the same experimental conditions. The oxidative stability was measured in duplicate for each sample and the IP (h) was recorded. The relative activity on the antioxidant compounds was expressed as the

protection factor (PF) where, PF = IP of oil with additives/IP of the control (no additive) (Frega et al., 1999).

3.2.14. Inhibition of strand breaking of supercoiled DNA

DNA strand breaking by hydroxyl radical was performed according to the method described by Johnson and Grossman (1977) and Hiramoto et al. (1994) with slight modifications. The reaction was carried out in 1 M phosphate buffer (pH 7.4). Reaction mixture contained 2 µL of phosphate buffer, 4 µL of a solution of extract at the indicated final concentration (3 or 6 mg of extract/mL), 2 µL of a solution of supercoiled plasmid pBR 322 DNA (4300 base pairs) at 100 µg/mL, 6 µL of 0.33 mM H_2O_2 and 6 µL of 0.33 mM $FeSO_4$ added in the order stated. The reaction was carried out in an Eppendorf tube (1 mL) and incubated at 37°C for 1 h. Simultaneously, the plasmid DNA was also incubated with the restriction endonuclease Hind III. (Hind III has one restriction site on the pBR 322 plasmid DNA thus producing one fragment having the original number of base pairs. The reaction mixture contained 8 µL of DNA (100 µg/mL), 2 µL of Hind III restriction enzyme, 2 µL of restriction buffer (X10) and 8 µL of distilled water). For identification, the base pair ladder DRIgest™ III was run along with the extracts (Appendix 2.1). After incubation, 2 µL of the loading dye (0.25% bromophenol blue/0.25% xylene cyanol/50% glycerol) were added and the whole mixture was loaded on to a 0.8% (w/v) agarose gel prepared in Tris/borate/EDTA (TBE) electrophoresis buffer (pH 8.3). Agarose gel electrophoresis was performed using TBE electrophoresis buffer at 116 V for 75 min. The gel was stained with 0.5 µg/mL ethidium bromide and bands were visualized under ultraviolet light. The images were analyzed using AlphaEase™ Stand Alone software (Alpha Innotech Corporation, San Leandro, CA). The

protective effects of the crude extracts were measured using the retention percentage of supercoiled DNA.

3.2.15. Analysis of phenolic composition using high performance liquid chromatography (HPLC)

The HPLC procedure described by Amarowicz and Weidner (2001) was used for determination of phenolic acids. A Shimadzu (Kyoto, Japan) HPLC system equipped with an LC-AD pump, an SPD-M10A diode array detector and an SCL-10A system controller was used for analytical and preparative HPLC of wheat crude extracts. Conditions for preparative HPLC were as follows: prepacked LiChrospher 100 RP-18 column (5 µm, 4 x 250 mm, Merck, Darmstadt, Germany); water/acetonitrile/acetic acid (88:10:2, v/v/v) as the mobile phase; flow rate of 1 mL/min; injection volume of 20 µL. The content of vanillic acid was calculated from chromatograms that were recorded at 260 nm while other phenolics were identified at 320 nm. Free phenolics and those liberated from soluble esters were isolated from the extract according to a previously described method (Naczk and Shahidi, 1989). An aqueous suspension of the extract (800 mg/20 mL) was adjusted to pH 2 using 6 M HCl and free phenolic acids were extracted 5 times into 20 mL of diethyl ether using a separatory funnel. The extract was evaporated to dryness under vacuum at room temperature. The aqueous solution was neutralized to pH 7 with NaOH and lyophilized. The residue was dissolved in 20 mL of 2 M NaOH and hydrolyzed for 4 h at room temperature under a nitrogen atmosphere. After acidification to pH 2 using 6 M HCl, phenolic acids released from soluble esters were extracted from the hydrolysate 5 times into 30 mL of diethyl ether using a separatory

funnel. The total free and esterified phenolic acid content was expressed as µg/g defatted material.

3.2.16. Statistical analysis

All analyses were performed in triplicate and data reported as mean ± standard deviation, unless otherwise stated. Analyses of variance were performed using General Linear Model of Minitab Release 14 Xtra for Windows (Minitab Inc., State College, PA). Significant differences ($P<0.05$) among means were determined using the Tukey's multiple range test at a fixed level of $\alpha = 0.05$. The realtionship between TPC and other variables within the observed data range were determined as Pearson correlation coefficients in bivariate correlations.

CHAPTER 4

Optimization of Extraction of Phenolic Compounds from Wheat Using Response Surface Methodology

4.1. Introduction

Phenolic compounds are common dietary phytochemicals found in fruits, vegetables and grains. Epidemiological studies have suggested that food phenolics may protect against degenerative diseases (Mazza, 2000). Most of the beneficial characteristics of phenolic compounds have been ascribed to their antioxidant activity which is a fundamental property important to life (Rice-Evans et al., 1997). Andreasen et al. (2000) have reported on the positive effects of a higher intake of whole grain foods in lowering the risk of coronary heart disease. It has also been suggested that adults would gain appreciable protection from coronary heart disease by consuming the recommended three servings of whole grains daily (Andreasen et al., 2000a). Thus, whole grains, rich in fibre and phytochemicals, are among the healthiest foods that individuals may consume and render a wide variety of health benefits (Andreasen et al., 2001). Plants and plant extracts have been used in traditional cures and herbal remedies for centuries throughout the world. Recently, there has been a renewed interest in secondary plant metabolites because of their potential preventive effects on the chronic diseases such as cardiovascular disease and cancer (Rowland, 1999). Hence, isolation, identification and quantification of phytochemicals in foods and evaluation of their potential health benefits have been in focus. However, *in vitro* and animal studies have shown that the action of some chemicals is likely to be achieved only at doses much higher than those that can be obtained from eating plants (Rowland,

1999). Thus, the extraction of the active ingredient is essential if they are to be of prophylactic or therapeutic value in human subjects (Rowland, 1999).

Many factors such as solvent composition, extraction time, extraction temperature (Wettasinghe and Shahidi 1999), solvent to solid ratio (Cacace and Mazza, 2003a) and extraction pressure (Cacace and Mazza, 2002), among others, may significantly influence the extraction efficacy. In general, optimization of a process could be achieved by either empirical or statistical methods; the former having limitations toward complete optimization. The traditional one-factor-at-a-time approach to process optimization is time consuming. Moreover, the interactions among various factors may be ignored hence the chance of approaching a true optimum is very unlikely. One-factor-at-a-time procedure assumes that various parameters do not interact, thus the process response is a direct function of the single varied parameter. However, the actual response of the process results from the interactive influence of various variables. Unlike conventional optimization, the statistical optimization procedures allow one to take interaction of variables into consideration (Haaland, 1989).

Response surface methodology (RSM), originally described by Box and Wilson (1951), enables evaluation of the effects of several process variables and their interactions on response variables. Thus, RSM is a collection of statistical and mathematical techniques that has successfully been used for developing, improving and optimizing processes (Myers and Montgomery, 2002). Response surface methodology has been used to model and optimize biochemical and biotechnological processes related to food systems (Parajo et al., 1995; Vasquez and Martin, 1998; Senanayake and Shahidi, 1999; Senanayake and Shahidi, 2002; Cacace and Mazza, 2003b; Tellez-Luis et al., 2003) including extraction of phenolic compounds from berries (Cacace and

Mazza, 2003b) and evening primrose meal (Wettasinghe and Shahidi, 1999), anthocyanins from black currants (Cacace and Mazza, 2003a) and sunflower hull (Gao and Mazza, 1996) and vitamin E from wheat germ (Ge et al., 2002), among others.

The extraction and purification of phytochemicals from natural sources is needed since these bioactives are often used in the preparation of dietary supplements, nutraceuticals, functional food ingredients, food additives, pharmaceuticals and cosmetic ingredients (Gao and Mazza, 1996).

4.2. Objectives

This work intended to optimize experimental conditions that results in the highest antioxidant capacity of crude wheat phenolic extracts was conducted. Whole grains and brans of soft and hard wheat were extracted with a number of polar solvents and their total antioxidant capacity (TAC) determined and optimum experimental conditions derived using RSM.

4.3. Materials and Methods

4.3.1. Preparation of samples

Details are given in Chapter 3, Section 3.1, Subsections 3.1.1. and 3.1.3.

4.3.2. Selection of appropriate extraction conditions

The initial step of the preliminary experiment was to select an appropriate extraction medium for wheat phenolics. Three different solvent systems, namely ethanol, methanol and acetone were examined. Crude phenolic compounds from whole wheat and bran of soft and hard wheat were extracted using a series of extraction media

varying in the range of 0 to 100% (v/v; water/ethanol, water/methanol or water/acetone). The crude phenolic extracts were prepared by extracting the ground wheat samples (6 g) with 100 mL of solvent for 20 min at a 80 °C water bath. Based on total antioxidant capacity (TAC), determined by Trolox equivalent antioxidant capacity (TEAC) assay and expressed as μM Trolox equivalents (TE), the best medium and its composition were chosen. The second step of the preliminary experiment was to determine the extraction temperature. Crude phenolics from wheat were extracted using the best solvent composition chosen in the previous step. The temperature of the water bath varied from 15 to 95 °C while the extraction time course was held constant at 20 min. Final step of the preliminary experiment was to select the appropriate extraction time course for extraction of phenolics. Using the solvent system from the first step, phenolics were extracted during various extraction times ranging from 15 to 105 min at a given temperature as determined from the second step. Based on the results the three levels (lower, middle, upper) of each process variable were determined for RSM.

4.3.3. Measurement of total antioxidant capacity

Experimental details are given in Chapter 3, section 3.2, subsection 3.2.2.

4.3.4. Experimental design

Optimization of extraction of phenolics from whole grain and bran of soft and hard wheat in aqueous ethanol was carried out using RSM (Montgomery, 2001; Myers and Montgomery, 2002). A three-factor and a three level face-centred cube design (FCD) consisting of seventeen experimental runs was employed including three replicates at the center point. The effects of unexplained variability in the observed

response due to extraneous factors were minimized by randomizing the order of experiments. The design variables were solvent composition (X_1, %, v/v, water/ethanol), extraction temperature (X_2, °C) and extraction time (X_3, min), while response variable was TAC.

4.3.5. Data analysis

The response surface regression (RSREG) procedure of statistical analysis system (SAS) and design expert (version 6.0.5) software were used to analyze the experimental data (Myers and Montgomery, 2002). Experimental data were fitted to a second-order polynomial model and regression coefficients obtained. The generalized second-order polynomial model used in the response surface analysis was as follows:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum\sum_{i<j=1}^{3} \beta_{ij} X_i X_j$$

where β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and X_i and X_j are the independent variables. The design expert software was used to generate response surfaces and contour plots while holding a variable constant in the second-order polynomial model. When the results showed a saddle point in response surfaces the ridge analysis of SAS RSREG procedure was used to compute the estimated ridge of the optimum response.

4.3.6. Verification of model

Optimal conditions for the extraction of phenolic compounds from wheat depended on solvent composition, extraction temperature and extraction time course were obtained using the predictive equations of RSM. The TAC was determined after

extraction of phenolic compounds under optimal conditions. The experimental and predicted values were compared in order to determine the validity of the model.

4.4. Results and Discussion

4.4.1. Selection of lower, middle and upper levels of the design variables

In general, efficiency of the extraction of a compound is influenced by multiple parameters such as temperature, time and solvent polarity, among others; their effects may be either independent or interactive (Montgomery, 2001). The influence of extraction variables such as solvent composition, temperature and time course on the recovery of phenolic compounds from wheat and its milling fractions has not yet been reported. The entire experiment was divided into three parts. The initial part included the determination of the lower, middle and upper levels of the three design variables employed in the RSM. These levels of independent variables were selected based on values obtained in preliminary experiments. Hence, the first step of the preliminary experiment was to select an appropriate medium for the extraction of wheat phenolics. Selection of appropriate conditions is crucial in the extraction of antioxidant compounds from plant materials. The extraction conditions may not be generalized due to the diverse nature of natural antioxidants existing in different plant materials (Wettasinghe and Shahidi, 1999). Consequently, RSM has shown to be a powerful tool in optimizing experimental conditions to maximize various responses (Cacace and Mazza, 2003a; Gao and Mazza, 1996; Liu et al., 2000; Wettasinghe and Shahidi, 1999). Thus, the effect of various organic solvents (ethanol, methanol and acetone) on TAC of wheat phenolic extracts showed that the response behaved more as a quadratic ($r = 0.68$ to 0.95) function than a linear ($r = 0.004$ to 0.32) one (Fig. 4.1 and Appendices 4.1 – 4.3).

Hence, TAC of crude phenolic extracts started to increase with an increased proportion of organic solvent in the extraction medium. Total antioxidant capacity reached a maximum followed by a decrease with a further increase in the proportion of the organic solvent in the extraction medium.

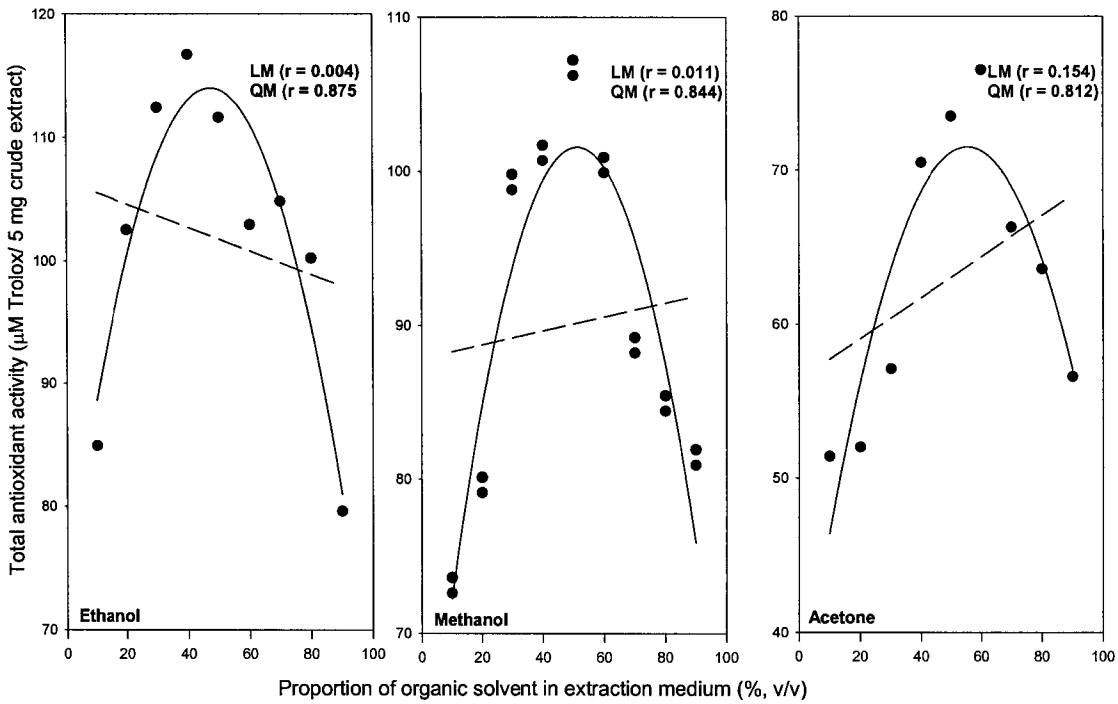


Figure 4.1. Effect of varying organic solvent on the total antioxidant capacity of soft wheat bran crude extract. Each data point represents the average of two determinations. LM and QM represent the linear and quadratic models, respectively.

A similar trend was observed for all three organic solvents employed. Furthermore, the trends were similar for all wheat extracts examined. Thus, the proportion of organic solvent in the extraction medium had a significant influence on the antioxidative properties of wheat extracts with respect to the organic solvent employed. A ratio of organic solvent with water at 1:1 (v/v) produced the highest TAC for all wheat extracts. However, aqueous ethanol yielded extracts with higher TAC compared to other aqueous

solvents from whole grain and bran of both soft and hard wheat and was considered the most effective solvent. Subsequently, the lower, middle and upper levels of the solvent composition were selected based on the above results and the values were 30, 50 and 70% ethanol, respectively, for each wheat fraction. Thus, a moderately polar solvent (50% ethanol) was chosen for determination of extraction temperature and extraction time.

With regard to extraction temperature, the TAC of wheat extracts increased with increasing temperature up to 60 °C and then began to decline (Fig. 4.2 and Appendices 4.4 – 4.6). Results indicated that mobilization of active compounds from the substrate may occur up to a certain level followed by their possible loss due to decomposition at higher temperatures. According to Wettasinghe and Shahidi (1999), high temperatures may mobilize certain antioxidants while promoting possible concurrent decomposition of antioxidants which were already mobilized at lower temperatures. It was also stated that the rate of extraction of thermally-stable antioxidants at elevated temperatures is higher than the rate of decomposition of less soluble antioxidants. This has been suggested by the relatively high antioxidant activities possessed by extracts prepared at higher temperatures. Increasing temperature may favour extraction by enhancing the solubility of phenolic compounds in the solvent. A major effect of the increase in extraction temperature may be to increase the rate of extraction, thereby, decreasing the extraction time (Cacace and Mazza, 2002). The regression analysis demonstrated that relationships between TAC and extraction temperature were more quadratic ($r = 0.82$ to 0.93) than linear ($r = 0.04$ to 0.27). All wheat extracts employed in RSM produced the highest TAC at about 60 °C which led to the selection of 40, 60 and 80 °C as the lower, middle and upper levels, respectively, of the variable for optimization.

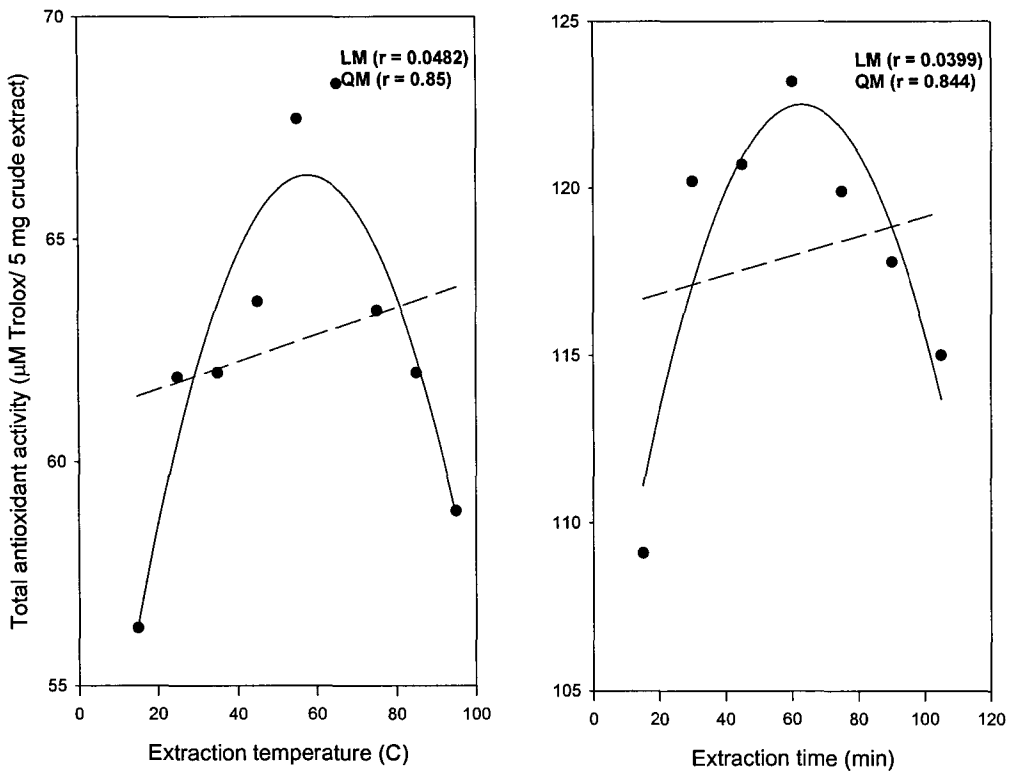


Figure 4.2. Effect of varying extraction temperature and extraction time on total antioxidant capacity of soft wheat bran crude extract. Each data point represents the average of two determinations. LM and QM represent the linear and quadratic model, respectively.

The selection of an appropriate extraction time was the final step in a series of preliminary experiments. Phenolic compounds were extracted from wheat samples by varying the time course of extraction using 50% aqueous ethanol while keeping the temperature constant at 60°C. Relationship between TAC and extraction time demonstrated a quadratic function (r = 0.69 to 0.97) rather than a linear (r = 8.73×10^{-5} to 2.23×10^{-4}) one, hence indicating trends similar to those observed for the other two variables (Fig 4.2 and Appendices 4.4 – 4.6). The results showed that TAC increased when extraction time increased from 15 to 60 min. Beyond 70 min, TAC decreased

sharply and reached a minimum at 105 min, possibly due to the decomposition of active compounds during the prolonged extraction time. Thus, the extraction time was another important parameter influencing the extraction of phenolic compounds and hence TAC. The best extraction time was approximately 60 min for whole grain and bran of both the soft and hard wheat examined in this study. Hence, the lower, middle and upper levels of extraction time chosen for RSM were 45, 60 and 75 min, respectively.

4.4.2. Fitting the models

The three factors and lower, middle and upper design points for RSM in coded and natural/uncoded values are shown in Table 4.1. In RSM, natural variables are transformed into coded variables that have been defined as dimensionless with a mean of zero and the same spread or standard deviation (Myers and Montgomery, 2002). Multiple regression equations were generated relating response variable to coded levels of the independent variables. Multiple regression coefficients were determined by employing the least squares technique (Myers and Montgomery, 2002) to predict quadratic polynomial models for TAC of wheat extracts. Analysis of variance (ANOVA) showed that the selected quadratic models adequately represented the data obtained for TAC. Table 4.2 shows the experimental design employed while Table 4.3 summarizes the data for TAC of all wheat extracts examined. The results of ANOVA for TAC with corresponding coefficients of multiple determination (R^2) for wheat fractions are shown in Table 4.4. The model was adequate and explained most of the variability for all wheat fractions. For the model fitted, the software used generated model coefficients, R^2-values, F-values and significant probabilities and hence one can justify the significance

Table 4.1. Independent variables and their coded and actual values used for optimization

Independent variable	Units	Symbol	Coded levels		
			-1	0	+1
Solvent composition	% (v/v)	X_1	30	50	70
Temperature	C	X_2	40	60	80
Time	Min	X_3	45	60	75

Table 4.2. Three-factor, three-level face-centered cube design used for RSM

Standard order[a]	Run order[b]	Factor 1 (X_1) Solvent composition (%)	Factor 2 (X_2) Temperature (C)	Factor 3 (X_3) Time (min)
1	10	70 (+1)	60 (0)	60 (0)
2	14	50 (0)	60 (0)	75 (+1)
3	2	70 (+1)	40 (-1)	45 (-1)
4	13	50 (0)	60 (0)	45 (-1)
5	12	50 (0)	80 (+1)	60 (0)
6	15	50 (0)	60 (0)	60 (0)
7	11	50 (0)	40 (-1)	60 (0)
8	8	70 (+1)	80 (+1)	75 (+1)
9	5	30 (-1)	40 (-1)	75 (+1)
10	6	70 (+1)	40 (-1)	75 (+1)
11	17	50 (0)	60 (0)	60 (0)
12	7	30 (-1)	80 (+1)	75 (+1)
13	4	70 (+1)	80 (+1)	45 (-1)
14	1	30 (-1)	40 (-1)	45 (-1)
15	3	30 (-1)	80 (+1)	45 (-1)
16	16	50 (0)	60 (0)	60 (0)
17	9	30 (-1)	60 (0)	60 (0)

[a] Nonrandomized, [b] Randomized

Table 4.3. Experimental data for the response total antioxidant capacity (μM TE) of soft and hard wheat extracts under different extraction conditions shown in Table 3.2

Standard order[a]	SWB[b]	SWW[c]	HWB[d]	HWW[e]
1	55.0	33.8	62.9	29.8
2	51.7	55.1	59.4	51.5
3	60.6	33.9	74.1	28.6
4	58.3	40.7	65.3	36.4
5	52.4	33.4	55.1	29.4
6	54.1	45.8	61.9	41.8
7	60.4	42.4	76.1	36.1
8	56.7	53.5	73.1	49.5
9	57.3	27.1	69.3	23.1
10	54.7	49.7	73.6	45.2
11	58.7	51.9	43.1	47.4
12	67.3	51.4	63.1	50.4
13	63.8	53.6	61.8	49.6
14	60.1	56.7	61.3	52.7
15	65.3	58.4	60.1	49.4
16	58.9	55.6	64.3	49.2
17	62.4	60.7	60.9	53.4

[a]Nonrandomized; [b]Soft wheat bran; [c]Soft whole wheat; [d]Hard wheat bran; [e]Hard whole wheat

Table 4.4. Regression coefficients of predicted quadratic polynomial models for the response total antioxidant capacity of whole grain and bran of soft and hard wheat

Coefficient	SWB[a]	SWW[b]	HWB[c]	HWW[d]
B_0	62.29**	55.17**	61.0***	49.54***
LINEAR				
B_1	-1.02	7.42***	-0.42	7.74***
B_2	3.14**	0.19	6.93***	0.11
B_3	-0.57	1.47	0.4	1.36
QUADRATIC				
B_{11}	-6.36***	-14.47***	11.03***	-14.54***
B_{22}	0.64	-1.22	-7.32	0.21
B_{33}	-0.41	2.28	1.13	2.46
CROSSPRODUCT				
B_{12}	-0.55	-1.97	-1.89	-1.61
B_{13}	0.45		2.01*	-0.46
B_{23}		3.88**	1.89	3.84**
$R^{2,e}$	0.89	0.92	0.94	0.94
C.V.[f]	3.5	8.8	4.49	7.93

[a]Soft wheat bran; [b]Soft whole wheat; [c]Hard wheat bran; [d]Hard whole wheat; [e]coefficient of multiple determination; [f]coefficient of variance; ***Significant at 0.1%, **Significant at 5%, *Significant at 10%

of each experimental variable. The maximum predictable response for TAC was obtained based on a total of 17 experiments required for determining 10 regression coefficients of the model (Table 4.4). In general, proceeding with exploration and optimization of a fitted response surface may produce poor or misleading results unless the model exhibits an adequate fit (Myers and Montgomery, 2002). This makes the checking of model adequacy essential (Table 4.5). A plot of experimental and theoretical values indicated an excellent fit ($r \geq 0.94$, $p<0.01$) for whole grain and bran of both soft and hard wheat.

A high proportion of variability was explained by the RSM models for TAC as indicated by R^2 of all wheat fractions (Table 4.4). The regression models were highly significant ($p<0.001$ or $p<0.05$) for all wheat fractions with a satisfactory coefficient of determination (R^2) that varied from 0.89 to 0.94 for TAC. Moreover, the coefficient of variation (C.V.) describes the extent to which the data were dispersed. The C.V. for TAC (Table 4.4) of each wheat fraction was within the acceptable range. Since C.V. is a measure expressing standard deviation as a percentage of the mean the small values of C.V. give better reproducibility. In general, a high C.V. indicates that variation in the mean value is high and does not satisfactorily develop an adequate response model (Daniel, 1991).

An ANOVA of regression parameters of the predicted response surface quadratic models for TAC of wheat fractions is shown in Table 4.6. The results indicated that both linear and quadratic parameters were highly significant ($p<0.001$ or $p<0.05$) for all wheat extracts. However, interactions did not produce a significant effect in each case. Thus, linear and quadratic effects of independent variables were the primary determining terms that could cause significant effects in the response while the interaction terms

Table 4.5. Analysis of variance for the response surface quadratic model for total antioxidant capacity

Source	DF[a]	Sum of Squares	Mean Square	F-value
SOFT WHEAT BRAN		14.36	1.8	0.17[b]
Lack of fit	8	20.54	10.27	
Pure error	2	34.9	3.49	
Total error	10			
SOFT WHOLE WHEAT				
Lack of fit	7	129.02	18.43	2.83[b]
Pure error	2	13.05	6.52	
Total error	9	142.06	15.78	
HARD WHEAT BRAN				
Lack of fit	5	47.62	9.52	1.91[b]
Pure error	2	9.95	4.97	
Total error	7	57.56	8.22	
HARD WHOLE WHEAT				
Lack of fit	7	70.42	10.06	10.06[b]
Pure error	2	11.23	5.61	
Total error	9	81.64	9.07	

[a]Degrees of freedom; [b]Insignificant

Table 4.6. Analysis of variance of the regression parameters of the predicted response surface quadratic models

Regression	DF[a]	Sum of Squares	$R^{2,b}$	F-value[c]
SOFT WHEAT BRAN				
Linear	3	112.24	0.36	7.85**
Quadratic	3	161.39	0.51	11.29**
Cross product	3	4.36	0.01	0.3
Total model	9	278.0	0.89	6.48**
SOFT WHOLE WHEAT				
Linear	3	572.53	0.34	9.87**
Quadratic	3	813.67	0.49	14.02**
Cross product	3	153.97	0.09	2.65
Total model	9	1540.18	0.91	8.85**
HARD WHEAT BRAN				
Linear	3	483.61	0.47	19.6***
Quadratic	3	402.84	0.39	16.33**
Cross product	3	89.4	0.09	3.62*
Total model	9	975.86	0.94	13.19**
HARD WHOLE WHEAT				
Linear	3	617.69	0.39	18.06**
Quadratic	3	732.71	0.47	21.42***
Cross product	3	140.32	0.09	4.1*
Total model	9	1490.73	0.95	14.53***

[a]Degrees of freedom; [b]Coefficient of multiple determination; [c]***Significant at 0.1%l, ** Significant at 5%, * Significant at 10%

were insignificant in most cases. The positive coefficients for X_1, X_2 and X_3 indicated linear effects that may increase the responses (Table 4.4). The quadratic effects of independent variables demonstrated both positive and negative effects. An ANOVA of independent variables shown in Table 4.7 indicates that solvent composition (X_1) was the most significant ($p<0.001$ or $p<0.05$) factor affecting TAC of all wheat fractions. The model indicated that the proportion of ethanol had significant linear effects on TAC of whole grains of both soft and hard wheat (Table 4.4). Hence, ethanol concentration showed the largest positive linear regression coefficient. However, with respect to the bran fraction of both soft and hard wheat, extraction temperature was associated with the largest positive coefficients for the response TAC (Table 4.4). Thus, ethanol concentration and/or temperature contributed significantly to the response. On the other hand, the extraction time had no significant effect on TAC of wheat extracts. By considering the regression coefficients obtained for independent and dependent variables, ethanol concentration and temperature were perhaps the most important factors that may significantly influence TAC. Park et al. (1998) found that solvent concentration plays a critical role in the extraction of soluble solids from various natural products. Similarly, Kwon et al. (2003) reported that solvent concentration was the most important factor contributing to the extraction of ginseng components using RSM.

4.4.3. Analysis of response surfaces

Since the models have shown a lack of fit to be insignificant the responses were sufficiently explained by the regression equation. The regression models allowed the prediction of the effects of the three parameters on TAC of wheat fractions. The relationship between independent and dependent variables is illustrated in a three

Table 4.7. Analysis of variance of the factors and the critical values obtained from ridge analysis of the response surface for total antioxidant capacity (μM TE)

	Analysis of variance				Critical values	
Source	DF[a]	Sum of Squares	Mean Square	F-value[b]	Coded[c]	Uncoded
SOFT WHEAT BRAN						
Solvent composition (X_1, %, v/v)	4	122.74	30.68	6.44**	-0.038	49
Temperature (X_2, °C)	4	102.44	25.61	5.37**	0.193	64
Time (X_3, min)	4	5.63	1.41	0.3	-0.034	60
SOFT WHOLE WHEAT						
Solvent composition (X_1, %, v/v)	4	1145.65	286.41	14.81**	0.178	54
Temperature (X_2, °C)	4	155.7	38.92	2.01	0.055	61
Time (X_3, min)	4	158.27	39.57	2.05	0.235	64
HARD WHEAT BRAN						
Solvent composition (X_1, %, v/v)	4	388.77	97.19	11.82**	-0.017	50
Temperature (X_2, °C)	4	680.72	170.18	20.69***	0.098	62
Time (X_3, min)	4	65.94	16.48	2.0	0.01	60
HARD WHOLE WHEAT						
Solvent composition (X_1, %, v/v)	4	1187.75	296.94	26.04***	0.178	54
Temperature (X_2, °C)	4	138.86	34.71	3.04*	0.055	61
Time (X_3, min)	4	154.28	38.57	3.38*	0.235	65

[a]Degrees of freedom; [b]***Significant at 1%l, ** Significant at 5%, * Significant at 10%, [c]Critical values obtained from ridge analysis

dimensional representation of the response surfaces and two-dimensional contour plots generated by the models for TAC. On the basis of coded data, canonical analysis for TAC demonstrated a saddle point as the stationary point for all wheat extracts examined.

Since analysis of the surface response revealed that the stationary point for TAC was a saddle, a ridge analysis was performed to determine the critical levels of the design variables that may produce the maximum response. The critical values in terms of coded and uncoded variables for TAC are given in Table 4.7.

Since time exhibited insignificant effects on TAC of wheat extracts under different circumstances the response surface and contour plots were generated as a function of solvent composition (30-70%) and temperature (40-80°C) while keeping the time constant at 60 min. Figure 4.3 depicts response surface and contour plots of the effects of the two variables, namely solvent composition and temperature on TAC of soft wheat bran extract. The solvent composition demonstrated quadratic effects on the response; hence, TAC increased up to about 50% organic solvent in the medium followed by a decline with its further increase. However, temperature demonstrated a linear effect on TAC. The effect of solvent composition and temperature on TAC of soft whole wheat extract is shown in Figure 4.4. Solvent composition displayed a quadratic effect on the response yielding a maximum between 50 and 60% ethanol concentration. When the time was kept constant at 60 min the temperature caused a linear increase in the response, at least with low proportions of organic solvent in the medium. Moreover, the response and contour plots generated for hard wheat bran showed that at lower and upper levels of temperature the solvent content influenced the response in a quadratic manner and hence TAC decreased with increasing proportion of organic solvent up to a

certain level (approximately 50%); but improved with further increase (Figure 4.5). Further, at lower and upper levels of solvent composition, as the temperature increased the response also displayed an increase. The effect of solvent composition and temperature at 60 min on TAC of hard whole wheat is shown in Figure 4.6. The solvent composition demonstrated quadratic effects on the response similar to those observed in soft wheat fractions while the effect of temperature was linear regardless of the proportion of ethanol in the medium. Solvent compositions towards the upper design point of the variable produced a greater response compared to that produced at the lower design point.

4.4.4. Verification of experiments

Verification experiments performed at the predicted conditions derived from ridge analysis of RSM demonstrated that experimental values were reasonably close to the predicted values confirming the validity and adequacy of the predicted models. Moreover, the verification experiments also proved that the predicted values of TAC for the model with each wheat fraction could be satisfactorily achieved within 95% confidence interval of experimental values (Table 4.8).

4.5. Conclusions

The conditions for extraction of phenolics from whole grain and bran of soft wheat and whole grain and bran of hard wheat were 54% aqueous ethanol (solvent composition, v/v), 61 °C, 64 min; 49%, 64 °C, 60 min; 54%, 61 °C, 65 min and 50%, 62 °C, 60min, respectively.

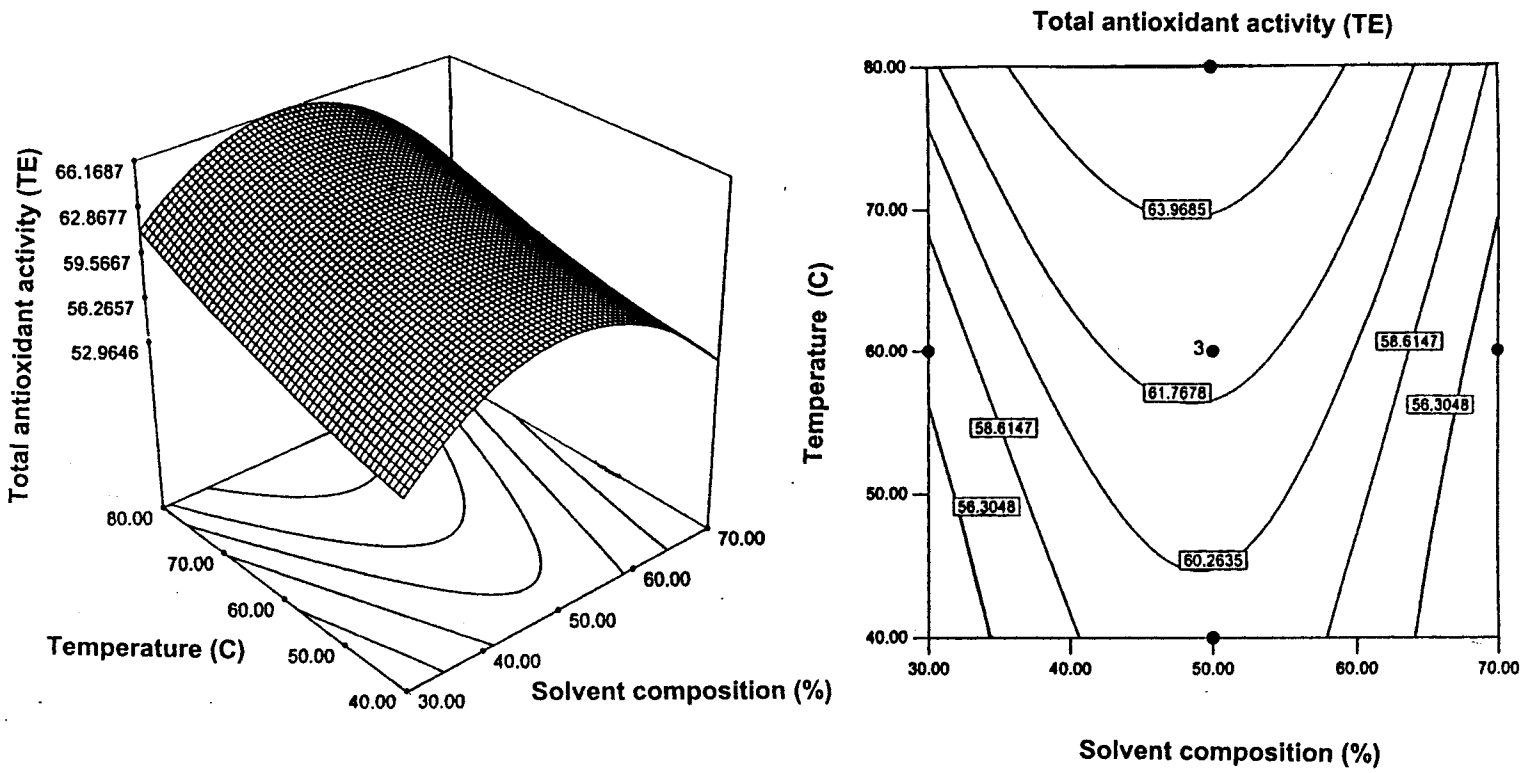


Fig. 4.3. Response surface and contour plots for the effects of solvent composition and temperature at a constant time course of 60 min on total antioxidant capacity (μM TE) of soft wheat bran.

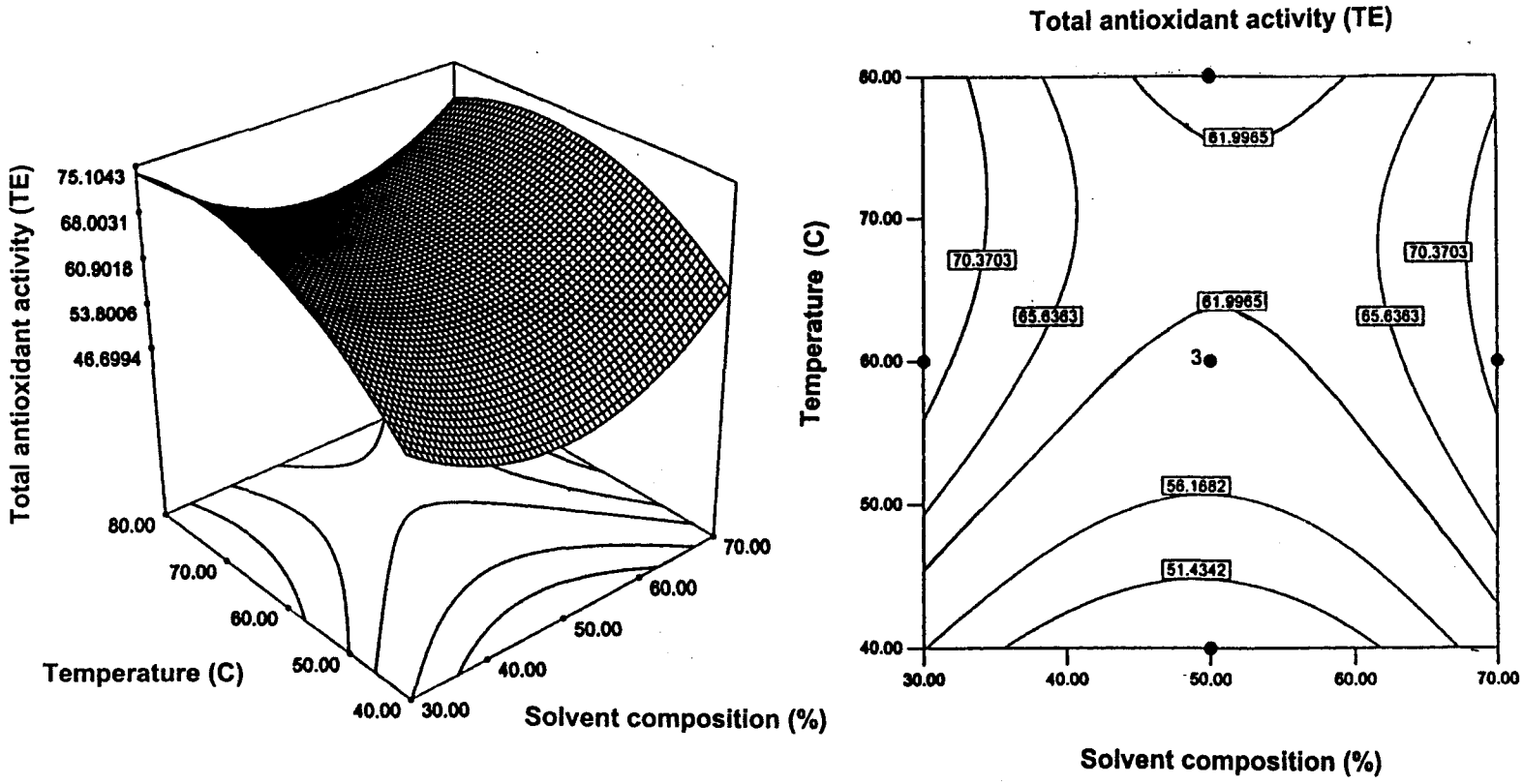


Fig. 4.4. Response surface and contour plots for the effects of solvent composition and temperature at a constant time course of 60 min on total antioxidant capacity (μM TE) of soft whole wheat.

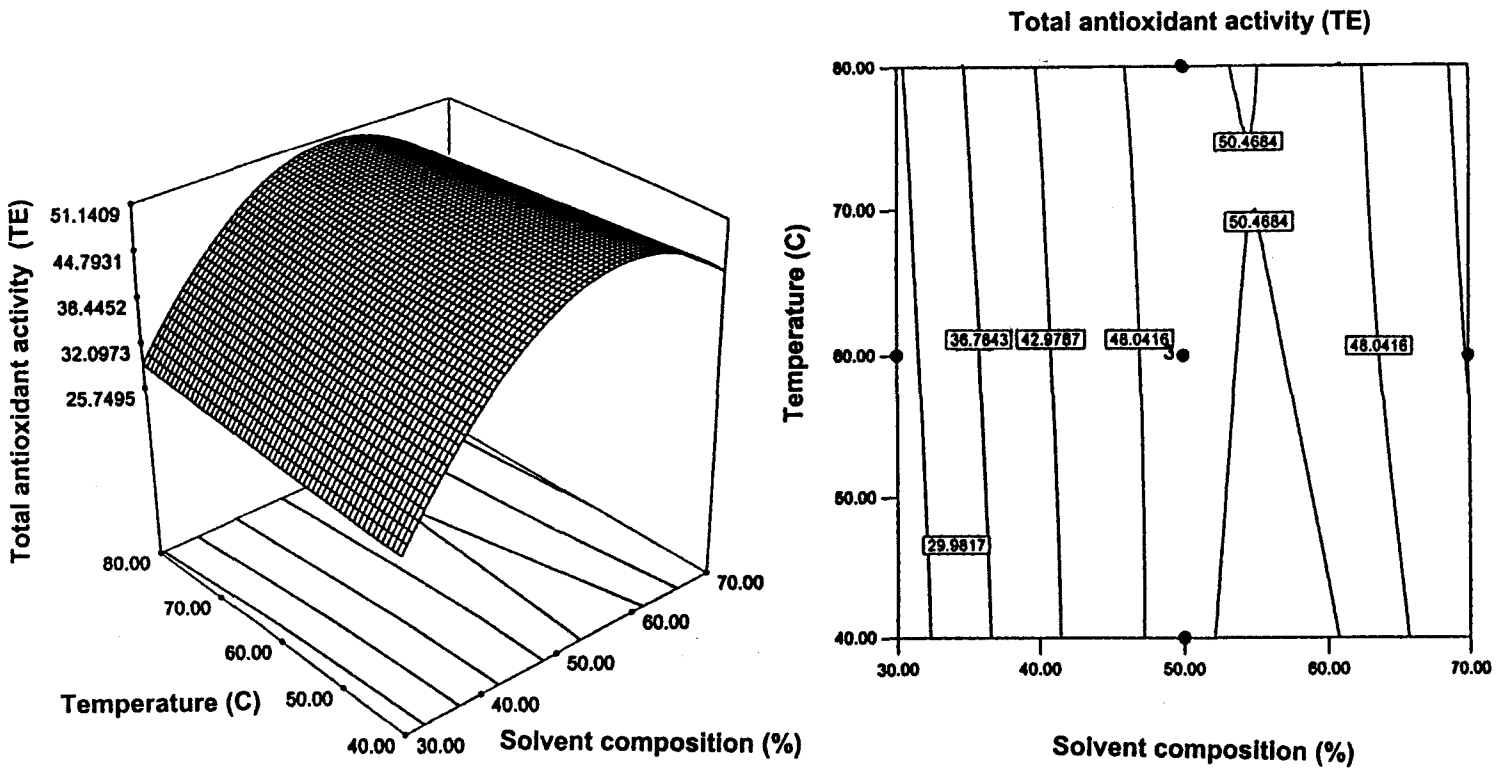


Fig. 4.5. Response surface and contour plots for the effects of solvent composition and temperature at a constant time course of 60 min on total antioxidant capacity (μM TE) of hard wheat bran.

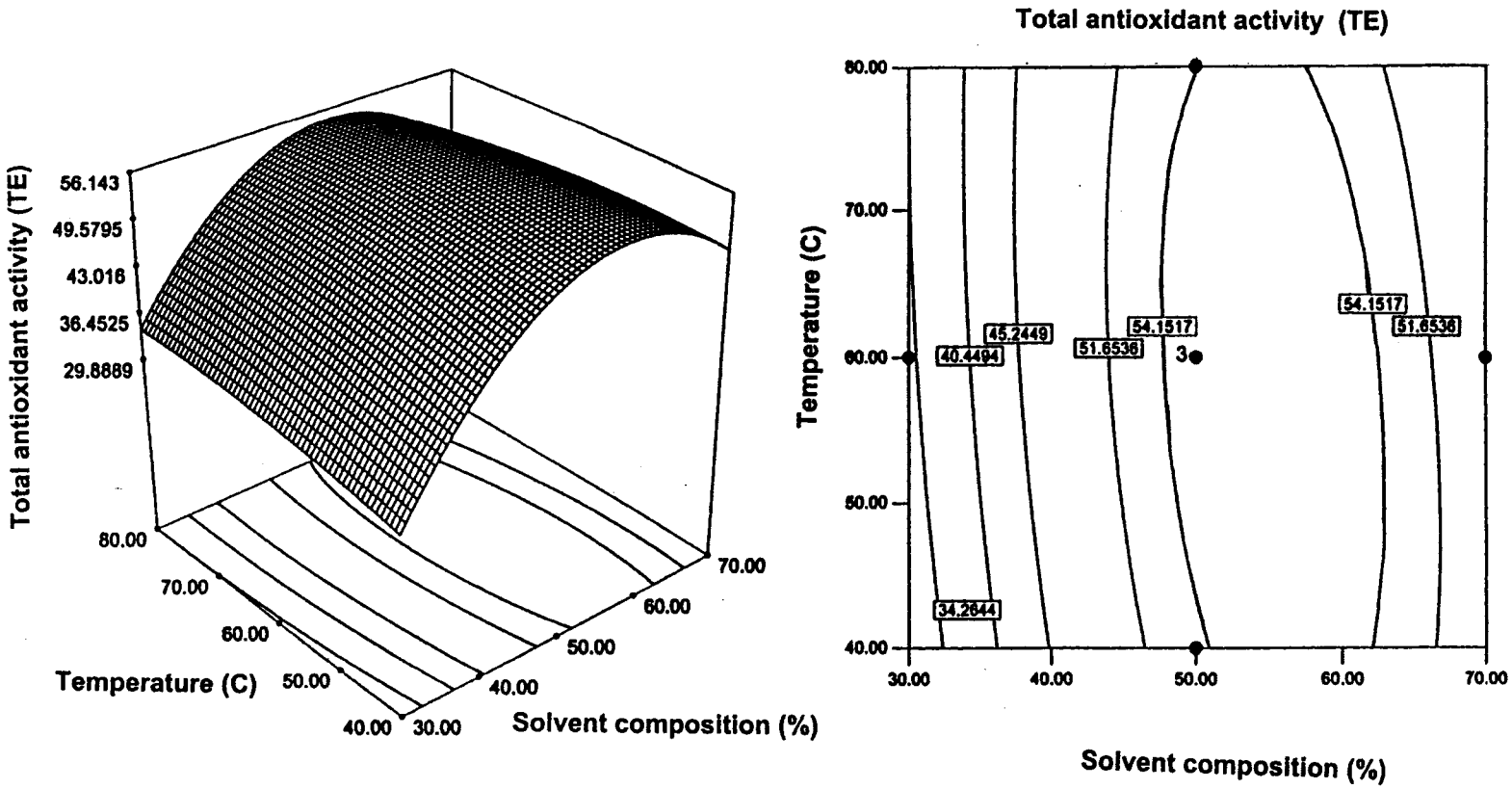


Fig. 4.6. Response surface and contour plots for the effects of solvent composition and temperature at a constant time course of 60 min on total antioxidant capacity (μM TE) of hard whole wheat.

Table 4.8. Comparison of predicted and experimental values for the response variable, total antioxidant capacity.

Wheat fraction	Eigen values	Stationary point	Predicted value[a]	Observed value[b]
SOFT WHEAT				
Bran	0.664, -0.411, -6.377	Saddle	63.0	61.3 ± 1.9 (17.6)[c]
Whole grain	3.163, -2.035, -14.548	Saddle	56.5	54.7 ± 3.2 (6.5)
HARD WHEAT				
Bran	11.172, 1.156, -7.481	Saddle	61.6	58.2 ± 2.7 (16.7)
Whole grain	3.583, -0.861, -14.581	Saddle	50.9	51.6 ± 1.3 (6.8)

[a]Predicted using ridge analysis of response surface quadratic model

[b]Mean ± standard deviation of triplicate determinations from different experiments

[c]Values in parenthesis are μmol Trolox equivalents/g defatted wheat

CHAPTER 5

Antioxidant Properties of Wheat Extracted with Aqueous Ethanol

5.1. Introduction

Phenolic and polyphenolic compounds are naturally present in essentially all plant materials including foods of plant origin and are ubiquitous in fruits, vegetables, cereals and nuts, among others (Arnous et al., 2002). Traditionally, attention has been paid to dietary antioxidants such as α-tocopherol, ascorbic acid and carotenoids. However, other food antioxidants such as amino acids, peptides, proteins, flavonoids and other phenolic compounds might also play a significant role as physiological and dietary antioxidants, thereby, augmenting the body's natural resistance to oxidative damage (Shahidi, 2000). Antioxidants may be defined as compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions (Velioglu et al., 1998). Plants contain a wide range of chemical classes of compounds with antioxidant activity. Considerable evidence suggests that a significant portion of the antioxidant activity of many plants is due to their constituent phytochemicals other than traditional vitamins such as β-carotene, ascorbic acid and α-tocopherol (Cao et al., 1996a). Epidemiological studies indicate that consumption of fruits and vegetables is inversely related to mortality from cancer and coronary heart disease (Hertog et al., 1995). There has been a growing interest in natural antioxidants derived from plants owing to the worldwide trend toward the use of natural additives in foods and cosmetics (Sanchez-Moreno et al., 2000). In fact, plant phenolics present in fruits and vegetables possess both antioxidant properties and potential health benefits (Shahidi and Wanasundara, 1992). Antioxidants, in general, are important not only for

food preservation, but also for the defense of the body against oxidative stress (Aruoma, 1998).

Oxidation of lipids, which is the main cause of quality deterioration in many food systems, may lead to off-flavour development, formation of toxic compounds and lowering of the quality and nutritional value of foods. Furthermore, lipid oxidation is also associated with aging, membrane damage, heart disease and cancer in humans (Ramarathnam et al., 1995). Although synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ), as well as propyl gallate (PG), have been widely used in retarding lipid oxidation, their safety has recently been questioned due to toxicity and possible carcinogenicity concerns (Sun and Fukuhara, 1997). Thus, development of safer natural antioxidants from extracts of oilseeds, spices and other plant materials that can replace synthetic antioxidants has been of interest (van Ruth et al., 2001). Additionally, there has also been interest in preserving endogenous antioxidants in food products both for stabilization and for nutritional purposes (Bryngelsson et al., 2002). Natural antioxidants may in some cases exhibit a wide range of biological effects including antibacterial, antiviral, anti-inflammatory, antiallergic, antithrombotic and vasodilatory activities. In fact, a fundamental property important for life is antioxidant activity and this property may give rise to anticarcinogenicity, antimutagenicity and antiaging activity, among others (Cook and Samman, 1996). The antioxidant activity of phenolic compounds is mainly attributed to their redox properties that allow them to act as reducing agents, hydrogen donors and quenchers of singlet oxygen. In addition, they may also possess metal chelation properties (Rice-Evans et al., 1995).

Different solvent systems including absolute ethanol (Zhou et al., 2004; Zhou and Yu, 2004; Yu et al., 2002 & 2003), 50% aqueous acetone (Zhou et al., 2004) and 80% aqueous methanol (Zielinski and Kozlowska, 2000) have been used to extract phenolics from wheat in order to determine their antioxidant potential, but under different extraction conditions. In Chapter 3, we demonstrated that approximately 50% aqueous ethanol afforded the highest antioxidant activity of wheat extracts using RSM. However, by considering the energy requirements for the removal of water, 80% aqueous ethanol was selected for extraction of phenolics from wheat. Ethanol is a non-toxic solvent. Moreover, presence of 20% water in the medium may still facilitate the extraction of polar compounds during the process; this system has frequently been used for extraction of phenolics and other bioactives. In order to preserve the integrity of any heat-labile wheat antioxidants extraction was carried out at a low temperature (4 °C) over a prolonged period.

5.2. Objectives

The antioxidant properties of whole wheat and different milling fractions, namely flour, germ, bran and shorts of soft and hard wheat were investigated by extracting phenolics into 80% aqueous ethanol. The antioxidant tests carried out determined, in general, the free radical scavenging and metal ion chelation properties of wheat phenolics.

5.3. Materials and methods

5.3.1. Preparation of samples

Details are given in Chapter 3, Section 3.1, Subsection 3.1.1.

5.3.3. Experimental

Details are in Chapter 3, Section 3.2, Subsections from 3.2.1 to 3.2.5 and 3.2.11.

5.4. Results and discussion

5.4.1. Total phenolic content (TPC) and total antioxidant capacity (TAC) of wheat

Under the experimental conditions employed, a higher TPC and antioxidant potential were observed compared to those of other similar studies (Zhou et al., 2004; Zhou and Yu, 2004; Yu et al., 2002 & 2003; Martinez-Tome et al., 2001; Zielinski and Kozlowska, 2000).

The extraction yield and TPC of 80% aqueous ethanolic extracts of whole wheat and different milling fractions, namely flour, germ, bran and shorts are shown in Table 5.1. The amount of total phenolics varied widely in different milling fractions and ranged from 819 ± 24 to 7222 ± 235 and 604 ± 23 to 7540 ± 276 µg ferulic acid equivalents/g of defatted soft and hard wheat, respectively. Miller et al. (2000) have reported that most of the antioxidants in grains are located in the bran and germ. Thus much higher TPC values were observed in the bran, germ, shorts and whole grain fractions than that in the flour portion which is common for both soft and hard wheat. According to Terao et al. (1993) ferulic acid is the dominant phenolic acid in rye, wheat and barley. Hence, the TPC was expressed as ferulic acid equivalents.

Andreasen et al. (2001) determined the TPC of rye whole grain and its milling fractions, flour and bran. They found that rye bran contained the highest amount of phenolics followed by whole grain and flour. The TPC was approximately 15 and 6-times more than that of the flour fraction in bran and whole grain, respectively. Emmons et al. (1999) found that the TPC was highest for oat pearling fractions indicating that many of

Table 5.1. Extraction yield (%, w/w), total phenolic content (TPC) and total antioxidant capacity (TAC) of soft and hard wheat

Milling fraction	Yield (%)	Total phenolic content[A] (μg/g defatted wheat)	Total antioxidant activity (μmol Trolox equivalents/g defatted wheat)
Soft wheat			
Whole grain	4.4 ± 0.4[a]	2006 ± 48[b]	5.2 ± 0.1[b]
Flour	3.4 ± 0.2[a]	819 ± 24[a]	2.3 ± 0.02[a]
Germ	10.2 ± 0.9[d]	7222 ± 235[ce]	23.9 ± 0.6[d]
Bran	9.8 ± 0.6[c]	6550 ± 186[d]	13.7 ± 0.4[c]
Shorts	8.2 ± 0.3[b]	4040 ± 156[c]	14.3 ± 0.3[c]
Hard wheat			
Whole grain	4.9 ± 0.5[a]	1990 ± 44[b]	5.5 ± 0.3[b]
Flour	3.8 ± 0.3[a]	604 ± 23[a]	2.4 ± 0.1[a]
Germ	12.0 ± 0.8[c]	7540 ± 276[e]	24.3 ± 0.4[e]
Bran	9.0 ± 0.9[b]	5340 ± 189[d]	11.7 ± 0.3[c]
Shorts	8.9 ± 0.7[b]	3760 ± 169[c]	14.1 ± 0.2[d]

[A]Total phenolic content was expressed as ferulic acid equivalents

Values are the mean of three determinations ± standard deviation

Values in each column having the same superscripts are not different ($p>0.05$); data for soft and hard wheat were treated separately

these phenolic compounds are located in the seed coat/aleurone/subaleurone layers. Thus, there is a declining concentration of these phenolics toward the interior of the kernel which is the starchy endosperm. Using histochemical studies it has been shown that the highest concentration of phenolics exists in the bran layers; especially, the aleurone cell walls (Fulcher, 1986). Results of the current study also indicated an asymmetric distribution of phenolic compounds in the wheat grain. Thus, the TPC of the

bran was 8 and 8.8 times higher than that of flour fraction, in soft and hard wheat, respectively.

The antioxidant activity of soft and hard wheat was tested using the TEAC assay. The TEAC assay is based on the scavenging of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) radical ($ABTS^{\bullet-}$) in the presence of an antioxidant. The ABTS radical has a characteristic long wavelength absorption spectrum showing maxima at 660, 734 and 820 nm (Prior and Cao, 1999). The radical-scavenging activity of wheat samples was expressed as TEAC values defined as the relative ability of an antioxidant to scavenge the $ABTS^{-\bullet}$ generated in the aqueous phase as compared with Trolox, which is a water-soluble analogue of vitamin E. The TAC of soft and hard wheat expressed as micromoles Trolox equivalents (TE)/g of defatted material are summarized in Table 5.1. Ferulic acid demonstrated a TAC of 60 ± 0.8 µmol at 100 ppm. For soft wheat TAC varied from 2.3 ± 0.02 to 23.9 ± 0.6 µmol TE/g defatted sample while this was 2.4 ± 0.1 to 24.3 ± 0.4 µmol TE/g of defatted hard wheat sample. The TAC observed indicated that ferulic acid possessed a much stronger TAC than those of soft and hard wheat samples. The TAC relative to Trolox indicated that wheat germ was more efficient than other wheat fractions examined. Thus, wheat germ exhibited the highest TAC followed by the shorts, bran, whole grain and flour for both soft and hard wheat. The correlations (r^2) between TPC and TAC were 0.922 ($p<0.05$) and 0.947 ($p<0.05$) for soft and hard wheat, respectively, demonstrating a strong association between the two parameters. In the whole grain, the bran portion is mixed with the starchy endosperm, therefore diluting the antioxidant capacity. Thus, the TAC in the bran was 6 and 4.9 times higher than that in the flour of soft and hard wheat, respectively. In oat the aleurone layer contained the

highest concentration of antioxidants. Therefore, the bran fraction possessed the highest antioxidant activity among different oat fractions (Handelman et al., 1999).

It has been reported that antioxidant activity does not necessarily correlate with high amounts of phenolics. Kahkonen et al. (1999) reported no significant correlation between TPC and antioxidant activity of plant extracts belonging to different subgroups such as berries, vegetables, cereals and herbs. Hence, both phenolic content and antioxidant activity have to be considered in evaluating the antioxidant potential of a plant extract (Kahkonen et al., 1999). On the other hand, Emmons et al. (1999) observed a good correlation between the TPC and antioxidant activity that indicates the responsibility of phenolic compounds for a large proportion of the antioxidant activity. This study also lends support to the findings of Emmons et al. (1999).

The Folin-Ciocalteu method measures the reducing capacity of a compound (Satué-Gracia et al., 1999). Therefore, the TPC determined according to this procedure may not represent the absolute amount of phenolic materials present. However, the values may be based on the chemical reducing capacity relative to an equivalent reducing capacity of a compound (Sanchez-Moreno et al., 2000); for instance, ferulic acid in the current study. Moreover, the Folin-Ciocalteu procedure is rather unspecific and hence any compound with a reducing capacity reacts with the Folin-Ciocalteu's reagent. Therefore, this method may lead to an overestimation of TPC that has been attributed to the proportionality of the molar response of Folin reagent to the number and relative position of hydroxyl groups on the phenolic compound (Satué-Gracia et al., 1999). Hence, the TPC measured by the Folin-Ciocalteu procedure may not give a full picture of the quantity or quality of phenolic constituents in the extracts. There may be interference arising from the chemical constituents other than phenolics such as sugars

or ascorbic acid (Shahidi and Naczk, 2004). Different phenolic compounds have been known to respond differently in the Folin-Ciocalteu's assay. Thus, antioxidant activity of a particular compound depends on its chemical structure (Satué-Gracia et al., 1997). Consequently, this may not always warrant the prediction of antioxidant activity on the basis of TPC (Kahkonen et al., 1999). Results of the current study indicate that the antioxidant capacity of phenolics was different among various milling fractions with strong correlation with TPC.

5.4.2. Free radical scavenging properties of soft and hard wheat phenolics

Free radical scavenging is an accepted mechanism for antioxidant action thereby inhibiting lipid oxidation; scavenging of DPPH radical was used in this work. This method has been used extensively to predict antioxidant activities owing to the relatively short time required for analysis (Chen et al., 1999). The DPPH radical scavenging capacity of soft and hard wheat is shown in Table 5.2. The DPPH radical scavenging capacity of ferulic acid, α-tocopherol and BHT was determined at 100 ppm concentration. The soft wheat fractions scavenged from 151 ± 4 to 1557 ± 18 µmol of DPPH radical/g defatted sample. The corresponding values for hard wheat were from 105 ± 2 to 1657 ± 18 µmol of DPPH radical/g defatted material, respectively. The scavenging of DPPH radical by the germ and bran of soft wheat was 10.3 and 8.7 times higher than that for the flour fraction, respectively. The corresponding values for hard wheat were 15.8 and 9.9 times, respectively. According to Shimada et al. (1992), the DPPH radical scavenging property is attributed to the hydrogen-donating ability of the compound. Thus, phenolic compounds in aqueous wheat extracts may donate a hydrogen atom from the phenolic hydroxyl groups and form a stable intermediate which

is incapable of initiating or propagating free radical chain reactions (Sherwin, 1978). The TPC and DPPH radical scavenging capacity correlated with one another with coefficients of 0.998 and 0.996 at the 1% level for soft and hard wheat, respectively.

Table 5.2. DPPH radical scavenging capacity[A] of soft and hard wheat

Milling fraction	μmol/g defatted wheat
Soft wheat	
Whole grain	393 ± 12[b]
Flour	151 ± 3.8[a]
Germ	1557 ± 18[e]
Bran	1309 ± 16[d]
Shorts	809 ± 7[c]
Hard wheat	
Whole grain	384 ± 6[b]
Flour	105 ± 2[a]
Germ	1657 ± 18[e]
Bran	1039 ± 16[d]
Shorts	722 ± 22[c]
Ferulic acid[B]	85.6 ± 0.4
α-Tocopherol[B]	89.5 ± 0.3
BHT[B]	75.7 ± 0.4

Values are the mean of three determinations ± standard deviation

Values in each column having the same superscripts are not different ($p>0.05$); data for soft and hard wheat were treated separately

[A]Original percentage data used for the calculation of scavenging capacity (μmol/g) of wheat samples are shown in Appendix 5.1

[B]DPPH radical scavenging (%) of reference antioxidants at 100 ppm

Amount of DPPH in the assay medium was 54 μmol

In our study ferulic acid demonstrated stronger DPPH radical scavenging activity compared to BHT and α-tocopherol. Graf (1992) reported that ferulic acid readily forms a resonance-stabilized phenoxyl radical while extended side chain conjugation also accounts for its potent antioxidant potential. On the other hand, ortho substitution with an electron donor methoxy group increases the stability of the phenoxyl radical and therefore increases the antioxidative efficiency (Terao et al., 1993).

The effects of wheat extracts on the control of autoxidation of linoleic acid were also determined. The ability of soft and hard wheat samples to inhibit the coupled oxidation of linoleic acid and β-carotene is shown in Table 5.3. The absorbance readings at 470 nm reflect the efficacy of inhibiting oxidation of β-carotene by wheat fractions/standards. Bran, germ, shorts and whole grain of both soft and hard wheat exhibited a significantly ($p<0.05$) higher antioxidative activity than the flour fraction. The inhibition capacity of β-carotene bleaching of germ and bran of soft wheat was 13 and 12.3 times higher than that of the flour, respectively. The corresponding values for hard wheat were about 19 and 14 times, respectively. Thus, different milling fractions exhibited various degrees of antioxidant activity. The soft wheat fractions retained 739 ± 12 to 9627 ± 206 nmol of β-carotene/g of defatted material while this varied from 507 ± 13 to 9651 ± 228 nmol/g defatted material for hard wheat fractions. Antioxidative components responsible for free radical scavenging were concentrated in the germ and bran fractions of the grain. Consequently, shorts, a milling fraction composed of varying proportions of germ and bran, also exhibited higher free radical scavenging properties than flour.

According to Singh et al. (2002) the mechanism involved in bleaching of β-carotene is a free radical-mediated phenomenon resulting from hydroperoxides of

Table 5.3. Retention[A] of β-carotene against bleaching by soft and hard wheat

Milling fraction	nmol/g defatted wheat
Soft wheat	
Whole grain	2375 ± 48[cb]
Flour	739 ± 12[a]
Germ	9627 ± 206[d]
Bran	9116 ± 100[d]
Shorts	4895 ± 129[bc]
Hard wheat	
Whole grain	2147 ± 70[b]
Flour	507 ± 13[a]
Germ	9651 ± 228[e]
Bran	7138 ± 144[d]
Shorts	4368 ± 44[c]
Ferulic acid[B]	92.6 ± 0.9
α-Tocopherol[B]	96.7 ± 0.7
BHT[B]	89.0 ± 0.2

Values are the mean of three determinations ± standard deviation

Values in each column having the same superscripts are not different ($p>0.05$); data for soft and hard wheat were treated separately

[A]Original inhibition percentage data used for the calculation of retention of β-carotene (nmol/g) are shown in Appendix 5.2

[B]Inhibition (%) of β-carotene-linoleate oxidation at 100 ppm

Amount of β-carotene in the assay medium was 838 nmol

linoleic acid oxidation. The free radical of linoleic acid formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups may attack the highly unsaturated β-carotene molecules. In this model system β-carotene undergoes rapid discoloration in the absence of an antioxidant as observed in the control. Upon oxidation

β-carotene loses its chromophore and changes in its characteristic orange colour may be monitored spectrophotometrically. Wheat extracts demonstrated considerable antioxidative activity by inhibiting the bleaching of β-carotene. Thus, wheat antioxidants may scavenge free radicals thereby inhibiting lipid peroxidation reactions.

In general, membrane lipids are rich in unsaturated fatty acids that are highly prone to oxidative processes and hence linoleic acid and arachidonic acid, in particular, form possible targets of lipid peroxidation (Braca et al., 2003). Therefore, it is important to maintain a balance between antioxidants and oxidants in living organisms and increased intake of dietary antioxidants may help in maintaining the adequate antioxidant status. The activity of the extracts may be attributed to their hydrogen-donating ability (Shimada et al., 1992). Free radicals have been shown to cause autoxidation of unsaturated lipids in food systems. In the presence of phenolic antioxidants, free radical chain of oxidation is intercepted by donating hydrogen from the phenolic hydroxyl groups to free radicals thereby forming a stable end product that does not initiate or propagate further oxidation of lipid. The results indicated that wheat fractions, especially germ and bran, are potential free radical scavengers and primary antioxidants that react with free radicals.

5.4.3. Iron (II)-chelation capacity of soft and hard wheat phenolics

Results for iron (II)-chelation capacity of phenolics of soft and hard wheat are summarized in Table 5.4. The chelation capacity of different milling fractions decreased in the order of germ > bran > shorts > whole grain > flour for both soft and hard wheat. Hence, the flour fraction showed the lowest chelation capacity while the germ exhibited the highest capacity for both soft and hard wheat. Iron (II)-chelation capacity of soft and

Table 5.4. Iron (II)-chelation activity[A] of soft and hard wheat

Milling fraction	µg EDTA equivalents/g defatted wheat
Soft wheat	
Whole grain	1544 ± 27[b]
Flour	617 ± 19[a]
Germ	4771 ± 105[e]
Bran	3996 ± 142[d]
Shorts	2883 ± 127[c]
Hard wheat	
Whole grain	1486 ± 44[b]
Flour	447 ± 15[a]
Germ	4459 ± 107[e]
Bran	3752 ± 85[d]
Shorts	2419 ± 98[c]
EDTA[B]	100.0 ± 0
Trolox[B]	0
Ferulic acid[B]	4.7 ± 0.7

Values are the mean of three determinations ± standard deviation

Values in each column having the same superscripts are not different ($p>0.05$); data for soft and hard wheat were treated separately

[A]Original percentage data used for the calculation of iron (II)-chelation activity (µg/g) of wheat samples are shown in Appendix 5.3.

[B]Iron (II) chelation (%) at 100 ppm extract as ferulic acid equivalents

hard wheat fractions varied from 617 ± 19 to 4771 ± 105 µg EDTA equivalents/g and 447 ± 15 to 4459 ± 107 µg EDTA equivalents/g defatted material, respectively. The germ fraction of soft and hard wheat, respectively, were 7.7 and 10 times more effective chelators than their flour fraction. The corresponding values for bran fraction were 6.5

and 8.4 folds, respectively. Thus, phenolic compounds of germ fraction exhibited the strongest chelation capacity of iron (II) among different fractions. It has been suggested that phenolic acids may either act synergistically or there may be other constituents that contribute to the antioxidant effect via iron (II)-chelation (Andreasen et al., 2001).

Among reference antioxidants, EDTA exhibited the strongest chelation capacity; EDTA at 100 ppm exhibited 100% chelation. On the contrary, Trolox did not chelate iron (II) at all while ferulic acid exhibited only 4.7% chelation capacity at 100 ppm concentration. According to Graf (1992), ferulic acid may not exert antioxidant activity via metal chelation.

It has been reported that high iron status or iron overload is positively correlated with coronary heart disease risk (Salonen et al., 1992). Hence, increased intake of phenolic compounds may maintain a relatively low iron status thereby reducing the risk of iron overload (Samman et al., 2001). The correlation between TPC and iron (II)-chelation capacity for soft wheat was 0.995 ($p<0.01$) while this was 0.99 ($p<0.01$) for hard wheat. This indicates the importance of phenolics in iron (II) chelation capacity of wheat.

5.4.4. Inhibition of oxidation of LDL by soft and hard wheat phenolics

Low density lipoprotein oxidation is a radical chain reaction that generates conjugated diene hydroperoxides as the initial main product. In general, formation of conjugated dienes may be easily determined spectrophotometrically (Puhl et al., 1994). It has been suggested that inhibition of copper-mediated LDL oxidation may arise due to free radical scavenging and/or metal ion chelation (Decker et al., 2001). The formation of conjugated dienes due to copper-induced LDL oxidation was completely inhibited by

Trolox at 100 ppm concentration while the inhibition by ferulic acid was 81.7 ± 0.4%. Trolox and ferulic acid may have demonstrated their protective effects by scavenging free radicals. Chelation of Cu^{2+} cannot be a possibility for Trolox and ferulic acid since they are not metal iron chelators. The inhibition of oxidation of LDL, expressed as μg protein retained/g defatted material, by soft and hard wheat samples is shown in Table 5.5. Among different fractions flour extracts were least effective while the germ extracts were most effective. The retention capacity decreased in the order germ > bran > shorts > whole grain > flour for both soft and hard wheat. The bran fractions also exhibited significantly higher retention capacities compared to flour, whole grain and shorts.

According to a study performed by Andreasen et al. (2001) the rye bran extract was more powerful than a flour extract in inhibiting LDL oxidation. The dilution of antioxidant compounds by the endosperm in the whole grain was apparent, and hence the bran possessed 3.3 and 2.9 fold higher effect in prevention of oxidation of LDL than that of the whole grain. Similar patterns were noticed in other assays as well.

The correlation value between TPC and inhibition of LDL oxidation for soft wheat was 0.99 ($p<0.01$) for both soft and hard wheat. Antioxidant activity of wheat fractions as measured by the coupled oxidation of linoleic acid and β-carotene and DPPH scavenging was significantly ($p>0.05$) correlated with inhibition of LDL oxidation. This explains the influence of phenolic compounds on the antioxidant potential of wheat.

At pH 7.4 of the LDL system, the hydroxyl groups may be partially ionized and compounds with OH groups in their chemical structures may act as metal chelators as well as free radical scavengers (Satué-Gracia et al., 1997). Thus, depending on their chemical structure, the phenolics may act as hydrogen-donors that scavenge free radicals or metal ion chelators that prevent metal ion catalyzed formation of initiating

radical species (Salah et al., 1995). It is unlikely that ferulic acid exerts antioxidant activity against copper-mediated LDL oxidation by chelating Cu^{2+} because ferulic acid is a very weak metal ion chelator (Graf, 1992).

Table 5.5. Inhibition[A] of oxidation of human LDL by soft and hard wheat

Milling fraction	µg protein/g defatted wheat
Soft wheat	
Whole grain	1925 ± 15[b]
Flour	756 ± 3[a]
Germ	7183 ± 65[e]
Bran	6311 ± 46[d]
Shorts	3664 ± 36[c]
Hard wheat	
Whole grain	1787 ± 2[b]
Flour	522 ± 4[a]
Germ	7290 ± 68[e]
Bran	5159 ± 25[d]
Shorts	3402 ± 15[c]
Trolox[B]	100 ± 0
Ferulic acid[B]	81.7 ± 0.4

Values are the mean of three determinations ± standard deviation

Values in each column having the same superscripts are not different ($p>0.05$); data for soft and hard wheat were treated separately

[A]Original inhibition (%) data uased for calculation of the amount of LDL retained (µg/g) by wheat samples are shown in Appendix 5.4

[B]Inhibition (%) of copper-induced LDL oxidation at 100 ppm.

5.5. Conclusions

Extraction conditions (80% aqueous ethanol, 4 °C, 16 h) employed enabled extraction of wheat phenolics at 75-80% of the antioxidant capacity compared to that under optimum conditions (approximately 49-54% aqueous ethanol, 60-61 °C, 60-65 min) as reported in Chapter 4.

CHAPTER 6

Effects of Simulated Gastric pH Conditions on the Extractability and the Antioxidant Activity of Wheat Phenolics

6.1. Introduction

Regular consumption of fruits, vegetables and whole grains is known to reduce the risk of a number of chronic diseases. Hence, dietary modification by increasing the daily intake of plant foods may have a significant impact on preventing chronic diseases (Dewanto et al., 2002). Whole grains, in particular, provide a wide range of nutrients and biologically-active constituents which reduce the incidence of various diseases (Slavin et al., 2000). In this regard, wheat (*Triticum aestivum* L.) is a staple food for a majority of the world's population and serves as a source of potentially health-enhancing components such as dietary fibre, phenolics, tocopherols and carotenoids (Abdel-Aal and Hucl, 2003) if consumed as whole grains. Many constituents in plant foods may contribute independently or synergistically to anticancer or cardioprotective agents by a variety of mechanisms (Rice-Evans et al., 1997). In general, plant foods such as cereals, fruits, vegetables, nuts and spices form the primary source of naturally-occurring antioxidants in the human diet (Sang et al., 2002). Antioxidants are believed to play a significant role in the body's defense system against ROS. The ROS are harmful byproducts generated during normal cellular metabolism. Consequently, there has been much interest in the antioxidant activity of phytochemicals present in the diet (Gutteridge and Halliwell, 2000). According to Vinson et al. (1998, 2001) most phytochemicals in fruits and vegetables are in the free or soluble conjugate forms of glycosides. In contrast, phenolic compounds in grains and oilseeds may exist in the free,

soluble conjugate and insoluble-bound forms; mostly in the insoluble-bound form associated with cell wall polysaccharides (Herrmann, 1989; Mueller-Harvey et al., 1986; Smith and Hartley, 1983). Among phenolic compounds, cinnamic and benzoic acid derivatives are antioxidant constituents universally present in plant foods. Ferulic acid is the major hydroxycinnamic acid derivative present in cereals (Smith and Hartley, 1983). The commonly existing trans-ferulic acid (4-hydroxy-3-methoxycinnamic acid) and trans-*p*-coumaric acid (4-hydroxycinnamic acid) are predominantly esterified to hemicellulose via covalent links to arabinofuranose in the heteroxylans (Mueller-Harvey et al., 1986). In general, digestion of cell wall materials is rather difficult and hence may survive gastrointestinal digestion to reach the colon. However, it has been shown that the colonic fermentation of such material may lead to the release of some of the bound phenolics and, hence, may exert their unique health benefits in the colon following absorption (Kroon et al., 1997). In the gut, the action of microbial esterases may lead to the release of monomeric hydroxycinnamtes and their absorption into the circulatory system has been demonstrated in humans (Kroon et al., 1997). Therefore, bound phenolic constituents may have a significant effect on human health following digestion (Miller et al., 2000). Grains are known to possess unique phytochemicals, including ferulic and caffeic acids in a more concentrated form, that are complimentary to those present in fruits and vegetables (Dewanto et al., 2002).

6.2. Objectives

The objectives of this work were to examine the effects of simulated pH conditions of the gastrointestinal tract on the extractability of phenolic compounds from

soft and hard wheat and the milling fractions, namely bran, germ, flour and shorts, and and to investigate the antioxidant activity of wheat phenolics using *in vitro* assays.

6.3. Materials and methods

6.3.1. Sample preparation

Details are given in Chapter 3, Section 3.1, Subsection 3.1.1.

6.3.2. Experimental

6.3.2.1. Extraction of crude phenolics

Crude phenolics were extracted into aqueous media according to the procedure described by Baublis et al. (2000). Wheat samples (10 g) were extracted with distilled deionized water (150 mL) for 30 min while continuously stirring the slurry. The resulting slurries were centrifuged at 7500 x g for 15 min at ambient temperature.

To determine simulated gastrointestinal pH conditions on antioxidant activity, wheat samples were first incubated at ambient temperature for 30 min (pH 6.5 – 7.0). The pH of the samples were decreased to 2 using 6M HCl and incubated at 37 °C for 30 min. Then the pH was raised to 6 using 4M NaOH and incubated for another 30 min at 37 °C. Finally the pH-adjusted wheat samples were centrifuged under the same conditions (15 min, 7500 x g). The resulting supernatants from both steps were collected separately and lyophilized to obtain the crude phenolic extract. The crude phenolic extracts were stored at – 20 °C in vacuum sealed pouches for further analysis.

Other experimental details are given in Chapter 3, Section 3.2, Subsection 3.2.2 to 3.2.5, 3.2.11, 3.2.15.

6.4. Results and discussion

6.4.1. Effects of simulated gastric pH changes on TPC

The yield of extracts and total phenolic content of whole wheat and its different milling fractions, namely flour, germ, and bran (non-treated) and after subjecting them to gastric pH conditions (treated) are shown in Table 6.1.

Table 6.1. Extraction yield (%) and total phenolic content (μg ferulic acid equivalents/g defatted material) of non-treated and treated soft and hard wheat samples

Milling fraction	Yield of extract		Total phenolic content	
	Non-treated[A]	Treated[B]	Non-treated[A]	Treated[B]
Soft wheat				
Whole grain	0.6 ± 0.04[a]	1.2 ± 0.02[b]	62 ± 4[a]	294 ± 17[b]
Flour	0.4 ± 0.02[a]	1.0 ± 0.03[b]	22 ± 1[a]	74 ± 6[b]
Germ	2.0 ± 0.1[a]	2.5 ± 0.02[b]	356 ± 8[a]	850 ± 28[b]
Bran	0.9 ± 0.03[a]	1.8 ± 0.04[b]	181 ± 12[a]	959 ± 43[b]
Hard wheat				
Whole grain	0.6 ± 0.03[a]	1.4 ± 0.06[b]	81 ± 4.2[a]	252 ± 11[b]
Flour	0.5 ± 0.01[a]	1.1 ± 0.04[b]	23 ± 0.9[a]	118 ± 9[b]
Germ	1.7 ± 0.01[a]	2.9 ± 0.02[b]	251 ± 7[a]	699 ± 23[b]
Bran	1.1 ± 0.02[a]	1.9 ± 0.02[b]	243 ± 2[a]	574 ± 17[b]

[A]Aqueous extracts of whole grains and their milling fractions

[B]Samples are subjected to simulated gastric pH treatment

Values are the mean of three determinations ± standard deviation

Values in each raw having the same superscript are not different ($p>0.05$); means of non-treated and treated samples were compared for significance; data for soft and hard wheat were treated separately

Both the yield and total phenolic contents increased significantly ($p<0.05$) following simulated gastric pH treatment. The increase in the yield of crude phenolic extracts of soft and hard wheat upon pH treatment was 1.25 - 2.5 and 1.7 - 2.3 fold, respectively, compared to untreated samples. The amount of total phenolics in different milling fractions varied from 22 ± 1 to 356 ± 8 and 23 ± 1 to 251 ± 7 µg FAE/g of defatted soft and hard wheat, respectively. The corresponding values following treatment under simulated gastric pH conditions were 74 ± 6 to 959 ± 43 and 118 ± 9 to 699 ± 23 µg FAE/g defatted material, respectively. In the soft wheat samples the TPC was increased by 3.5 to 5.3 fold while this increase was 2.4 to 5.1 fold for hard wheat samples. The extractability of free and esterified phenolic compounds may be enhanced due to acidification of wheat samples. According to Baublis et al. (2000), treatment simulating gastrointestinal pH conditions brings about a dramatic increase in antioxidant activity of aqueous extracts of wheat-based ready-to-eat breakfast cereals. These authors reported that acid conditions alters the activity, composition and/or concentration of water-soluble low-molecular-weight antioxidants. Moreover, gastric conditions may influence phenolic composition because phenolics are commonly esterified to sugars or acids (Baubilis et al., 2000). Baubilis et al. (2000) further proposed the release of low-molecular-weight compounds such as phytates and/or a decrease in the activity of prooxidants in the cereal extracts. It can also be suggested that pH treatment resulted in partial hydrolysis leading to the release of some of the bound phenolics present in wheat samples. However, this possibility has not yet been verified. The HPLC analysis of crude phenolic extracts prepared from non-treated wheat samples and after subjecting to gastric pH treatment revealed a marked increase in the content of phenolic acids (Table 6.2). The extraction of hydrocolloidal materials such as hemicellulose, proteins,

peptides, amino acids and soluble sugars in water has also been suggested (Lehtinen and Laakso, 1997). The bound phenolic acids in wheat samples are not available for extraction, but may be released under simulated digestion conditions. Kroon et al. (1997) reported that acid conditions in the gastrointestinal tract may release the phenolics esterified to carbohydrates from wheat bran. Most antioxidants/phenolics in cereals are in the bran (Kern et al., 2003; Andreasen et al., 2001; Miller et al., 2000; Emmons et al., 1999). In this study both bran and germ samples of soft and hard wheat possessed greater antioxidative components when compared to endosperm, that constitute the flour fraction.

Table 6.2. Phenolic acid content (µg/g) of non-treated and treated soft and hard wheat samples as determined by HPLC

Milling fraction	Vanillic		Ferulic	
	Non-treated	Treated	Non-treated	Treated
Soft wheat				
Whole grain	Trace	0.19	Trace	0.4
Flour	0	Trace	0	Trace
Germ	0.6	3.88	3.78	5.68
Bran	0.38	2.41	0.92	1.98
Hard wheat				
Whole grain	Trace	0.35	Trace	0.94
Flour	o	Trace	0	Trace
Germ	0.48	1.07	0.14	4.21
Bran	0.65	1.16	0.59	1.6

Values indicate a single determination

6.4.2. Effects of simulated gastric pH conditions on total antioxidant capacity (TAC)

The antioxidant capacity of wheat samples was examined using the Trolox equivalent antioxidant capacity (TEAC) assay. The total antioxidant capacity of defatted wheat samples expressed as micromoles of TE/g is shown in Table 6.3. Treatment under simulated gastric conditions had a dramatic effect on TAC of wheat, reflecting a significant ($p<0.05$) increase in TAC. For soft wheat TAC varied from 0.4 ± 0.01 to 4.4 ± 0.1 µmol TE/g and 1.0 ± 0.02 to 6.3 ± 0.08 µmol TE/g for non-treated and treated wheat

Table 6.3. Total antioxidant capacity (µmol TE/g defatted material) of non-treated and treated soft and hard wheat samples

Type of extract	Non-treated[A]	Treated[B]
Soft wheat		
Whole grain	0.6 ± 0.01[a]	1.5 ± 0.09[b]
Flour	0.4 ± 0.01[a]	1.0 ± 0.02[b]
Germ	4.4 ± 0.1[a]	6.3 ± 0.08[b]
Bran	1.2 ± 0.03[a]	2.5 ± 0.08[b]
Hard wheat		
Whole grain	0.5 ± 0.02[a]	2.0 ± 0.02[b]
Flour	0.3 ± 0.01[a]	1.1 ± 0.02[b]
Germ	1.1 ± 0.01[a]	6.4 ± 0.1[b]
Bran	1.4 ± 0.01[a]	3.7 ± 0.06[b]

[A]Aqueous extracts of whole grains and their milling fractions

[B]Samples are subjected to simulated gastric pH treatment

Results are the mean of three determinations ± standard deviation

Values in each raw having the same superscript are not significantly different ($p>0.05$); data for soft and hard wheat were treated separately

samples, respectively. The corresponding values for hard wheat varied from 0.3 ± 0.01 to 1.4 ± 0.3 µmol TE/g and 1.1 ± 0.02 to 6.4 ± 0.1 µmol TE/g defatted material,

respectively. The germ fraction, except that of hard wheat produced the highest TAC while the flour fractions had the lowest TAC under both conditions examined. In non-treated hard wheat bran produced the highest TAC. Thus, the antioxidant activities of phenolics were different among various milling fractions.

In oat the aleurone layer or the outermost layer of the endosperm contained the highest concentration of antioxidants. Since the aleurone layer is often removed with the bran during milling, the bran fraction possessed the greatest antioxidant capacity among different oat fractions. This has mainly been attributed to the release of a portion of bound phenolics upon hydrolysis resulting from extreme pH changes (Handelman et al., 1999). The TAC of wheat samples represents antioxidant activity of free and soluble conjugates of phenolic acids while TAC of wheat samples subjected to simulated gastric pH conditions may represent mainly the antioxidant activity of free phenolics and soluble phenolic esters while bound phenolics might also make some contribution, if hydrolysis had occurred as anticipated. The TPC and TAC of wheat samples of both soft and hard wheat showed a strong correlation (r^2 = 0.96, $p<0.05$). Hard wheat samples after subjecting to pH adjustment also reflected a strong correlation (r^2 = 0.95, $p<0.05$) between the two parameters. However, the correlation of TPC and TAC of treated soft wheat samples was weak (r^2 = 0.68). Hence, there was no consistency in the correlation between TPC and TAC. The method of extraction, type of compounds and procedures employed for evaluation of antioxidant activity may vary greatly in different studies, thus making it difficult to compare the results. The inconsistent relationships between TAC and TPC of wheat extracts may also be attributed to the poor specificity of the Folin-Ciocalteu procedure for phenolics as described in Chapter 4. For instance, extractable proteins may be included in the measurement (Shahidi and Naczk, 2004) and hence the

TPC could be overestimated. Moreover, phenolics do not exert the same antioxidant activity; some may demonstrate strong antioxidative properties while others may demonstrate poor activity. They may also display antagonistic or synergistic effects among themselves or with other constituents in the extracts (Rice-Evans et al., 1997). Thus, antioxidant activity of the extracts may originate from the combined action of phenolic constituents and other compounds such as extractable proteins, among others (Zielinski and Kozlowska, 2000). Cereal proteins have been known to exert strong antioxidant properties (Iwami et al., 1987) and some of the water-soluble proteins that may also be present in the extracts examined in this study may have contributed to antioxidant activity of the extracts. This may be explained by considering the phenolic acid composition of the flour fraction of both hard and soft wheat. In both non-treated soft and hard wheat phenolic acids were not detected, at the concentration examined. However, according to Folin-Ciocalteu procedure the soft and hard wheat samples contributed 22 ± 1 and 23 ± 1 µg FAE/ g, respectively, to the TPC. This may be due to the presence of other components that reacted with the Folin-Ciocalteu's reagent.

Baublis et al. (2000) reported the antioxidative capacity of extracts from high bran, whole grain and refined ready-to-eat (RTE) breakfast cereals in a phosphatidylcholine liposome model system. They observed that high bran and whole grain RTE cereals had a higher antioxidant activity than refined RTE cereals. Furthermore, when aqueous extracts were subjected to simulated gastrointestinal pH treatment, the antioxidative capacities of treated extracts were greater than those of their untreated counterparts. The results of this study lend support to the findings of Baublis et al. (2000) for RTE breakfast cereals.

6.4.3. Effects of simulated gastric pH conditions on free radical scavenging

The antioxidant potential of wheat extracts was evaluated using the stable DPPH radical. The DPPH scavenging capacity of soft and hard wheat samples and after simulated gastric pH treatment is shown in Table 6.4. The DPPH scavenging capacity of reference compounds such as ferulic acid, α-tocopherol and BHT at 100 ppm is also shown in Table 6.4.

Ferulic acid, α-tocopherol and BHT scavenged DPPH radical efficiently at 100 ppm concentration. The defatted soft and hard wheat samples (non-treated), scavenged 1.8 ± 0.01 to 63.6 ± 1.1 µmol/g and 1.9 ± 0.03 to 40.1 ± 0.06 µmol/g DPPH radical, respectively, while the corresponding values increased from 7.8 ± 0.1 to 168.3 ± 1.0 µmol/g and 12.3 ± 0.1 to 126.4 ± 2 µmol/g, respectively, when wheat samples were subjected to simulated gastric pH conditions. Thus, the highest DPPH radical scavenging activity was rendered by wheat samples of both soft and hard, when they were treated to mimic gastric conditions. Ferulic acid, α-tocopherol and BHT scavenged the DPPH radical efficiently at a 100 ppm concentration. In this study, the correlation between TPC and DPPH scavenging ability was strong (r^2 = 0.98 or 0.99. $p<0.05$).

The antioxidant activity of wheat extracts as measured by the inhibition of bleaching of β-carotene after 2h of assay period is presented in Table 6.5. The absorbance readings at 470 nm reflected the efficacy of inhibiting oxidation of β-carotene by wheat samples/standards. Bran, germ and whole grain of both soft and hard wheat exhibited a significantly ($p<0.05$) higher antioxidative activity than the flour fraction. In both types of wheat examined, the antioxidant capacity was significantly enhanced when the samples were subjected to simulated gastric pH conditions. Different milling fractions exhibited various degrees of antioxidant activity.

Table 6.4. DPPH scavenging capacity[A] (μmol/g defatted material) of non-treated and treated soft and hard wheat samples

Type of extract	Non-treated[B]	Treated[C]
Soft wheat		
Whole grain	6.5 ± 0.03[a]	32.4 ± 0.1[b]
Flour	1.8 ± 0.01[a]	7.8 ± 0.08[b]
Germ	63.6 ± 1.1[a]	168.3 ± 1.0[b]
Bran	24.0 ± 0.06[a]	164.8 ± 0.8[b]
Hard wheat		
Whole grain	8.9 ± 0.1[a]	31.0 ± 0.4[b]
Flour	1.9 ± 0.03[a]	12.3 ± 0.1[b]
Germ	40.1 ± 0.06[a]	126.4 ± 2.0[b]
Bran	30.3 ± 0.03[a]	78.1 ± 1.1[b]
Ferulic acid[D]	46.2 ± 0.3	
α-Tocopherol[D]	48.3 ± 0.3	
BHT[D]	40.9 ± 0.4	

[A]Original inhibition (%) data uased for calculation of the amount of DPPH scavenged (μmol/g) by wheat samples are shown in Appendix 6.1

[B]Aqueous extracts of whole grains and their milling fractions

[C]Samples are subjected to simulated gastric pH treatment

[D]Antioxidant activity of reference compounds were expressed at 100 ppm

Values are the mean of three determinations ± standard deviation

Values in each raw having the same superscript are not different ($p>0.05$); data for soft and hard wheat were treated separately

The antioxidant activity of reference compounds were determined at a 100 ppm concentration. Even at this level, reference antioxidants exhibited a stronger activity than that of samples; α-tocopherol showed the strongest activity against β-carotene bleaching. Wheat extracts especially those of bran and germ exhibited considerable

Table 6.5. Inhibition[A] of β-carotene bleaching (nmol/g defatted material) by non-treated and treated soft and hard wheat samples

Type of extract	Non-treated[B]	Treated[C]
Soft wheat		
Whole grain	52 ± 1[a]	292 ± 10[b]
Flour	12 ± 0.3[a]	51 ± 2[b]
Germ	396 ± 4[a]	1034 ± 6[b]
Bran	189 ± 3[a]	1207 ± 14[b]
Hard wheat		
Whole grain	91 ± 2[a]	228 ± 4[b]
Flour	13 ± 1[a]	76 ± 2[b]
Germ	278 ± 3[a]	832 ± 15[b]
Bran	295 ± 6[a]	672 ± 11[b]
Ferulic acid[D]	92.6 ± 1.9	
α-Tocopherol[D]	96.7 ± 2.7	
BHT[D]	89.0 ± 1.2	

[A]Original inhibition (%) data uased for calculation of the amount of β-carotene retained (nmol/g) by wheat samples are shown in Appendix 6.2

[B]Aqueous extracts of whole grains and their milling fractions

[C]Samples are subjected to simulated gastric pH treatment

[D]Antioxidant activity of reference compounds were expressed as a percentage (%) at 100 ppm

Values are the mean of three determinations ± standard deviation

Values in each raw having the same superscript are not different ($p>0.05$); data for soft and hard wheat were treated separately

antioxidative activity by inhibiting β-carotene bleaching in the medium. The correlation between TPC and β-carotene bleaching by all wheat samples were excellent ($r^2 = 0.99$, $p<0.01$). The germ and bran samples demonstrated a significantly ($p<0.05$) higher

antioxidant activity than that of whole wheat and flour. Results of the β-carotene-linoleate assay indicated that the increase in activity was 5.6, 4.3, 2.6 and 6.3 fold for whole grain, flour, germ and bran of soft wheat, respectively, when the samples were subjected to treatment under simulated gastric pH conditions. The corresponding values for hard wheat were 2.5, 5.8, 3.0 and 2.3, respectively.

6.4.4. Effects of simulated gastric pH conditions on iron (II)-chelation capacity

Iron (II)-chelation capacity of non-treated and treated soft and hard wheat samples is given in Table 6.6. Iron (II)-chelation capacity was increased when samples were subjected to simulated digestion prior to extraction. Thus, treated samples of soft and hard wheat produced greater iron (II)-chelation capacity than that of their untreated counterparts. The chelation capacity of different milling fractions decreased in the order of bran > germ > whole grain > flour for both soft and hard wheat irrespective of the sample treatment. Hence, bran showed the highest chelation capacity while the flour had the lowest effect in each instance.

As expected, EDTA exhibited the strongest chelation capacity and at 100 ppm there was a complete chelation of iron (II). On the contrary, Trolox did not chelate iron (II) at all while ferulic acid exhibited only 4.7% chelation capacity at 100 ppm phenolics. According to Graf (1992), ferulic acid does not exert antioxidant activity via metal chelation. The TPC and iron (II) chelation capacity demonstrated a poor correlation (r^2 = 0.644) for non-treated soft wheat samples. However, hard wheat samples showed an excellent correlation ($r^2 > 0.97$, $p<0.05$) between TPC and iron (II)-chelation capacity. In addition to phenolic compounds, phytic acid may play a role in the iron (II) chelation assay. Phytic acid is found in high concentrations in the seeds of grains, pulses and

oleaginous products. In cereals, phytic acid is present at approximately 1-2% of seed weight.

Table 6.6. Iron (II)-chelation capacity[A] (μg EDTA equivalents/g defatted material) of non-treated and treated soft and hard wheat samples

Type of extract	Non-treated[B]	Treated[C]
Soft wheat		
Whole grain	19.1 ± 0.9[a]	110.0 ± 3.0[b]
Flour	6.9 ± 0.6[a]	28.2 ± 1.7[b]
Germ	53.7 ± 1.8[a]	239.0 ± 4.7[b]
Bran	85.4 ± 2.0[a]	345.0 ± 4.0[b]
Hard wheat		
Whole grain	23.4 ± 2.1[a]	87.7 ± 3.4[b]
Flour	6.9 ± 1.0[a]	42.5 ± 1.5[b]
Germ	58.9 ± 1.5[a]	193.2 ± 5.8[b]
Bran	61.2 ± 1.1[a]	200.5 ± 4.7[b]
EDTA[D]	100.0 ± 0	
Trolox[D]	0	
Ferulic acid[D]	4.7 ± 0.7	

[A]Original inhibition (%) data uased for calculation of the amount of iron chelated (μg/g) by wheat samples are shown in Appendix 6.3

[B]Aqueous extracts of whole grains and their milling fractions

[C]Samples are subjected to simulated gastric pH treatment

[D]Iron chelation capacity of reference compounds were expressed as a percentage (%) at 100 ppm

Values are the mean of three determinations ± standard deviation

Values in each raw having the same superscript are not different ($p>0.05$); data for soft and hard wheat were treated separately

This amount may even reach 3-6% in some cereals (Alabaster et al., 1996). In wheat, a greater proportion of phytic acid is distributed in the outer layers such as pericarp and aleurone (Cheryan, 1980). Phytic acid content of refined and whole wheat flours ranges from 2 to 4 mg/g and 6 to 10 mg/g, respectively (Febles et al., 2002). It has been shown that phytate is a potent chelator of free iron. According to Jayarajah et al. (1997) phytate was released when samples of wheat bran were incubated with acidified water (pH 5.1) at room temperature. Thus, under conditions employed in this study, possible release of phytates may have contributed to iron chelation.

6.4.5. Effects of simulated gastric pH conditions on inhibition of LDL oxidation

Oxidative modification of LDL plays a major role in the pathogenesis of coronary heart disease (Steinberg et al., 1989). Kinsella et al. (1993) reported the importance of dietary antioxidants in the inhibition of LDL cholesterol oxidation thereby reducing the risk of atherogenesis and coronary heart disease. Numerous studies have reported the antioxidant activities of various crude plant extracts in *in vitro* LDL models (Singh et al., 2002; Hu and Kitts, 2001; Emmons et al., 1999; Heinonen et al., 1998). Inhibition of copper-induced LDL oxidation by wheat phenolics is summarized in Table 6.6. Inhibition of LDL oxidation was significantly ($p<0.05$) higher following treatment of samples of both soft and hard wheat under simulated gastric pH condition. The increase was 5.3, 3.9, 3.1 and 6.0 fold for whole grain, flour, germ and bran, respectively, for soft wheat. The corresponding values for hard wheat were 3.6, 5.9, 3.0 and 2.6, respectively. A strong correlation existed between the content of phenolic acids in the extracts of rye and the antioxidant activity against copper-mediated LDL oxidation (Andreasen et al., 2001). Results of the current study indicated that samples of germ and bran which possessed

Table 6.7. Inhibition[A] of oxidation of LDL (μg protein/g defatted material) by non-treated and treated soft and hard wheat samples

Type of extract	Non-treated[B]	Treated[C]
Soft wheat		
Whole grain	63 ± 1[a]	334 ± 3[b]
Flour	21 ± 1[a]	81 ± 1[b]
Germ	335 ± 7[a]	1034 ± 7[b]
Bran	194 ± 4[a]	1163 ± 12[b]
Hard wheat		
Whole grain	78 ± 1[a]	280 ± 7[b]
Flour	20 ± 1[a]	118 ± 2[b]
Germ	275 ± 3[a]	831 ± 19[b]
Bran	257 ± 4[a]	676 ± 10[b]
Ferulic acid[D]	81.7 ± 2.4	
Trolox[D]	100 ± 0	

[A]Original inhibition (%) data uased for calculation of the amount of LDL retained (μg/g) by wheat samples are shown in Appendix 6.4

[B]Aqueous extracts of whole grains and their milling fractions

[C]Samples are subjected to simulated gastric pH treatment

[D]Inhibition of copper-induced oxidation of LDL by reference compounds was expressed as a percentage at 100 ppm

Values are THE mean of three determinations ± standard deviation

Values in each raw having the same superscript are not different ($p>0.05$); data for soft and hard wheat were treated separately

higher TPC than that of whole grain and flour, demonstrated the highest inhibition against LDL oxidation despite the treatment of samples. The flour fraction exhibited the lowest inhibition against copper-induced LDL oxidation. The correlation coefficient

between TPC and inhibition of LDL was strong ($r^2 \geq 0.99$, $p<0.01$) for all wheat samples examined.

Natella et al. (1999) reported that inhibition of copper-catalyzed oxidation represents the association of both chelation of metal ions and scavenging of free radical species in the LDL system. The main phenolic compound in rye bran was ferulic acid and this inhibited LDL oxidation by approximately 34% at a 40 μM concentration (Andreasen et al., 2001). It was also reported that rye bran extracts had a much higher antioxidant activity than what can be explained from the concentration of the individual phenolic acids (Andreasen et al., 2001), thus reflecting their synergistic action in the assay medium. Therefore, compounds other than ferulic acid must have contributed to the observed antioxidant activity. Ferulic acid exhibited 81.7% inhibition against copper-induced LDL oxidation at 100 ppm.

6.5 Conclusions

The antioxidant activity of wheat phenolics was significantly enhanced when wheat samples were subjected to simulated gastrointestinal pH treatment prior to extraction.

CHAPTER 7

Antioxidant Properties of Free, Soluble-esters and Insoluble-bound Phenolic Acids of Wheat

7.1. Introduction

In general, grains are a major source of phytoestrogens such as lignans (Zielinski and Kozlowska, 2000; Kris-Etherton et al., 2002), phytate and tannins (Kris-Etherton et al., 2002) and sterols (Shahidi, 1997). They are also a rich source of low-molecular-weight phenolic compounds such as ferulic, caffeic, *p*-hydroxybenzoic, protocatechuic, *p*-coumaric, gentisic, sinapic, vanillic and syringic acids, among others (Emmons et al., 1999; Kahkonen et al., 1999).

Phenolic acids may form both ester and ether linkages owing to their bifunctional nature through reactions involving their carboxylic and hydroxyl groups, respectively. This allows phenolic acids to form cross-links with cell wall macromolecules (Yu et al., 2001). Bound phenolics may be released by alkali, acid or enzymatic treatment of samples prior to extraction (Sosulski et al., 1982; Krygier et al., 1982 a & b; Andreasen et al., 2001; Zupfer et al., 1998; Bartolome and Gomez-Cordoves, 1999). In barley, most phenolic acids exist in the bound form with other grain components such as starch, cellulose, β-glucan and pentosans (Yu et al., 2001). A similar observation was made for phenolic acids in ground rye grain that were released upon enzymatic hydrolysis (Andreasen et al., 1999). With respect to the histological distribution of arabinoxylans, their concentration increases from the centre to the periphery of the endosperm. Thus, they are mainly concentrated in the seed coats of cereals (Lempereur et al., 1997). The increased concentration of ferulates in the outer layers may be implicated in resistance

to both insect and fungal pathogens. Thus, cross linking of phenolic compounds may provide a physical barrier to invasive disease development and consumption by insects (Zupfer et al., 1998).

Cereal grains are one of the most important food groups and their fibres are known to render health benefits that may be attributed to the nature of cell wall polymers and chemical architecture (Bunzel et al., 2003). Epidemiological data have shown that consumption of wheat bran is associated with a reduced risk of colorectal and gastric cancer (Jacobs et al., 1995). Cereals contain a high amount of hydroxycinnamates (HCA) that may exert potential health benefits (Andreasen et al., 2001). In cereals HCA and their dimers exist mainly as esters bound to arabinoxylan. In many studies phenolic antioxidants of wheat have been tested in their free form only (Yu et al., 2002, 2003; Zhou et al., 2004; Zhou and Yu, 2004). In order to understand the total antioxidant activity of cereals, it is imperative to consider the contribution from their bound phenolics since under normal conditions phenolics occur mainly in the bound form (Andreasen et al., 2001; Sosulski et al., 1982; Krygier et al., 1982 a & b; Andreasen et al., 2001; Zupfer et al., 1998; Bartolome and Gomez-Cordoves, 1999). Ferulic acid is the major phenolic acid in many cereals and exists predominantly in the seed coat (Watanabe et al., 1997) while traces may be present in the starchy endosperm (Pussayanawin et al., 1988).

Oxidative stress arises when the generation of oxidative species exceeds the capacity of the antioxidant system in the body (Halliwell, 1996). The enhancement of naturally-occurring endogenous antioxidant defense system through a balanced diet by consuming plant-derived foods may protect the body against oxidative stress.

7.2. Objectives

The objectives of this work were to study the antioxidant capacity of free, soluble esters and insoluble-bound phenolic acids isolated from soft and hard whole wheat, bran and flour.

7.3. Materials and methods

7.3.1. Preparation of samples

Details are given in Chapter 3, Section 3.1, Subsection 3.2.1.

7.3.2. Experimental

7.3.2.1. Extraction of free, esterified and bound phenolics

The free phenolics, soluble esters and insoluble bound phenolic acids in wheat extracts were isolated according to the procedure described by Krygier et al. (1982a) with slight modifications. The defatted samples (2 g) were extracted six times with 40 mL of methanol-acetone-water (7:7:6, v/v/v) at room temperature using a Polytron homogenizer (Brinkman, 15 sec, 10,000rpm). The mixtures were then centrifuged (5000 x g, 15 min) and supernatants were collected and combined. The solvent was evaporated at 30 °C under vacuum to approximately 40 mL. Concentrated supernatants were extracted with diethyl ether; phenolic acids so extracted were labelled as free phenolics. The supernatants with esterified phenolic acids were then treated with 30 mL of 4N NaOH for 4 h at room temperature. The samples were flushed with nitrogen and closed to be airtight. The resultant hydrolysate was acidified to pH 2 using 6M HCl, and extracted six times with diethyl ether. The ether extracts were then combined and evaporated to dryness at 30 °C under vacuum. The phenolic acids extracted were those

liberated from their esters and labelled as esterified phenolic acids. The leftover meal after extractions was treated with 20 mL of 4M NaOH for 4 h. The samples were flushed with nitrogen and the samples were then acidified to pH 2 with 6 N HCl followed by centrifugation (5000 x g, 15 min). The mixture was extracted six times with diethyl ether. The ether extracts were combined and evaporated to dryness under vacuum at 30 °C. The phenolic acids so extracted were labelled as bound phenolics. Free, esterified and insoluble-bound phenolics were dissolved separately in 2 mL of methanol and stored at -20 °C until used within one week.

Other experimental details are given in Chapter 3, Section 3.2, Subsections 3.2.2 to 3.2.4, 3.2.10 to 3.2.15. It should also be noted here that in all these assays phenolic extracts already dissolved in methanol were used whereas in other experiments (Chapters 4-6 & 8-9) the extracts were in the form of lyophilized extracts.

7.4. Results and discussion

7.4.1. Total phenolic content (TPC) and total antioxidant capacity (TAC) of free, esterified and insoluble-bound phenolic fractions

The content of free, esterified and insoluble-bound phenolics of whole grain, flour and bran of hard and soft wheat samples are shown in Table 7.1; the percentage contribution of each type of phenolic to TPC is shown in Fig. 7.1. The content of insoluble-bound phenolics was 1.4, 2.3 and 8.3 fold higher than that of esterified phenolics for flour, whole grain and bran of hard wheat, respectively. The corresponding values for soft wheat were 1.7, 1.8 and 8.5 fold, respectively. The content of bound phenolics of flour was 2.4 and 2.9 times greater than that of free phenolics in hard and

soft wheat, respectively. In whole wheat, the content of bound phenolics was 6.1 and 4.5 fold compared to that of free phenolics for hard and soft wheat examined, respectively. The corresponding values for bran were 13.4 and 12.4 fold. Thus, the content of bound phenolics was significantly higher than that of free and esterified fractions, especially for wheat bran.

Table 7.1. Free, esterified and bound phenolic contents (μg ferulic acid equivalents/g defatted material) of whole grains, flour and bran of hard and soft wheat

Milling fraction	Free	Esterified	Bound	Total
HWF	137 ± 4	234 ± 13	328 ± 14	699
SWF	161 ± 6	278 ± 12	464 ± 16	903
HWW	353 ± 16	954 ± 34	2149 ± 43	3456
SWW	478 ± 12	1196 ± 59	2144 ± 52	3818
HWB	846 ± 31	1365 ± 63	11303 ± 126	13514
SWB	981 ± 47	1432 ± 42	12186 ± 149	14599

Values are the mean of three determinations ± standard deviation
Abbreviations: HWF, hard wheat flour; SWF, soft wheat flour; HWW, hard whole wheat; SWW, soft whole wheat; HWB, hard wheat bran; SWB, soft wheat bran

Andreasen et al. (2001) determined TPC of flour, whole grain and bran of rye where bound phenolics were released from cell wall materials after being subjected to enzymatic treatment with α-amylase followed by saponification with NaOH. The phenolic acids were then extracted with ethyl acetate. The TPC in whole grain and bran of rye was 5.6 and 14.8-fold, respectively, compared to the TPC of rye flour. Moreover, the antioxidant activity of bran extracts was significantly higher than that of whole grain and flour against copper-induced oxidation of LDL. The amount of phenolic compounds

present in cereals was very low; amounting to 0.2-1.3 mg gallic acid equivalents/g of crude phenolic extract (Kahkonen et al., 1999). The low antioxidant activity of cereal extracts even at a 5000 ppm level against oxidation of methyl linoleate was also reported by Kahkonen et al. (1999). The low TPC in the above study may possibly be due to the inclusion of only free phenolics in the analysis.

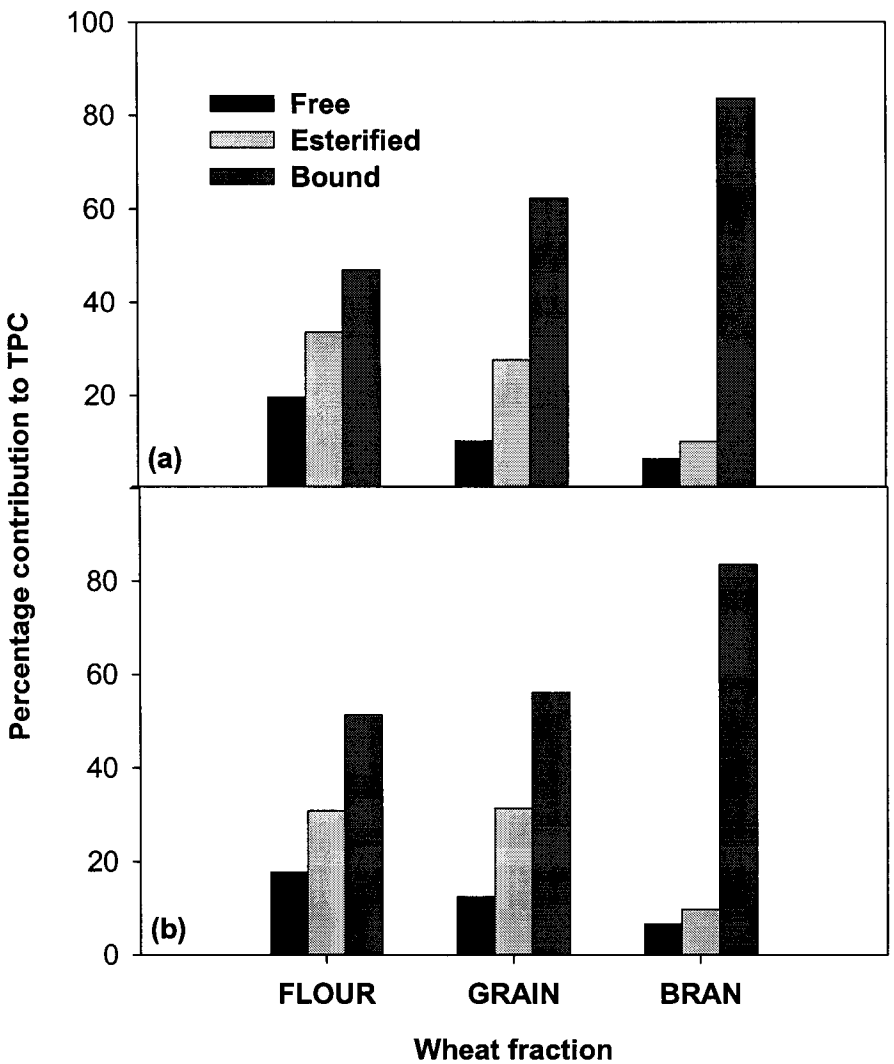


Fig. 7.1. Percentage contribution of free, esterified and bound phenolics to total phenolic content (TPC) of (a) hard and (b) soft wheat

However, in cereals the phenolic compounds exist primarily in the bound form in association with cell wall materials (Xing and White, 1997; Andreasen et al., 2001; Adom and Liu, 2002; Sosulski et al., 1982; Krygier et al 1982 a&b; Adom et al., 2003). This study also demonstrated the significant contribution of bound phenolic compounds to the TPC of whole grains, flour and bran of soft and hard wheat. Thus, it is apparent why cereals could exhibit low TPC when they tested only for free phenolic acids. A similar conclusion was reached by Dewanto et al. (2002) for corn phenolics and dominance of bound phenolics. Sosulski et al. (1982) reported that 69.2% of TPC was due to insoluble-bound phenolics. They also reported that levels of soluble conjugated phenolic acids in the cereal flours were 2-5 times greater than that of the free phenolic acids.

Antioxidant compounds, in general, are located in the bran fraction while endosperm also exhibits considerable antioxidant activity. It is unlikely that bound or insoluble phenolics are extracted from whole grains under normal extraction conditions (Martinez-Tome et al., 2004). The TPC of whole grains and bran has been reported in several studies (Yu et al., 2002; Zhou et al., 2004; Zhou and Yu, 2004; Martinez-Tome et al., 2004). In all these studies only free and esterified phenolics have been extracted and included in the analysis. Thus, the true TPC may be underestimated in many studies dealing with whole grains. Adom and Liu (2002) determined the content of free and bound phenolics in the whole grains of wheat, corn, rice and oat. Bound phenolics contributed 90, 87, 71 and 58% to the TPC in wheat, corn, rice and oat, respectively. In our study, bound phenolics contributed approximately 40, 60 and 80% in flour, whole grain and bran of wheat, respectively (Fig. 7.1). The free phenolics of the wheat

fractions ranged from 5 to 20% while esterified phenolics contributed 10 to 30% to TPC (Fig. 6.1).

The TAC of extracts in µmol TE/g of defatted wheat types is shown in Table 7.2. The percentage contribution of free, esterified and bound phenolics of wheat extracts to TAC is shown in Fig. 7.2. The TAC of wheat fractions of both hard and soft wheat samples revealed the significance of bound phenolics as >80% of TAC was due to bound phenolics (Fig. 7.2). The contribution of free phenolics to TAC was less than 2% in all wheat fractions examined while the contribution of esterified phenolics was significantly higher compared to that of free phenolics, but significantly less than that of bound phenolics. Bound phenolics in the bran fraction contributed 8.6 and 7.8 times higher to the TAC than that of the total of free and soluble esters for hard and soft wheat, respectively. The corresponding values for flour were 5.1 and 5.2 times, respectively. This difference, was remarkable with whole grains and the contribution of bound phenolics to TAC of hard and soft wheat, respectively, was 22 and 18.5 times higher than that of free and esterfied phenolics (Table 7.2). Thus, this study showed the importance of bound phenolics in the TAC of wheat samples.

The alkaline hydrolysis of wheat samples released phenolic acid esters linked to various cell wall polymers (Sosulski et al., 1982). Since cell wall materials are resistant to digestion they may survive gastrointestinal digestion and reach the colon. Eventually, digestion of such materials will occur in the colon due to microbial action, thus releasing the bulk of the bound phytochemicals. Ferulic and diferulic acids from cereal brans are released due to the action of colonic microflora in humans and rats (Kroon et al.,1997; Buchanan et al., 1996). Thus, bound phenolic compounds may render their health benefits in the colon and beyond following absorption. These compounds have

demonstrated potent antioxidant properties and their absorption into the blood stream has been demonstrated (Kroon et al., 1997). The reduced risk of colorectal cancer associated with increased consumption of wheat bran may explain its possible health effects in the colon (Jacobs et al., 1995).

Table 7.2. Total antioxidant capacity (μmol Trolox equivalents/g defatted material) of free, esterified and bound phenolic fractions of whole grains, flour and bran of hard and soft wheat

Milling fraction	Free	Esterified	Bound	Total
HWF	12 ± 0.2	90 ± 1.3	516 ± 12	618
SWF	15 ± 0.4	105 ± 2.2	628 ± 17	748
HWW	75 ± 6	412 ± 13	10718 ± 134	11205
SWW	86 ± 9	549 ± 16	11582 ± 183	12217
HWB	162 ± 11	3117 ± 57	28270 ± 221	31549
SWB	192 ± 9	4009 ± 63	32765 ± 279	36966

Values are the mean of three determinations ± standard deviation
Abbreviations: HWF, hard wheat flour; SWF, soft wheat flour; HWW, hard whole wheat; SWW, soft whole wheat; HWB, hard wheat bran; SWB, soft wheat bran

7.4.2. Antioxidant potential of free, esterfied and bound phenolics

There was a positive association between TPC and TAC as well as other *in vitro* models used to determine antioxidant potential. Plant phenolics are diverse in their chemical structures and characteristics and demonstrate a wide range of effects both *in vivo* and *in vitro* (Cook and Samman, 1996).

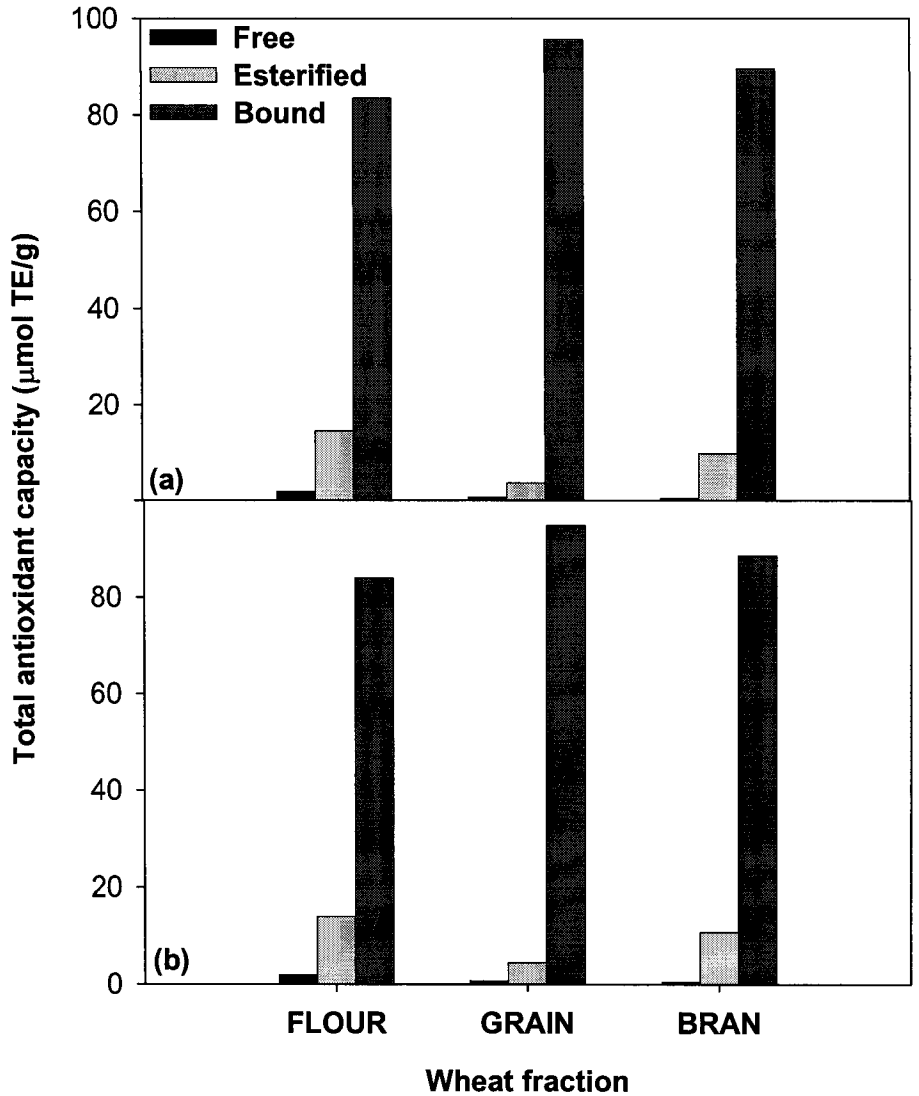


Fig. 7.2. Total antioxidant capacity (μmol Trolox equivalents/g of defatted wheat) of free, esterified and bound phenolics of (a) hard and (b) soft wheat

7.4.2.1. DPPH radical scavenging capacity of wheat phenolics

The scavenging of the stable DPPH radical was used in the evaluation of antioxidant activity of free, esterified and bound phenolic fractions of soft and hard

wheat. Scavenging of DPPH radical allows evaluation of the hydrogen-donating potency of phenolic compounds (Blois, 1958).

In the DPPH assay bound phenolics of flour, whole grain and bran of hard and soft wheat exhibited 62.7 to 87% scavenging of total radicals (Table 7.3). The scavenging capacity of DPPH rdical was 13-14.9 and 25.9-32.9 times higher in the whole grains and bran, respectively, compared to that of the flour. Hence, antioxidants that react directly with the DPPH radical reside mainly in the bran fraction. Since, there was dilution of the antioxidants present in the bran by the endosperm, the antioxidant activity of whole grain was significantly lower than that of the bran. The DPPH radical scavenging assay demonstrated the efficiency of wheat phenolics as hydrogen donors.

Table 7.3. Scavenging capacity (μmol/g defatted material) of DPPH radical of free, esterified and bound phenolic fractions of whole grains, flour and bran of hard and soft wheat

Milling fraction	Free	Esterified	Bound	Total
HWF	1.4 ± 0.02	5.4 ± 0.2	12.5 ± 0.4	19.3
SWF	3.0 ± 0.1	6.5 ± 0.3	16.0 ± 0.5	25.5
HWW	10.4 ± 0.7	27.0 ± 0.4	249.5 ± 1.2	286.9
SWW	12.4 ± 0.9	29.1 ± 0.7	288.9 ± 1.4	330.4
HWB	41.1 ± 0.9	107.5 ± 1.3	486.0 ± 1.1	634.6
SWB	44.3 ± 1.2	115.0 ± 1.6	502.2 ± 1.7	661.5

Values are mean of three determinations ± standard deviation

Abbreviations: HWF, hard wheat flour; SWF, soft wheat flour; HWW, hard whole wheat; SWW, soft whole wheat; HWB, hard wheat bran; SWB, soft wheat bran

7.4.2.2. Oxygen radical absorbance capacity of wheat phenolics

The ORAC values of wheat extracts were also determined and expressed as μmol Trolox equivalents/g of defatted material (Table 7.4). The ORAC of wheat extracts was in the order of bran > whole grain > flour. The ORAC of bran was approximately 37 and 36.7 times higher than that of the flour of hard and soft wheat, respectively. The corresponding values for whole grains were 9 and 8.8 times. Contribution of bound phenolics in ORAC, especially bran of hard and soft wheat, was also significant. To the best of our knowledge this is the first report on a determination of ORAC of bound phenolics of wheat.

Table 7.4. Oxygen radical absorbance capacity (ORAC, μmol Trolox equivalents/g defatted material) of free, esterified and bound phenolic fractions of whole grains, flour and bran of hard and soft wheat

Milling fraction	Free	Esterified	Bound	Total
HWF	31 ± 2.0	71 ± 1.3	276 ± 1.9	378
SWF	38 ± 0.9	79 ± 1.0	300 ± 2.2	417
HWW	86 ± 1.6	342 ± 3.1	2978 ± 13	3406
SWW	98 ± 1.0	392 ± 3.3	3180 ± 24	3670
HWB	380 ± 2.1	3100 ± 23	10550 ± 114	14030
SWB	440 ± 1.3	3500 ± 33	11350 ± 174	15290

Values are the mean of three determinations ± standard deviation

Abbreviations: HWF, hard wheat flour; SWF, soft wheat flour; HWW, hard whole wheat; SWW, soft whole wheat; HWB, hard wheat bran; SWB, soft wheat bran

Free radicals $ABTS^{\bullet-}$ and $DPPH^{\bullet}$ are commonly used to assess antioxidant activity in *in vitro* assays, although both of these radicals are foreign to biological

systems (Awika et al., 2003). ORAC is based on free radical scavenging where different radical generators are used in producing free radicals. Recently, peroxyl radical (ROO•) has been adopted as a standard due to its common existence in biological systems (Cao et al., 2001). Thus, it has been stated that ORAC mimics the antioxidant activity of phenolics in biological systems since it uses biologically-relevant free radicals and integrates both inhibition time and percentage of ROS by the antioxidant. Thus, ORAC is based on hydrogen atom transfer and in the presence of an antioxidant ROO• can abstract a hydrogen atom from the antioxidant, and the reaction between ROO• and florescein is retarded or inhibited (Ou et al., 2002). This method also demonstrated the hydrogen-donating properties of wheat phenolics (Table 6.4).

7.4.2.3. Evaluation of antioxidant potential of wheat phenolics using photochemi-luminescence (PCL)

The antioxidative potential of free, esterified and bound phenolics of whole grains, flour and bran of hard and soft wheat as measured by PCL method is shown in Table 7.5. The highest inhibition of PCL was exhibited by phenolic fractions derived from the bran while flour exhibited the weakest antioxidant effect. The inhibition of PCL varied from 13.4 to 237.8 and 14.2 to 269.4 μmol α-tocopherol equivalents/g defatted material, respectively, for bran of hard and soft wheat. The contribution from bound phenolics of hard and soft wheat bran was >84%. The PCL assay is based on temporary inhibition of a photo-induced, superoxide radical anion-mediated chemiluminescence originating from oxidation of luminol (Popov and Lewin, 1994). In the presence of an antioxidant, chemiluminescence may be delayed and the resulting integral in the ACL system is indicative of the radical-scavenging potential of the

compound. The method is quite sensitive and hence allows detection of low concentrations of antioxidants relative to a reference compound (Vichi et al., 2001). In PCL, both hydrophilic and hydrophobic antioxidants present in the extracts can be evaluated. This study reports for the first time the antioxidant activity of whole grains, flour and bran of wheat using the PCL method.

Table 7.5. Antioxidant activity (μmol α-tocopherol equivalents/g defatted material) of whole grains, flour and bran of hard and soft wheat as evaluated by photochemiluminescence in ACL system

Milling fraction	Free	Esterified	Bound	Total
HWF	3.1 ± 0.2	8.4 ± 0.7	31.6 ± 1.1	43.1
SWF	3.4 ± 0.3	9.3 ± 0.9	36.9 ± 0.9	49.6
HWW	5.8 ± 0.1	13.2 ± 1.1	89.4 ± 1.4	108.4
SWW	6.1 ± 0.4	14.6 ± 0.7	97.6 ± 1.0	118.3
HWB	13.4 ± 0.9	29.1 ± 0.8	237.8 ± 2.0	280.3
SWB	14.2 ± 1.0	33.5 ± 0.9	269.4 ± 1.7	317.1

Values are the mean of three determinations ± standard deviation

Abbreviations: HWF, hard wheat flour; SWF, soft wheat flour; HWW, hard whole wheat; SWW, soft whole wheat; HWB, hard wheat bran; SWB, soft wheat bran

7.4.2.4. Evaluation of antioxidant potential of wheat phenolics using the Rancimat

The influence of free, esterified and bound phenolics of wheat on oxidation of stripped corn oil (SCO) was determined at 100 °C using the Rancimat method. The bound phenolics were more effective in extending the induction period and reducing autoxidation of virtually antioxidant-free corn oil at elevated temperatures than free and esterified phenolics (Table 7.6). The protection factor obtained for the bran fraction of

Table 7.6. Oxidative stability (expressed as a protection factor) of stripped corn oil (SCO) in the presence of free, esterified and bound phenolic extracts of whole grains, flour and bran of hard and soft wheat as evaluated by the Rancimat

Milling fraction	Free	Esterified	Bound
HWF	1.12	1.31	1.82
SWF	1.16	1.33	1.89
HWW	1.19	1.61	2.19
SWW	1.21	1.73	2.36
HWB	1.9	2.26	3.93
SWB	2.1	2.4	4.43

Values are the average of two determinations

Abbreviations: HWF, hard wheat flour; SWF, soft wheat flour; HWW, hard whole wheat; SWW, soft whole wheat; HWB, hard wheat bran; SWB, soft wheat bran

both hard and soft wheat was remarkable and indicated the concentration of bioactive components in the external layers of the grain. Oxidation of lipids which is the main cause of quality deterioration in many food systems, may lead to off-flavour development and formation of toxic compounds that compromise the quality and nutritional value of foods. Moreover, lipid oxidation products have been known to be associated with aging, membrane damage, heart disease and cancer (Ramarathnam et al., 1995). Although synthetic antioxidants such as BHT, BHA and TBHQ have been widely used in retarding lipid oxidation, their safety has recently been questioned (Buxiang and Fukuhara, 1997). Thus, there is much interest in the development of safer antioxidants using natural extracts from oilseeds, spices and other plant materials (van Ruth et al., 2001). In general, phenolic acids, flavonoids and anthocyanins, among others, have been

recognized to confer stability to vegetable oils against autoxidation (van Ruth et al., 2001).

7.4.2.5. Inhibition of oxidation of LDL by wheat phenolics

An increased level of LDL is associated with increased risk of atherosclerosis and cardiovascular disease. LDL is the major cholesterol carrier in the blood and LDL may not form atherosclerotic plaques in its native form, however, oxidative modification of LDL is the key factor in pathogenesis of atherosclerosis (Esterbauer et al., 1992). Hence, dietary antioxidants that inhibit LDL oxidation may prevent atherosclerosis as well as cardiovascular diseases (Esterbauer et al., 1992). The bound phenolics of whole grain and bran and esterified phenolics of bran, of hard and soft wheat completely inhibited (100%) copper-induced oxidation of LDL at concentrations tested. Among various fractions of phenolics of flour of hard and soft wheat, only the bound phenolics exhibited ≤ 50% inhibition in the LDL assay (Appendix 7.1). Low concentration of bioactive compounds in the flour fraction is responsible for its low activity.

The inhibition of oxidation of LDL, expressed as μg protein retained/g of defatted soft or hard wheat, are shown in Table 7.7. Bran of both hard and soft wheat exhibited the highest retention capacities followed by whole grains and flour. The bound phenolics of bran, whole grain and flour contributed significantly compared to free and esterified phenolics.

The free phenolic fraction possessed the lowest inhibition of oxidation of LDL among three types of phenolics. The inhibition of LDL oxidation by bound phenolics were 5.3 and 5.1 times higher than that of free phenolics for hard and soft wheat bran,

Table 7.7. Inhibition of oxidation of LDL (μg protein/g defatted material) by free, esterified and bound phenolic extracts of whole grains, flour and bran of hard and soft wheat

Milling fraction	Free	Esterified	Bound
HWF	1,472	3,584	6,502
SWF	1,997	4,634	7,373
HWW	3,315	4,551	25,600
SWW	3,795	4,691	25,600
HWB	4,845	12,800	25,600
SWB	4,978	12,800	25,600

Values are the average of determinations

Abbreviations: HWF, hard wheat flour; SWF, soft wheat flour; HWW, hard whole wheat; SWW, soft whole wheat; HWB, hard wheat bran; SWB, soft wheat bran

respectively. The corresponding values for whole wheat and flour were 7.2 and 6.7 times and 4.4 and 3.7 times, respectively. Moreover, retention capacity of bound phenolics of soft and hard wheat bran was 3.9 and 3.5 times higher than that of bound phenolics of the flour, respectively. Results indicated the significance of bound phenolics of wheat against LDL oxidation.

7.4.2.6. Effects of wheat phenolics on HO$^{\bullet}$-mediated DNA scission

Table 6.8 shows the effects of free, esterified and bound phenolic extracts of whole grains, flour and bran of hard and soft wheat in inhibiting DNA strand cleavage by the Fenton reaction-mediated hydroxyl radical (HO$^{\bullet}$). The results are expressed as the amount of DNA retained by wheat (μg DNA/g defatted wheat). The free, esterified and bound phenolic extracts of whole grains, flour and bran of hard and soft wheat differed

considerably in their ability to protect DNA from nicking by HO$^{\bullet}$. The HO$^{\bullet}$ cleaved supercoiled plasmid pBR DNA completely into nicked circular and linear DNA in the absence of antioxidants (Fig. 7.3, Lane 2). The bound phenolics of bran of hard and soft wheat were most effective in inhibiting nicking, and retained 1660 and 1740 µg DNA/g of defatted wheat bran, respectively (Table 7.8). Moreover, the esterified phenolics of bran and bound phenolics of whole grain of both wheat types inhibited DNA scission by retaining 560 to 590 µg DNA/g. The free phenolics of flour demonstrated weak activity against protecting DNA from oxidation and the retention capacities varied from 110 to 150 µg DNA/g for hard and soft wheat, respectively.

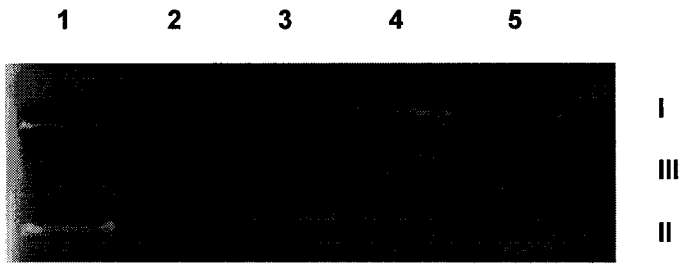


Fig. 7.3. Agarose gel electrophoresis of supercoiled DNA treated with hydroxyl radical in the presence of free, esterified and bound phenolics of flour of hard wheat (Lane 1: Supercoiled DNA; Lane 2: Supercoiled DNA + OH$^{\bullet}$; Lane 3; Supercoiled DNA + OH$^{\bullet}$ + bound phenolics; Lane 4: Supercoiled DNA + OH$^{\bullet}$ + esterified phenolics; Lane 5: Supercoiled DNA + OH$^{\bullet}$ + free phenolics; Form I: Supercoiled DNA; Form II: Nicked open circular DNA; Form III: Linear DNA).

Antioxidant properties of a compound may be evaluated by monitoring HO$^{\bullet}$-induced single strand breaks in DNA in the presence of that compound (Hiramoto et al., 1996). According to Schneider et al. (1988), ascorbate and Fe (III) together form H_2O_2

Table 7.8. Retention ((μg DNA/g defatted material) of pBR 322 supercoiled DNA against hydroxyl radical mediated single strand scission by free, esterified and bound phenolic fractions of whole grains, flour and bran of hard and soft wheat

Milling fraction	Free	Esterified	Bound
HWF	110	240	920
SWF	150	290	880
HWW	270	450	1300
SWW	290	470	1380
HWB	430	590	1660
SWB	470	560	1740

Values are the average of two determinations

Abbreviations: HWF, hard wheat flour; SWF, soft wheat flour; HWW, hard whole wheat; SWW, soft whole wheat; HWB, hard wheat bran; SWB, soft wheat bran

and $HO^{\bullet}$ generating system that readily induces DNA strand cleavage. The bound phenolics of bran effectively prevented the cleavage of supercoiled DNA induced by the Fenton reactants, demonstrating their $HO^{\bullet}$ scavenging activity. In general, $HO^{\bullet}$ is a highly reactive ROS formed in biological systems. It can react with biological molecules such as lipids, sugars, phospholipids, proteins and nucleic acids, among others (Halliwell and Gutteridge, 1984).

7.4.2.7. Iron (II)-chelation capacity of wheat phenolics

The iron (II)-chelating capacity of free, esterified and bound phenolics of whole grains, flour and bran of hard and soft wheat samples were determined by measuring the iron-ferrozine complex (Table 7.9). Despite the significant role in oxygen transport, respiration and as a co-factor in the activity of several enzymes, iron is an extremely

Table 7.9. Iron (II)-chelation capacity (μg EDTA equivalents/g defatted material) of free, esterified and bound phenolic fractions of whole grains, flour and bran of soft and hard wheat

Milling fraction	Free	Esterified	Bound
HWF	304 ± 15	914 ± 41	4270 ± 101
SWF	398 ± 21	1074 ± 56	4802 ± 122
HWW	639 ± 43	1829 ± 99	7064 ± 135
SWW	794 ± 39	2158 ± 81	8310 ± 111
HWB	1238 ± 76	1945 ± 76	9459 ± 205
SWB	1563 ± 64	2487 ± 33	10572 ± 221

Values are mean of three determinations ± standard deviation

Abbreviations: HWF, hard wheat flour; SWF, soft wheat flour; HWW, hard whole wheat; SWW, soft whole wheat; HWB, hard wheat bran; SWB, soft wheat bran

reactive metal that catalyzes oxidative changes in lipids, proteins and nucleic acids, among others (Decker and Hultin, 1990). Any compound that can chelate ferrous ions may render them inactive or poorly active in the Fenton reaction (Smith et al., 1992) as in DNA scission assay. In this study, the bound phenolics of bran of hard and soft wheat demonstrated considerable chelating properties and were 9459 and 10,572 μg EDTA equivalents/g of defatted material, respectively. The free phenolics of soft and hard wheat did not chelate Fe (II) efficiently. Fe (II)-chelation capacity of free, esterified and bound phenolics may also be explained by considering the TPC of these extracts. Thus, there was a positive association between TPC and the Fe (II)-chelation property. When wheat samples were subjected to alkaline hydrolysis the phenolic aglycons are released and they may participate in Fe (II)-chelation. When the phenolic acids exist in esterified or bound form their Fe (II)-chelating properties may be hindered.

In general, glycosylation of phenolic compounds is known to diminish their antioxidant activity as measured by the aqueous radical-trapping capacity (Rice-Evans et al., 1996) as the OH group is not available for reaction. The same phenomenon may be applied to Fe (II)-chelation as well.

7.5. Conclusions

The content of bound phenolics in the hard and soft wheat samples was significantly higher than that of free and esterified phenolics. This resulted in a much higher contribution of bound phenolics to antioxidant capacity compared to that of free and esterified phenolic fractions.

CHAPTER 8

Effects of Pearling on the Antioxidant Activity of Wheat

8.1. Introduction

Pearling is a debranning process that involves sequential removal of bran layers from wheat kernels by friction and abrasion operations. Thus, pearling results in wheat grains that may contain different proportions of bran, that is always less compared to unprocessed wheat grains, depending on the level of pearling. The bran portion accumulated during pearling is referred to as the 'by-product' and the grains are referred to as 'pearled grains'.

Oxidative stress may result from an imbalance in the body between oxidants and antioxidants due to a decrease in natural cell antioxidant capacity or an increase in the amount or overproduction of ROS in organisms (Halliwell, 1996). Consumption of foods rich in antioxidants may lead to scavenge of free radicals and ROS that could otherwise cause oxidative damage to biomolecules such as lipids, proteins and nucleic acids (Aruoma, 1998). Increased consumption of plant-derived phenolics has been associated with a reduced risk of degenerative diseases such as cardiovascular diseases, cancer and other chronic diseases (Kushi et al., 1999). Grains are a major source of phenolic compounds such as hydroxycinnamic acids (HCA) and hydroxybenzoic acids (HBA) known for their antioxidant activity (Peterson, 2001; Handelman et al., 1999; Emmons et al., 1999). In general, phenolic acids have been recognized as potent antioxidants (Graf, 1992; Natella et al., 1999). Several ferulic acid dehydrodimers have been shown to possess antioxidant activity in different *in vitro* models (Garcia-Cornesa et al., 1997 a & b and 1999). The HCA derivatives are the most notable phenolics acids strengthening

their potential role as nutritional antioxidants (Natella et al., 1999). Phenolic acids, that are known to influence the flavour, taste and colour of foods, have gained much attention due to their antioxidative, anti-inflammatory, antimutagenic and anticancer properties as well as the ability to modulate some key enzyme functions in the cell (Ho et al., 1992).

The addition of antioxidants to food systems may increase their shelf life thereby reducing the wastage and nutritional loss by inhibiting and delaying oxidation (Tsuda et al., 1994). Recently, much attention has been paid for in replacing synthetic antioxidants with natural alternatives, primarily plant phenolics (Takeoka and Dao, 2003). This has led to the isolation and characterization of effective natural antioxidants (Reische et al., 2002). In fact plant phenolics, present in fruits and vegetables, are of much interest because of their antioxidant properties and health benefits (Shahidi and Wanasundara, 1992).

According to Dietrych-Szostak and Oleszek (1999), buckwheat grains may be stored for a long time without any symptoms of chemical changes owing to the presence of several natural antioxidants that stabilize them during storage. However, the concentration of natural antioxidants may vary considerably depending on many factors, including variety, location and environmental conditions, among others. Processing of cereals may also have a significant effect on their antioxidant activity since the bran fraction alone exhibits the highest antioxidant activity. This may be explained by localization of phenolics in grains; the outer layers such as husk, pericarp, testa and aleurone layers contain the highest concentration of total phenolics (Xing and White, 1997). According to Xing and White (1997), approximately 80% of the trans-ferulic acid in both rye and wheat grains was found in the bran. Moreover, according to Onyeneho

and Hettiarachchy (1992) the freeze-dried bran fraction of durum wheat exhibited a stronger antioxidant activity than extracts of other milling fractions.

The United States Department of Agriculture (USDA) has recommended daily consumption of 6-11 servings of grain products, that form the base of the USDA food guide pyramid. Although, many of the protective bioactive compounds present in whole grains are also found in fruits and vegetables, some of the compounds, including phenolic ferulates, are unique to whole grains (Dewanto et al., 2002).

8.2. Objectives

This work intended to study the effect of sequential removal of outermost layers (pearling) on the antioxidant activity and phenolic composition of wheat grains using several *in vitro* models. Moreover, the antioxidant activity and phenolic constituents of wheat by-products were determined in order to evaluate their potential for possible use as value-added nutraceutical components.

8.3. Materials and methods

8.3.1. Sample preparation

Two wheat cultivars, CWAD (Canadian Western Amber Durum; *Triticum turgidum* L. var. durum) and CWHRS (Canadian Western hard red spring; *Triticum aestivum* L.), grown in Manitoba (crop year 2002) and Saskatchewan (crop year 2001), respectively, were used. Wheat grains were "debranned" using a pearler (Model TM05C, Satake, Missisauga, ON) to various levels. Starting from unprocessed grain (subsamples of 4.5 kg were used), kernels were pearled from 10 to 50% in 10% increments and pearled wheat and corresponding by-products were collected separately

at each level. Pearled-grain and their by-products were ground, if necessary, in a coffee grinder (Model CBG5 series, Black and Decker Canada Inc., Brockville, ON) and passed through a sieve of mesh size 16 (Tylor Test Sieve, Mentor, OH). All samples were defatted by blending the ground material with hexane (1:5, w/v, 5 min, x3) in a Waring blender (Model 33BL73, Waring Products Dividion, Dynamics Corp. of America, New Hartford, CT) at ambient temperature. Defatted wheat samples were air-dried for 12 h and stored in vacuum packaged polyethylene pouches at -20 °C until used for further analysis within a week period.

8.3.2. Experimental

Details are given in Chapter 3, Section 3.2, Subsections 3.2.1 to 3.2.16.

8.4. Results and discussion

8.4.1. Total phenolic content (TPC) and total antioxidant capacity (TAC) of pearled wheat grains and their by-products

It is known that whole grains provide benefits to humans because of their unique phytochemical composition. However, the antioxidant activity of wheat pearling fractions and their by-products has not been studied. The extraction yield, TPC and TAC of pearling fractions and corresponding by-products of two wheat cultivars, namely CWAD and CWHRS, are shown in Table 8.1. The TPC in the 10% pearled grains and by-products, respectively, was 2.4 and 4.1 times higher than that for pearled grains and by-products of 50% pearling level for CWAD cultivar. The corresponding values for CWHRS were 2.5 and 4 times. The effects were similar for TAC as well; the TAC was decreased from 3.1 ± 0.04 to 1.9 ± 0.03 μmol TE/g and 4.5 ± 0.04 to 1.6 ± 0.02 μmol

Table 8.1. Extraction yield (%, w/w), total phenolic content (TPC; µg ferulic acid equivalents/g defatted material) and total antioxidant capacity (TAC; µmol TE/g defatted material) of pearled grain- and by-products of two wheat cultivars, CWAD and CWHRS

Degree of	Crude yield		TPC		TAC	
Pearling (%)	Pearled grain	By-product	Pearled grain	By-product	Pearled grain	By-product
CWAD						
10	5.6 ± 0.6^{b}	10.6 ± 0.5^{d}	566 ± 22^{e}	2173 ± 32^{e}	3.1 ± 0.04^{e}	9.3 ± 0.07^{e}
20	5.4 ± 0.4^{b}	10.0 ± 0.7^{cd}	367 ± 11^{d}	1740 ± 20^{d}	2.7 ± 0.03^{d}	7.3 ± 0.05^{d}
30	5.0 ± 0.5^{ab}	9.2 ± 0.5^{bc}	340 ± 25^{c}	856 ± 18^{c}	2.4 ± 0.04^{c}	5.7 ± 0.06^{c}
40	5.2 ± 0.7^{ab}	8.6 ± 0.6^{b}	302 ± 10^{b}	697 ± 9^{b}	2.5 ± 0.02^{b}	4.7 ± 0.03^{b}
50	4.2 ± 0.6^{a}	7.4 ± 0.3^{a}	235 ± 17^{a}	533 ± 22^{a}	1.9 ± 0.03^{a}	3.9 ± 0.02^{a}
CWHRS						
10	5.2 ± 0.2^{c}	10.8 ± 0.6^{c}	1014 ± 10^{e}	3208 ± 76^{e}	4.5 ± 0.5^{e}	11.7 ± 0.09^{e}
20	5.1 ± 0.7^{c}	10.1 ± 0.4^{bc}	852 ± 15^{d}	2697 ± 20^{d}	3.7 ± 0.3^{d}	9.7 ± 0.07^{d}
30	4.8 ± 0.5^{b}	9.2 ± 0.8^{b}	648 ± 19^{c}	1638 ± 46^{c}	3.1 ± 0.3^{c}	8.1 ± 0.07^{c}
40	3.8 ± 0.5^{ab}	7.8 ± 0.5^{a}	502 ± 15^{b}	1222 ± 23^{b}	2.0 ± 0.5^{b}	6.6 ± 0.06^{b}
50	3.4 ± 0.5^{a}	6.8 ± 0.6^{a}	408 ± 10^{a}	802 ± 14^{a}	1.6 ± 0.2^{a}	5.2 ± 0.02^{a}

Values are mean of three determinations ± standard deviation

Values in each column with the same superscript are not different (P>0.05); data for CWAD and CWHRS were treated separately

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

TE/g, respectively, for CWAD and CWHRS from 10 to 50% pearling. The decrease was more significant for by-products than that for pearled grains, and the corresponding values were 9.3 ± 0.07 to 3.9 ± 0.02 μmol TE/ g and 11.7 ± 0.09 to 5.2 ± 0.02 μmol TE/g. The cultivar, CWHRS, yielded a significantly ($p<0.05$) higher TPC and TAC compared to CWAD. The grains of both wheat cultivars pearled to 10% level possessed higher TPC and TAC compared to the other pearled wheat grains. The TPC and TAC were decreased with each level of pearling; the 10% pearled grains had the highest values when compared to a higher pearling level. The pearled wheat samples examined in this study contained both bran and germ remnants. As the degree of pearling increased there was a dilution effect on the existing phytochemicals as the grains were composed mainly of the endosperm. Since wheat grain is asymmetrical in shape, pearling did not yield uniform removal of the external layers. In general, the bran portion is approximately 14% of the grain while germ is only 1% (Dexter and Wood, 1996). Therefore, the by-product at 10% level of pearling may include bran layers while at 20% level the by-product contains bran and endosperm including the aleurone layer, the outermost layer of the endosperm. The aleurone layer alone has been shown to contribute significantly to the TPC and hence the antioxidant capacity of wheat (Zhou et al., 2004). This phenomenon can be attributed to the higher antioxidant capacity of by-products resulting from 20% pearling than those resulting from 30-50%. Theoretically, beyond 20% of pearling the by-product should be composed of endosperm alone. However, contamination always exists due to the asymmetry of the grain. The by-products always had a greater TPC and TAC compared to the respective pearled grains.

Phenolic compounds were concentrated in the bran fraction although endosperm also possessed some phenolics that contributed to TPC. The industrial pearling process

can significantly lower the antioxidative phytochemicals, and render polished wheat grains with low antioxidative capacity. Thus, a significant portion of the antioxidative compounds usually end up in the by-product following pearling. Martinez-Tome et al. (2004) observed high antioxidant activity in oat and wheat bran. In Chapter 6 it was demonstrated that whole wheat and its milling fractions, especially bran, are rich sources of phenolic compounds. Peterson et al. (2001) determined antioxidant activity of pearling fractions of oat and observed higher antioxidant activities in the short-pearling-time fractions. The authors pearled oat from 5 to 180 sec and the longer the pearling time the lower the antioxidant activity of the sample was. Longer pearling times led to the removal of external layers leaving starchy endosperm in the pearled products. Peterson (2001) also reported that antioxidative components were concentrated in the outer layers of the wheat kernel. According to Emmons et al. (1999) extracts of oat pearling fractions possessed higher TPC and antioxidant activity than those prepared from the flour fractions. Thus this study also lends further support to these findings, and suggests the degree of pearling should be kept at a minimum in order to retain a high TPC and TAC in the products.

8.4.2. Free radical and ROS scavenging capacity of phenolics of pearled wheat grains and their by-products

Tables 8.2 and 8.3 summarize the results for scavenging of the DPPH radical and ROS such as those of H_2O_2, $HO^{\bullet}$ and $O_2^{-\bullet}$, respectively. Pearled grains at 10% scavenged 2.1 and 2.2 times higher amount of DPPH radical than those at 50% level for CWAD and CWHRS, respectively. The corresponding values for by-products were 2.1 and 1.7 times, respectively. Scavenging of ROS also demonstrated similar activity

trends (Table 8.3). The pearling had a significant effect on the antioxidant potential of wheat. The scavenging activity decreased as the external layers were sequentially removed from the grains. Thus, products at a low pearling level possessed higher free radical and ROS activity compared to those products subjected to a higher level of pearling. The by-products demonstrated superior activities against scavenging of DPPH radicals and ROS in comparison to pearled grains. The varietal differences played a significant role in the antioxidant properties of wheat. Hence, the CWHRS cultivar, in general, performed better than the CWAD cultivar at each level of pearling tested. However, the scavenging pattern of the DPPH radical and ROS was very similar in both cultivars examined. Thus, results of the DPPH radical-scavenging assay corresponded well with those of the other ROS scavenging assays such as those of H_2O_2, $HO^{\cdot}$ and $O_2^{-\cdot}$. Moreover, free radical and ROS scavenging capacities were directly related to TPC and TAC.

Table 8.2. DPPH radical-scavenging capacity (μmol/g defatted material) of pearled grains and by-products of two wheat cultivars, CWAD and CWHRS

Degree of	CWAD		CWHRS	
pearling (%)	Pearled wheat	By-product	Pearled wheat	By-product
10	183 ± 0.4^{e}	404 ± 0.3^{e}	203 ± 0.5^{e}	412 ± 0.4^{e}
20	158 ± 0.7^{d}	369 ± 0.9^{d}	196 ± 0.7^{d}	406 ± 0.3^{d}
30	136 ± 0.9^{c}	300 ± 0.9^{c}	180 ± 0.3^{c}	326 ± 0.5^{c}
40	133 ± 0.3^{b}	260 ± 0.4^{b}	136 ± 0.5^{b}	297 ± 0.3^{b}
50	99 ± 0.3^{a}	192 ± 0.7^{a}	118 ± 0.7^{a}	239 ± 0.3^{a}

Values are the mean of three determinations ± standard deviation

Values in each column with the same superscript are not different (P>0.05)

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

Table 8.3. ROS scavenging capacity (hydroxyl radical; μmol/g defatted material, superoxide radical anion; nmol/g defatted wheat, hydrogen peroxide; μmol/g defatted wheat) of pearled grains and by-products of two wheat cultivars, CWAD and CWHRS

Degree of	CWAD		CWHRS	
pearling (%)	Pearled wheat	By-product	Pearled wheat	By-product
HYDROXYL RADICAL				
10	19.0 ± 0.7^{e}	45.4 ± 1.4^{d}	19.6 ± 0.6^{e}	47.6 ± 0.6^{e}
20	17.4 ± 0.2^{d}	39.6 ± 0.9^{c}	18.5 ± 0.4^{d}	40.8 ± 0.4^{d}
30	15.7 ± 0.3^{c}	38.6 ± 0.5^{c}	17.2 ± 0.3^{c}	38.6 ± 0.4^{c}
40	15.1 ± 0.2^{b}	31.0 ± 0.4^{b}	13.2 ± 0.1^{b}	31.5 ± 0.3^{b}
50	11.7 ± 0.2^{a}	25.8 ± 0.6^{a}	10.9 ± 0.1^{a}	26.3 ± 0.4^{a}
SUPEROXIDE RADICAL ANION				
10	151 ± 1^{d}	404 ± 4^{e}	200 ± 2^{e}	510 ± 6^{e}
20	139 ± 2^{c}	351 ± 5^{d}	180 ± 2^{d}	452 ± 3^{d}
30	125 ± 2^{b}	297 ± 2^{c}	169 ± 2^{c}	383 ± 6^{c}
40	122 ± 1^{b}	257 ± 5^{b}	118 ± 1^{b}	322 ± 3^{b}
50	103 ± 1^{a}	193 ± 1^{a}	104 ± 1^{a}	269 ± 2^{a}
HYDROGEN PEROXIDE				
10	288 ± 7^{d}	580 ± 15^{e}	284 ± 6^{e}	510 ± 6^{e}
20	269 ± 3^{c}	523 ± 10^{d}	258 ± 7^{d}	452 ± 3^{d}
30	249 ± 3^{b}	448 ± 4^{c}	237 ± 4^{c}	383 ± 6^{c}
40	240 ± 7^{b}	413 ± 3^{b}	181 ± 3^{b}	322 ± 3^{b}
50	189 ± 2^{a}	349 ± 5^{a}	160 ± 2^{a}	269 ± 2^{a}

Values are the mean of three determinations ± standard deviation.

Values in each column with same superscript are not different (P>0.05); data for different ROS were treated separately.

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

The deoxyribose method evaluates the activity of $HO^{\bullet}$ to damage carbohydrates. The highly reactive $HO^{\bullet}$ is generated by a mixture of ascorbate and $FeCl_3$-EDTA at pH 7.4, where ascorbate greatly increases the rate of $HO^{\bullet}$ production by reducing iron thereby maintaining Fe^{2+} supply (Aruoma, 1996). The deoxyribose assay evaluates the ability of wheat extracts to scavenge $HO^{\bullet}$, where wheat antioxidants compete with deoxyribose for $HO^{\bullet}$ and inhibit $HO^{\bullet}$-mediated deoxyribose degradation. Thus, Fenton reaction-mediated $HO^{\bullet}$ attack deoxyribose and other simple carbohydrates that result in the formation of thiobarbituric acid reactive substances (TBARS), that can be detected as a pink chromogen at 532 nm (Morelli et al., 2003).

In general, H_2O_2 is generated *in vivo* by several oxidase enzymes (Halliwell et al., 1995). Hydrogen peroxide may act directly or indirectly as a messenger molecule in the synthesis and activation of several inflammatory mediators (Sprong et al., 1998). Moreover, $O_2^{-\bullet}$ are generated in living cells under normal metabolism and is converted to H_2O_2 by dismutation under physiological conditions. Hydrogen peroxide may then give rise to a hydroxyl radical, by itself or in combination with $O_2^{-\bullet}$, in the presence of trace amounts of Fe (II) by the Fenton reaction or Haber-Weiss reaction (Halliwell and Gutteridge, 1989). The scavenging capacity of wheat extracts for $O_2^{-\bullet}$ was much less than that for other free radicals and ROS. The $O_2^{-\bullet}$ scavenging properties of wheat extracts may be attributed to both neutralization of $O_2^{-\bullet}$ via hydrogen donation and inhibition of xanthine oxidase by various phenolic compounds present in the extract (Rice-Evans et al., 1996).

Free radicals have a deleterious role in food and biological systems hence substances with antioxidant properties especially those that scavenge free radicals and ROS are of great significance. In general, excessive formation of free radicals may lead

to food quality deterioration. In particular, $O_2^{\cdot-}$ plays a role in the formation of other ROS such as H_2O_2, $HO^{\cdot}$ and singlet oxygen. These ROS may induce oxidative damage in biological molecules such as lipids, proteins and DNA (Aruoma, 1998). Thus, wheat antioxidants can play a significant role in neutralizing free radicals and ROS.

8.4.3. Reducing power of phenolics of pearled wheat grains and their by-products

Effects of pearling on the antioxidant potential of wheat fractions were determined by measuring their ability to function as reducing agents. The reducing power of wheat extracts was expressed as μmol ascorbic acid equivalents/g of defatted wheat (Table 8.4). The reducing power of CWAD and CWHRS varied from 107 ± 9 to 274 ± 13 and 172 ± 13 to 372 ± 25 μmol ascorbic acid equivalents/g of pearled wheat, respectively. The corresponding values for by-products were 258 ± 17 to 807 ± 21 and 387 ± 29 to 1110 ± 53 μmol ascorbic acid equivalents/g of wheat, respectively.

Table 8.4. Reducing power (μmol ascorbic acid equivalents/g defatted material) of pearled grains and by-products of two wheat cultivars, CWAD and CWHRS

Degree of	CWAD		CWHRS	
pearling (%)	Pearled wheat	By-product	Pearled wheat	By-product
10	274 ± 13^{d}	807 ± 21^{e}	372 ± 25^{e}	1110 ± 53^{e}
20	199 ± 12^{c}	643 ± 14^{d}	288 ± 16^{d}	828 ± 46^{d}
30	141 ± 8^{b}	483 ± 10^{c}	256 ± 14^{c}	577 ± 37^{c}
40	132 ± 10^{b}	326 ± 19^{b}	198 ± 12^{b}	452 ± 35^{b}
50	107 ± 9^{a}	258 ± 17^{a}	172 ± 13^{a}	387 ± 29^{a}

Values are the mean of three determinations ± standard deviation

Values in each column with the same superscript are not different (P>0.05)

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

At the 10% level of pearling the by-products of CWAD and CWHRS, respectively, demonstrated 2.9 and 3.0 times higher reducing power than their pearled grains. At 50% these values were 2.4 and 2.3. The trends were much similar to those observed for DPPH radical and ROS scavenging by wheat extracts. Thus, the pearling of wheat grains significantly affected the phenolic content and thereby the reducing power of different pearled products. Data obtained for reducing power exhibited a positive correlation with the TPC (r^2=0.97 and 0.98 at $p<0.01$ for pearled grain and by-product of CWAD. Corresponding values for CWHRS are r^2=0.99 and 0.98 at $p<0.01$) and TAC (r^2=0.93 at $p<0.05$ and 0.99 at $p<0.01$ for pearled grain and by-product of CWAD. Corresponding values for CWHRS are r^2=0.98 and 0.98 at $p<0.01$) of the wheat samples examined. This method has been further described in Chapter 8.

8.4.4. Oxygen radical absorbance capacity (ORAC) of phenolics of pearled wheat grains and their by-products

The antioxidant activity of wheat fractions measured by the ORAC procedure showed effective scavenging of peroxyl radical especially by the by-products at 10-20% pearling (Table 8.5). Zhou et al. (2004) determined antioxidant activity of bran and aleurone layer of a Swiss red wheat variety and found that the aleurone layer had a higher antioxidant activity than the bran. Thus, the aleurone layer alone exhibited 7-8 fold higher activity than the bran.

According to Hendelman et al. (1999) the ORAC of oat varied from 2.08 to 8.13 μmol/g. These authors found that bran and flour had similar antioxidant activity, due to the mixing of the bran with the starchy endosperm. However, the aleurone layer possessed the highest ORAC value. In contrast, this study demonstrated that the by-

products at 10 and 20% pearling, that contain mainly bran and aleurone portions, possessed much higher ORAC than all pearled grains and by-products at 30-50% pearling and wheat had a superior antioxidant activity, in the ORAC assay, than oat (Hendelman et al., 1999). However, ORAC was determined using two different methods in the two studies, and hence comparison becomes rather difficult.

Table 8.5. Oxygen radical absorbance capacity (ORAC, μmol/g defatted material) of pearled grains and by-products of two wheat cultivars, CWAD and CWHRS

Degree of	CWAD		CWHRS	
pearling (%)	Pearled wheat	By-product	Pearled wheat	By-product
10	86 ± 3^{e}	195 ± 5^{e}	87 ± 3^{e}	207 ± 4^{e}
20	76 ± 2^{d}	173 ± 3^{d}	79 ± 2^{d}	189 ± 3^{d}
30	67 ± 3^{c}	146 ± 3^{c}	73 ± 1^{c}	163 ± 1^{c}
40	62 ± 1^{b}	137 ± 2^{b}	58 ± 2^{b}	138 ± 4^{b}
50	47 ± 1^{a}	101 ± 2^{a}	41 ± 1^{a}	115 ± 1^{a}

Values are the mean of three determinations ± standard deviation.
Values in each column with the same superscript are not different (P>0.05)
Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

8.4.5. Iron (II)-chelation activity of phenolics of pearled wheat grains and their by-products

Iron (II)-chelation may render important antioxidative effects by retarding metal-catalyzed oxidation (Kehrer, 2000). The effective iron (II) chelators may also afford protection against oxidative damage by removing iron (II) that may otherwise participate in $HO^{\bullet}$-generating Fenton type reactions. Minimizing iron (II) may afford protection against oxidative damage by inhibiting production of ROS and lipid peroxidation.

Therefore, iron (II)-chelating activity of pearled wheat grains and by-products was determined by measuring the formation of iron-ferrozine complex. Table 8.6 summarizes the chelating effects of wheat fractions on iron (II).

The iron (II)-chelating capacity by pearled grains of CWAD and CWHRS, respectively, varied from 618 ± 69 to 782 ± 35 and 620 ± 47 to 823 ± 42 µg EDTA equivalents/g of defatted wheat. The corresponding values for the by-products were 692 ± 21 to 1173 ± 59 703 ± 38 to 1206 ± 68 µg EDTA equivalents/g, respectively. The iron

Table 8.6. Iron (II)-chelation capacity (µg EDTA equivalents/g defatted material) by pearled grains and by-products of two wheat cultivars, CWAD and CWHRS

Degree of	CWAD		CWHRS	
pearling (%)	Pearled wheat	By-product	Pearled wheat	By-product
10	782 ± 35^{b}	1173 ± 59^{b}	823 ± 42^{b}	1206 ± 68^{c}
20	673 ± 47^{a}	1021 ± 68^{b}	651 ± 64^{a}	1073 ± 72^{c}
30	629 ± 44^{a}	778 ± 40^{a}	642 ± 18^{a}	821 ± 56^{b}
40	631 ± 72^{a}	724 ± 37^{a}	633 ± 29^{a}	766 ± 41^{b}
50	618 ± 69^{a}	692 ± 21^{a}	620 ± 47^{a}	703 ± 38^{a}

Values are the mean of three determinations ± standard deviation

Values in each column with the same superscript are not different (P>0.05)

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

chelation properties of the two cultivars were not significantly different from each other. The iron (II)-chelation capacity of pearled wheat grains of both CWAD and CWHRS cultivars differed significantly at 10% level of pearling. Moreover, the by-products of both cultivars at 10 and 20% level of pearling demonstrated significantly higher iron (II)-chelation activity compared to the by-products derived from 30 to 50% degree of pearling. The iron (II) chelating activity of wheat may be attributed to their endogenous

chelating agents including phenolic acids. Metal-chelating properties have been reported for amino acids and short peptides that can be easily extracted into an aqueous medium (Yee and Shipe, 1981). Shahidi (2000) reported that constituents such as amino acids, peptides and proteins might play a significant role as physiological and dietary antioxidants thereby augmenting the antioxidant properties against oxidative damage. The antiradical properties exhibited by wheat extracts were also directly proportional to their iron (II)-chelating properties.

8.4.6. Effects of wheat phenolic extracts on oxidative stability of oils and fats

Since fats and oils are susceptible to oxidative deterioration, addition of antioxidants may prevent development of off-flavours and undesirable compounds. Since the use of synthetic antioxidants has been questioned due to toxicity and possible carcinogenecity (Sies, 1993) there is considerable interest in developing plant-derived natural antioxidants, especially from edible plants (Hu and Kitts, 2000). The results of the Rancimat method suggested that wheat phenolics enhanced oxidative stability of seal blubber oil (SBO) and stripped corn oil (SCO) compared to the control that had no additives (Table 8.7). The by-products at 10-20% pearling level produced most noticeable effects especially in the SBO system. Thus, protection factor (PF) of by-products of CWHRS cultivar varied from 1.72 to 2.48 in SBO system. Phenolic compounds are effective antioxidants for polyunsaturated fatty acids (PUFA) as they can transfer a hydrogen atom to lipid peroxyl radicals with ease. The stable aryloxyl radical formed does not act as a chain initiater, hence lipid peroxidation is interrupted (Fisch et al., 2003).

Wheat phenolics may prevent the propagation of peroxidation by scavenging free radicals and function as antioxidants. Wheat extracts, especially those of by-products, enhanced the oxidative stability of SCO and SBO. One of the most important parameters that influences lipid oxidation is the degree of unsaturation of its fatty acid constituents (Frega et al., 1999). The major fatty acids in SCO were palmitic, oleic and linoleic acids; the latter accounted for approximately 59% of the total fatty acids (Appendix 8.1).

Table 8.7. Oxidative stability (expressed as a protection factor) of seal blubber oil (SBO) and stripped corn oil (SCO) in the presence of extracts of pearled grain and by-products of two wheat cultivars CWAD and CWHRS as evaluated by Rancimat

Degree of pearling (%)	Pearled grains		By-products	
	SBO	SCO	SBO	SCO
CWAD				
10	1.49 ± 0.09	1.33 ± 0.08	1.93 ± 0.06	1.66 ± 0.06
20	1.51 ± 0.1	1.27 ± 0.1	1.64 ± 0.05	1.53 ± 0.08
30	1.46 ± 0.04	1.29 ± 0.06	1.58 ± 0.06	1.43 ± 0.05
40	1.4 ± 0.03	1.31 ± 0.02	1.59 ± 0.08	1.38 ± 0.03
50	1.43 ± 0.03	1.29 ± 0.05	1.49 ± 0.03	1.41 ± 0.04
CWHRS				
10	1.73 ± 0.08	1.46 ± 0.1	2.48 ± 0.03	1.93 ± 0.05
20	1.66 ± 0.1	1.38 ± 0.05	1.91 ± 0.06	1.61 ± 0.05
30	1.63 ± 0.04	1.37 ± 0.08	1.74 ± 0.1	1.54 ± 0.03
40	1.54 ± 0.09	1.36 ± 0.03	1.7 ± 0.1	1.47 ± 0.05
50	1.58 ± 0.08	1.38 ± 0.06	1.72 ± 0.07	1.47 ± 0.7

Values are the average of two determinations

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

The total saturated and monounsaturated fatty acids made up about 13 and 26% of the SCO, respectively, while PUFA content was approximately 60%. In SBO, on the other hand, PUFA content was much less than that of SCO. However, more than 90% of PUFA were made up of highly unsaturated fatty acids such as eicosapentaenoic, docosahexaenoic and docosapentaenoic acids which are extremely susceptible to oxidation. The Rancimat method is commonly used in the evaluation of the antioxidative efficiency of natural and synthetic antioxidants (Aparicio et al., 1999). In general, oxidation proceeds slowly under normal conditions until it reaches a point after which the rate starts to increase sharply (Velasco et al., 2004). In the Rancimat method, the elevated temperatures and presence of excess oxygen or air allow one to obtain results within a relatively short period. The differences were not quite significant for 30-50% pearled samples. In the presence of chain-breaking antioxidants such as phenolic compounds in wheat extracts, the antioxidant will donate a hydrogen atom and consequently the free radical chain reaction will be terminated.

8.4.7. Inhibition of photochemiluminescence (PCL) by wheat phenolics

The antioxidative activity of the pearled grains and their by-products was measured by the PCL method and results are shown in Table 8.8. In this method the radical-scavenging capacity is evaluated by measuring the inhibition of photo-induced, chemiluminescent autoxidation of luminol (Popov and Lewin, 1996). The antioxidant activity was highest for the by-products resulting from 10% pearling of both CWAD and CWHRS. In the ACW system, the pearled grains of CWAD and CWHRS at 10% pearling demonstrated 2 and 2.9 times stronger antioxidant activity than those pearled to

50%, respectively. The corresponding values for the ACL system were 2.3 and 2.5 times. Moreover, the by-products were much stronger than the pearled grains.

Table 8.8. Antioxidant activity of pearled grains and by-products of two wheat cultivars CWAD and CWHRS as evaluated by photochemiluminescence in ACW (µmol ascorbic acid equivalents/g defatted material) and ACL (µmol tocopherol equivalents/g defatted material) systems

Degree of Pearling (%)	Pearled grains		By-products	
	ACW	ACL	ACW	ACL
CWAD				
10	3.77 ± 0.03^{e}	4.09 ± 0.07^{e}	11.1 ± 0.09^{e}	13.71 ± 0.07^{e}
20	3.09 ± 0.04^{d}	3.35 ± 0.03^{d}	9.03 ± 0.06^{d}	8.47 ± 0.06^{d}
30	2.52 ± 0.04^{c}	2.71 ± 0.04^{c}	6.56 ± 0.03^{c}	6.74 ± 0.05^{c}
40	2.39 ± 0.02^{b}	2.22 ± 0.03^{b}	5.92 ± 0.03^{b}	6.26 ± 0.04^{b}
50	1.88 ± 0.04^{a}	1.78 ± 0.02^{a}	4.54 ± 0.04^{a}	5.05 ± 0.02^{a}
CWHRS				
10	4.82 ± 0.06^{e}	4.47 ± 0.04^{e}	12.6 ± 0.06^{e}	15.49 ± 0.08^{e}
20	4.14 ± 0.05^{d}	3.64 ± 0.04^{d}	10.4 ± 0.02^{d}	10.44 ± 0.06^{d}
30	2.75 ± 0.04^{c}	3.0 ± 0.03^{c}	8.28 ± 0.03^{c}	9.14 ± 0.02^{c}
40	2.05 ± 0.07^{b}	2.16 ± 0.05^{b}	5.66 ± 0.05^{b}	7.05 ± 0.03^{b}
50	1.63 ± 0.05^{a}	1.81 ± 0.03^{a}	4.58 ± 0.04^{a}	2.09 ± 0.03^{a}

Values are the mean of three determinations ± standard deviation.

Values in each column with the same superscript are not different (P>0.05)

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

Superoxide radical anions ($O_2^{\bullet-}$) are produced by means of a photosensitizer (S) as shown in the equation, $S + h\nu + O_2 \longrightarrow [S^{*}O_2] \longrightarrow S^{\bullet+} + O_2^{\bullet-}$. The

free radicals are then detected by their reaction with a chemiluminogenic substance and by measuring the chemiluminescence produced. Luminol acts as the radical generator and detector (Fisch et al., 2003). In the presence of an antioxidant compound the intensity of PCL is attenuated due to scavenging of $O_2^{\bullet-}$. Hence, the antiradical properties of the additive may be quantified and expressed in equivalent concentration units of a reference compound; for instance, ascorbic acid or tocopherol equivalents in water- and lipid-based systems, respectively. Hence, the PCL method allows determination of the effects of both hydrophilic and hydrophobic compounds using the same system (Popov and Lewin, 1999). The PCL method has been used to assess the antioxidant activity of plant extracts and blood plasma (Amarowicz et al., 1999; Vichi et al., 2001; Amarowicz et al., 2003; Gahler et al., 2003; Lee et al., 2004; Popov and Lewin, 1999). This study is the first report on antioxidant activity of wheat pearlings using the PCL method. With many fractions the hydrophobic antioxidants demonstrated greater activity than hydrophilic constituents and hence values of ACL analysis were higher than those of ACW analysis.

8.4.8. Effects of pearling on inhibition of LDL oxidation by phenolics of pearled wheat grains and their by-products

Dietary antioxidants that prevent LDL from oxidation are of great importance in protection against atherosclerosis (Esterbauer et al., 1992). Cereals such as wheat (Lempereur et al., 1997 & 1998), rye (Andreasen et al., 2000 a & b, 2001) and barley (Zupfer et al., 1998) have been shown to be rich sources of phenolic acids. Hydroxycinnamic acids and ferulic acid dehydrodimers are most common among cereal

phenolic acids (Andreasen et al., 2000 a & b; Lempereur et al., 1997 & 1998; Zupfer et al., 1998).

The activity of wheat samples in preventing the oxidation of human LDL was greatest for by-products at 10-20% pearling. The inhibition of oxidation of human LDL by wheat decreased with sequential removal of the bran layers (Table 8.9). There was a significant difference in the inhibition of LDL oxidation by the extracts, with an increase in pearling degree. The highest activity of 10-20% by-products may be attributed to the presence of a greater proportion of bran and/or aleurone layer in these fractions than in the pearlings from 30-50%. It was shown in Chapter 4 that 80% aqueous ethanolic extracts of whole wheat and their milling fractions were quite efficient in inhibiting copper- induced oxidation of LDL. Low density lipoprotein is known to contain

Table 8.9. Inhibition of oxidation of LDL (μg protein/g defatted material) by pearled grains and by-products of two wheat cultivars, CWAD and CWHRS

Degree of	CWAD		CWHRS	
pearling	Pearled wheat	By-product	Pearled wheat	By-product
10	680 ± 5^{e}	1524 ± 7^{e}	843 ± 6^{e}	1587 ± 9^{e}
20	619 ± 6^{d}	1378 ± 9^{d}	727 ± 8^{d}	1510 ± 8^{d}
30	538 ± 5^{c}	1168 ± 8^{c}	658 ± 3^{c}	1359 ± 11^{c}
40	551 ± 3^{b}	969 ± 4^{b}	588 ± 2^{b}	1057 ± 4^{b}
50	441 ± 5^{a}	778 ± 5^{a}	517 ± 5^{a}	898 ± 5^{a}

Values are the mean of three determinations ± standard deviation.

Values in each column with the same superscript are not different (P>0.05)

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

endogenous antioxidants such as α-tocopherol that may contribute to antioxidative activity in copper-induced LDL oxidation assay. There may be synergism between the well known chain-breaking antioxidant α-tocopherol and the hydrogen donors (Rice-Evans et al., 1996) in the wheat samples. Thus the phenolic hydrogen donors in the wheat extracts may spare tocopherols from consumption and revert the tocopheryl radical directly to tocopherol. Moreover, phenolic antioxidants may scavenge other radicals thereby preventing them from attacking tocopherol (Rice-Evans et al., 1996). Another factor that may bring about antioxidant action in the LDL system is interaction of wheat phenolic compounds with the lipoprotein of LDL. Thus, the phenolic antioxidant may bind with the apo-lipoprotein B preventing the copper catalyst from binding to LDL. Moreover, the phenolic-protein binding may promote the access of phenolics to lipids (Satue-Gracia et al., 1997). Several antioxidant mechanisms such as hydrogen donation, metal chelation and protein binding may explain the antioxidant activity of phenolics against *in vitro* LDL oxidation (Satue-Gracia et al., 1997). The ability of an antioxidant to inhibit cooper-induced LDL may also be attributed to efficient removal of copper from the surface of LDL (Decker et al., 2001). Thus, dietary antioxidants may play a significant role in retarding the development of atherosclerosis that may otherwise lead to coronary heart disease.

8.4.9. Influence of pearling of wheat on $HO^{\bullet}$ mediated supercoiled DNA scission

The supercoiled pBR DNA (form I) is converted into a nicked open circular form (form II) and a linear form (form III) upon strand breakage. It has been shown that the $HO^{\bullet}$ can effectively induce single strand breaks in DNA (Breen and Murphy, 1995). Monitoring of single strand breaks in DNA induced by $HO^{\bullet}$ may be used in the evaluation

of antioxidant properties of a compound against the radical (Hiramoto et al., 1996). Wheat antioxidants present in pearled fractions differed in their ability to protect DNA from nicking by the Fenton reaction mediated HO$^{\bullet}$. The HO$^{\bullet}$ cleaved supercoiled plasmid pBR 322 DNA completely into nicked circular and linear DNA in the absence of any protection (Fig. 8.1, Lane 2).

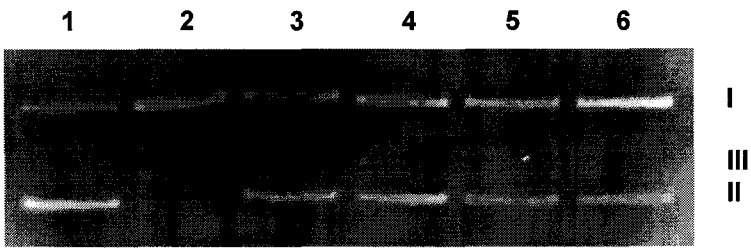


Fig 8.1. Agarose gel electrophoresis of supercoiled DNA treated with hydroxyl radical in the presence of extract of by-product at 10% pearling of grains of CWHRS cultivar (Lane 1: Supercoiled DNA; Lane 2: Supercoiled DNA + OH$^{\bullet}$; Lane 3; Supercoiled DNA + OH$^{\bullet}$ + 6 mg/ mL extract; Lane 4: Supercoiled DNA + OH$^{\bullet}$ + 3 mg/ mL extract; Lane 5: Supercoiled DNA + OH$^{\bullet}$ + 2 mg/ mL extract; Lane 6: Supercoiled DNA + OH$^{\bullet}$ + 1 mg/ mL extract. Form I: Supercoiled DNA; Form II: Nicked open circular DNA; Form III: Linear DNA).

Wheat antioxidants demonstrated a dose-dependent activity against HO$^{\bullet}$-mediated cleavage of DNA (Fig. 8.1, Lanes 3-6). The by-products of wheat pearling fractions were more effective than pearled grain products in preventing HO$^{\bullet}$ damage. In the presence of wheat antioxidants DNA was converted mostly to nicked circular DNA and rarely to linear DNA and fragments.

Table 8.10 shows the dose-dependent effects of wheat antioxidants on inhibiting DNA strand breakage by Fenton reaction-mediated ROS while Table 8.11 presents the

Table 8.10. Inhibition (%) of hydroxyl radical-mediated pBR 322 supercoiled DNA scission by extracts of pearled grains and by-products over 1-6 mg/mL concentration

Degree of pearling	Pearled grains (mg/ mL)				By-products (mg/ mL)			
	1	2	3	6	1	2	3	6
CWAD								
10	40	48	52	70	61	69	77	84
20	35	42	50	64	58	63	68	74
30	33	40	48	58	51	57	63	70
40	30	41	47	55	48	50	56	71
50	31	37	42	52	42	48	55	66
CWHRS								
10	44	50	56	72	66	73	82	91
20	38	51	53	67	61	67	72	83
30	39	46	53	62	50	60	70	76
40	36	43	48	60	49	54	63	74
50	33	42	50	55	43	49	59	69

Values are the average of two determinations.

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

Table 8.11. Retention capacity (μg DNA/g defatted material) of pBR 322 supercoiled DNA against hydroxyl radical mediated scission by pearled grains and by-products of two wheat cultivars, CWAD and CWHRS

Degree of	CWAD		CWHRS	
pearling	Pearled wheat	By-product	Pearled wheat	By-product
10	327	742	312	819
20	288	617	285	699
30	242	537	248	583
40	238	509	190	481
50	182	407	156	391

Values are average of two determinations

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

amount of DNA retained by pearled grains and by-products of wheat. Thus, by-products of pearling were more efficient than pearled grains in retaining DNA against $HO^{\bullet}$-mediated oxidation. Moreover, the effects were significantly reduced with increased degree of pearling of the grains and resultant by-products. The extracts derived from by-products at 10% pearling of CWHRS at 6 mg/mL concentration rendered more than 90% protection against DNA scission (Table 8.10). The inhibitory activity of wheat antioxidants may be attributed to their scavenging of $HO^{\bullet}$ or chelation of iron (II). The oxidizing agents can damage deoxyribose as well as DNA. When deoxyribose is attacked by $HO^{\bullet}$, it leads to the production of toxic malonaldehyde which is highly reactive and leads to cross-linking with DNA and proteins (Gutteridge, 1984).

8.4.10. Phenolic acid composition of pearled wheat grains and by-products

The HPLC analysis of 80% aqueous ethanolic extracts of wheat revealed that vanillic, *p*-coumaric, ferulic and sinapic acids were the major phenolic acids present in

both pearled-grains and their by-products (Table 8.12). The content of phenolic acids liberated from soluble esters was significantly higher than that of free phenolics in the wheat fractions. In the current study most of the pearled grains and by-products possessed sinapic acid as the major phenolic compound. However, sinapic acid was not always present in the free phenolic acid fraction. The by-products contained higher amounts of phenolic acids than the pearled grain products. In fact, the by-product resulting from 10% pearling possessed the highest amount of phenolics in both cultivars.

Although HCA were more prevalent than HBA in the wheat extracts studied, ferulic acid was not the main phenolic present in total phenolics as shown in several studies (Lempereur et al., 1997; Zupfer et al., 1997). Meanwhile, vanillic acid was the only benzoic acid derivative present in the wheat system tested. The bran that was represented by the by-product at 10% pearling possessed the highest content of phenolic acids in both cultivars.

Andreasen et al. (2000 a & b) have shown that rye bran is a rich source of phenolics and hence, the concentration of ferulic acid and its dehydrodimers was approximately 10-20 times higher in the bran than that in the endosperm. Recently, antioxidant activity of simple phenolic acids, namely HCA and HBA, has been studied in different model systems. In general, HCA had a superior antioxidant activity than the

Table 8.12. Phenolic acid content (μg/g defatted material) of selected pearled grains and by-products of two wheat cultivars, CWAD and CWHRS

Wheat fraction	Content (μg/g crude extract)							
	Vanillic		*p*-Coumaric		Ferulic		Sinapic	
	Free	Ester	Free	Ester	Free	Ester	Free	Ester
CWAD								
Whole grain	1.24±0.06	5.39±0.19	0.12±0.001	0.74±0.06	0.43±0.01	4.46±0.12	0.19±0.02	21.1±1.61
10 % Pearled grain	1.01±0.06	3.7±0.11	0	1.06±0.06	1.12±0.06	4.42±0.17	0	4.59±0.34
30% Pearled grain	0.65±0.02	1.81±0.04	0.17±0.01	0.7±0.04	1.18±0.04	2.23±0.08	0	2.52±0.21
Flour	0.34±0.01	1.5±0.05	0.1±0.005	0.38±0.02	0.75±0.03	2.2±0.1	0	1.25±0.1
10% By-product	9.75±0.32	25.44±0.85	0.95±0.04	8.9±0.42	4.98±0.21	27.24±0.95	0.74±0.05	16.54±1.27
30% By-product	2.3±0.09	10.12±0.37	0.28±0.01	1.66±0.09	2.85±0.09	11.68±0.37	0	2.48±0.18
CWHRS								
Whole grain	0.16±0.01	5.56±0.16	0.11±0.05	1.46±0.05	0.54±0.05	5.72±0.22	0.05±0.005	18.36±1.4
10 % Pearled grain	1.25±0.05	4.99±0.16	0.21±0.01	0.99±0.05	1.77±0.05	6.81±0.21	0.1±0.01	11.49±0.88
30% Pearled grain	0.77±0.05	3.07±0.1	0.14±0.005	0.43±0.02	1.68±0.05	3.74±0.14	0	8.54±0.62
Flour	0.28±0.08	1.36±0.04	0.08±0.004	0.2±0.01	0.44±0.02	1.56±0.04	0	3.2±0.02
10% By-product	5.29±0.22	24.62±0.86	0.54±0.02	5.4±0.22	2.05±0.11	21.6±0.76	0.43±0.04	48.06±3.56
30% By-product	Trace	8.28±0.28	Trace	1.29±0.09	Trace	16.83±0.55	0	6.9±0.55

Values are mean of three determinations ± standard deviation.

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

corresponding HBA (Andreasen et al., 2001) due to the better delocalization and hence stability of the resultant free radicals upon donation of a hydrogen atom. Moreover, the antioxidant activity improved as the number of hydroxyl or methoxy groups increased in the molecule (Natella et al., 1999). Although caffeic and sinapic acids have a higher antioxidant activity than ferulic and *p*-coumaric acids, ferulic acid is most dominant in cereal grains (Lempereur et al., 1997; Zupfer et al., 1998). Thus, free HCA showed decreasing antioxidant activity against LDL oxidation in the order of caffeic > sinapic > ferulic > *p*-coumaric (Natella et al., 1999).

The compositional studies of wheat extracts revealed the presence of these HCA except caffeic acid. Sinapic acid effectively retarded LDL oxidation at 20 – 40 μM concentration (Andreasen et al., 2001). The greater antioxidant activity of sinapic acid against copper-mediated LDL oxidation is related to its ability to complex copper ions (Natella et al., 1999). Hence, sinapic acid may exert its antioxidant activity against LDL oxidation via metal chelation (Andreasen et al., 2001). It is very unlikely that *p*-coumaric and ferulic acids exert antioxidant activity via metal chelation (Graf, 1992). H owever, ferulic acid readily forms a resonance-stabilized phenoxyl radical that accounts for its potent antioxidant activity (Graf, 1992). In a lipid model system phenolics may either donate hydrogen atoms to a lipid free radical, and neutralize them or they may chelate metal ions (Rice-Evans et al., 1996). The electron-donating ability of methoxy groups on the phenyl ring confers a better stability to the resultant phenoxyl radical upon hydrogen abstraction from the parent compound (Satue-Gracia et al., 1997). Rye bran extracts have demonstrated much higher antioxidant activity than what can be explained from concentration of individual phenolic acids. Hence, phenolic acids

may act synergistically or there may be other components in the extract that may contribute to the antioxidant activity (Andreasen et al., 2001).

8.5. Conclusions

With increased removal of external layers of wheat the phenolic content and antioxidant activity decreased significantly resulting in lower values with higher degrees of pearling. The trend was common to both pearled grains and by-products of wheat.

CHAPTER 9

Antioxidant and Free Radical Scavenging Activities of Whole Wheat and Milling Fractions

9.1. Introduction

In general, grains, vegetables and fruits contain a wide variety of phytochemicals that are biologically active (Caragay, 1992). Plant-derived antioxidants may function as reducing agents, and scavengers of free radicals and metal ions, among others (Rice-Evans et al., 1996). The antioxidant activity of plasma has been shown to increase after consumption of foods high in antioxidants. Thus, in the human body phytochemicals may combat oxidative stress by maintaining a balance between oxidants and antioxidants (Temple, 2000). This is particularly important because under severe oxidative stress excessive formation of ROS and free radicals can damage biomolecules, such as DNA, proteins, lipids and carbohydrates, and lead to numerous disease conditions (Halliwell, 1996).

Cereals have been known to contain a high amount of HCA that render potential health benefits (Andreasen et al., 2001). Commercial processing of cereals may lead to products with low value fractions such as hulls and polish waste. In general, hulls are removed prior to food production. However, the low value fractions may serve as potential sources of natural antioxidants at relatively high concentrations (Bryngelsson et al., 2002). In oats antioxidant compounds are mostly concentrated in the bran as compared to that in the endosperm as shown by *in vitro* assays (Peterson, 2000; Peterson et al., 2001). Emmons et al. (1999) demonstrated higher antioxidant activity and TPC in three oat pearling fractions containing different levels of bran layers

compared to those of the flour extracts. According to Yu et al. (2000) bran extracts of three different wheat varieties exhibited significant antioxidant properties against free radical scavenging and metal ion chelation. Moreover, whole grains of three hard wheat varieties exhibited antioxidant activity against lipid peroxidation in a fish oil model system (Yu et al., 2002). The 'Akron' variety of wheat was highly effective in scavenging DPPH radical and chelating Fe (II). Zhou and Yu (2004) reported the effects of growing conditions employed on antioxidant activity of a wheat variety grown in different locations. These authors reported that TPC, scavenging of DPPH radical and chelation of Fe (II) were significantly influenced by agronomic practices and environmental conditions. The antioxidant properties of whole grains, bran and aleurone layer of a Swiss red wheat variety was studied using free radical scavenging and metal ion chelation capacity. Thus, aleurone, bran and grains differed significantly in their antioxidant potential, TPC and phenolic acid composition. Moreover, the aleurone layer exhibited the highest antioxidant activity, TPC and content of phenolic acids (Zhou et al., 2004). Ferulic acid was reported to be the predominant phenolic acid accounting for approximately 57 to 77% of total phenolic acids present in wheat on a weight basis. Ferulic acid content was positively correlated with scavenging of free radicals and TPC and hence may be used as a potential marker of wheat antioxidants (Zhou et al., 2004). Plant phenolic compounds including phenolic acids, flavonoids and anthocyanins, among others, have also been recognized as confering stability against autoxidation of vegetable oils (van Ruth et al., 2001). There is much interest in the use of crude phenolic extracts from fruits, herbs, vegetables, cereals and other plant materials in the food industry because they have been shown to retard oxidative degradation of lipids thereby improving the quality and nutritional value of food (Kahkonen et al., 1999).

9.2. Objectives

The objectives of this study was to examine the effects of milling on the antioxidant potential of whole grains and their milling fractions, namely bran, flour, feed flour and shorts of two wheat cultivars under *in vitro* conditions.

9.3. Materials and methods

9.3.1. Sample preparation

Whole wheat grains and milling fractions, namely bran, flour, shorts and feed flour of two cultivars, CWAD (Canadian Western Amber Durum; *Triticum turgidum* L. var. durum) and CWHRS (Canadian Western hard red spring; *Triticum aestivum* L.) that were grown in Manitoba (crop year 2002) and Saskatchewan (crop year 2001), respectively, were obtained from Canadian Grain Commission, Winnipeg, Manitoba. Whole grains and their milling fractions, when necessary, were ground in a coffee grinder (Model CBG5 series, Black and Decker Canada Inc., Brockville, ON) and passed through a mesh size 16 sieve (Tylor Test Sieve, Mentor, OH). Wheat flour was used as such for the extraction of crude phenolics. All samples were defatted by blending the ground material with hexane (1:5, w/v, 5 min, X3) in a Waring blender (Model 33BL73, Waring Products Division, Dynamics Corp. of America, New Hartford, CT) at ambient temperature. Defatted wheat samples were air dried for 12 h and stored in vacuum packaged polyethylene pouches at -20 °C for further analysis.

9.3.2. Experimental

Experimental details are given in Chapter 3, Section 3.2, Subsections 3.2.1 to 3.2.16.

9.4. Results and discussion

9.4.1. Total phenolic content (TPC) and total antioxidant capacity (TAC) of wheat milling fractions

The yield of crude extracts, total phenolic content (TPC) and total antioxidant capacity (TAC) of whole wheat grains and milling fractions of cultivars CWAD and CWHRS are shown in Table 9.1. The cultivar CWAD is mainly cultivated for production of semolina and pasta (Miller et al., 1988) while CWHRS cultivar is a common bread wheat. The amount of extractable matter varied significantly among whole grains and their milling fractions and hence produced varying extraction yields. The differences were also significant between the two cultivars examined. The TPC of different fractions ranged from 140 to 2279 μg FAE/g of defatted wheat and 216 to 3437 μg FAE/g of defatted wheat, respectively, for CWAD and CWHRS. In both cultivars the bran fraction possessed the highest TPC. The order of TPC was bran > shorts > feed flour > whole grain > flour, for CWAD and CWHRS. With CWAD there was an additional fraction, semolina, that possessed the lowest TPC. The TAC expressed as μM Trolox equivalents (TE) demonstrated a positive correlation with the TPC values (r^2=1.0 and 0.99 at $p<0.01$ for CWAD and CWHRS, respectively). Both TPC and TAC were higher in the outermost layers of the wheat grain. Hence, bran was significantly higher in TPC and TAC compared to those of the endosperm that form the flour fraction.

Semolina is also a product derived from endosperm. Semolina is obtained from durum wheat, for instance the cultivar CWAD, examined in this study. Semolina possessed the lowest TPC and TAC, with respect to the fractions examined with the CWAD cultivar. The higher TPC and TAC of shorts and feed flour compared to that of flour and semolina can be attributed to the presence of various proportions of bran and

Table 9.1. Extraction yield (%, w/w), total phenolic content (TPC; µg FAE/g defatted material) and total antioxidant capacity (TAC; µmol TE/ g defatted material) of milling fractions of two wheat cultivars, CWAD and CWHRS.

Milling fraction	Crude yield		TPC		TAC	
	CWAD	CWHRS	CWAD	CWHRS	CWAD	CWHRS
Whole grain	6.2 ± 0.4^{b}	5.4 ± 0.4^{a}	769 ± 41^{c}	1291 ± 31^{b}	4.24 ± 0.03^{c}	4.99 ± 0.06^{b}
Bran	10.6 ± 0.7^{de}	10.2 ± 0.8^{c}	2279 ± 61^{f}	3437 ± 86^{d}	10.32 ± 0.08^{f}	12.79 ± 0.09^{e}
Flour	4.2 ± 0.9^{c}	4.0 ± 0.5^{a}	210 ± 11^{b}	216 ± 16^{a}	2.24 ± 0.04^{b}	2.35 ± 0.02^{a}
Shorts	10.0 ± 1.1^{e}	9.8 ± 0.7^{c}	1920 ± 22^{e}	3146 ± 97^{c}	8.8 ± 0.06^{e}	11.53 ± 0.05^{d}
Feed flour	9.0 ± 0.8^{d}	8.1 ± 0.3^{b}	1404 ± 64^{d}	2033 ± 83^{b}	6.78 ± 0.03^{d}	8.96 ± 0.04^{c}
Semolina	5.2 ± 0.6^{a}	-	140 ± 13^{a}	-	1.69 ± 0.02^{a}	-

Values are the mean of three determinations ± standard deviation

Values in each column with the same superscript are not different ($p>0.05$)

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

germ in them. In whole grains, on the other hand, the antioxidants get diluted due to the endosperm. The Trolox equivalent antioxidant capacity (TEAC) values, have been used to rank the antioxidant activity of unknown mixtures (van den Berg et al., 1999). Thus, the TEAC of a compound determines its antioxidant activity relative to that of Trolox. Another study on milling and sieving to obtain bran-rich and starch-rich fractions of oat revealed a higher TPC and antioxidant activity in the bran-rich fraction than those in the starch-rich fraction (Gray et al., 2000).

9.4.2. Scavenging of free radicals and reactive oxygen species (ROS) by wheat phenolics

The capacity of wheat samples to scavenge the stable DPPH radical is shown in Table 9.2 while Table 9.3 summarizes the results for quenching of biologically important oxygen species such as hydroxyl ($HO^{\bullet}$) and superoxide anion radicals ($O_2^{\bullet-}$) as well as hydrogen peroxide (H_2O_2). The ability to scavenge DPPH radicals by wheat fractions was in the order of bran > shorts > feed flour > whole grain > flour, for both wheat cultivars. In addition, the CWAD semolina fraction possessed the lowest scavenging capacity for these reactive oxygen species.

The wheat samples demonstrated significant scavenging capacity against $HO^{\bullet}$ as measured by the deoxyribose assay (Table 9.3). Superoxide anion radical scavenging capacity of wheat fractions was measured using the xanthine-xanthine oxidase system and the results were reported as nmol $O_2^{\bullet-}$ scavenged/g of defatted material (Table 9.3). While the efficiency of wheat fractions in scavenging $HO^{\bullet}$ and H_2O_2 was excellent, they were not effective in scavenging $O_2^{\bullet-}$. The samples of wheat especially those of bran, shorts, feed flour and whole grain demonstrated very high activity against scavenging of

$HO^{\bullet}$ and H_2O_2 (Table 9.3). With respect to CWAD, the H_2O_2 scavenging varied from 263 ± 6 to 728 ± 12 µmol/g of defatted wheat. The corresponding values for CWHRS were 264 ± 4 to 597 ± 16 µmol/g of defatted wheat. The flour and semolina fractions demonstrated the lowest scavenging capacity against H_2O_2. The ability to scavenge free radicals and ROS by wheat fractions was assessed using several *in vitro* antioxidant models. The ethanolic extracts of wheat could scavenge free radicals and ROS at the stage of initiation of a chain reaction thereby terminating the reaction. The results indicated wheat antioxidants, particularly those present in the bran, were efficient free radical scavengers.

Table 9.2. DPPH radical scavenging capacity (µmol/g of defatted material) of whole grains and their milling fractions of two wheat cultivars, namely CWAD and CWHRS

Milling fraction	CWAD	CWHRS
Whole grain	210 ± 1.4^{c}	218 ± 1.5^{b}
Bran	415 ± 1.9^{e}	429 ± 1.8^{d}
Flour	96 ± 1.6^{a}	100 ± 2.4^{a}
Shorts	342 ± 3.0^{d}	406 ± 3.1^{c}
Feed flour	318 ± 2.0^{d}	338 ± 3.5^{c}
Semolina	114 ± 1.3^{b}	-

Values are the mean of three determinations ± standard deviation

Values in each column with the same superscript are not different ($p>0.05$)

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

Table 9.3. ROS scavenging capacity (hydroxyl radical; μmol/g defatted material, superoxide radical anion; nmol/g defatted wheat, hydrogen peroxide; μmol/g defatted wheat) of whole grains and milling fractions of two wheat cultivars, CWAD and CWHRS

Milling fraction	CWAD	CWHRS
HYDROXYL RADICAL SCAVENGING		
Whole grain	24.1 ± 0.2^{d}	22.5 ± 0.4^{b}
Bran	47.8 ± 1.2^{e}	48.1 ± 0.5^{c}
Flour	21.1 ± 0.8^{b}	18.3 ± 0.3^{a}
Shorts	50.9 ± 2.0^{e}	43.8 ± 0.8^{c}
Feed flour	34.4 ± 0.7^{c}	33.3 ± 0.9^{b}
Semolina	13.4 ± 0.4^{a}	-
SUPEROXIDE ANION RADICAL SCAVENGING		
Whole grain	242 ± 3^{c}	249 ± 2^{d}
Bran	439 ± 6^{e}	497 ± 2^{e}
Flour	149 ± 2^{b}	174 ± 2^{a}
Shorts	444 ± 7^{d}	421 ± 4^{c}
Feed flour	332 ± 2^{e}	319 ± 1^{b}
Semolina	87 ± 2^{a}	-
HYDROGEN PEROXIDE SCAVENGING		
Whole grain	333 ± 7^{c}	324 ± 3^{b}
Bran	633 ± 7^{e}	621 ± 0^{c}
Flour	301 ± 4^{b}	264 ± 4^{a}
Shorts	712 ± 18^{cd}	597 ± 0^{c}
Feed flour	728 ± 12^{d}	493 ± 0^{c}
Semolina	263 ± 6^{a}	-

Values are the mean of three determinations ± standard deviation.

Values in each column with the same superscript are not different ($p>0.05$).

Assays are treated separately

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

9.4.3. Reducing power of wheat phenolics

Table 9.4 shows the reducing power of phenolic compounds of whole grains and milling fractions of two wheat cultivars, CWAD and CWHRS, as determined by the potassium ferricyanide reduction method. The bran fraction of CWHRS possessed a strong (1068 µmol/g of defatted wheat ascorbic acid equivalents) reducing power. The reducing power of different milling fractions were significantly ($p<0.05$) different from one another. The flour and semolina had the weakest reducing power among all fractions.

Table 9.4. Reducing power (µmol ascorbic acid equivalents/g defatted material) of whole grains and milling fractions of two wheat cultivars, CWAD and CWHRS

Milling fraction	CWAD	CWHRS
Whole grain	367 ± 18^{b}	405 ± 14^{b}
Bran	949 ± 23^{e}	1088 ± 24^{e}
Flour	99 ± 4^{a}	131 ± 12^{a}
Shorts	811 ± 17^{d}	1009 ± 23^{d}
Feed flour	620 ± 26^{c}	777 ± 26^{c}
Semolina	100 ± 9^{a}	-

Values are the mean of three determinations ± standard deviation

Values in each column with the same superscript are not different ($p>0.05$)

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

The reducing power of the bran layers, in general, was better than that of the endosperm. Hence, the bran alone, shorts and feed flour exhibited the highest reducing power. The reducing power of samples of the CWHRS cultivar was significantly higher than that of CWAD. Shimada et al. (1992) reported that reducing power of a compound may be attributed to its hydrogen-donating ability. These authors reported that ascorbic

acid is a strong reductone that can readily donate a hydrogen atom to a free radical, thus terminating free radical reactions.

Duh (1998) also stated that reductones are efficient reducing agents and their efficiency is attributed to their hydrogen-donating ability. The wheat extracts examined in this study demonstrated good reducing capacity thereby acting as efficient reductones. The results on reducing power demonstrate the electron donor properties of wheat extracts thereby neutralizing free radicals by forming stable products. The outcome of the reducing reaction is to terminate the radical chain reactions that may otherwise be very damaging (Yen and Chen, 1995).

9.4.4. Oxygen radical absorbance capacity (ORAC) of wheat phenolics

The ORAC values of wheat fractions were expressed as µmol TE/g of defatted wheat. The ORAC values of wheat samples ranged from 45 ± 0.7 to 301 ± 5 and 45 ± 2 to 310 ± 3 µmol TE/g of for CWAD and CWHRS cultivars of wheat, respectively (Table 9.5). The ORAC of wheat fractions was in the order of bran > shorts > feed flour > whole grain > flour. A general agreement in the rank order with regard to ORAC was noted between CWAD and CWHRS. Hence, bran possessed the highest ORAC while flour and semolina fractions had the lowest ORAC. The ORAC values for samples of CWHRS were much higher than those of CWAD. The ORAC of wheat bran was approximately 6.8 folds higher than those of flour fractions for both CWAD and CWHRS. A positive correlation existed between TPC and ORAC (r^2=0.97 and 0.99 at $p<0.01$ for CWAD and CWHRS, respectively) in this study.

According to Handelman et al. (1999) the ORAC of oat milling fractions varied from 2.08 to 8.13 µmol TE/g sample. The ORAC values observed in this study were

much higher than those observed for oat. However, we used florescein as the florescence probe while Handelman et al. (1999) used R-phycoerythrin as the target molecule. In contrast to our study, Handelman et al. (1999) reported similar antioxidant potential for bran and flour fractions of oat, due to contamination of commercial oat bran samples with endosperm that diluted the TAC. Zhou et al. (2004) have determined antioxidant activity of bran and aleurone layer of a Swiss red wheat variety and found that aleurone layer exerted 7-8 times more antioxidant activity, as determined by the ORAC assay, than that of the bran. Thus, consumption of whole grains may provide full advantage of all antioxidative compounds present in wheat.

Table 9.5. Oxygen radical absorbance capacity (ORAC, μmol/g defatted material) of whole grains and milling fractions of two wheat cultivars, CWAD and CWHRS

Milling fraction	CWAD	CWHRS
Whole grain	100 ± 1^{b}	95 ± 5^{b}
Bran	301 ± 5^{e}	310 ± 3^{d}
Flour	45 ± 2^{a}	54 ± 2^{a}
Shorts	246 ± 8^{d}	304 ± 10^{d}
Feed flour	199 ± 3^{c}	234 ± 6^{c}
Semolina	48 ± 2^{a}	-

Values are the mean of three determinations ± standard deviation.
Values in each column with the same superscript are not different ($p>0.05$).
Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

9.4.5. Iron (II)-chelation capacity of wheat phenolics

The iron (II)-chelating capacity of both whole wheat grains and their milling fractions was determined by measuring the iron-ferrozine complex and results are summarized in Table 9.6. In the iron (II)-chelation assay, bran demonstrated superior

chelating properties over the other fractions. Flour and semolina had the lowest iron (II)-chelation capacity. Results indicated the presence of iron (II) chelating agents such as phenolics in association with bran layers. The ethanolic extracts of flour possessed greater iron (II)-chelation capacity than the other fractions (Appendix 5.3). However, iron chelation capacities were then expressed on the weight basis of the crude extract. In this chapter, on the other hand, iron (II)-chelation capacity was expressed on the basis of defatted wheat and hence the existing difference in the observed trend. The extraction yields of various milling fractions were different thus causing the above deviation.

Table 9.6. Iron (II) chelation capacity (μg EDTA equivalents/g defatted material) of whole grains and their milling fractions of two wheat cultivars, CWAD and CWHRS

Milling fraction	CWAD	CWHRS
Whole grain	759 ± 36^{c}	794 ± 17^{b}
Bran	1276 ± 58^{f}	1316 ± 53^{d}
Flour	595 ± 12^{a}	616 ± 13^{a}
Shorts	1121 ± 31^{e}	1102 ± 49^{c}
Feed flour	903 ± 39^{d}	744 ± 34^{b}
Semolina	679 ± 18^{b}	-

Values are the mean of three determinations ± standard deviation

Values in each column with the same superscript are not different ($p>0.05$)

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

9.4.6. Inhibition of copper-induced LDL oxidation by wheat phenolics

The inhibition of copper-induced oxidation of human LDL by whole wheat grains and milling fractions thereof are presented in Table 9.7. The retention capacity for

CWAD varied from 401 to 1603 µg protein/g while in CWHRS it ranged from 464 to 1673 µg protein/g of defatted wheat. In CWAD cultivar, the bran was 4 and 3.7 times more effective than the flour and semolina fractions, respectively, in inhibiting LDL against copper-induced oxidation. The bran of CWHRS was 3.6 times more powerful than its flour fraction in the LDL system. The fractions that contained a higher proportion of bran exhibited a better antioxidant activity than those with a higher proportion of endosperm.

Table 9.7. Inhibition of oxidation of LDL (µg protein/g defatted material) by whole grains and their milling fractions of two wheat cultivars, CWAD and CWHRS

Milling fraction	CWAD	CWHRS
Whole grain	834 ± 5^{c}	866 ± 10^{b}
Bran	1603 ± 4^{f}	1673 ± 5^{e}
Flour	401 ± 3^{a}	464 ± 4^{a}
Shorts	1426 ± 4^{e}	1589 ± 8^{d}
Feed flour	1123 ± 7^{d}	1285 ± 4^{c}
Semolina	429 ± 3^{b}	-

Values are the mean of three determinations ± standard deviation

Values in each column with the same superscript are not different ($p>0.05$)

9.4.7. Inhibition of photochemiluminescence (PCL) by wheat phenolics

The antioxidative potential of whole grains and their milling fractions as measured by PCL method are shown in Table 9.8. The highest inhibition was observed with the extracts derived from the bran of both wheat cultivars. The inhibition of PCL varied from 0.9 to 14.7 and 1.5 to 15.6 µmol tocopherol equivalents/g of defatted wheat for CWHRS and CWAD, respectively, in the ACL system. The corresponding values for the ACW system were 1.1 to 12.1 and 1.8 to 13.1 µmol ascorbic acid equivalents/g of defatted wheat, respectively. The inhibition of PCL varied significantly among different

milling fractions and was in the decreasing order of bran > shorts > feed flour > whole grain > flour. Moreover, in the CWAD cultivar semolina produced the lowest inhibition. Similar to wheat pearling fractions (Chapter 8) the content of hydrophobic antioxidants in wheat milling fractions exceeded those of the hydrophilic antioxidants.

Table 9.8. Antioxidant activity of whole grains and milling fractions of two wheat cultivars, CWAD and CWHRS, as evaluated by photochemiluminescence in ACW (μmol ascorbic acid equivalents/g defatted material) and ACL (μmol tocopherol equivalents/g defatted material) systems

Milling fraction	CWAD		CWHRS	
	ACW	ACL	ACW	ACL
Whole grain	5.0 ± 0.02^{c}	6.1 ± 0.06^{c}	5.2 ± 0.05^{b}	6.8 ± 0.04^{b}
Bran	12.1 ± 0.07^{f}	14.7 ± 0.08^{f}	13.1 ± 0.09^{e}	15.6 ± 0.09^{e}
Flour	1.6 ± 0.03^{b}	1.3 ± 0.03^{b}	1.8 ± 0.02^{a}	1.5 ± 0.01^{a}
Shorts	10.6 ± 0.06^{e}	10.2 ± 0.06^{e}	11.2 ± 0.03^{d}	13.5 ± 0.08^{d}
Feed flour	9.3 ± 0.04^{d}	8.6 ± 0.05^{d}	9.8 ± 0.07^{c}	10.3 ± 1.0^{c}
Semolina	1.1 ± 0.02^{a}	0.9 ± 0.01^{a}	-	-

Values are the mean of three determinations ± standard deviation

Values in each column with the same superscript are not different ($p>0.05$)

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

9.4.8. Effects of wheat phenolics on oxidative stability of oils and fats

The influence of wheat phenolic extracts on the oxidative stability of seal blubber oil (SBO), stripped corn oil (SCO) and a shortening sample was determined using the Rancimat method. All wheat extracts produced a protection factor (PF) greater than 1 in each oil/fat model (Table 9.9) and hence oxidative stability was significantly improved by wheat extracts compared to the control that did not contain any additive. Wheat

Table 9.9. Oxidative stability (expressed as a protection factor) of seal blubber oil (SBO), stripped corn oil (SCO) and vegetable shortening in the presence of extracts of whole grains and their milling fractions of two wheat cultivars, CWAD and CWHRS as evaluated by Rancimat

Milling fraction	CWAD			CWHRS		
	SBO	SCO	SHORTENING	SBO	SCO	SHORTENING
Whole grain	1.67 ± 0.1^{b}	1.47 ± 0.05^{b}	1.31 ± 0.05^{b}	1.81 ± 0.04^{b}	1.66 ± 0.15^{b}	1.33 ± 0.03^{b}
Bran	2.59 ± 0.2^{e}	1.96 ± 0.06^{d}	2.12 ± 0.1^{d}	2.93 ± 0.3^{d}	2.26 ± 0.2^{c}	2.32 ± 0.1^{c}
Flour	1.36 ± 0.08^{a}	1.15 ± 0.04^{a}	1.17 ± 0.06^{a}	1.42 ± 0.1^{a}	1.24 ± 0.03^{a}	1.27 ± 0.06^{a}
Shorts	2.54 ± 0.09^{d}	1.74 ± 0.07^{c}	1.96 ± 0.08^{c}	2.74 ± 0.1^{c}	2.05 ± 0.05^{c}	2.21 ± 0.04^{c}
Feed flour	2.56 ± 0.2^{d}	1.76 ± 0.04^{c}	2.04 ± 0.13^{c}	2.59 ± 0.1^{c}	1.94 ± 0.07^{c}	2.12 ± 0.1^{c}
Semolina	1.31 ± 0.07^{a}	1.15 ± 0.1^{a}	1.12 ± 0.03^{a}	-	-	-

Values are the mean of three determinations ± standard deviation

Values in each column with the same superscript are not different ($p>0.05$)

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

extracts, especially those of bran, shorts and feed flour, enhanced the oxidative stability of SBO, SCO and shortening. The PF varied significantly ($p<0.05$) among different fractions in both cultivars.

The first step in lipid oxidation is the abstraction of a hydrogen atom from a fatty acid and production of $ROO^{\cdot}$ upon reaction with oxygen. In the presence of an antioxidant, abstraction of hydrogen from fatty acid is suppressed and hence theformation of hydroperoxides is retarded (Torel et al., 1986). Thus, protection against $ROO^{\cdot}$ formation by wheat extracts may arise due to their hydrogen donating ability. In the presence of wheat extracts the induction periods were prolonged to different extents. Thus, the additives delayed the onset of the propagation phase of a free radical chain reaction (Martinez-Tome et al., 2001). In the present study, wheat extracts were added to oil/fat at a proportion of 2.5% (w/w); this is considerably higher than the amount used in other *in vitro* assays. Martinez-Tome et al. (2001) determined the oxidative stability of olive oil at 120 °C after adding wheat or oat bran at a 20% (w/w) level. The differences in the results can be attributed to the discrepancy between the concentration of additives used in the reaction medium. The PF for samples derived from wheat bran and oat bran ranged from 1.23 to 1.91 and 1.47 to 2.11, respectively (Martinez-Tome et al., 2001). In another study, wheat demonstrated better antioxidant properties than oat in stabilizing sunflower oil at 110 °C (Lehner et al., 2000). Results of this study indicated that wheat extracts possessed antioxidative properties and their inclusion in lipid materials would extend the shelf-life of products. Obviously, the effect is due to the antioxidant components, especially phenolics, present in the wheat extracts.

9.4.9. Influence of wheat phenolics on HO•-mediated supercoiled DNA scission

Tables 9.10 and 9.11 summarize the results of inhibition of DNA strand cleavage by Fenton reaction mediated HO• upon introduction of wheat extracts. The whole grains and milling fractions of two wheat cultivars significantly differed in their ability to protect DNA from nicking by HO•. The antioxidant activity of wheat extracts against HO•-mediated DNA scission was concentration-dependent (Fig 9.1, Lanes 3-6).

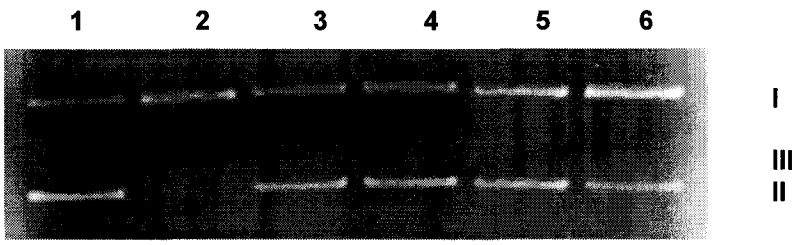


Fig 9.1. Agarose gel electrophoresis of supercoiled DNA treated with hydroxyl radical in the presence of extract of bran CWAD cultivar (Lane 1: Supercoiled DNA; Lane 2: Supercoiled DNA + OH•; Lane 3: Supercoiled DNA + OH• + 6 mg/ mL extract; Lane 4: Supercoiled DNA + OH• + 3 mg/ mL extract; Lane 5: Supercoiled DNA + OH• + 2 mg/ mL extract; Lane 6: Supercoiled DNA + OH• + 1 mg/ mL extract. Form I: Supercoiled DNA; Form II: Nicked open circular DNA; Form III: Linear DNA).

The extracts of bran, shorts and feed flour of the wheat cultivar CWHRS were most effective against DNA nicking and their efficiency ranged from 90 to 95%. The corresponding extracts of the CWAD wheat cultivar inhibited DNA scission by 87 to 90%. The flour extracts of CWAD and CWHRS demonstrated 29 to 49 and 32 to 52% inhibition, respectively. The extract derived from semolina fraction of CWAD possessed the lowest inhibiting power against DNA nicking and was 26 to 47%. Results of this assay may lend support to the results obtained using the deoxyribose assay. The poor capacity of flour and semolina in preventing DNA nicking indicates

Table 9.10. Inhibition (%) of hydroxyl radical mediated pBR 322 supercoiled DNA scission by extracts of whole grains and their milling fractions of two wheat cultivars over 1-6 mg/mL concentration

Milling fractions	CWAD (mg/mL)				CWHRS (mg/mL)			
	1	2	3	6	1	2	3	6
Whole grain	42	49	56	74	46	53	62	78
Bran	66	70	81	89	69	77	88	95
Flour	29	34	39	49	32	39	44	52
Shorts	57	64	83	90	60	70	84	92
Feed flour	61	66	78	87	61	72	81	90
Semolina	26	31	41	47	-	-	-	-

Values are the average of two determinations.

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

Table 9.11. Retention capacity (μg DNA/g defatted material) of pBR 322 supercoiled DNA against hydroxyl radical mediated scission by whole grains and milling fractions of two wheat cultivars, CWAD and CWHRS

Milling fraction	CWAD	CWHRS
Whole grain	395	351
Bran	786	808
Flour	172	174
Shorts	743	751
Feed flour	608	653
Semolina	204	-

Values are average of two determinations

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

that Fe (II) chelation was not a possible mechanism in this assay. The retention capacity of DNA by wheat fractions is summarized in Table 9.11. This also showed that the bran had the highest retention capacity followed by the shorts, feed flour, whole grains and flour for both wheat cultivars.

9.4.10. Phenolic acid composition of wheat as determined by HPLC

The phenolic acids identified using HPLC analysis of whole wheat and milling fractions included a benzoic acid derivative, namely vanillic acid, and three phenylpropanoids, namely *p*-coumaric, ferulic and sinapic acids; the latter being predominant in all fractions examined. Free and esterified phenolic acids were extracted into 80% aqueous ethanol. Sinapic acid was the predominant phenolic acid especially in the whole grain and bran of wheat followed by ferulic, vanillic and *p*-coumaric acids.

Table 9.12. Phenolic acid composition (free and esterified) of crude extracts of whole grain, bran and flour of two wheat cultivars, CWAD and CWHRS

Milling fraction	Content (µg/g crude extract)							
	Vanillic		*p*-Coumaric		Ferulic		Sinapic	
	Free	Esterified	Free	Esterified	Free	Esterified	Free	Esterified
CWAD								
Whole grain	1.24±0.06	5.39±0.19	0.12±0.06	0.74±0.06	0.43±0.01	46±0.122	0.19±0.002	1.08±1.61
Bran	8.48±0.32	26.08±0.85	0.64±0.03	3.6±0.21	7.84±0.31	34.24±1.17	1.27±0.11	65.83±4.98
Flour	0.55±0.01	1.26±0.04	0.08±0.004	0.59±0.04	0.63±0.02	1.85±0.08	0	1.05±0.08
CWHRS								
Whole grain	0.16±0.005	5.56±0.13	0.11±0.005	1.46±0.01	0.54±0.001	5.72±0.05	0.05±0.005	18.36±1.4
Bran	5.92±0.2	26.93±0.76	0.71±0.03	6.22±0.04	2.14±0.01	31.72±1.02	1.02±0.08	122.1±9.18
Flour	0.28±0.008	1.36±0.05	0.08±0.004	0.2±0.001	0.44±0.002	1.56±0.001	0	3.2±0.02

Each value is the mean ± standard deviation of three determinations.

Abbreviations: CWAD, Canadian Western amber durum; CWHRS, Canadian Western hard red spring

However, in flour of the CWAD cultivar ferulic acid was the predominant phenolic acid. Sinapic acid contents of bran fraction of CWAD and CWHRS, respectively, were approximately 45 and 63% of the total content of phenolic acids. In each fraction examined the content of esterified phenolics was higher than that of free phenolic acids. The extracts of CWHRS cultivar possessed a higher total content of phenolic acids compared to that of the CWAD cultivar.

9.5. Conclusions

Asymmetric distribution of antioxidative components in wheat grain was evident. The concentration of bioactive constituents was greater in the external layers; thus the bran fraction alone demonstrated a higher antioxidant activity than that of the other milling fractions.

CHAPTER 10

Summary and Conclusions

The optimum conditions for the extraction of wheat phenolics were determined using response surface methodology. A face-centred cube design was used to investigate the effects of three independent variables on the total antioxidant capacity. The high correlation of the model demonstrated that a second order polynomial model could be used to optimize the extraction of phenolic compounds from wheat for maximizing the total antioxidant capacity. Aqueous ethanol was found to be most effective in extracting phenolic compounds from whole grain and bran of both soft and hard wheat. Hence, the conditions for extraction of phenolics from whole grain and bran of soft wheat and whole grain and bran of hard wheat were 54% aqueous ethanol (solvent composition, v/v), 61 °C, 64 min; 49%, 64 °C, 60 min; 54%, 61 °C, 65 min and 50%, 62 °C, 60 min, respectively. Under optimized conditions the experimental values agreed with those predicted by ridge analysis. The experimental conditions allow a fast, quantitative and maximum extraction of phenolic compounds from wheat. In future work, this solvent system can be used in the extraction of wheat phenolics for further evaluaition of their biological properties. This confirms the validity and adequacy of the predicted models. The verification experiments proved that the predicted values for the model could be satisfactorily achieved within 95% confidence interval of experimental values.

Although approximately 50% aqueous ethanol was found to be the best solvent system for extraction of phenolics, the economy of the process as related to costs associated with the removal of water from the extracts makes this solvent system

unattractive for possible industrial use and hence the content of water was reduced to 20% (80% aqueous ethanol). The latter solvent system containing 20% water was thought to be adequate for enhancing the extraction of polar phenolic compounds from the raw material. This phenomenon would benefit from future verification. Moreover, instead of short term extraction at high temperatures, a prolonged extraction (16 h) was performed at 4 °C. The use of low temperature may allow preservation of any heat-labile compounds present in the samples. Thus, extraction conditions employed enabled extraction of wheat phenolics at 75-80% of their antioxidant capacity compared to that under optimum conditions.

In general, the efficacy of an antioxidant depends strongly on the oxidation conditions and substrate, and hence, a single method may produce just one approximation of the possibilities of an extract to act as an antioxidant. Several *in vitro* antioxidant assays were employed for determination of antioxidant capacity. The results showed that wheat antioxidants behaved differently in different assays. Antioxidant capacities depend not only on the chemical structures of the active components involved, but also on the model system empolyed for their evaluation. Moreover, the extraction method, identification of compounds and evaluation of antioxidant activity vary greatly among different studies, thus making it difficult to compare the results.

The antioxidative activity was significantly enhanced when wheat samples were subjected to simulated gastrointestinal pH treatment prior to extraction. The low pH may have improved extractability of the phenolic compounds from wheat. The simulated gastrointestinal conditions may also solubilize some phenolics bound to cell wall polymers. Moreover, possible release of some phytates may contribute to enhanced antioxidant activity of wheat. However, these phenomena were not verified in this study

and this may perhaps be carried out in the future research. Among different fractions of wheat examined, bran and germ exhibited the highest antioxidative capacities while the endosperm showed the lowest.

The content of bound phenolics in the hard and soft wheat samples examined was significantly higher than that of free and esterified phenolics in this study. The contribution of bound phenolics to the total antioxidant capacity and antioxidant potential, as determined by several *in vitro* assays was much higher compared to that of free and esterified phenolic fractions. Therefore, consideration of free and esterified phenolics above would lead to underestimation of phenolics as the bound fraction is not usually available for determination under normal conditions. Thus, results of this work emphasize the significance of bound phenolics in determination of total phenolic content of wheat. It would be appropriate to carry out a simulated digestion study using simulated gastric juices rather than just adjusting of the pH of samples in order to investigate the effect of true-to-nature digestion on the release of bound phenolics from wheat. The bioavailability of wheat phenolics in humans also needs to be fully evaluated.

This work further demonstrated that ethanolic extracts of pearled wheat grains and their by-products had multiple antioxidant effects in *in vitro* assays. The 10% by-product from wheat pearling demonstrated the greatest antioxidant activity. With subsequent removal of external layers, i.e. higher degree of pearling, the phenolic content and antioxidant activity decreased significantly. The trend was common to both pearled grains and by-products of wheat samples examined. There was a dilution of antioxidative constituents with endosperm at higher degree of pealing, as pearling removes the external layers, including the bran and aleurone layer which are the

outermost layers of the endosperm. Asymmetrical distribution of antioxidative components in the wheat grain was evident. In the milling fractions, the concentration of bioactive constituents was greater in the external layers; thus the bran fraction alone demonstrated a higher antioxidant activity than that of other milling fractions. The shorts and feed flour fractions are also known to contain bran and germ fractions in different proportions. Thus, uniformity cannot be expected with the shorts and feed flour. The antioxidant activity may vary depending on the actual composition of these fractions.

This work processing of cereals may thus have a significant effect on their antioxidant activity. Concentration of grain antioxidants will be drastically reduced during the refining process. As phenolic compounds are found to be concentrated in the outermost layers, the bran fractions resulting from pearling and milling may be used as a natural source of antioxidants and as a value-added product in the preparation of functional food ingredients or for enrichment of certain food commodities.

The different activities of various pearling and milling fractions of wheat can also be due to the existing differences in the composition of phenolics present. Moreover different wheat varieties contain different antioxidant profiles that could in turn contribute to their antioxidant activity. Results of the current indicated the low content of antioxidant components in the endosperm, and hence the flour and semolina fractions possessed the lowest antioxidant potential. Further, the commercial wheat mixtures possessed a higher amount of total phenolics compared with the pure varieties examined. The HPLC analysis of phenolics in this study was rather preliminary. This necessitates the importance of determining full phytochemical composition of wheat in future studies. Thus, the content and composition of diferulates, flavonoids and amides of hydroxycinnamic acid amides in wheat will be investigated.

Consumption of wheat as whole grains or the use of wheat bran as a dietary supplement may render maximum health benefits associated with wheat. Wheat phenolics may function as potential candidates in disease prevention. However, further research is needed in order to evaluate their activity under *in vivo* conditions and to elucidate their impact on human health. Results of this work contribute to the growing body of evidence that recognizes the role of phenolics as antioxidants. It also demonstrates that retention of by-products using minimal processing might be important in providing wheat products with full complement of their most active phytochemicals that are usually lost in processed wheat flour.

BIBLIOGRAPHY AND REFERENCES

Abdalla, A. E. and Roozen, J. P. 1999. Effect of plant extracts on the oxidative stability of sunflower oil and emulsion. Food Chemistry 64: 323-329.

Abdel-Aal, E-S, M. and Hucl, P. 2003. Comparison and stability of anthocyanins in blue-grained wheat. Journal of Agricultural and Food Chemistry 51: 2174-2180.

Abu-Amasha, R.; Croft, K. D.; Puddey, I. B.; Proudfoot, J. M. and Beilin, L. J. 1996. Phenolic content of various beverages determines the extent of inhibition of human serum and low-density lipoprotein oxidation *in vitro*: identification and mechanism of action of some cinnamic acid derivatives from red wine. Clinical Science 91: 449-458.

Abuja, P. M.; Murkovic, M. and Pfannhauser, W. 1998. Antioxidant and prooxidant activities of elderberry (*Sambucus nigra*) extract in low-density lipoprotein oxidation. Journal of Agricultural and Food Chemistry 46: 4091-4096.

Adom, K. K. and Liu, R. H. 2002. Antioxidant activity of grains. Journal of Agricultural and Food Chemistry 50: 6182-6187.

Adom, K. K.; Sorrells, M. E. and Liu, R. H. 2003. Phytochemical profiles and antioxidant activity of wheat varieties. Journal of Agricultural and Food Chemistry 51: 7825-7834.

Alabaster, O.; Tang, Z. and Shivapurkar, N. 1996. Dietary fiber and chemopreventive modelation of colon carcinogenesis. Mutation Research 350: 185-197.

Amarowicz, R. and Shahidi, F. 1996. A rapid chromatographic method for separation of individual catechins from green tea. Food Research International 29: 71-76.

Amarowicz, R. and Weidner, S. 2001. Content of phenolic acids in rye caryopses determined using DAD-HPLC method. Czech Jounal of Food Sciences 19: 201-205.

Amarowicz, R.; Raab, B. and Karamac, M. 1999. Antioxidative activity of an ethanolic extract of evening primrose. Nahrung 43: 216-217.

Amarowicz, R.; Raab, B. and Shahidi, F. 2003. Antioxidant activity of phenolic fractions of rapeseed. Journal of Food Lipids 10: 51-62.

Ames, B. N.; Gold, L. S. and Willett, W. C. 1995. Review: The causes and prevention of cancer. Proceedings of the National Academy of Sciences of the United States of America 92: 5258-5265.

Andreasen, M. F.; Christensen, L. P.; Meyer, A. S. and Hansen, A. 2000b. Ferulic acid dehydridimers in rye (*Secale cereale* L.) Journal of Cereal Science 31: 303-308.

Andreasen, M. F.; Christensen, L. P.; Meyer, A. S. and Hansen, A. 1999. Release of hydroxycinnamic and hydrobenzoic acids in rye by commercial plant cell wall degrading enzyme preparations. Journal of the Science of Food and Agriculture 79: 411-413.

Andreasen, M. F.; Christensen, L. P.; Meyer, A. S. and Hansen, A. 2000a. Content of phenolic acids and ferulic acid dehydrodimers in 17 rye (*Secale cereale* L.) varieties. Journal of Agricultural and Food Chemistry 48: 2837-2842.

Andreasen, M. F.; Landbo, A-K.; Christensen, L. P.; Hansen, A. and Meyer, A. S. 2001. Antioxidant effects of phenolic rye (*Secale cereale* L.) extracts monomeric hydroxycinnamates, and ferulic acid dehydrodimers on human low density lipoprotein. Journal of Agricultural and Food Chemistry 49: 4090-4096.

Anjum, F. M. and Walker, C. E. 1991. A review on the significance of starch and protein to wheat kernel hardness. Journal of the Science of Food and Agriculture 56: 1-13.

AOCS. 1990. Official methods and recommended practices of the American Oil Chemists' Society. 4th ed., American Oil Chemists' Society, Champaign, IL.

Aparicio, R.; Roda, L.; Albi, M. A. and Gutierrez, F. 1999. Effects of various compounds on vergin olive oil stability measured by Rancimat. Journal of Agricultural and Food Chemistry 47: 4150-4155.

Arnason, J. T.; Gale, J.; Conilh de Beyssac, B.; Sen, A.; Miller, S. S,; Philogene, B. J. R.; Lambert, J. D. H.; Fulcher, R. G.; Serratos, A. and Mihm, J. 1992. Role of phenolics in resistance of maize grain to the stored grain insects, *Prostephanus truncates* (Horn) and *Sitophilus zeamais* (Motsch). Journal of Stored Product Research 28: 119-126.

Arnous, A.; Makris, D. P. and Kefalas, P. 2002. Correlation of pigment and flavonol content with antioxidant properties in selected aged regional wines from Greece. Journal of Food Composition and Analysis 15: 655-665.

Aruoma, O. I. 1996. Characterization of drugs as antioxidant prophylactics. Free Radical Biology and Medicine 20: 675-705.

Aruoma, O. I. 1998. Free radicals, oxidative stress, and antioxidants in human health and disease. Journal of the American Oil Chemists' Society 75: 199-212.

Aruoma, O. I. 1999. Antioxidant actions of plant foods: use of oxidative DNA damage as a tool for studying antioxidant efficacy. Free Radical Research 30: 419-428.

Aruoma, O. I. and Halliwell, B. 1998. DNA and free radicals: Techniques, mechanisms and applications. OICA International, Saint Lucia.

Aruoma, O. I.; Spencer, J. P. E. and Mahmood, N. 1999. Protection against oxidative damage and cell death by the natural antioxidant ergothioneine. Food and Chemical Toxicology 37: 1043-1054.

Awika, J. M.; Dykes, L.; Gu, L.; Rooney, L. W. and Prior, R. L. 2003. Processing of sorghum (*Sorghum bicolor*) and sorghum products alters procyanidin oligomer and polymer distribution and content. Journal of Agricultural and Food Chemistry 51: 5516-5521.

Azuma, K.; Nakayama, M.; Koshioka, M., Ippoushi, K.; Yamaguchi, Y.; Kohata, K.; Yamauchi, Y.; Ito, H. and Higashio, H. 1999. Phenolic antioxidants from the leaves of Corchorus olitorius. Journal of Agricultural and Food Chemistry 47: 3963-3966.

Balentine, D. A.; Albano, M. C. and Nair, M. G. 1999. Role of medicinal plants, herbs and spices in protectineg human health. Nutrition Reviews 7: S41-S45.

Baranowski, J. D.; Davidson, P. M.; Nagel, C. W. and Branen, A. L. 1980. Inhibition of Saccharomyces cerevisiae by naturally occurring hydroxycinnamates. Journal of Food Science 45: 592-594.

Bartolome, B. and Gomez-Cordoves, C. 1999. Barley spent grain: release of hydroxycinnamic acids (ferulic and *p*-coumaric acids) by commercial enzyme preparations. Journal of the Science of Food and Agriculture 79: 435-439.

Baublis, A.; Decker, E. A. and Clydesdale, F. M. 2000. Antioxidant effect of aqueous extracts from wheat based ready-to-eat breakfast cereals. Food Chemistry 68: 1-6.

Beckman, K. B. and Ames, B. N. 1998. The free radical theory of aging matures. Physiological Reviews 78: 547-581.

Belderok, B. 2000. Developments in bread-making processes. Plant Foods for Human Nutrition 55: 1-14.

BeMiller, J. N. and Whistler, R. L. 1996. Carbohydrates. In: Food Chemistry. Ed. O. R. Fennema. Dekker, New York, NY. pp 178-185.

Benzie, I. F. F. and Strain, J. J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. Analytical Biochemistry 239: 70-76.

Benzie, I. F. F. and Strain, J. J. 1999. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods in Enzymalogy 299: 15-27.

Benzie, I. F. F. and Szeto, Y. T. 1999. Total antioxidant capacity of teas by the ferric reducing/antioxidant power assay. Journal of Agricultural and Food Chemistry 47: 633-636.

Bewly, J. D. and Black, M. 1994. Seeds: Physiology of development and germination. Plenum Press, New York, NY.

Blois, M. S. 1958. Antioxidant determinations by the use of a stable free radical. Nature 26: 1199-1200.

Box, G. E. P. and Wilson, K. B. 1951. On the experimental attainment of optimum conditions. Journal of the Royal Statistical Society 13: 1-45.

Braca, A.; Politi, M.; Sanogo, R.; Sanou, H.; Morelli, I.; Pizza, C. and De Tommasi, N. 2003. Chemical composition and antioxidant activity of phenolic compounds from wild and cultivated *Sclerocarya birrea* (Anacardiaceae) leaves. Journal of Agricultural and Food Chemistry 51: 6689-6695.

Bravo, L. 1998. Polyphenols: chemistry, dietary sources, metabolism and nutritional significance. Nutrition Reviews 56: 317-333.

Bravo, L.; Abia, R. and Saura-Calixto, F. 1994. Polyphenols as dietary fiber associated compounds, comparative study on *in vivo* and *in vitro* properties. Journal of Agricultural and Food Chemistry 42: 1481-1487.

Breen, A. P. and Murphy, J. A. 1995. Reactions of oxyradicals with DNA. Free Radical Biology and Medicine 18: 1033-1078.

Brielmann, H. L. Jr. 1999. Phytochemicals: the chemical components of plants. In: Natural products from plants. Eds. P. B. Kaufman, L. J. Cseke, S. Warber, J. A. Duke & H. L. Brielmann. CRC Press, Boca Raton, FL. pp 1-36.

Bryngelsson, S.; Dimberg, L. H. and Kamal-Eldin, A. 2002. Effects of commercial processing on levels of antioxidants in oats (*Avena sativa* L.). Journal of Agricultural and Food Chemistry 50: 1890-1896.

Buchanan, C. J.; Wallace, G. and Fry, S. C. 1996. *In vivo* release of ^{14}C-labelled phenolic groups from intact dietary spinach cell walls during passage through the rat intestine. Journal of the Science of Food and Agriculture 71: 459-469.

Buege, J. A. and Aust, S. D. 1978. Microsomal lipid peroxidation. Methods in Enzymology 52: 302-310.

Bunzel, M.; Ralph, J.; Funk, C. and Steinhart, H. 2003a. Isolation and identification of a ferulic acid dehydrodimer from saponified maize bran insoluble fiber. European Food Research and Technology 217: 128-133.

Bunzel, M.; Ralph, J.; Kim, H., Lu, F.; Ralph, S. A.; Marita, J. M.; Hatfield, R. D. and Steinhart, H. 2003b. Sinapate dehydrodimers and sinapate-ferulate heterodimers in cereal dietary fiber. Journal of Agricultural and Food Chemistry 51: 1427-1434.

Bunzel, M.; Ralph, J.; Martia, J. M.; Hatfield, R. D.; Steinhart, H. 2001. Diferulates as structural components in soluble and insoluble cereal dietary fibre. Journal of the Science of Food and Agriculture 81: 653-660.

Bushuk, W. 1998. Wheat breeding for end-product use. Euphytica 100: 137-145.

Butt, V. S. and Lamb, C. J. 1981. Oxygenases and the metabolism of plant products. In: Biochemistry of plants. Ed. E. E. Conn. Academic Press, New York, NY. pp 627-665.

Buxiang, S. and Fukuhara, M. 1997. Effects of co-administration of butylated hydroxytoluene, butylated hydroxyanisole and flavonoids on the activation of mutagens and drug-metabolizing enzymes in mice. Toxicology 26: 61-72.

Cacace, J. E. and Mazza, G. 2002. Extraction of anthocyanins and other phenolics from black currants with sulfured water. Journal of Agricultural and Food Chemistry 50: 5939-5946.

Cacace, J. E. and Mazza, G. 2003a. Optimization of extraction of anthocyanins from black currants with aqueous ethanol. Journal of Food Science 68: 240-248.

Cacace, J. E., and Mazza, G. 2003b. Mass transfer process during extraction of phenolic compounds from milled berries. Journal of Food Engineering 59: 379-389.

Cao, G.; Giovanoni, M. and Prior, R. L. 1996b. Antioxidant capacity decreases during growth but not aging in rat serum and brain. Archives of Gerontology and Geriatrics 22: 27-37.

Cao, G.; Muccitelli, H. U.; Sanchez-Moreno, C. and Prior, R. L. 2001. Anthocyanins are absorbed in glycated forms in elderly women: A pharmacokinetic study. American Journal of Clinical Nutrition 73: 920-926.

Cao, G.; Sofic, E. and Prior, R. L. 1996a. Antioxidant capacity of tea and common vegetables. Journal of Agricultural and Food Chemistry 44:3426-3431.

Cao, G.; Sofic, E. and Prior, R. L. 1997. Antioxidant and prooxidant behaviour of flavonoids: structure-activity relationships. Free Radical Biology and Medicine 22: 749-760.

Cao, G.; Verdon, C. P.; Wu, A. H. B.; Wang, H. and Prior, R. L. 1995. Automated assay of oxygen radical absorbance capacity with the COBAS FARA II. Clinical Chemistry 41: 1738-1745.

Caragay, A. B. 1992. Cancer-preventive foods and ingredients. Food Technology 46: 65-68.

Carter, P. 1971. Spectrophotometric determination of serum iron at the submicrogram level with a new reagent (ferozine). Analytical Biochemistry 40: 450-458.

Cassidy, A. 1996. Physiological effects of phytoestrogens in relation to cancer and other human health risks. Proceedings of the Nutrition Society. 55: 399-418.

Castelluccio, C.; Bolwell, G. P.; Gerrish, C. and Rice-Evans, C. 1996. Differential distribution of ferulic acid to the major plasma constituents in relation to its potential as an antioxidant. Biochemical Journal 316: 691-694.

Castelluccio, C.; Paganga, G.; Melikian, N.; Bolwell, G. P.; Pridham, J.; Sampson, J. and Rice-Evans, C. 1995. Antioxidant potential of intermediates in phenylpropanoid metabolism in higher plants. FEBS Letters 368: 188-192.

Chait, A.; Brazg, R. L.; Tribble, D. L. and Krauss, R. M. 1993. Susceptibility of small, dense, low-density lipoproteins to oxidative modification in subjects with the atherogenic lipoprotein phenotype, pattern B. American Journal of Medicine 94: 350-356.

Chen, J. H. and Ho, C.-T. 1997. Antioxidant activities of caffeic acid and its related hydroxycinnamic acid compounds. Journal of Agricultural and Food Chemistry 45: 2374-2378.

Chen, K. and Frei, B. 1997. The effect of histidine modification on copper-dependent lipid peroxidation in human low-density lipoprotein. Redox Reports 3: 175-181.

Chen, X. and Ahn, D. U. 1998. Antioxidant activities of six natural phenolics against lipid oxidation induced by Fe^{2+} or ultraviolet light. Journal of the American Oil Chemists' Society 75: 1717-1721.

Chen, Y.; Wang, M.; Rosen, R. T. and Ho, C-T. 1999. 2,2-Diphenyl-1-picrylhydrazyl radical-scavenging active components from *Polygonum multiflorum* Thunb. Journal of Agricultural and Food Chemistry 47: 2226-2228.

Cheryan, M. 1980. Phytic acid interactions in food systems. CRC Critical Reviews in Food Science Nutrition 13: 296-335.

Chevolleau, S.; Mallet, J. F.; Ucciani, E.; Gamisans, J. and Gruber, M. 1992. Antioxidant activity in leaves of some Mediterraniean plants. Journal of the American Oil Chemists' Society 69: 1269-1271.

Chidambara Murthy, K. N.; Singh, R. P. and Jayaprakasha, G. K. 2002. Antioxidant activities of grape (*Vitis vinifera*) pomace extracts. Journal of the Agricultural and Food Chemistry 50: 5909-5914.

Chu, Y.; Chang, C. and Hsu, H. 2000. Flavonoid content of several vegetables and their antioxidant activity. Journal of the Science of Food and Agriculture 80: 561-566.

Cipollini, M. L. and Levey, D. J. 1997. Secondary metabolites of fleshy vertebrate-dispersed fruits: adaptive hypotheses and implications for seed dispersal. American Naturalist 150: 346-373.

Cook, N. C. and Samman, S. 1996. Flavonoids–Chemistry, metabolism, cardioprotective effects and dietary sources. Journal of Nutrition and Biochemistry 7: 66-76.

Corbellini, M.; Canevar, M. G.; Mazza, L.; Ciaffi, M.; Lafiandra, D. and Borghi, B. 1997. Effect of duration and intensity of heat shock during grain filling on dry matter and protein accumulation, technological quality, and protein composition in bread and durum wheat. Australian Journal of Plant Physiology 24: 245-260.

Coupland, J. N. and McClements, D. J. 1996. Lipid oxidation in food emulsions. Trends in Food Science and Technology 7: 83-91.

Cseke, L. J. and Kaufman, P. B. 1999. Regulation of metabolite synthesis in plants. In: Natural products from plants. Eds. P. B. Kaufman, L. J. Cseke, S. Warber, J. A. Duke & H. L. Brielmann. CRC Press, Boca Raton, FL. pp 91-121.

Cuppett, S. 1998. Plant production of biochemical compounds. INFORM 9: 588-590,592-595.

Daniel, W. W. 1991. Biostatistics: A Foundation for Ananlysis in the Health Sciences. 5th ed. John Wiley & Sons Inc., New York, NY.

Dávalos, A.; Gómez-Cordovés, C. and Bartolomé, B. 2004. Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. Journal of Agricultural and Food Chemistry 52: 48-54.

De Graaf, J.; Hak-Lemmers, H. L. M.; Hectors, M. P. C.; Demacker, P. N. M.; Hendricks, J. C. M. and Stalenhoef, A. F. H. 1991. Enhanced susceptibility to in vitro oxidation of the dense low density lipoprotein subfraction in healthy subjects. Arteriosclerosis and Thrombosis 11: 298-306.

Decker, E. A. and Hultin, H. O. 1990. Factors influencing catalysis of lipid oxidation by the soluble fraction of mackerel muscle. Journal of Food Science 55: 947-950, 953.

Decker, E. A.; Ivanov, V.; Zhu, B-Z. and Frei, B. 2001. Inhibition of low-density lipoprotein oxidation by carnosine and histidine. Journal of Agricultural and Food Chemistry 49: 511-516.

Delange, R. J. and Glazer, A. N. 1989. Phycoerythrin fluorescence-based assay for peroxyl radicals: a screen for biologically relevant protective agents. Analytical Biochemistry 177: 300-306.

deMan, J. M.; Tie, F. and deMan, L. 1987. Formation of short chain volatile organic acids in the automated AOM method. Journal of the American Oil Chemists' Society 64: 993-996.

Dewanto, V.; Wu, X. and Liu, R. H. 2002. Processed sweet corn has higher antioxidant activity. Journal of Agricultural and Food Chemistry 50: 4959-4964.

Dexter, J. E. 1993. End-use quality implications of grading factors in wheat. In: Grains and Oilseeds: Handling, Marketing, Processing. Ed. E. Bass. Canadian International Grains Institute, Winnipeg, MN. pp 739-777.

Dexter, J. E. and Edwards, N. M. 1998a. The implications of frequently encountered grading factors on the processing quality of common wheat. Association of Operative Millers Bulletin, June. pp 7115-7122.

Dexter, J. E. and Edwards, N. M. 1998b. The implications of frequently encountered grading factors on the processing quality of durum wheat. Association of Operative Millers Bulletin, October. pp 7165-7171.

Dexter, J. E. and Marchylo, B. A. 2001. http://www.grainscanada.gc.ca/pubs/conf-paper/Dexter/trends/qualreq-e.pdf

Dexter, J. E. and Matsuo, R. R. 1979. Effect of starch on pasta dough rheology and spaghetti cooking quality. Cereal Chemistry 56: 190-195.

Dexter, J. E. and Wood, P. J. 1996. Recent applications of debranning of wheat before milling. Trends in Food Science and Technology 7: 35-40.

Dick, J. W. and Matsuo, R. R. 1988. Durum wheat and pasta products. In: Wheat, Chemistry and Technology. Ed. Y. Pomeranz. American Association of Cereal Chemists, St. Paul, MN. pp 507-547.

Dietrych-Szostak, D. and Oleszek, W. 1999. Effect of processing on the flavonoid content in buckwheat (*Fagopyrum esculentum* Möench) grain. Journal of Agricultural and Food Chemistry 47: 4384-4387.

Dittrich, R.; El-massry, F.; Kunz, K.; Rinaldi, F.; Peich, C. C.; Beckmann, M. W. and Pischetsrieder, M. 2003. Maillard reaction products inhibit oxidation of human low-density lipoproteins *in vitro*. Journal of Agricultural and Food Chemistry 51: 3900-3904.

Dizdaroglu, M. 1991. Chemical determination of free-radical induced damage to DNA. Free Radical Biology and Medicine 10: 225-242.

Donovan, J. L.; Bell, J. R.; Kasim-Karakas, S.; German, J. B.; Walzem, R. L.; Hansen, R. J. and Waterhouse, A. L. 1999. Catechin is present as metabolites in human plasma after consumption of red wine. Journal of Nutrition 129: 1662-1668.

Duh, P-D. 1998. Antioxidant activity of Budrock (*Arctium laooa* Linn): its scavenging effect on free radical and active oxygen. Journal of the American Oil Chemists' Society 75: 455-461.

El-Alim, S. S. L. A.; Lugasi, A.; Hovari, J. and Dworschak, E. 1999. Culinary herbs inhibit lipid oxidation in raw and cooked minced meat patties during storage. Journal of the Science of Food and Agriculture 79: 277-285.

El-Saadani, M.; Esterbauer, H.; El-Sayed, M.; Goher, M.; Nassar, A. Y. and Juergens, G. 1989. A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. Journal of Lipid Research 30: 627-630.

Emmons, C. L. and Peterson, D. M. 1999. Antioxidant activity and phenolic contents of oat groats and hulls. Cereal Chemistry 76: 902-906.

Emmons, C. L.; Peterson, D. M. and Paul G. L. 1999. Antioxidant capacity of oat (*Avina sativa* L.) extracts. 2. *In vitro* antioxidant activity and contents of phenolic and tocol antioxidants. Journal of Agricultural and Food Chemistry 47: 4894-4898.

Erikkson, C. F. 1982. Lipid oxidation catalysts and inhibitors in in raw materials and processed foods. Food Chemistry 9: 3-11.

Esterbauer, H. and Ramos, P. 1995. Chemistry and pathophysiology of oxidation of LDL. Reviews of Physiology Biochemistry and Pharmacology 127: 33-64.

Esterbauer, H. J.; Gebicki, J.; Puhl, H. and Juergens, G. 1992. The role of lipid peroxidation and antioxidants on oxidative modification of LDL. Free Radical Biology and Medicine 13: 341-390.

Esterbauer, H.; Schaur, R. G. and Zollner, H. 1991. Chemistry an biochemistry of 40hydroxynonenal, malonaldehyde, and related aldehydes. Free Radical Biology and Medicine 11: 81-128.

Esterbauer, H.; Striegl, G.; Puhl, J. and Rotheneder, M. 1989. Continuous monitoring of *in vitro* oxidation of human low density lipoprotein. Free Radical Research and Communications 6: 67-75.

Evars, A. D. and Bechtel, D. B. 1988. Microscopic structure of the wheat grain. In: Wheat Chemistry and Technology. Eds. G. Fabriani & C. Lintas. American Association of Cereal Chemists, St. Paul, MN. pp 47-92.

Febles, C. I.; Arias, A.; Hardisson, A.; Rodriguez-Alvarez, C. and Sierra, A. 2002. Phytic acid level in wheat flours. Journal of Cereal Science 36: 19-23.

Feillet, P. 1988. Protein and enzyme composition of durum wheat. In: Durum, Chemistry and Technology. Eds. G. Fabriani & C. Lintas. American Association of Cereal Chemists, St. Paul, MN. pp 93-120.

Finney, K. F.; Yamazaki, W. T.; Youngs, V. L. and Rubenthaler, G. L. 1987. Quality of hard, soft and durum wheats. In: Wheat and wheat improvement. Ed. E. G. Heyne. Agronomy Monograph No. 13, 2nd edition, ASA-CCSA-SSSA, Madison, WI. pp 677-748.

Fisch, K. M.; Bohm, V.; Wright, A. D. and Konig, G. M. 2003. Antioxidative meroterpenoids from the brown alga *Cystoseira crinita*. Journal of Natural Products 66: 968-975.

Fogliano, V.; Verde, V.; Randazzo, G., Ritieni, A. 1999. Methods for measuring antioxidant activity and its application to monitoring the antioxidant capacity of wines. Journal of Agricultural and Food Chemistry 47: 1035-1040.

Fortini, S. 1988. Some specific aspects of durum wheat and pasta cooking quality evaluation in Europe. In: Durum, Chemistry and Technology. Eds. G. Fabriani & C. Lintas. American Association of Cereal Chemists, St. Paul, MN. pp 229-233.

Fraga, C. C.; Shigenaga, M. K.; Park, J. W.; Degan, P. and Ames, B. N. 1990. Oxidative damage to DNA during aging: 8-hydroxy-2'-deoxyguanosine in rat organ DNA and urine. Proceedings of the National Academy of Sciences of the United States of America 87: 4533-4537.

Frankel, E. N. and Meyer, A. S. 2000. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. Journal of the Science of Food and Agriculture 80: 1925-1941.

Frankel, E. N.; Huang, S. W.; Aeschbach, R. and Prior, E. 1996. Antioxidant activity of a rosemary extract and its constituents, carnosic acid, carnosol and rosmarinic acid in bulk oil and oil-in-water emulsion. Journal of Agricultural and Food Chemistry 44: 131-135.

Frankel, E. N.; Kanner, J.; German, J. B.; Parks, E. and KInsella, J. E. 1993. Inhibition of human LDL oxidation by phenolic substances in red wine. Lancet 341: 454-457.

Frankel, E. N.; Waterhouse, A. L. and Teissedre, P. L. 1995. Principal phenolic phytochemicals in selected California wines and their antioxidant activity in inhibiting oxidation of human low density lipoproteins. Journal of Agricultural and Food Chemistry 43: 890-894.

Frega, N.; Mozzon, M. and Lercker, G. 1999. Effects of free fatty acids on oxidative stability of vegetable oil. Journal of the American Oil Chemists' Society 76: 325-329.

Frei, B. and Gaziano, J. M. 1993. Content of antioxidants, preformed lipid peroxides, and cholesterol as predictors of the susceptibility of human LDL to meat ion-dependent and –independent oxidation. Journal of Lipid Research 34: 2135-2145.

Fulcher, R. G. 1996. Morphological and chemical organization of the oat kernel. In Oats Chemistry and Technology, Ed by Webster FH. American Association of Cereal Chemists, St. Paul, MN, pp 47-74.

Gahler, S.; Otto, K. and Bohm, V. 2003. Alterations of vitamin C, total phenolics, and antioxidant capacity as affected by processing tomatoes to different products. Journal of Agricultural and Food Chemistry 51: 7962-7968.

Gao, L. and Mazza, G. 1996. Extraction of anthocyanin pigments from purple sunflower hulls. Journal of Food Science 61: 600-603.

Garcia-Conesa, M. T.; Plumb, G. W.; Kroon, P. A.; Wallace, G. and Williamson, G. 1997a. Antioxidant properties of ferulic acid dimers. Redox Reports 3: 239-244.

Garcia-Conesa, M. T.; Plumb, G. W.; Waldron, K. W.; Ralph, J. and Williamson, G. 1997b. Ferulic acid dehydrodimers from wheat bran: Isolation, purification and antioxidant properties of 8-O-4'-diferulic acid. Redox Reports 3: 319-323.

Garcia-Conesa, M. T.; Wilson, P. D.; Plumb, G. W.; Ralph, J. and Williamson, G. 1999. Antioxidant properties of 4,4'-dihydroxy-3,3'-dimethoxy-β,β'-bicinnamic acid (8,8'-diferulic acid, noncyclic form). Journal of the Science of Food and Agriculture 79: 379-384.

Ge, Y.; Ni, Y.; Yan, H,; Chen, Y. and Cai, T. 2002. Optimization of the supercritical fluid extraction of natural vitamin E from wheat germ using response surface methodology. Journal of Food Science 67: 239-243.

Ghesson, A.; Provan, G. J.; Russell, W. R.; Scobbie, L.; Richardson, A. J. and Stewart, C. 1999. Hydroxycinnamic acids in the digestive tract of livestock and humans. Journal of the Science of Food and Agriculture 79: 373-378.

Ghiselli, A.; Serafini, M.; Maiani, G.; Azzini, E. and Ferro-Luzzi, A. 1995. A fluorescence-based method for measuring total plasma antioxidant capability. Free Radical Biology and Medicine 18: 29-36.

Gieseg, S. P. and Esterbauer, H. 1994. Low density lipoprotein is saturable by pro-oxidant copper. FEBS Letters 343: 188-194.

Giessauf, A.; Steiner, E. and Esterbauer, H. 1992. Early destruction of tryptophan residues of apolipoprotein B is a vitamin E-independent process during copper-mediated oxidation of LDL. Biochimica et Biophysica Acta 1256: 221-232.

Giovannelli, L.; Testa, G.; De Filippo, C.; Cheyneir, V.; Clifford, M. N. and Dolara, P. 2000. Effect of complex polyphenols and tannins from red wine on DNA oxidative damage of rat colon mucosa *in vivo*. European Journal of Nutrition 39: 207-212.

Giroux, M. J. and Morris, 1997. A glycine to serine change in puroindoline b is associated with wheat grain hardness and low levels of starch-surface fraibilin. Theoretcal and Applied Genetics 95: 857-864.

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

Grabber, J. H.; Ralph, J. and Hatfield, R. D. 1998a. Diferulate cross-links impede the enzymatic degradation of non-lignified maize walls. Journal of the Science of Food and Agriculture 77: 193-200.

Grabber, J. H.; Ralph, J. and Hatfield, R. D. 1998b. Ferulate cross-links limit the enzymatic degradation of synthetically lignified primary walls of maize. Journal of Agricultural and Food Chemistry 46: 2609-2614.

Graf, E. 1992. Antioxidant potential of ferulic acid. Free Radical Biology and Medicine 13, 435-448.

Gray, D. A.; Auerbach, S.; Hill, S.; Wang, R.; Campbell, G. M.; Webb, C. and South, B. 2000. Enrichment of oat antioxidant activity by dry milling and sieving. Journal of Cereal Science 32: 89-98.

Graybosch, R. A.; Peterson, C. J.; Shelton, D. R. and Baenziger, P.S. 1996. Genotypic and environmental modification of wheat flour protein composition in relation to end-use quality. Crop Science 36: 296-300.

Gutteridge, J. M. C. 1984. Reactivity of hydroxyl and hydroxyl-like radicals discriminated by release of thiobarbituric acid-reactive material from deoxy sugars, nucleosides and benzoate. Biochemical Journal 224: 761-767.

Gutteridge, J. M. C. and Halliwell, B. 2000. Free radicals and antioxidants in the year 2000: A historical look to the future. Annals of the New York Academy of Sciences 899, 136-147.

Haaland, P.D. 1989. Experimental Design in Biotechnology. New York: Marcel Dekker.

Hakala, P.; Lampi, A.-M.; Ollilainen, V.; Werner, U.; Murkovic, M.; Wähälä, K.; Karkola, S. and Phronen, V. 2002. Steryl phenolic acid esters in cereals and their milling fractions. Journal of Agricultural and Food Chemistry 50: 5300-5307.

Halder, J. and Bhaduri, A. N. 1998. Protective role of black tea against oxidation of human red blood cells. Biochemistry and Biophysics Research Communications 244: 903-907.

Halliwell, B. 1996. Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. Free Radical Research 25: 57-74.

Halliwell, B. and Gutteridge, J. M. C. 1984. Lipid peroxidation, oxygen radicals, cell damage and antioxidant therapy. Lancet 1: 1396-

Halliwell, B. and Gutteridge, J. M. C. 1989. Free radicals in Biology and medicine. Clarendon Press, Oxford, UK.

Halliwell, B.; Gutteridge, J. M. C. and Cross, C. E. 1992. Free radicals, antioxidants, and human disease-where are we now? Journal of Laboratory and Clinical Medicine 119: 598-620.

Halliwell, B.; Gutteridge, J. M. C. and Grootveld, M. 1987. Methods for the measurement of hydroxyl radicals in biochemical systems: Deoxyribose degradation and aromatic hydroxylation. Methods in Biochemical Analysis 33: 59-90.

Halliwell, B.; Murcia, M. A.; Chirico, S. and Aruoma, O. I. 1995. Free radicals and antioxidants in food and *in vivo*: what do they do and how do they work? Critical Reviews in Food Science and Nutrition 35: 7-20.

Handelman, G.; Cao, G.; Walter, M. F.; Nightingale, Z. D.; Paul, G. L.; Prior, R. L. and Blumberg, J. B. 1999. Antioxidant capacity of oat (*Avena sativa* L.) extracts. 1.

Inhibition of low-density lipoprotein oxidation and oxygen radical absorbance capacity. Journal of Agricultural and Food Chemistry 47: 4888-4893.

Haraguchi, H.; Ishikawa, H. and Kubo, I. 1997. Antioxidative action of diterpenoids from *Podocarpus nagi*. Plant Medica – Natural Products and Medicinal Plant research 63: 213-215.

Harborne, J. B. 1988. The flavonoids: recent advances. In: Plant pigments. Ed. T. W. Goodwin. Academic Press, London, UK. pp 299-343.

Harborne, J. B. 1991a. Flavonoid pigments. In: Herbivores; their interaction with secondary metabolites. Vol 1. The Chemical participants. Eds. G. A. Rosenthal & M. R. Berenbaum. Academic Press, San Diego, CA. pp 389-429.

Harborne, J. B. 1991b. The chemical defense of plant defense. In: Plant defenses against mammalian herbivory. Eds. R. T. Palo & C. T. Robbins. CRC Press, Boca Raton, FL. pp 45-59.

Harborne, J. B. 1994. The Falvonoids. Advances in research since 1986. Chapman & Hall, London, UK.

Harborne, J. B. 1999a. An overview of antinutritional factors in higher plants. In: Secondary plant products; Antinutritional and beneficial actions in animal feeding. Eds. J. C. Caygill & I. Muller-Harvey. Nottingham University Press, Nottingham, UK. pp 7-16.

Harborne, J. B. 1999b. Classes and functions of secondary products from plants. In: Chemicals from plants, perspectives on plant secondary products. Eds. N. J. Walton & D. E. Brown. Imperial College Press, London, UK. pp 1-25.

Harman, D. 1995. Role of antioxidant nutrients in aging: overview. Journal of the American Aging Association 18: 51-62.

Harris, P. J. and Ferguson, L. R. 1993. Dietary fibre: its composition and role in protection against colorectal cancer. Mutation Research 290: 97-110.

Hartley, R. D.; Morrison, W. H., III; Himmelsbach, D. S. and Borneman, W. S. 1990. Cross-linking of cell wall phenolic arabinoxylans in graminaceous plants. Phytochemistry 29: 3705-3709.

Haslam, E. 1989. Plant polyphenols: Vegetable tannins revisited. University Press, Cambridge, UK.

Hatcher, D. W. and Kruger, J. E. 1997. Simple phenolic acids in flours prepared from Canadian wheat: relationship to ash content, color, and polyphenol oxidase activity. Cereal Chemistry 74: 337-343.

He, Y. and Shahidi, F. 1997. Antioxidant activity of green tea and its catechins in a fish meat model system. Journal of Agricultural and Food Chemistry 45: 4262-4266.

Heilmann, J.; Calis, I.; Kirmizibekmez, H.; Schuhly, W.; Harput, S. and Sticher, O. 2000. Radical scavenger activity of phenylethanoid glycosides in FMLP stimulated human polymorphonuclear leukocytes: structure-activity relationship. Planta Medica – Natural Products and Medicinal Plant Research 66: 746-748.

Heilmann, J.; Resch, M. and Bauer, R. 1999. Antioxidant activity of constituents from Atractylodes lancea. Pharmaceutical and Pharmacological Letters. 8: 69-71.

Heinonen, I. M.; Meyer, A. S. and Frankel, E. N. 1998. Antioxidant activity of berry phenolics on human low-density lipoprotein and liposome oxidation. Journal of Agricultural and Food Chemistry 46: 4107-4112.

Herrmann, K. 1989. Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in food. Critical Reviews in Food Science and Nutrition 28: 315-347.

Hertog, M. G. L. 1996. Epidemiological evidence on potential health properties of flavonoids. Proceedings of the Nutrition Society 55: 383-398.

Hertog, M. G. L.; Feskens, E. J. M.; Hollman, P. C. H. and Katan, M. R. 1994. Dietary antioxidant flavonoids and cancer risk in the Zutphen elederly study. Nutririon and Cancer 22: 175-184.

Hertog, M. G. L.; Feskens, E. J. M.; Hollman, P. C.; Katan, M. B. and Kromhout, D. 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen elderly study. Lancet 342: 1007-1011.

Hertog, M. G. L.; Kromhout, D.; Aravanis, C.; Blackburn, H.; Buzina, R.; Fidanza, F.; Giampaoli, S.; Jansen, A.; Menotti, A.; Nedeljkovic, S.; Pekkarinen, M.; Simic, B. S.; Toshima, H.; Feskens, E. J. M.; Hollman, P. C. H. and Katan, M. B. 1995. Flavonoid intake and long-term risk of coronary heart and cancer in the seven countries study. Archives in Internal Medicine 155, 381-386.

Hertog, M. G. L.; Vries, A de.; Ocke, M. C.; Schouten, A.; Bueno-de-Mesquita, H. B. and Verhagen, H. 1997. Oxidative DNA damage in humans: comparison between high and low habitual fruit and vegetable consumption. Biomarkers 2: 259-262.

Hiramoto, K.; Kido, K. and Kikugawa, K. 1994. DNA breaking by maillard products of glucose-amino acid mixtures formed in an aqueous system. Journal of Agricultural and Food Chemistry 42: 689-694.

Hiramoto, K.; Ojima, N.; Sako, K. and Kikugawa, K. 1996. effect of plant phenolics on the formation of the spin-adduct of hydroxyl radical and the DNA strand breaking by hydroxyl radical. Biological and Pharmaceutical Bulletin 19: 558-563.

Ho, C-T.; Chen, Q.; Shi, H.; Zhang, K. Q. and Rosen, R. T. 1992. Antioxidative effects of polyphenol extract prepared from various Chinese teas. Prev. Med. 21: 520-525.

Hochstein, P. and Atallah, A. S. 1988. The nature of oxidants and antioxidant systems in the inhibition of mutation and cancer. Mutation Research 202: 363-375.

Hodis, H. N.; Kramsch, D. M.; Avogaro, P.; Bittolo-Bon, G.; Cazzolato, G.; Hwang, J.; Peterson, H. and Sevanian, A. 1994. Biochemical and cytotoxic characteristics of an in vitro circulating oxidized low-density lipoprotein (LDL). Journal of Lipid Research 35: 669- 677.

Hopia, A. I.; Huang, S. W.; Schwarz, K.; German, J. B. and Frankel, E. N. 1996. Effect of different lipid systems on antioxidant activity of rosemary constituents carnosol and carnosic acid with or without α-tocopherol. Journal of Agricultural and Food Chemistry 44: 2030-2036.

Hu, C. and Kitts D. D. 2001. Free radical scavenging capacity as related to antioxidant activity and ginsenoside composition of Asian and North American ginseng extracts. Journal of the American Oil Chemists' Society 78: 249-255.

Hu. C. and Kitts, D. D. 2000. Studies on the antioxidant activity of Echinacea root extract. Journal of Agricultural and Food Chemistry 48: 1466-1472.

Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A. and Deemer, E. K. 2002. Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated β-cyclodextrin as the solubility enhancer. Journal of Agricultural and Food Chemistry 50: 1815-1821.

Huang, S. W. and Frankel, E. N. 1997. Antioxidant activity of tea catechins in different lipid systems. Journal of Agricultural and Food Chemistry 45: 3033-3038.

Huang, S. W.; Frankel, E. N. and German, B. J. 1994. Antioxidant activity of α- and γ tocopherols in bulk oils and oil-in-water emulsions. Journal of Agricultural and Food Chemistry 42: 2108-2114.

Huang, S. W.; Hopia, A.; Schwarz.; Frankel, E. N. and German, J. B. 1996. Antioxidant activity of α-tocopherol and Trolox in different lipid substrates: Bulk oi vs oil-in-water emulsions. Journal of Agricultural and Food Chemistry 44: 444-452.

Ishii, T. 1997. Structure and functions of ferulated polysaccharides, review. Plant Science 127: 111-127.

Iwami, K.; Hattori, M. and Ibuki, F. 1987. Prominent antioxidant effect of wheat gliadin on linoleate peroxidation in powder model systems at high water activity. Journal of Agricultural and Food Chemistry 35: 628-631.

Jacobs, D. R.; Slavin, J. and Marquart, L. 1995. Whole-grain intake and cancer: A review of the literature. Nutrition and Cancer 24: 221-299.

Jayarajah, C. N.; Tang, H-R.; Robertson, J. A. and Selvendran, R. R. 1997. Dephytinisation of wheat bran and the consequences for fibre matrix non-starch polysaccharides. Food Chemistry 58: 5-12.

Johnson, P.H. and Grossman, L.I. 1977. Electrophoresis of DNA in agarose gels. Optimizing separations of conformational isomers of bouble- and single- stranded DNAs. Biochemistry 16: 4217-4225.

Kagan, V. E.; Serbinova, E. A.; Forte, T.; Scita, G. and Packer, L. 1992. Recycling of vitamin E in human low density liproteins. Journal of Lipid Research 33: 385-397.

Kähkönen, M. P.; Hopia, A. I.; Vuorela, H. J.; Rauha, J-P.; Pihlaja, K.; Kujala, T. S.; Heinonen, M. 1999. Antioxidant activity of plant extracts containing phenolic compounds. Journal of Agricultural and Food Chemistry 47: 3954-3962.

Kaldy, M. S.; Rubenthaler, G. I.; Kereliuk, G. R.; Berhow, M. A. and Vandercook, C. E. 1991. Relationships of selected flour constituents to baking quality in soft white wheat. Cereal Chemistry 68: 508-512.

Kanner, J.; Frankel, E.; Granit, R.; German, B. and Kinsella, J. E. 1994. Natural antioxidants in grapes and wines. Journal of Agricultural and Food Chemistry 42: 64-69.

Kansci, G.; Genot, G.; Meynier, A. and Gandemer, G. 1997. The antioxidant activity of carnosine and its consequences on the volatile profiles of liposomes during iron/ascorbate-induced phospholipids oxidation. Food Chemistry 60: 165-175.

Kanski, J.; Aksenova, M.; Stoyanova, A. and Butterfield, D. A. 2002. Ferulic acid antioxidant protection against hydroxyl and peroxyl radical oxidation in synaptosomal and neuronal cell culture systems *in vitro*: structure-activity studies. Journal of Nutritional Biochemistry 13: 273-281.

Kehrer, J. P. 2000. The Haber-Weiss reaction and mechanisms of toxicity. Toxicology 149: 43-50.

Kern S. M.; Bennett, R. N.; Mellon, F. A.; Kroon, P. A. and Garcia-Conesa, M-T. 2003. Absorption of hydroxycinnamates in humans after high-bran cereal consumption. Journal of Agricultural and Food Chemistry 51: 6050-6055.

Kerry, N. L. and Abbey, M. 1997. Red wine and fractionated phenolic compounds prepared from red wine inhibit low density lipoprotein oxidation *in vitro*. Atherosclerosis 135: 930102.

Kim, D-O.; Lee, K. W.; Lee, H. J. and Lee, C. Y. 2002. Vitamin C equivalent antioxidant capacity (VCEAC) of phenolic phytochemicals. Journal of Agricultural and Food Chemistry 50: 3713-3717.

Kim, S. Y.; Kim, S. K.; Kim, M. J.; Oh, M. J. and Jung, M. Y. 1994. Antioxidant activities of selected oriental herb extracts. Journal of American Oil Chemists Society 71: 633-640.

Kinsella, J. E.; Frankel, E.; German, B. and Kanner, J. 1993. Possible mechanisms for the protective role of antioxidants in wine and plant foods. Food Technology 47 (4): 85-89.

Kitts, D. D.; Wijewickreme, A. N. and Hu, C. 2000. Antioxidant properties of North American ginseng extract. Molecular and Cellular Biochemistry 203: 1-10.

Kochian, L. V. and Garvin, D. F. 1999. Agricultural approaches to improving phytonutrient content in plants: an overview. Nutrition Reviews 57: S13-S18.

Koo, W. W. and Taylor, R. D. 2003. 2003 Outlook of the U.S. and world wheat industries, 2002-2012. Agribusiness & Applied Economics Report No. 517. Centre for Agricultural Policy and Trade Studies, Department of Agribusiness and Applied Economics, North Dakota State University, ND.

Krinsky, N. I. 1994. The biological properties of carotenoids. Pure Applied Chemistry 50: 292-302.

Kris-Etherton, P. M.; Hecker, K. D.; Bonanome, A.; Coval, S. M.; Binkoski, A. E.; Hilpert, K. F.; Griel, A. E.; Etherton, A. E. 2002. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. American Journal of Medicine 113: 71S-88S.

Kroon, P. A. and Williamson, G. 1999. Hydroxycinnamates in plants and food: current and future perspectives, review.Journal of the Science of Food and Agriculture 79: 355-361.

Kroon, P. A.; Faulds, C. B.; Ryden, P.; Robertson, J. A. and Williamson, G. 1997. Release of covalently bound ferulic acid from fiber in the human colon. Journal of Agricultural and Food Chemistry 45: 661-667.

Krygier, K.; Sosulski, F. and Hodge, L. 1982a. Free, esterified, and insoluble bound phenolic acids. 1. Extraction and purification procedure. Journal of Agricultural and Food Chemistry 30: 330-334.

Krygier, K.; Sosulski, F. and Hogge, L. 1982b. Free, esterified and insoluble-bound phenolic acids. 2. Composition of phenolic acids in rapeseed flour and hulls. Journal of Agricultural and Food Chemistry 30: 334-336.

Kuo, C-C.; Chiang, W.; Liu, G-P.; Chien, Y-L.; Chang, J-Y.; Lee, C-K.; Lo, J-M.; Huang, S-L.; Shih, M-C. and Kou, Y-H. 2002. 2,2'-Diphenyl-1-picrylhydrazyl radical-scavenging active components from Adlay (*Coix lachrymal-jobi* L. var. ma-yuen Stapf) hulls. Journal of Agricultural and Food Chemistry 50: 5850-5855.

Kurulich, A. C.; Tsau, G. J., Brown, A.; Howard, L.; Klein, B. P.; Jeffery, E. H.; Kushad, M.; Wallig, M. A. and Juvik, J. A. 1999. Carotene, tocopherol and ascorbate contents in subspecies of Brassical oleracea. Journal of Agricultural and Food Chemistry 47: 1576-1581.

Kushi, L. H.; Meyer, K. A. and Jacobs, Jr. D. R. 1999. Cereals, Legumes, and chronic disease risk reduction: Evidence from epidemiologic studies. American Journal of Clinical Nutrition 70: 451S-458S.

Kwon, J. H.; Belanger, J. M. R. and Pare, J. R. J. 2003. Optimization of microwave-assisted extraction (MAP) for ginseng components by response surface methodology. Journal of Agricultural and Food Chemistry 51: 1807-1810.

Lai, S. –M.; Gray, J. I.; Booren, A. M.; Rhonda, L. C. and John, L. G. 1995. Assessment of off-flavour development in restructured chicken nuggets using hexanal and TBARS measurements and sensory evaluation. Journal of the Science of Food and Agriculture 67: 447-452.

Laranjinha, J. A. N; Almeida, L. M. and Madeira, V. M. C. 1994. Reactivity of dietary phenolic acids with peroxyl radicals: antioxidant activity upon low density lipoprotein peroxidation. Biochemical Pharmacology 48: 487-494.

Laranjinha, J.; Almeida, L. and Madeira, V. 1995a. Reduction of ferrylmyoglobin by dietary phenolic derivatives of cinnamic acid. Free Radical Biology and Medicine 19: 329-338.

Laranjinha, J.; Vieira, O.; Madeira, V. and Almeida, L. 1995b. Two related antioxidants with opposite effects on vitamin E content in low density lipoproteins oxidized by ferrylmyoglobin: consumption vs regeneration. Archives of Biochemistry and Biophysics 323: 373-381.

Larson, R. A. 1988. The antioxidants of higher plants. Phytochemistry 27: 967-978.

Lass, A.; Witting, P.; Stocker, R. and Esterbauer, H. 1996. Inhibition of copper- and peroxyl radical-induced LDL lipid oxidation by ebselen: Antioxidant actions in addition to hydroperoxide-reducing activity. Biochimica et Biophysica Acta – Lipids and Lipid Metabolism 1303: 111-118.

Lee, J.; Renita, M.; Fioritto, R. J.; Martin, S. St.; Schwartz, S. J. and Vodovotz, Y. 2004. Isoflavone characterization and antioxidant activity of Ohio soybeans. V Journal of Agricultural and Food Chemistry 52: 2647-2651.

Lee, S. K.; Mei, L. and Decker, E. A. 1996. Lipid oxidation in cooked turkey as affected by added antioxidant enzymes. Journal of Food Science 61: 726-728, 795.

Lehtinen, P. and Laakso, S. 1997. Antioxidative-like effect of different cereals and cereal fractions in aqueous suspension. Journal of Agricultural and Food Chemistry 45: 4606-4611.

Lempereur, I.; Rouau, X.; Abecassis, J. 1997. Genetic and agronomic variation in arabinoxylan and ferulic acid contents of durum wheat (*Triticum durum* L.) grain and its milling fractions. Journal of Cereal Science 25: 103-110.

Lempereur, I.; Surget, A. and Rouau, X. 1998. Variability in dehydrodiferulic acid composition of durum wheat (*Triticum durum* Desf.) and distribution in milling fractions. Journal of Cereal Science 28: 251-258.

Lintas, C. 1988. Carbohydrates of durum wheat. In: Durum, Chemistry and Technology. Eds. G. Fabriani & C. Lintas. American Association of Cereal Chemists, St. Paul, MN. pp 121-138.

Liu, F. F.; Ang, C. Y. W. and Springer, D. 2000. Optimization of extraction conditions for active components in Hypericum perforatum using response surface methodology. Journal of Agricultural and Food Chemistry 48: 3364-3371.

Macheix, J-J.; Fleuriet, A. and Billot, J. 1990. Fruit phenolics. CRC Press, Boca Raton, FL.

Madhujith, T.; Amarowicz, R. and Shahidi, F. 2004. Phenolic antioxidants in beans and their effects on inhibition of radical-induced DNA damage. Journal of the American Oil Chemists' Society 81: 691-696.

Madsen, H. L. and Bertelsen, G. 1995. Spices as antioxidants. Trends in Food Science and Technology 6: 271-276.

Maillard, M. N.; Soum, M. H.; Boivin, P. and Berset, C. 1996. Antioxidative activity of barley and malt – Relationship with phenolic content. Lebensm.-Wiss. Technology 29: 238-244.

Maillard, M-N. and Berset, C. 1995. Evolution of antioxidant activity during kilning: role of insoluble bound phenolic acids of barley and malt. Journal of Agricultural and Food Chemistry 43: 1789-1793.

Marinova, E. and Yanishlieva, N. 1992. Effect of temperature on the autooxidative action of inhibitor in lipid autooxidation. Journal of the Science of Food and Agriculture 60: 313-318.

Marinova, E. M. and Yanishlieva, N. 1997. Antioxidative activity of extracts from selected species of the family Lamiaceae in sunflower oil. Food Chemistry 58: 245-248.

Martinez-Tome, M.; Jimenez, A. M.; Ruggieri, S.; Frega, N.; Strabbioli, R. and Murcia, A. 2001. Antioxidant properties of Mediterranean spices compared with common food additives. Journal of Food protection 64: 1412-1419.

Martinez-Tome, M.; Murcia, M. A.; Frega, N.; Ruggieri, S.; Jimenez, A. M.; Roses, F. and Parras, P. 2004. Evaluation of antioxidant capacity of cereal beans. Journal of Agricultural and Food Chemistry 52: 4690-4699.

Masuda, T.; Inaba, Y.; Maekawa, T.; Takeda, Y.; Yamaguchi, H.; Nakamoto, K.; Kuninaga, H.; Nishizato, S. and Nonaka, A. 2003. Simple detection method of powerful antiradical compounds in raw extracts of plants and its application for the identification of antiradical plant constituents. Journal of Agricultural and Food Chemistry 51: 1831-1838.

Mazza, G. (2000). Health aspects of natural colors: In: Natural Food and Colorants Science and Technology. Eds. G. J. Lauro & F. J. Francis. Marcel Dekker, New York, NY. pp 289-314.

Mazza, G.; Fukumoto, L.; Delaquis, P.; Girard, B. and Ewert, B. 1999. Anthocyanins, phenolics and color of Cabernet Franc, Merlot and Pinot Noir wines from British Colombia. Journal of Agricultural and Food Chemistry 47: 4009-4017.

Mendiratta, S. K.; Anjaneyulu, A. S. R.; Lakshmanan, V.; Naveena, B. M. and Bisht, G. S. Tenderizing and antioxidant effect of ginger extract on sheep meat. Journal of Food Science and Technology 37: 651-655.

Meyer, A. S.; Donovan, J. L.; Pearson, D. A.; Waterhouse, A. L. and Frankel, E. N. 1998. Fruit hydroxycinnamic acids inhibit human low-density lipoprotein oxidation *in vitro*. Journal of Agricultural and Food Chemistry 46: 1783-1787.

Miller, H. E. 1971. A simplified method for the evaluation of antioxidants. Journal of the American Oil Chemists' Society 48: 91-97.

Miller, H. E.; Rigelhof, F.; Marquart, L.; Prakash, A. and Kanter, M. 2000. Antioxidant content of whole grain breakfast cereals, fruits and vegetables. Journal of American College of Nutrition 19: 312S-319S.

Miller, N. J. and Rice-Evans, C. A. 1997. Cinnamates and hydroxybenzoates in the diet: Antioxidant activity assessed using the $ABTS^{\bullet+}$ radical cation. British Food Journal 99: 57-62.

Miller, N. J.; Rice-Evanc, C. A.; Davies, M. J.; Gopinathan, V. and Milner, A. 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clinical Science 84: 407-412.

Montgomery, D. C. 2001. Design and Analysis of Experiments. 5th ed. John Wiley & Sons, Inc. New York, NY.

Morelli, R.; Russo-Volpe, S.; Bruno, N. and Lo Scalzo, R. 2003. Fenton-dependent damage to carbohydrates: free radical scavenging activity of some simple sugars. Journal of Agricultural and Food Chemistry 51: 7418-7425.

Mueller-Harvey, I.; Harley, R. D.; Harris, P. J. and Curzon, E. H. 1986. Linkage of *p*-coumaroyl and feruloyl groups to cell-wall polysaccharides of barley straw. Carbohydrate Research 148: 71-85.

Murphy, A.; Kerry, J. P.; Buckley, J. and Gray, I. 1998. The antioxidative properties of rosemary oleoresin and inhibition of off-flavours in precooked roast beef slices. Journal of the Science of Food and Agriculture 77: 235-243.

Myers, R. H. and Montgomery, D. C. 2002. Response Surface Methodology: Process and Product Optimization Using Designed Experiments. 2nd Ed. John Wiley & Sons, Inc. New York, NY.

Naczk, M. and Shahidi, F. 1989. The effect of methanol-ammonia-water treatment on the content of phenolic acids of canola. Food Chemistry 31: 159-164.

Nardidni, M.; D'Aquino, M.; Tomassi, G.; Gentili, V.; Di Felice, M. and Scaccini, C. 1995. Inhibition of human low-density lipoprotein oxidation by caffeic acid and other hydroxycinnamic acid derivatives. Free Radical Biology and Medicine 19: 541-552.

Nardini, M.; Natella, F.; Gentili, V.; DiFelice, M. and Scaccini, C. 1997. Effect of caffeic acid dietary supplementation on the antioxidant defense system in rat: an in vivo study. Archives of Biochemistry and Biophysics 342: 157-160.

Natella, F.; Nardini, M.; DiFelice, M.; Scaccini, C. 1999. Benzoic and cinnamic acid derivatives as antioxidants: Structure-activity relation. Journal of Agricultural and Food Chemistry 47: 1453-1459.

Nawar, W. W. 1996. Lipids. In: Food Chemistry, 3rd Edition, Fennema, O. R. Marcel Dekker Inc., New York, NY, pp 225-314.

Neilsen, S. E.; Young, J. F.; Daneshvar, B.; Lauridsen, S. T.; Knuthsen, P.; Sandström, B. and Dragsted, L. O. 1999. Effect of parsley (*Petroselium crispum*) intake on urinary apigenin excretion, blood antioxidant enzymes and biomarkers for oxidative stress in human subjects. British Journal of Nutrition 81: 447-455.

Nenadis, N.; Boyle, S.; Bakalbassis, E. G. and Tsimidou, M. 2003a. An experimental approach to structure-activity relationships of caffeic and dihydrocaffeic acids and related monophenols. Journal of the American Oil Chemists' Society 80: 451-458.

Nenadis, N.; Zhang, H-Y. and Tsimidou, M. Z. 2003b. Structure-antioxidant activity relationship of ferulic acid derivatives: effect of carbon side chain characteristic groups. Journal of the Agricultural and Food Chemistry 51: 1874-1879.

Nishikimi, M.; Rao, N. A. and Yagi, K. 1972. The occurrence of superoxide anion in the rection of reduced phenazine methosulphate and molecular oxygen. Biochemistry and Biophysics Research Communications 46: 849-854.

Nunez-Delicado, E.; Sanchez-Ferrer, A. and Garcai-Carmona, F. 1997. Cyclodextrins as secondary antioxidants: synergism with ascorbic acid. Journal of Agricultural and Food Chemistry 45: 2830-2835.

Ohta, T.; Nakano, T.; Egashira, Y. and Sanada, H. 1997. Antioxidant activity of ferulic acid β-glucuronide in the LDL oxidation system. Bioscience, Biotechnology and Biochemistry 6: 1942-1943.

Olthof, M. R.; Hollman, P. C. H; and Katan, M. B. 2000. Chlorogenic acid and caffeic acid are absorbed in humansine concentrations in humans. Journal of Nutrition 131: 66-71.

Onyeneho, S. N. and Hettiarachchi, N. S. 1992. Antioxidant activity of durum wheat bran. Journal of Agricultural and Food Chemistry 40: 1496-1500.

Orth, R. A. and Shellenberger, J. A. 1988. Origin, production, and utilization of wheat. In: Wheat, Chemistry and Technology. Ed. Y. Pomeranz. American Association of Cereal Chemists, St. Paul, MN. pp 1-14.

Ou, B.; Hampsch-Woodill, M and Prior, R. L. 2001. Development and validation of an improved oxygen radical absorbance capcity assay using fluorescein as the fluorescent probe. Journal of Agricultural and Food Chemistry 49: 4619-4626.

Ou, B.; Hampsch-Woodill, M.; Flanagan, J.; Deemer, E. K.; Prior, R. L. and Huang, D. 2002. Novel fluorometric assay for hydroxyl radical prevention capacity using fluoroscein as the probe. Journal of Agricultural and Food Chemistry 50: 2772-2777.

Oyaizu M. 1986. Studies on products of browning reaction: antioxidative activities of products of browning reaction prepared from glucosamine. Japanese Journal of Nutrition 44:307-315.

Ozawa, T. 1997. Genetic and functional changes in mitochondria associated with aging. Physiological Reviews 77: 425-464.

Parajo, J. C.; Santos, V.; Dominguez, H. and Vazquez, M. 1995. NH_4OH-based pretreatment for improving the nutritional quality of single-cell protein (SCP). Applied Biochemistry and Biotechnology, 55: 133-150.

Park, N. Y.; Lee, G. D.; Jeong, Y. J. and Kwon, J. H. 1998. Optimization of extraction conditions for physicochemical properties of ethanol extracts from *Chrysanthemum boreale*. Journal of the Korean Society for Food Science and Nutrition 27 : 585-590.

Peterson, C. J.; Graybosch, R. A.; Baenziger, P. S. and Grombacher, A. W. 1992. Genotype and environment effects on quality characteristics of hard red winter wheat. Crop Science 32: 98-103.

Peterson, D. M. 2001. Oat antioxidants. Journal of Cereal Science 33: 115-129.

Peterson, D. M.; Emmons, C. L. and Hibbs, A. H. 2001. Phenolic antioxidants and antioxidant activity in pearling fractions of oat groats. Journal of Cereal Science 33: 97-103.

Pins, J. J.; Pereira, M. A.; Jacobs, D. R. Jr.; Marquart, L. and Keenan, J. M. 2001. Whole grains, cereal fiber, and chronic diseases: Experimental evidence and possible biologic mechanisms. In: CRC Handbook of Dietary Fiber in Human Nutrition. Ed. G. A. Spiller. CRC Press, New York, NY. pp 481-498.

Pinzino, C.; Capocchi, A.; Galleschi, L.; Saviozzi, F.; Nanni, B. and Zandomeneghi, M. 1999. Aging, free radicals and antioxidants in wheat seeds. Journal of Agricultural and Food Chemistry 47: 1333-1339.

Poehlman, J. M. and Sleper, D. A. 1995. Breeding Field Crops. Iowa State University Press, Ames, IA.

Pomeranz, Y. and Afework, S. 1984. The effect of kernel size in plump and shrunken kernels and of sprouting on kernel hardness in wheat. Journal of Cereal Science 2: 119-126.

Pomeranz, Y. and Williams, P. C. 1990. Wheat hardness: Its genetic, structural, and biochemical background, measurement, and significance. In: Advances in cereal science and technology, Vol X. Ed. Y. Pomeranz. American Association of Cereal Chemists, St. Paul, MN. pp 471-548.

Popov, I. and Lewin, G. 1999. Antioxidative homeostasis: characterization by means of chemiluminescent technique. Methods in Enzymology 300: 437-456.

Popov, I. N. amd Lewin, G. 1994. Photochemiluminescent detection of antiradical activity: II. Testing of nonenzymic water-soluble antioxidants. Free Radical Biology and Medicine 17: 267-271.

Popov, I. N. and Lewin, G. 1996. Photochemiluminescent detection of antiradical activity: IV. Testing of lipid-soluble antioxidants. J. Biochem. Biophys. Methods. 31: 1-8.

Prior, R. L. and Cao, G. 1999. *In vivo* total antioxidant capacity: comparison of different analytical methods. Free Radical Biology and Medicine. 27: 1173-1181.

Prior, R. L. and Cao, G. 2000. Antioxidant phytochemicals in fruits and vegetables: Diet and health implications. Horticultural Science 35: 588-592.

Prior, R. L.; Hoang, H.; Gu, L.; Wu, X.; Bacchiocca, M.; Howard, L.; Hampsch-Woodill, M.; Huang, D.; Ou, B. and Jacob, R. 2003. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity ($ORAC_{FL}$)) of plasma and other biological and food samples. Journal of Agricultural and Food Chemistry 51: 3273-3279.

Puhl, H.; Waeg, G. and Esterbauer, H. 1994. Methods to determine oxidation of low density lipoproteins. Methods in Enzymology 233: 425-441.

Pussayanawin, V.; Wetzel, D. L. and Fulcher, R. G. 1988. Fluorescence detection and measurement of ferulic acid in wheat milling fractions by microscopy and HPLC. Journal of Agricultural and Food Chemistry 36: 515-520.

Ramarathnam, N.; Osawa, T.; Ochi, H. and Kawakishi, S. 1995. The contribution of plant food antioxidants to human health. Trends in Food Science and Technology 6: 75-82.

Re, R.; Pellegrini, N.; Proteggente, A; Pannal, A.; Yang, M. and Rice-Evans, C. A. 1999. Antioxidant activity applying an improved ABTS radical cation decolrorization assay. Free Radical Biology and Medicine 26: 1231-1237.

Reische, D. W.; Lillard, D. A. and Eitenmiller, R. R. 2002. Antioxidants. In: Food Lipids. Eds. C. C. Akoh and D. B. Min. Marcel Dekker, New York, NY. pp 489-516.

Retsky, K. L.; Chen, K.; Zeind, J. and Frei, B. 1999. Inhibition of copper-induced LDL oxidation by vitamin C is associated with decreased copper-binding to LDL and 2-oxo-histidine formation. Free Radical Biology and Medicine 26: 90-98.

Rice-Evans, C. A. and Miller, N. 1997. Measurment of the antioxidant status of dietary constituents, low density lipoproteins and plasma. Prostaglandins, Leukotrienes and Essential Fatty Acids 57: 499-505.

Rice-Evans, C. A.; Miller, N. J. and Paganga, G. 1997. Antioxidant properties of phenolic compounds. Trends in Plant Science 2:152-159.

Rice-Evans, C. A.; Miller, N. J.; Bolwell, P. G.; Bramley, P. M. and Pridham, J. B. 1995. The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Radical Research 22: 375-384.

Rice-Evans, C. A.; Miller, N. J.and Paganga, G. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology and Medicine 20: 933-956.

Roberts, M. F. and Wink, M. 1999. Alkaloids: Biochemistry, ecological functions and medical applications. Plenum, New York, NY.

Roberts. M. F. and Strack, D. 1999. Biocxhemistry and physiology of alkaloids and betalains. In: Biochemistry of plant secondary metabolism. Ed. M. Wink. Annual plant reviews Vol. 2. Sheffield Academic Press, Sheffield, UK. pp 17-78.

Rowland, I. 1999. Optimal Nutrition: fibre and phytochemicals. Proceedings of the Nutrition Society 58: 415-420.

Ruch, R. J.; Cheng, S. T. and Klauring, J. E. 1989. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis 10: 1003-1008.

Salah, N.; Miller, N. J.; Paganga, G.; Tijburg, L.; Bolwell, G. P. and Rice-Evans, C. A. 1995. Polyphenolic flavonols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. Archives of Biochemistry and Biophysics 322: 339-346.

Salonen, Y.; Ylä-Herttuala, S.; Yamamoto, R.; Butler, S.; Korpela, H.; Salonen, R.; Nyyssönen, K,; Palinski, W. and Witztum, J. L. 1992. Autoantibody against oxidized LDL and progression of carotid atherosclerosis. Lancet 339: 883-887.

Samman, S.; Sandstrom, B.; Toft, M. B.; Bukhave, K.; Jensen, M.; Sorensen, S. S. and Hansen, M. 2001. Green tea or rosemary extract added to food reduces nonheme-iron absorption. American Journal of Clinical Nutrition 73: 607-612.

Sanchez-Moreno, C. 2002. Review: methods used to evaluate the free radical scavenging activity in foods and biological systems. Food Science and Technology International 8: 121-137.

Sanchez-Moreno, C. and Larrauri, J. A. 1998. Main methods used in lipid oxidation determination. Food Science and Technology International 4: 391-400.

Sanchez-Moreno, C.; Jimenez-Escrig, A. and Saura-Calixto, F. 2000. Study of low-density lipoprotein oxidizability indexes to measure the antioxidant activity of dietary polyphenols. Nutrition Research 20: 941-953.

Sang, S.; Lapsley, K.; Jeong, W-S.; Lachance, P. A.; Ho, C-T.; Rosen, R. T. 2002. Antioxidative phenolic compounds isolated from almond skins (*Prunus amygdalus* Batsch). Journal of Agricultural and Food Chemistry 50: 2459-2463.

Satue-Gracia, M. T.; Heinonen, M. and Frankel, E. N. 1997. Anthocyanins as antioxidants on human low-density lipoprotein and lecithin-liposome systems. Journal of Agricultural and Food Chemistry 45: 3362-3367.

Schneider, H.; Schwiertz, A.; Collins, M. D. and Blaut, M. 1999. Anaerobic transformation of quercetin-3-glucoside by bacteria from the human intestinal tract. Archives of Microbiology 171: 81-91.

Scott, B. C.; Butler, J.; Halliwell, B. and Aruoma, O. I. 1993. Evaluation of the antioxidant actions of ferulic acid and catechins. Free Radical Research and Communications 19: 241-253.

Senanayake, S. P. J. N. and Shahidi, F. 1999. Enzyme-assisted acidolysis of borage (*Borago officinalis* L.) and evening primrose (*Oenothera biennis* L.) oils: Incorporation of omega-3 polyunsaturated fatty acids. Journal of Agricultural and Food Chemistry 47: 3105-3112.

Senanayake, S. P. J. N. and Shahidi, F. 1999. Oxidative deterioration of borage and evening primrose oils as assessed by NMR spectroscopy. Journal of Food Lipids 6: 195-203.

Senanayake, S. P. J. N. and Shahidi, F. 2002. Lipase-catalyzed incorporation of docosahexaenoic acid (DHA) into borage oil: optimization using response surface methodology. Food Chemistry 77: 115-123.

Serafini, M.; Maiani, G. and Ferro-Luzzi, A. 1998. Alcohol-free red wine enhances plasma antioxidant capacity in humans. Journal of Nutrition 128: 1003-1007.

Shahidi, F. 1992. Prevention of lipid oxidation in muscle foods by nitrite and nitrite-free compositions. In: Lipid oxidation in Food, Angelo, A. J. St., Ed.; ACS symposium series 500, American Chemical Society, Washington DC, pp 161-182.

Shahidi, F. 1996. Natural Antioxidants. Chemistry, Health Effects and Applications. AOCS Press, Champaigne, IL.

Shahidi, F. 2000. Antioxidants in food and food antioxidants. Nahrung 44: 158-163

Shahidi, F. and Hong, C. 1991. Evaluation of malonaldehyde as a marker of oxidative rancidity in meat products. Journal of Food Biochemistry 15: 97-105.

Shahidi, F. and Naczk, M. 2004. Phenolics in Food and Nutraceuticals. CRC Press, New York, NY.

Shahidi, F. and Pegg, R. B. 1994a. Hexanal as an indicator of meat flavour deterioration. Journal of Food Lipids 1: 177-186.

Shahidi, F. and Pegg, R. B. 1994b. Hexanal as an indicator of the flavour deterioration of meat and meat products. In: Lipids in Food Flavours, Ho, C. T., Hartman, T. G., Eds.; ACS symposium series 558, American Chemical Society: Washington, DC.

Shahidi, F. and Wanasundara, P. K. J. P. D. 1992. Phenolic antioxidants. Critical Reviews in Food Science and Nutrition 32: 67-103.

Shahidi, F. and Wanasundara, U. N. 1998. Methods of measuring oxidative rancidity in fats and oils. In: Food Lipids Chemistry Nutrition and Biotechnology, Akoh, C. C., Min, D. B., Eds.; Marcel Dekker: New York, NY, pp 377-398.

Shahidi, F.; Rubun, L. J. and Wood, D. F. 1987. Control of lipid oxidation on cooked ground pork with antioxidants and dinitrosyl ferrohemochrome. Journal of Food Science 52: 564-567.

Sherwin, E. R. 1978. Oxidation and antioxidants in fat and oil processing. Journal of the American Oil Chemists' Society 55: 809-814.

Shimada, K.; Fijikawa, K.; Yahara, K. and Nakamura, T. 1992. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsions. Journal of Agricultural and Food Chemistry 40: 945-948.

Sies, H. 1991. Oxidative stress II: Oxidants and antioxidants. Academic press, London.

Sies, H. 1993. Strategies of antioxidant defense. European Journal of Biochemistry 215: 213-219.

Singh, K.; Khanna, A. K. and Chander, R. 1999. Hepatoprotective activity of ellagic acid against carbon tetrachloride induced hepatotoxicity in rats. Indian Journal of Experimental Biology 37: 1025-1026.

Singh, R. P.; Murthy, K. N. C.; Jayaprakasha, G. K. 2002. Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using in vitro models. Journal of Agricultural and Food Chemistry 50: 81-86.

Singleton, V. L. and Rossi, J. A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture 16: 144-158.

Singleton, V. L.; Timberlake, C. F. and Lea, A. G. H. 1978. The phinolic cinnamates of white grapes and wine. Journal of the Science of Food and Agriculture 29: 403-410.

Siriwardhana, S. S. K. W. and Shahidi, F. 2002. Antiradical activity of extracts of almond and its by-products. Journal of the American Oil Chemists' Society 79: 903-908.

Slavin, J. L.; Jacobs, D. and Marquart, L. 2000. Grain processing and nutrition. Critical Reviews in Food Science and Nutrition 40: 309-328.

Slavin, J.; Jacobs, D. and Marquart, L. 1997. Whole-grain consumption and chronic disease: protective mechanisms. Nutrition and cancer 27: 14-21.

Smith, M. M. and Hartley, R. D. 1983. Occurrence and nature of ferulic acid substitution of cell wall polysaccharides in graminaceous plants. Carbohydrate Research 118: 65-80.

Soler-Rivas, C.; Espín, J. C. and Wichers, H. J. 2000. An easy fast test to compare total free radical scavenger capacity of foodstuffs. Phytochemical Analysis 11: 330-338.

Sosulski, F.; Krzysztof, K. and Lawrence, H. 1982. Free, esterified, and insoluble-bound phenolic acids. 3. Composition of phenolic acids in cereal and potato flours. Journal of Agricultural and Food Chemistry 30: 337-340.

Sprong, R.C.; Winkelhuyzen-Janssen, A.; Aarsman, C.; van Oirschot, J.; van der Bruggen, T. and van Asbeck, B. 1998. Low-dose N-acetylcysteine protects rats against endotoxin-mediated oxidative stress, but high dose increases mortality. American Journal of Respiratory and Critical Care Medicine 157: 1283-1293.

Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C. and Witzum, J. L. 1989. Beyond cholesterol-modification of low density lipoprotein that increases its atherogenecity. New England Journal of Medicine 320: 915-924.

Stenvert, N. L. and Kingswood, K. 1977. The influence of the physical structure of the protein matrix on wheat hardness. Journal of the Science of Food and Agriculture 28: 11-19.

Strack, D. 1997. Phenolic metabolism. In: Plant biochemistry. Eds. P. M. Dey & J. B. Harborne, Academic Press, London, UK. pp 387-416.

Sun, B. and Fukuhara, M. 1997. Effects of co-administration of butylated hydroxitoluene, butylated hydroxyanisole and flavonoids on the activation of mutagens and drug-metabolizing enzymes in mice. Toxicology 122: 61-72.

Sun, R. C.; Tomkinson, J.; Zhu, W. and Wang, S. Q. 2000. Delignification of maize stems by peroxymonosulfuric acid, peroxyformic acid, peracetic acid, and hydrogen peroxide. 1. Physicochemical and structural characterization of the solubilized lignins. Journal of Agricultural and Food Chemistry 48: 1253-1262.

Sun, R. C.; Xiao, B. and Lawther, J. M. 1998. Fractional and structural characterization of ball-milled and enzyme lignins from wheat straw. Journal of Applied Polymer Science 68: 1633-1641.

Takeoka, G. R. and Dao, L. T. 2003. Antioxidant constituents of almond (Prunus dulcis (Mill.) D.A. Webb) hulls. Journal of Agricultural and Food Chemistry 51: 496-501.

Teissedre, P. L.; Frankel, E. N.; Waterhouse, A. L.; Peleg, H. and German, J. B. 1996. Inhibition of in vitro human LDL oxidation by phenolic antioxidants from grapes and wines. Journal of the Science of Food and Agriculture 70: 55-61.

Telez-Luis, S. J., Moldes, A. B., Alonso, J.L. & Vazquez, M. 2003. Optimization of lactic acid production by *Lactobicillus delbrueckii* through response surface methodology. Journal of Food Science 68: 1454-1458.

Temple, N. J. 2000. Antioxidants and disease: more questions than answers. Nutrition Research 20: 449-459.

Terao, J.; Karasawa, H.; Arai, H.; Nagao, A.; Suzuki, T. and Takama, K. 1993. Peroxyl radical scavenging activity of caffeic acid and its related phenolic compounds in solution. Biosciience, Biotechnology and Biochemistry 57: 1204-1205.

Tim, M. J. and Watts, B. M. 1958. Protection of cooked meats with phosphates. Food Technology 12: 240-243.

Tipples, K. H.; Kilborn, R. H. and Preston, K. R. 1994. Bread-wheat quality defined. In: Wheat Production, Properties, and Quality. Eds. W. Bushuk & V. F. Rasper. 8th World Congress of Food Science and Technology. Sep. 29-Oct. 4, 1992. Toronto, ON. pp 25-36.

Torel, J.; Cillard, J. and Cillard, P. 1986. Antioxidant activity of flavonoids and reactivity with peroxyl radical. Phytochemistry 25: 383-385.

Tsuda, T.; Watanabe, M.; Ohshima, K.; Norinobu, S.; Choi, S.; Kawakishi, S. and Osawa, T. 1994. Antioxidative activity of the anthocyanin pigments cyaniding 3-O-β-D-glucose and cyaniding. Journal of Agricultural and Food Chemistry 42: 2407-2410.

van Acker, S. A. B. E.; van den Berg, D-J.; Tromp, M. N. J. L.; Griffioen, D. H.; van Bennekom, W. P.; van der Vijgh, W. J. F. and Bast, A. 1996. Structural aspects of antioxidant activity of flavonoids. Free Radical Biology and Medicine 20: 331-342.

van den Berg, R.; Haenen, G. R. M. M.; van den Berg, H. and Bast, A. 1999. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. Food Chemistry 66: 511-517.

van Ruth, S. M.; Shaker, E. S. and Morrissey, P. A. 2001. Influence of methanolic extracts of soybean seeds and soybean oil on lipid oxidation in linseed oil. Food Chemistry 75: 177-184.

Vasquez, M. and Martin, A. 1998. Optimization of *Phaffia rhodozyma* continuous culture through response surface methodology. Biotechnology and Bioengineering 57: 314-320.

Velasco, J.; Andersen, M. L. and Skibsted, L. H. 2004. Evaluation of oxidative stability of vegetable oils by monitoring the tendency to radical formation. A comparison of electron spin resonance spectroscopy with the Rancimat method and differential scanning calorimetry. Food Chemistry 85: 623-632.

Velioglu, Y. S.; Mazza, G.; Gao, L. and Oomah, B. D. 1998. Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. Journal of Agricultural and Food Chemistry 46: 4113-4117.

Vichi, S.; Zitterl-Eglseer, K.; Jugl, M. and Franz, C. 2001. Determination of the presence of antioxidants deriving from sage and oregano extracts added to animal fat by means of assessment of the radical scavenging capacity by photochemiluminescence analysis. Nahrung 45: 101-104.

Vinson, J. A.; Hao, Y.; Su, X. and Zubik, L. 1998. Phenol antioxidant quantity and quality in foods: vegetables. Journal of Agricultural and Food Chemistry 46, 3630-3634.

Vinson, J. A.; Su, X.; Zubik, L. and Bose, P. 2001. Phenol antioxidant quantity and quality in foods: fruits. Journal of Agricultural and Food Chemistry 49: 5315-5321.

Wanasundara, J. K. P. D. and Shahidi, F. 1996. Optimization of hexametaphosphate-assisted extraction of flaxseed proteins using response surface methodology. Journal of Food Science 61: 604-607.

Wanasundara, U. N. and Shahidi, F. 1993. Application of NMR spectroscopy to assess oxidative stability of canola and soybean oils. Journal of Food Lipids 1: 15-24.

Wang, H.; Cao, G. and Prior, R. L. 1996. Total antioxidant capacity of fruits. Journal of Agricultural and Food Chemistry 44: 701-705.

Wang, M.; Jin, Y. and Ho, C-T. 1999. Evaluation of resveratrol derivatives as potential antioxidants and identification of a reaction product of resveratrol and 2,2-diphenyl-1-picrylhydrazyl radical. Journal of Agricultural and Food Chemistry 47: 3974-3977.

Watanabe, M.; Ohshita, Y. and Tsushida, T. 1997. Antioxidant compounds from buckwheat (*Fagopyrum esculentum* Möench) hulls. Journal of Agricultural and Food Chemistry 45: 1039-1044.

Wayner, D. D. M.; Burton, G. W.; Ingold, K. U. and Locke, S. J. 1985. Quantitative measurement of the total, peroxyl radical-trapping antioxidant capacity of human blood plasma by controlled peroxidation. FEBS Letters 187: 33-37.

Wayner, D. D. M.; Burton, G. W.; Ingold, K. U.; Barclay, L. R. C. and Locke, S. J. 1987. The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical trapping antioxidant activity of human blood plasma. Biochimica et Biophysica Acta 924: 408-419.

Weisburger, J. H. 1999. Mechanisms of action of antioxidants as exemplified in vegetables, tomatoes, and tea. Food and Chemical Toxicology 37: 943-948.

Weisburger, J. H.; Rivenson, A.; Aliaga, C.; Reinhardt, J.; Kelloff, G. J.; Boone, C. W.; Steele, V. E.; Balentine, D. A.; Pittman, B. and Zang, E. 1998. Effect of tea extracts, polyphenols, and epigallocatechin gallate on azoxymethane-induced colon cancer. Proceedings of the Society for Experimental Biology and Medicine 217: 104-108.

Weisburger, J. H.; Rivenson, A.; Reinhardt, J.; Aliaga, C.; Braley, J.; Pittman, B. and Zang, E. 1998. Effect of black tea on azoxymethane-induced colon cancer. Carcinogenesis 19: 229-232.

Wende, G.; Buchanan, C. J. and Fry, S. C. 1997. Hydrolysis and fermentation by rat gut microorganisms of 2-O-β-D-xylopyranosyl-(5-O-feruloyl)-L-arabinose derived from grass cell wall arabinoxylan. Journal of the Science of Food and Agriculture 73: 296-300.

Wettasinghe, M. and Shahidi, F. 1996. Oxidative stability of cooked comminuted lean pork as affected by alkali and alkali-earth halides. Journal of Food Science 61: 1160-1164.

Wettasinghe, M. and Shahidi, F. 1997. Oxidative stability, cooking yield and texture of pork treated with a low-sodium salt. Journal of Muscle Food 8: 373-382.

Wettasinghe, M. and Shahidi, F. 1999. Evening primrose meal: A source of natural antioxidants and scavenger of hydrogen peroxide and oxygen-derived free radicals. Journal of Agricultural and Food Chemistry, 47: 1801-1812.

Williams, P. C. 1993. The world of wheat. In: Grains and Oilseeds: Handling, Marketing, Processing. Canadian Grain Commission, Winnipeg, MN. pp 557-602.

Wilson, D. O. Jr. and MacDonald, M. B. Jr. 1986. The lipid peroxidation model of seed ageing. Seed Science and Technology 14: 269-300.

Wink, M. 1993. Production and application of phytochemicals from an agricultural perspective. In: Phytochemistry and agriculture. Eds. T. a. van Beck & H. Breteler. Clarendon Press, London, UK. pp 171-213.

Wink, M. 1999a. Introduction: biochemistry, role and biotechnology of secondary emtabolites. In: Functions of plant secondary metabolites and their exploitation in biotechnology. Ed. M. Wink. CRC Press, Boca Raton, FL. pp 1-16.

Wink, M. 1999b. Introduction: biochemistry, role and biotechnology of secondary metabolites. In: Biochemistry of plant secondary metabolism. Ed. M. Wink. CRC Press, Boca Raton, FL. pp 1-15.

Wink, M. and Schimmer, O. 1999. Modes of action of defensive secondary metabolites and their exploitation in biotechnology. Ed. M. Wink. CRC Press, Boca Raton, FL. pp 17-133.

Xing, Y. and White, P. J. 1997. Identification and function of antioxidants from oat groats and hulls. Journal of the American Oil Chemists' Society 74: 303-308.

Yamaguchi, T.; Takamura, H.; Matoba, T. and Terao, J. 1998. HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1-diphenyl-2-picrylhydrazyl. Bioscience Biotechnology and Biochemistry 62: 1201-1204.

Yanishlieva, N. V. and Marinova, E. M. 1995. Effects of antioxidants on the stability of triacylglycerols and methyl esters of fatty acids of sunflower oil. Food Chemistry 54: 377-382.

Yee, J. J. and Shipe, W. F. 1981. Using enzymatic proteolysis to reduce copper-protein catalysis of lipid peroxidation. Journal of Food Science 46: 966-967.

Yen, G-C. and Duh, P-D. 1994. Scavenging effect of methanolic extracts of peanut hulls on free-radical and active-oxygen species. Journal of Agricultural and Food Chemistry 42: 629-632.

Yen, W. J. and Chen, B. H. 1995. Isolation of xanthophylls from Taiwanese orange peels and their effects on the stability of soybean oil. Food Chemistry 53: 417-426.

Yu, J.; Vasanthan, T, and Tamelli, F. 2001. Analysis of phenolic acids in barley by high-performance liquid chromatography. Journal of Agricultural and Food Chemistry 49: 4352-4360.

Yu, L.; Haley, S.; Perret, J.; Harris, M.; Wilson, J. and Qian, M. 2002. Free radical scavenging properties of wheat extracts. Journal of Agricultural and Food Chemistry 50: 1619-1624

Yu, L.; Perret, J.; Harris, M.; Wilson, J. and Haley, S. 2003. Antioxidant properties of bran extracts from "Akron" wheat grown at different locations. Journal of Agricultural and Food Chemistry 51: 1566-1570.

Zhang, H. Y.; Ge, N. and Zhang, Z. Y. 1999. Theoretical elucidation of activity differences of five phenolic antioxidants. Acta Pharmacologica Sinica 20: 363-366.

Zhou, K. and Yu, L. 2004. Antioxidant properties of bran extracts from Trego wheat grown at different locations. Journal of Agricultural and Food Chemistry 52: 1112-1117.

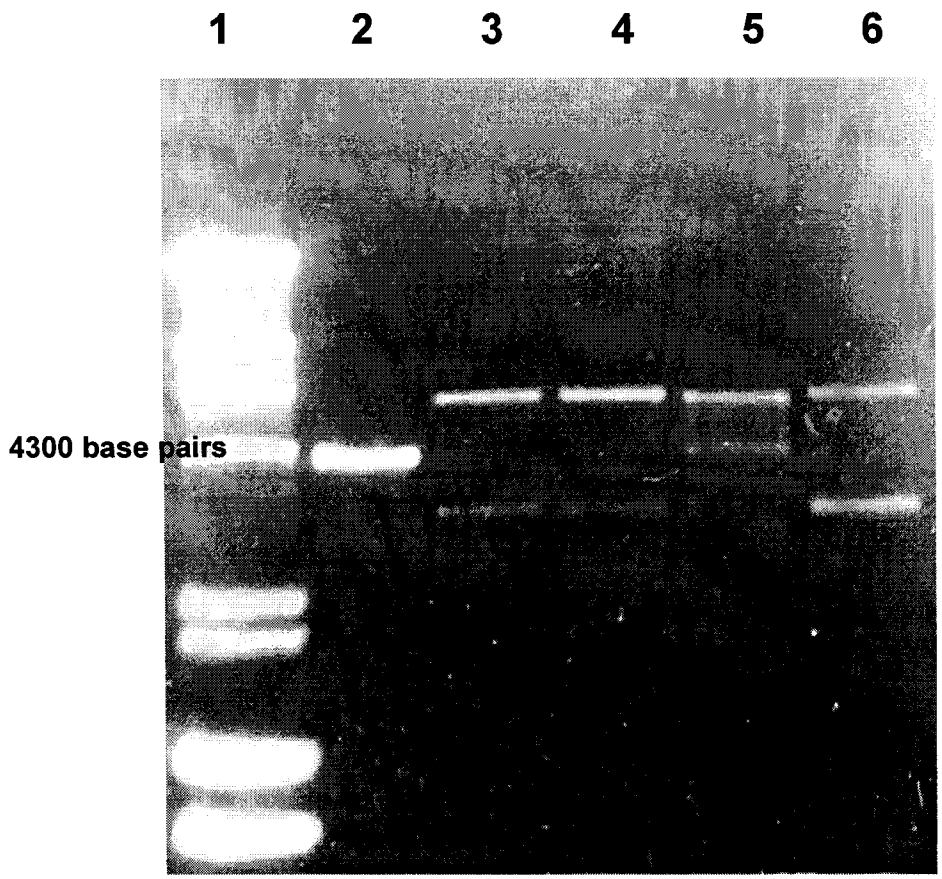
Zhou, K.; Laux, J. J. and Yu, L. 2004. Comparison of Swiss red wheat grain and fractions for their antioxidant properties. Journal of Agricultural and Food Chemistry 52:1118-1123.

Zieliński, H. and Kozłowska, H. 2000. Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions. Journal of Agricultural Food Chemistry 48: 2008-2016.

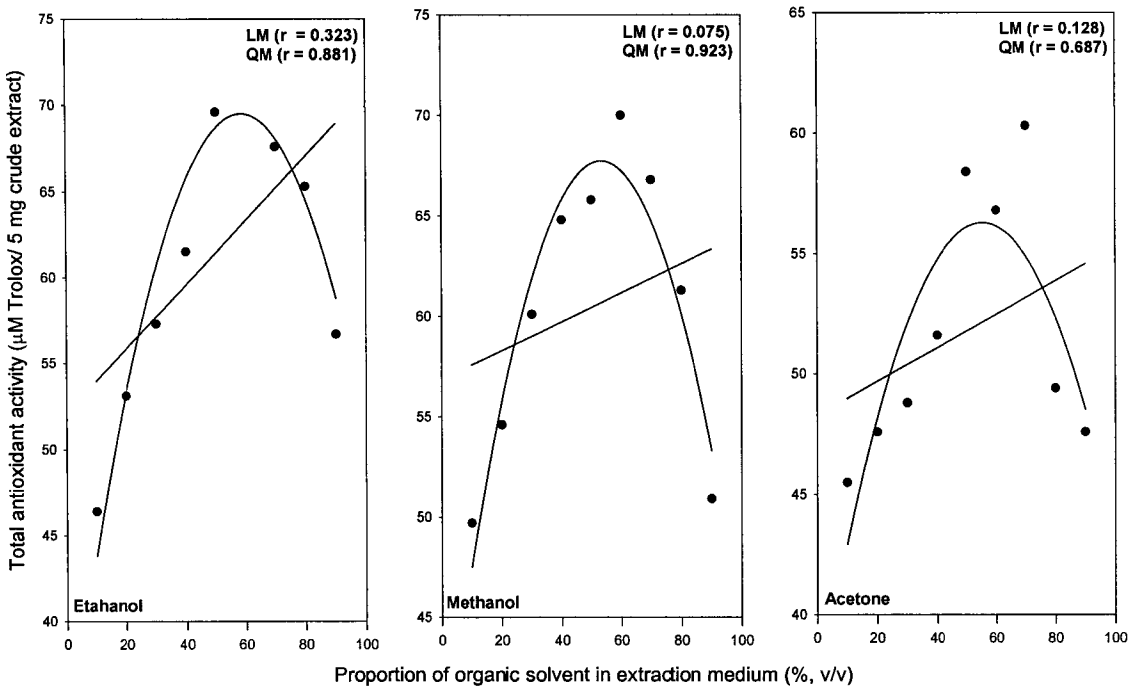
Zupfer, J. M.; Churchill, K. E.; Rasmusson, D. C. and Fulcher, R. G. 1998. Variation in ferulic acid concentration among diverse barley cultivars measured by HPLC and microspectrophotometry. Journal of Agricultural and Food Chemistry 46: 1350-1354.

Appendix 2.1. Proximate composition of soft and hard wheat samples

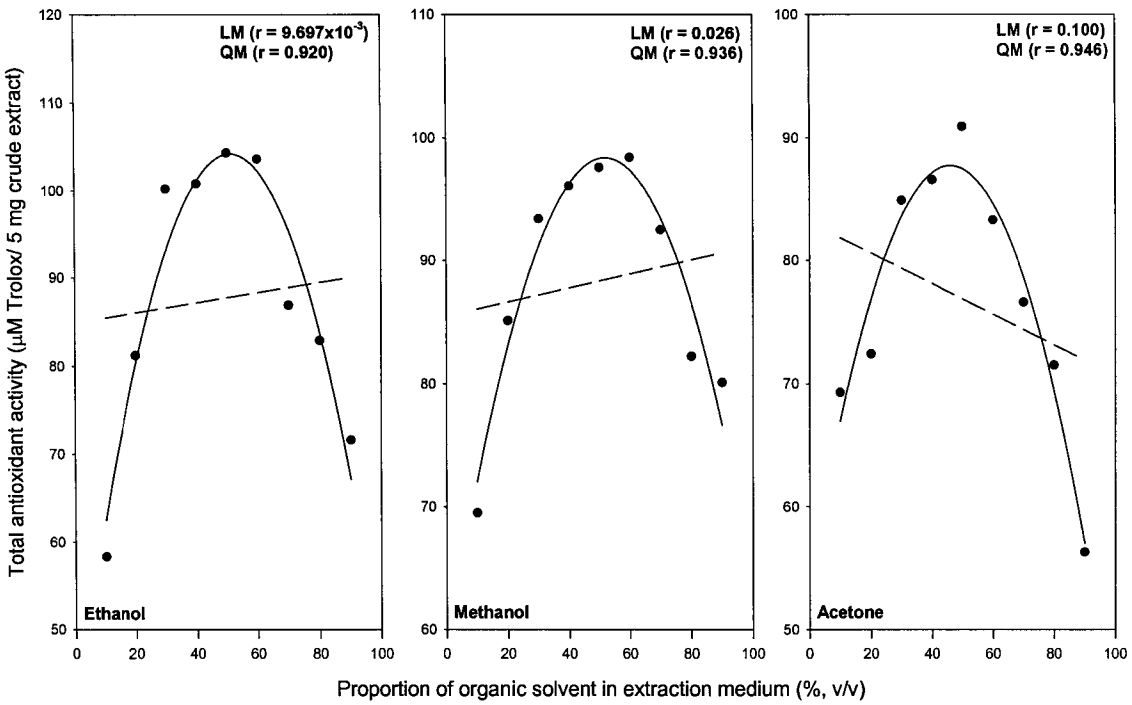
Milling fraction	Moisture	Ash	Protein	Lipid
Soft wheat				
Whole grain	10.9 ± 0.3	1.9 ± 0.2	10.7 ± 0.4	2.5 ± 0.2
Flour	10.5 ± 0.3	0.5 ± 0.01	9.4 ± 0.2	0.9 ± 0.1
Germ	11.2 ± 0.5	4.1 ± 0.3	22.9 ± 0.6	5.5 ± 0.5
Bran	9.6 ± 0.2	6.3 ± 0.4	14.3 ± 0.4	3.2 ± 0.3
Shorts	10.8 ± 0.4	4.7 ± 0.5	17.7 ± 0.3	3.6 ± 0.4
Hard wheat				
Whole grain	9.6 ± 0.1	1.7 ± 0.1	15.4 ± 0.5	2.8 ± 0.4
Flour	10.3 ± 0.2	0.6 ± 0.05	13.9 ± 0.4	1.1 ± 0.1
Germ	11.6 ± 0.3	4.4 ± 0.3	26.1 ± 0.7	6.8 ± 0.4
Bran	9.3 ± 0.3	6.8 ± 0.2	16.9 ± 0.2	3.5 ± 0.3
Shorts	10.3 ± 0.4	4.6 ± 0.2	19.3 ± 0.5	3.7 ± 0.4



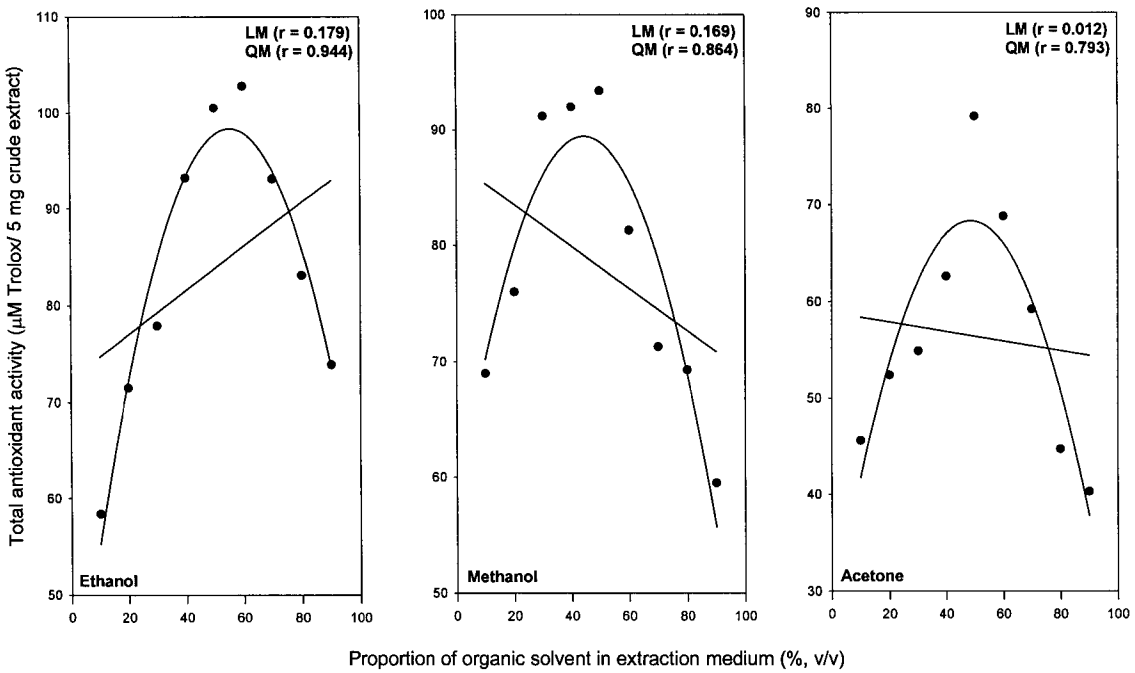
Appendix 3.1. Agarose gel electrophoresis of supercoiled DNA, wheat phenolics and base pair ladder DRIgest™ III (Lane 1: DNA marker; Lane 2: Supercoiled DNA incubated with restriction enzyme resulting linear DNA; Lanes 3 & 4: supercoiled DNA + hydroxyl radical + wheat extract; Lane 5: supercoiled DNA + hydroxyl radical; Lane 6: supercoiled DNA)



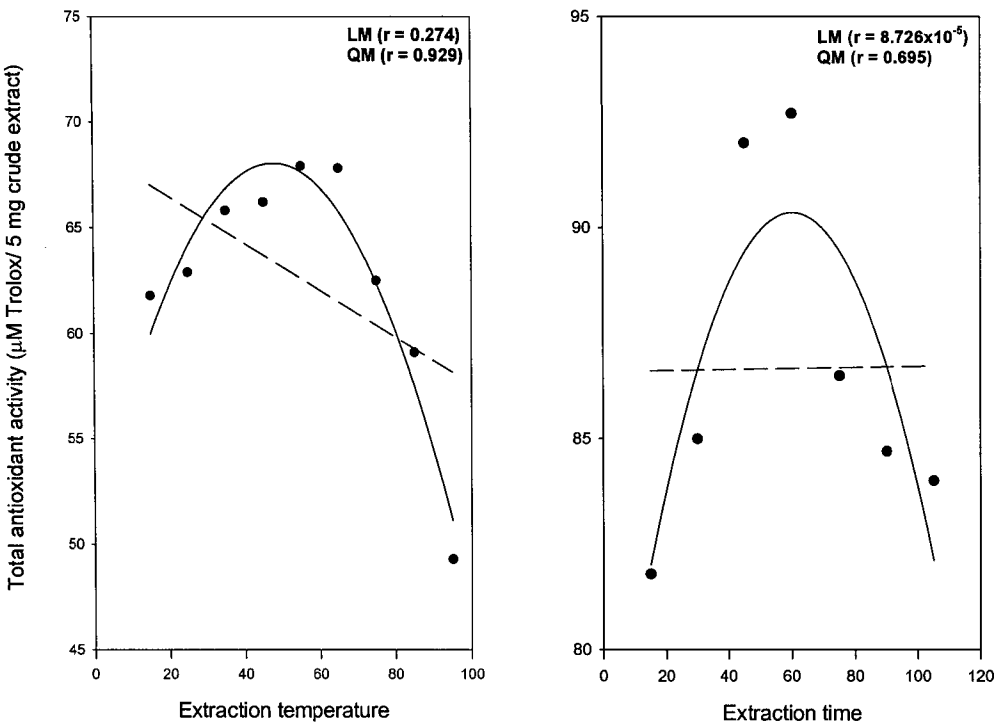
Appendix 4.1. Effect of varying solvent on total antioxidant activity of soft whole wheat crude extract. Each data point represents the average of two determinations. LM and QM represent the linear and quadratic models, respectively.



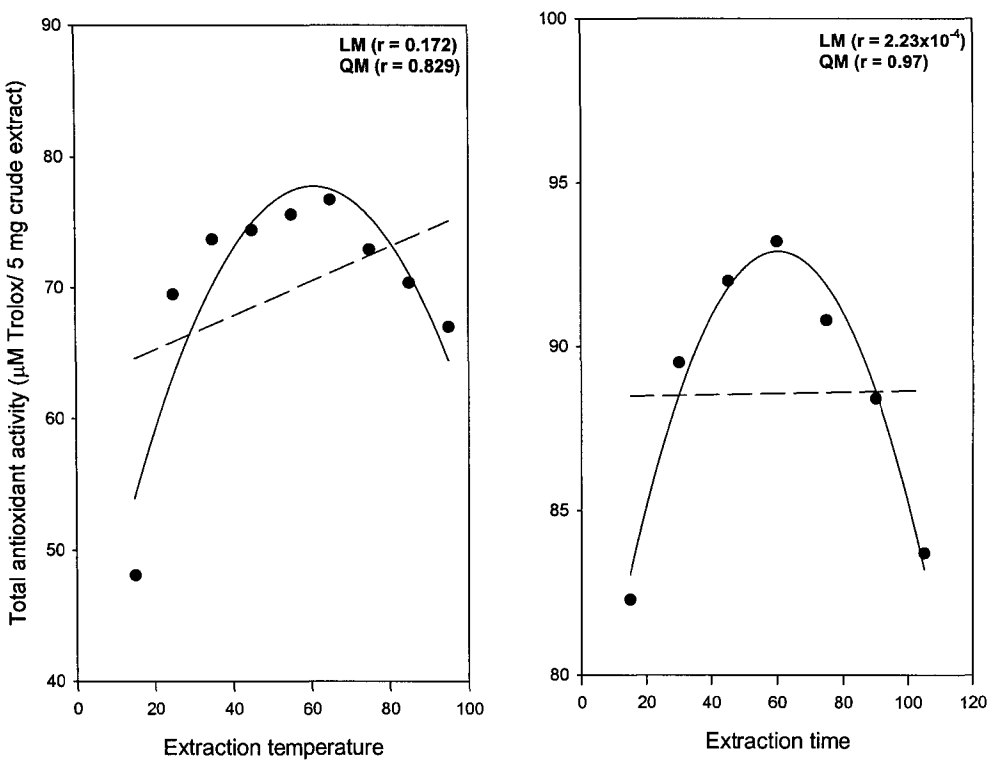
Appendix 4.2. Effect of varying solvent on total antioxidant activity of hard wheat bran crude extract. Each data point represents the average of two determinations. LM and QM represent the linear and quadratic models, respectively.



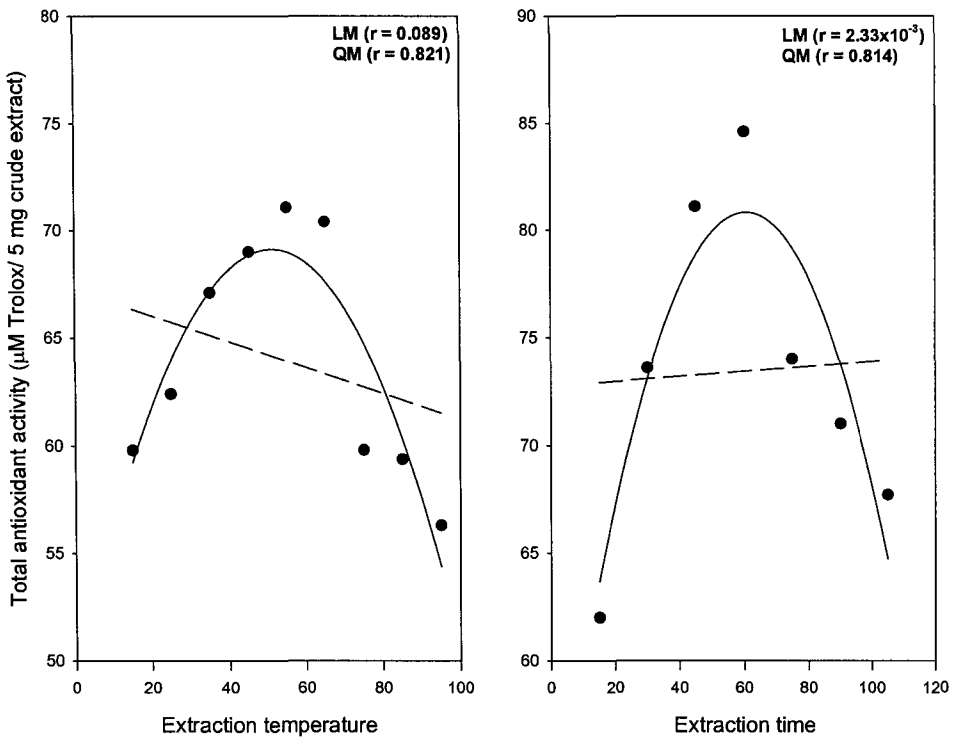
Appendix 4.3. Effect of varying solvent on total antioxidant activity of hard whole wheat crude extract. Each data point represents the average of two determinations. LM and QM represent the linear and quadratic models, respectively.



Appendix 4.4. Effect of varying extraction temperature and extraction time on total antioxidant activity of soft whole wheat crude extract. Each data point represents the average of two determinations. LM and QM represent the linear and quadratic models, respectively.



Appendix 4.5. Effect of varying extraction temperature and extraction time on total antioxidant activity of hard wheat bran crude extract. Each data point represents the average of two determinations. LM and QM represent the linear and quadratic models, respectively.



Appendix 4.6. Effect of varying extraction temperature and extraction time on total antioxidant activity of hard whole wheat crude extract. Each data point represents the average of two determinations. LM and QM represent the linear and quadratic models, respectively.

Appendix 5.1. Percentage radical scavenging capacity of soft and hard wheat extracts at 100 ppm as ferulic acid equivalents

Milling fraction	Scavenging capacity (%)
SOFT WHEAT	
Whole grain	70.4 ± 2.3
Flour	67.3 ± 1.7
Germ	79.7 ± 0.9
Bran	73.7 ± 0.9
Shorts	74.2 ± 1.2
HARD WHEAT	
Whole grain	71.3 ± 1.2
Flour	64.6 ± 1.0
Germ	81.3 ± 0.9
Bran	71.8 ± 1.1
Shorts	70.9 ± 2.2

Appendix 5.2. Retention of β-carotene (%) in the assay medium by soft and hard wheat extracts at 100 ppm as ferulic acid equivalents after 2 h

Milling fraction	Retention of β-carotene (%)
SOFT WHEAT	
Whole grain	68.6 ± 1.4
Flour	53.2 ± 1.0
Germ	79.4 ± 1.7
Bran	82.7 ± 0.9
Shorts	72.3 ± 1.9
HARD WHEAT	
Whole grain	64.3 ± 2.1
Flour	50.1 ± 1.3
Germ	76.3 ± 1.8
Bran	79.5 ± 1.6
Shorts	69.1 ± 0.7

Appendix 5.3. Iron (II) chelation activity (%) of soft and hard wheat extracts at 100 ppm as ferulic acid equivalents

Milling fraction	Chelation capacity (%)
SOFT WHEAT	
Whole grain	91.3 ± 2.3
Flour	95.2 ± 3.1
Germ	98.6 ± 3.3
Bran	92.5 ± 1.8
Shorts	91.3 ± 1.9
HARD WHEAT	
Whole grain	89.1 ± 1.9
Flour	91.6 ± 1.6
Germ	78.4 ± 1.3
Bran	89.6 ± 1.4
Shorts	76.6 ± 2.1

Appendix 5.4. Inhibition (%) of oxidation of Cu^{2++}-induced human LDL by soft and hard wheat extracts at 100 ppm as ferulic acid equivalents

Milling fraction	Inhibition of oxidation (%)
SOFT WHEAT	
Whole grain	93.2 ± 0.7
Flour	91.2 ± 0.4
Germ	99.3 ± 0.9
Bran	96.6 ± 0.7
Shorts	90.7 ± 0.9
HARD WHEAT	
Whole grain	89.7 ± 1.0
Flour	86.4 ± 0.7
Germ	97.2 ± 0.9
Bran	96.3 ± 1.0
Shorts	90.2 ± 0.4

Appendix 6.1. DPPH radical scavenging capacity (%) of non-treated and treated soft and hard wheat extracts at 100 ppm as ferulic acid equivalents

Milling fraction	Non-treated	Treated
SOFT WHEAT		
Whole grain	38.9 ± 0.7	40.8 ± 0.1
Flour	31.5 ± 1.0	39.0 ± 0.4
Germ	66.2 ± 0.3	73.3 ± 0.4
Bran	49.2 ± 0.9	63.6 ± 0.3
HARD WHEAT		
Whole grain	40.7 ± 0.3	45.5 ± 0.6
Flour	29.8 ± 0.3	38.7 ± 0.3
Germ	59.5 ± 0.6	67.0 ± 0.9
Bran	46.2 ± 1.4	50.4 ± 0.7

Appendix 6.2. Retention of β-carotene in the assay medium by non-treated and treated soft and hard wheat extracts at 100 ppm as ferulic acid equivalents

Milling fraction	Non-treated	Treated
SOFT WHEAT		
Whole grain	50.4 ± 1.2	59.4 ± 1.9
Flour	33.4 ± 0.8	40.9 ± 1.0
Germ	66.4 ± 0.7	72.6 ± 0.4
Bran	62.2 ± 0.8	75.1 ± 0.9
HARD WHEAT		
Whole grain	49.0 ± 1.2	54.1 ± 0.9
Flour	33.3 ± 1.7	38.5 ± 1.0
Germ	66.4 ± 0.7	71.0 ± 1.3
Bran	72.3 ± 1.4	70.1 ± 1.1

Appendix 6.3. Iron (II) chelation capacity (%) by non-treated and treated soft and hard wheat extracts at 100 ppm as ferulic acid equivalents

Milling fraction	Non-treated	Treated
SOFT WHEAT		
Whole grain	84.7 ± 1.0	98.1 ± 1.0
Flour	79.7 ± 2.1	91.3 ± 1.3
Germ	70.9 ± 1.3	78.2 ± 1.7
Bran	82.8 ± 1.4	94.7 ± 2.7
HARD WHEAT		
Whole grain	87.0 ± 0.7	98.7 ± 2.3
Flour	79.7 ± 1.1	91.7 ± 2.5
Germ	76.3 ± 2.4	86.4 ± 1.8
Bran	81.7 ± 1.8	92.9 ± 1.7

Appendix 6.4. Inhibition (%) of Cu^{2++}-induced oxidation of human LDL by non-treated and treated soft and hard wheat extracts at 100 ppm as ferulic acid equivalents

Milling fraction	Non-treated	Treated
SOFT WHEAT		
Whole grain	81.7 ± 2.0	90.8 ± 0.9
Flour	78.3 ± 2.1	87.4 ± 1.2
Germ	88.6 ± 1.8	97.3 ± 0.7
Bran	85.6 ± 2.1	96.9 ± 1.0
HARD WHEAT		
Whole grain	77.4 ± 2.0	88.9 ± 2.3
Flour	68.5 ± 2.1	80.2 ± 1.6
Germ	88.2 ± 1.6	95.1 ± 2.2
Bran	84.7 ± 1.5	94.2 ± 1.4

Appendix 8.1. Fatty acid composition of stripped corn oil, seal blubber oil and shortening

Fatty acid	SCO[a]	SBO[b]	Shortening[c]
SATURATED			
14:0		5.5 ± 0.2	0.3 ± 0
15:0		0.4 ± 0.01	-
16:0	10.4 ± 0.1	9.4 ± 0.3	18.4 ± 0.4
18:0	2.2 ± 0	1.1 ± 0.03	10.7 ± 0.1
20:0	0.5 ± 0.01		0.4 ± 0
22:0			0.4 ± 0
Total	13.1	16.4	30.2
MONOUNSATURATED			
14:1		0.9 ± 0.01	
16:1		19.5 ± 0.2	0.1 ± 0
18:1	26.4 ± 0.3	24.7 ± 0.2	40.6 ± 0.4
20:1		11.5 ± 0.1	
22:1		0.5 ± 0	
Total	26.0	57.1	40.7
POLYUNSATURATED			
18:2 n-6	58.9 ± 0.3	2.0 ± 0.01	27.0 ± 0.2
18:3 n-6			0.2 ± 0
18:3 n-3	1.0 ± 0	0.8 ± 0.02	1.7 ± 0
20:3		0.5 ± 0	
20:5 n-3		8.6 ± 0.04	
22:1	0.5 ± 0		
22:5 n-3		4.6 ± 0.3	
22:6 n-3		9.7 ± 0.1	
Total	60.4	26.2	28.9

[a]stripped corn oil, [b]seal blubber oil, [c]shortening (fluffo® all vegetable shortening, J. M. Smucker Canada Inc., Toronto, ON, Ingredients: partially hydrogenated vegetable and palm oils, hydrogenated modified palm oil, mono- and diglycerides)

Values are the mean of three determinations ± standard deviation